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Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines

Immunization, Vaccines and Biologicals

World Health Organization

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Preface

In 1982, with the aim of providing guidance for the standardization of tests needed to ensure the safety and efficacy of vaccines, the World Health Organization (WHO) issued a document entitled Manual of details of tests required on final vaccines used in the WHO Expanded Programme on Immunization (unpublished document BLG/UNDP/82.1 Rev.1). This document was updated in 1985 (unpublished document BLG/UNDP/82.1 Rev. 1 Corr. 1). As a result of changes in some of the potency tests and the development of alternative techniques for testing the potency of bacterial vaccines, and in an attempt to present the information in a more useful way for testing laboratories, details of the potency tests for live viral vaccines and bacterial vaccines were revised and reissued provisionally in two documents, Laboratory methods for the titration of live virus vaccines using cell culture techniques (unpublished document BLG/EPI/89.1) and Laboratory methods for the testing for potency of diphtheria (D), tetanus (T), pertussis (P) and combined vaccines (unpublished document BLG/92.1). These two documents were field tested in workshops worldwide. They were revised in the light of comments received and combined to produce the manual, WHO/BLG/95.1. With the establishment of the Global Training Network, the document was revised in light of its relevance as a curriculum material for quality control test methodologies. In 1997, WHO publish the Manual of laboratory methods-For testing vaccines used in the WHO Expanded Programme on Immunization (WHO/VSQ/97.04) with the contribution of the following scientists: JH Bruning, F van Nimwegen, JW van der Gun and JL di Fabio, R Dobbelaer, P Knight, J Lyng, CFM Hendriksen, FR Marsman, HJM van de Donk, JG Kreeftenberg, P Knight, G Nyerges, M Weiz Bentzon, F Marsman. This Manual was widely used by the National Control Laboratories and vaccine manufacturers and has served as a training material in WHO Global Training Network programme since its establishment.

Recently, the amendment on potency testing of diphtheria vaccine and tetanus vaccine (TRS 927), and revised recommendations for whole cell pertussis vaccines (TRS 941) were adopted by the Expert Committee on Biological Standardization, in 2003 and 2005, respectively. Taking into account recent developments in quality control methods, a need for updating the manual was recognized. Revised Manual should complement recommendations, providing details of testing and related statistical analysis. A meeting of the DTP working group was convened in July 2006 in Geneva Switzerland to review current potency and safety testing methods for the diphtheria, tetanus and pertussis vaccine, attended by the following people: Dr Maria Baca-Estrada, Biologics and Genetic Therapies Directorate, Health Canada, Canada; Dr. Marie-Emmanuelle Behr-Gross, European Pharmacopoeia Department, European Directorate for the Quality of Medicines and HealthCare (EDQM), France; Dr Mike Corbel, National Institute for Biological Standards & Control (NIBSC), UK; Dr Roland Dobbelaer, Scientific Institute of Public Health - Louis Pasteur (SIPH), Belgium; Dr Rose Gaines-Das, NIBSC, UK; Dr Sunil Gairola, Serum Institute of India Ltd., India; Dr Elwyn

Griffiths, Biologics and Genetic Therapies Directorate, Health Canada, Canada; Dr Yoshinobu Horiuchi, National Institute of Infectious Diseases (NIID), Japan; Dr Teeranart Jivapaisarnpong, Division of Biological Products, Department of Medical Sciences, Thailand; Dr Frederic Mortiaux, GlaxoSmithKline Biologicals, Belgium; Dr Olga Perelygina, L.A. Tarassevich State Research Institute for Standardization and Control of Medical Biological Preparations, The Russian Federation; Dr Rachel Preneta, NIBSC, UK; Ms Sonia Prieur, Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS), France; Dr. Guy Rautmann, EDQM, France; Dr Michael P. Schmitt, Centre for Biologics Evaluation Research, Food and Drug Administration, USA; Dr Dorothea (Thea) Sesardic, NIBSC, UK; Dr Motohide Takahashi, NIID, Japan; Dr Randi Winsnes, Norwegian Medicines Agency, Norway; Dra Sri Wahyuningsih, National Quality Control Laboratory of Drug and Food (NQCL), Indonesia; Dr Dorothy Xing, NIBSC, UK; Dr Shumin Zhang, National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), People's Republic of China; Dr Hans Kreeftenberg, Netherlands Vaccine Institute, Netherlands; Dr David Wood, World Health Organization (WHO), Switzerland; Dr Joelle Davaud, WHO, Switzerland; Dr Nora Dellepiane, WHO, Switzerland; Dr Ivana Knevici, WHO, Switzerland; Dr Dianliang Lei, WHO, Switzerland; Dr Tiequn Zhou, WHO, Switzerland; Dr Carmen Rodriguez Hernandez, WHO, Switzerland.

In this meeting the current situation of quality control of DTP vaccines was reviewed and the drafting group was set up to revise the Manual with the following members: Dr Mike Corbel, Dr Thea Sesardic, Dr Dorothy Xing, Dr Roland Dobbelaer, Dr Rose Gaines-Das, Dr Yoshinobu Horiuchi, Dr Teeranart Jivapaisarnpong, Dr Hans Kreeftenberg, Dr Randi Winsnes, and Dr Dianliang Lei. The parts of general introduction, testing for diphtheria vaccines, testing for tetanus vaccines, testing for whole cell pertussis vaccines and statistical analysis were revised by the drafting group. The revised Manual was reviewed by the DTP working group in its meeting in March 2007 in Geneva. Finally the working group agreed on the revised version.

The General introduction was revised by Dr R Dobbelaer, Dr M J Corbel. The Testing of diphtheria vaccines and Testing of tetanus vaccines were drafted by Dr T Sesardic and Dr R Winsnes. The Testing of pertussis vaccine was drafted by Dr D Xing, Dr Y Horiuchi and Dr T Jivapaisarnpong. And the Statistical analysis was drafted by Dr R Gaines-Das and Dr Y Horiuchi with contribution from Dr H Kreeftenberg on Validation of in house specific procedures and 'transferability' of IU. The Manual was finally compiled and reviewed by Dr Dianliang Lei.

Acknowledgements are due to the DTP working group and the drafting group for their contribution to the revision of the Manual.

Contents

Chapter I: General

I.1 General introduction

I.1.1 Scope

This Manual contains a description of a number of selected test methods that have been used successfully by manufacturers and national control authorities/national control laboratories in establishing and monitoring the quality, potency and safety of vaccines that contain Diphtheria, Tetanus and/or Pertussis (whole cell) components. Selection of the methods has been based on the tests mentioned in the corresponding WHO Requirements/Recommendations. The descriptions are intended to provide information complementary to the WHO Recommendations, thus facilitating the introduction and routine performance of the methods in vaccine quality control laboratories. The level of detail provided in this Manual is intended to be adequate for this purpose.

The reliability of the results that are obtained in any testing programme depends heavily on the correct execution of the tests by adequately trained staff and on the quality of the reagents used, including the reference materials and laboratory animals. Furthermore, as the tests in this Manual are biological assays, the correct calibration and use of reference materials is of vital importance as well as the appropriate statistical methods used for data evaluation and monitoring. Quality Control has to be performed under a Quality Assurance system including provisions for adequate training of staff, maintenance and calibration of equipment, environmental regulation, for appropriate validation of tests and for ensuring traceability of results. Therefore this Manual includes sections on each of these important elements.

I.1.2 Quality assurance

I.1.2.1 General principles.

Quality assurance is the term used to describe the combination of organized activities performed to ensure that the output (whether product or data) of a process meets the quality criteria and specifications required for its intended purpose.

For the vaccine manufacturer, quality assurance is the sum of all the procedures used to ensure that the vaccine produced will be both safe and effective and in compliance with the terms of the manufacturing and product marketing authorizations. The manufacturer is therefore responsible for ensuring that production is undertaken in accordance with the principles of good manufacturing practice covering the facilities, personnel, raw materials, production and control, storage and distribution procedures.

Total control of quality involves the organized effort within an entire manufacturing facility to design, produce, maintain and assure the specific quality of each lot of vaccine distributed. It is a facility-wide activity and represents the cumulative responsibility of all components of the manufacturing organization.

For the national control authority, the quality assurance system applies to all the evaluation activities that ensure the quality, safety and efficacy of vaccines. Such activities include approval of manufacturing processes and facilities, product marketing authorization, batch control testing and document review, monitoring of distribution systems and post-marketing performance. The regulatory authority must ensure that a vaccine manufacturer is capable of producing a safe and effective product and that the product continues to meet specifications on an ongoing basis.

I.1.2.2 Specific application

With respect to the application of the test methods described in this manual, it is important that all of the testing activities are conducted under a quality assurance system to ensure the reliability and consistency of the test results. A laboratory working under such a quality assurance system will be capable of producing accurate and reproducible test data, and can be relied upon in its day-to-day operations to maintain a consistently acceptable standard of performance. This is essential to maintain confidence in the quality of data produced, without which effective decisions on product quality cannot be made or defended. A full description of the activities included in laboratory quality systems is beyond the scope of this manual. The reader is referred to the reference list for further information. Globally recognized quality assurance systems have been developed that specifically address the activities of testing laboratories. ISO 17025 *(1)* covers general aspects such as quality policy, management of internal and external documents, internal and external complaint procedures and procedures for corrective actions, internal and external audit systems, training of staff, requirements for testing facilities, sample receipt and recording, management of reagents, test samples and reference preparations, data reporting, data monitoring, equipment maintenance and calibration, environmental monitoring, trend monitoring and participation in external quality assurance programmes. While it is desirable that testing laboratories should operate under a quality system subject to independent accreditation such as ISO 17025, it is recognized that this has serious resource implications and will not be achievable in many instances. It is recommended however, that all testing laboratories operate a quality system that complies with the basic principles of this standard. The key areas that should be addressed include the following.

- **Personnel:** The work should be undertaken by experienced laboratory staff thoroughly trained for the task. Where specific tests are performed infrequently, training must be updated at regular intervals to ensure maintenance of competence. For tests performed in animals, personnel appropriately trained in animal care and handling are also required. Staff performing test procedures on animals must be trained appropriately and shown to be capable of achieving valid test performance. Training records must be maintained for all staff.
- **Facilities:** Adequate laboratory space and services (running water, electricity, and gas) should be available, with emergency backup where the external supply is uncertain. The working environment must be comfortable, safe and contaminant-free. As infectious agents and toxins will need to be used, the accommodation must be adequate for protection of the operators, the public and the environment.

When tests are performed in laboratory animals, suitable animal accommodation that complies with animal health and safety standards and provides adequate disease security is absolutely essential. Laboratories must be provided with adequate and safe means of waste disposal for chemicals, infectious materials and animal waste.

- **Instruments and equipment** must be of adequate quality and suitable for the purpose. They must be used correctly and handled and maintained so that the accuracy required to ensure good quality results is achievable. Performance criteria for each instrument or piece of equipment must be established. Up-to-date records of use and of regular maintenance and calibration must be kept and should be readily available for inspection.
- **Test samples** must be received, recorded, handled, and stored according to standardized procedures to ensure that the quality of information gathered from them is accurate and truly representative.
- **Reagents, solutions and reference materials** must all be of high quality and appropriate for the specific analytical methods. Water is considered to be a reagent and must meet the requirements acceptable for other analytical reagents. Labeling and documentation of reagents and assignment of appropriate shelf life is part of the monitoring and maintenance of quality assurance programmes. Reference materials may be validated at source, but storage, handling and processing conditions must be controlled. Procedures for safe disposal of wastes must be developed and implemented.
- **Methods** must be appropriate for their intended use and compatible with the nature of the samples to be tested. They must be available in writing in a Methods Manual and written in the form of a standard operating procedures (SOP) in a clear and unambiguous manner, such that an experienced analyst, unfamiliar with the method, is able to use the procedure and interpret the results. SOPs should follow a pre-established format which includes the following:
  - title
  - date of authorization
  - reference(s)
  - scope
  - basic principles
  - apparatus and reagents
  - procedural details
  - safety precautions
  - calculations and statistics
  - quality assurance.

  Non-compendial methods will require validation. Details of this process must be recorded.

  Only one version of an SOP should be in use at any one time. Previous versions should be removed from circulation and archived. Unauthorised modifications must not be permitted. If circumstances require deviation from the standard procedure during the course of a test, this must be recorded.

- **Audit** of the quality system should be carried out regularly. Ideally this should be done independently. Audit records should be maintained together with any corrective actions or recommendations.
- **Trend monitoring** of data should be performed and any anomalies noted and investigated.

I.1.3 References

1. ISO/IEC 17025; 2005 General requirements for the competence of testing and calibration laboratories. International Standards Organisation, Geneva. 2005.

I.2. Standardization in vaccine testing

I.2.1 Introduction

The term **standardization** as used in this manual encompasses all the measures that should be undertaken to obtain results that are fully comparable in respect of both time and place, such as comparability of results of a certain test within one laboratory performed at different times, and by different operators, and comparability of results of a certain test performed in different laboratories.

To achieve such comparability of results it is essential to use standardized equipment, materials and reagents, including reference materials, in accordance with a standard operating procedure. Most of the tests described in this manual provide for vaccine samples to be tested in parallel with a working reference preparation which, if applicable, has been calibrated against the corresponding international standard or international reference reagent. In addition and whenever applicable, the use of a control vaccine is recommended. Individual data should be recorded for the continuous evaluation of reference stability and test conditions. This can be done by using a Shewhart control chart. Calibration of the working reference preparation should be repeated periodically to avoid over- or under-estimation of the samples under test.

I.2.2 Quality control charts

In order to evaluate properly the data generated in a quality control programme, values for each of the controls in each test run and test results should be listed on charts to permit a trend analysis. Such charts provide a visual representation of performance of the assay, enable comparisons to made, and clearly show any deviations, trends and shifts. A chart should be prepared for each control material. The most commonly used chart for internal quality control is the Shewhart control chart, also called the x chart, which is prepared in the following way.

The collection of data should be a continuous process, which should be done regularly by capturing the raw data in the specified document (e.g. Excel, word data sheets or a computer program). The test results should be obtained from the raw data for a particular test/parameter for which trend analysis needs to be performed, for a specified time period/interval and compiled data should be made available for analysis.

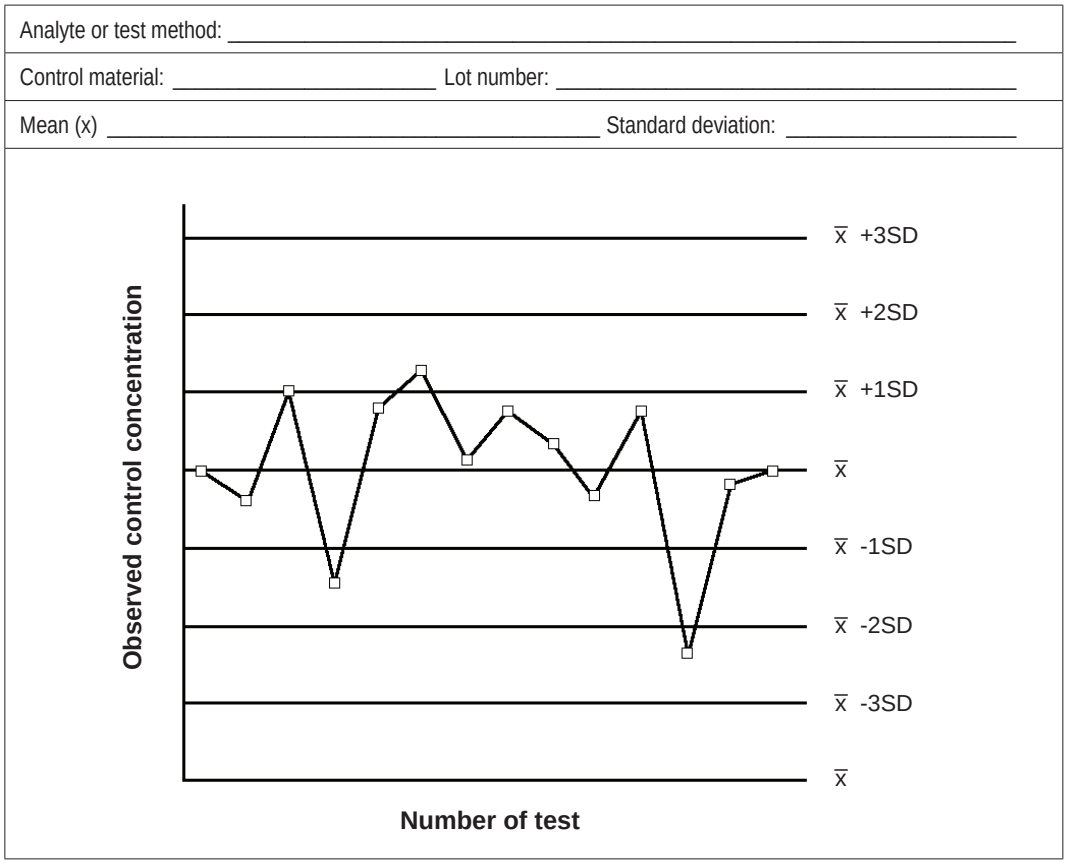
As a minimum, at least 20 values/data points of each quality parameter should be collected and the mean, standard deviation (SD), mean and ± 2 and 3 SD values for this data should be calculated. In case 20 data points/values are not available, the trends may be established by using the maximum number of data points/values available. These estimates may be revised on a regular basis or when more data is available and new cumulative values should be used to revise the previously established trends, until a minimum of 20 data points/values are completed. Subsequently, all the values should fall within these established trends and the comments should be given, based on these established trends.

The chart displays concentration on the y-axis (vertical) versus time on the x-axis (horizontal). Horizontal lines are drawn for the mean and for the upper and lower control limits, which are calculated from the standard deviation. The two upper control limits are the mean plus three and two standard deviations (+3 SD and +2 SD) and the lower control limits are the mean minus two and three standard deviations (-2 SD and -3 SD), respectively (see Figure I.2.1). The individual results from subsequent assays are then plotted on the chart after every valid run.

The graph is an extension of a Gaussian normal distribution curve (bell curve) laid on its side. Statistically, 95% of the control values are expected to fall within 2 SD of the calculated mean value, and the data generated by the different runs should be randomly distributed around the mean. Results falling outside the range of 2 SD (warning limit) should be cause for alert, especially if the results are from two consecutive runs. Any value falling outside mean ± 3 SD (action or rejection limit), but within the specification, shall be considered as "out of trend" and should be notified immediately to all the concerned staff. All such results should also be explained under remarks, in the footnote of the chart. In such case an investigation shall be conducted and may lead to rejection of that particular run or readjustment of the assay. The chart should be closed by putting up prepared by, approved by and authorized by signatures with applicable dates, at the bottom. Whenever, the trend shows a shift of at least 7 continuous values, the revision of the 'Established Trends" and/or "Specifications", may be considered, after a thorough investigation and analysis, provided there is no impact on the quality of the product. These are general terms—in some cases the acceptability of a test may be within ±1 SD.

Deviations are most frequently caused by random error. However, if the direction of several consecutive results points downwards or upwards from the mean line this probably indicates the presence of a systematic error or a stability problem that should be corrected. Precise test criteria should be set to increase the probability of error detection, taking care that the probability of false rejection is limited.

Figure I.2.1: The Shewhart control chart



I.3. Role of Biostatistics in Vaccine Potency Testing

Section V of this manual provides guidance on the statistical aspects of the bioassays used for vaccine potency testing. The Introduction to Section V gives an overview of the role of biostatistics and describes the scope of the Section. The Introduction is recommended for anyone involved with planning, carrying out or using the results of bioassays. Although numerical examples of assays in Section V are limited to those described in the manual, the introductory chapter discusses the general principles of standardization, assay design and analysis, assay validation and combination of potency estimates. The application of the 3Rs in the context of vaccine potency testing is considered. There are also comments on the use of statistical software and suggested further reading.

I.4. The use of laboratory animals in the quality control of vaccines

Bioassays performed on living animals are still the method of choice for evaluating the safety and potency of many bacterial vaccines. There is a strong movement towards reducing or eliminating the use of live animals in pharmaceutical testing and this has led to the introduction of replacement procedures in some situations. However, while the ultimate aim of eliminating the use of animals in testing is commendable, this should not lead to the introduction of procedures that are not effective for the primary purpose of assuring safety and efficacy. Before any new procedure is introduced it should be validated against the standard method to ensure at least non-inferiority of performance. Nevertheless, it should be noted that significant advances have been made in the introduction of less severe procedures for testing diphtheria and tetanus vaccines. Most bio-assays use either guinea-pigs or mice often in quite large numbers. In contrast to physicochemical tests, which can measure physical or chemical parameters with high precision, bioassays in animals can measure biological activity with very high specificity and sensitivity although often with low precision and substantial variability. Generally, responses are easily observable and quantal, meaning that the results obtained are "positive or negative", or "alive or dead". In addition, particular physiological responses can be measured, such as change in body temperature, production of antibodies, or body weight gain or loss. The main shortcoming of such tests is the result of individual variation between animals which can result in low precision and poor reproducibility.

More than any other system used for testing, animals have to be handled and maintained appropriately to generate accurate, reliable, and reproducible results. It is essential to be aware of all the factors that may affect the biological functions of the test animals, and thus interfere with the outcome of a potency, safety or toxicity test.

This section contains information on the maintenance of animals, measures to be taken to ensure their quality and other factors that may influence outcome of test results.

I.4.1 Environment

The environment—temperature, humidity, illumination, sound—in an animal house can have a great influence on the quality of the animals. For animal houses in tropical and subtropical countries, air conditioning is recommended, since it promotes environmental stability. Changes in ambient temperature will affect the temperature regulation system of animals and thus disturb metabolism and behaviour, resulting in varying experimental results. Relatively high temperatures will increase the survival of environmental or commensal opportunistic pathogens, which may produce clinical or sub-clinical infection and interfere with tests. The optimum ambient temperature for mice is 20–26 °C.

High relative humidity favours the production of ammonia in animal cages, facilitating respiratory diseases by depressing the defence system of the respiratory tract. A low relative humidity can lead to increased incidence of diseases such as ringtail in rats and mice. Relative humidity should be maintained at 30–70%.

Effective ventilation is necessary to promote comfort and thus reduce stress. The air should be changed 10–15 times per hour, with no recirculation of air between rooms.

To achieve a regular diurnal lighting cycle all year round, illumination with 12 hours daylight or fluorescent light (light intensity 130-325 Lux) and 12 hours darkness is recommended. Cameron (*1*) investigated the effect of illumination on the pertussis mouse weight gain test. Experiments in which mice were administered injections of saline only showed that lighting as well as temperature had an effect on weight gain; both prolonged lighting and higher temperature resulted in an increase in weight gain. Cameron suggested the use of a reference vaccine in potency testing to overcome these effects.

I.4.2 Nutrition

Commercially prepared laboratory animal food is preferred to freshly prepared food, since the latter carries a greater risk of introducing microbiological contamination into the animal facilities.

Van Ramshorst (*2*) investigated the influence of food on the antibody titre of guinea-pigs after immunization with a combined vaccine against diphtheria, tetanus, pertussis and poliomyelitis. During the immunization period one group received food A, the other food B, both commercially available guinea-pig foods. Food A gave a slightly higher weight gain (Table I.4. 1). The data demonstrated clearly the enhancing or suppressive effect of food on the immune response of animals emphasizing the need to use a reference vaccine in potency testing.

Table I.4.1: Influence of diet on antibody titre and weight gain[a]

	Antibody titre	
	Food A	Food B
Tetanus[b]	0.02 IU/ml	0.56 IU/ml
Diphtheria[b]	0.055 IU/ml	0.44 IU/ml
Pertussis[c]	< 1/1	¼
Weight gain (g/week)	47	39

[a] From reference (2).
[b] Arithmetic mean value (n = 18).
[c] Median pertussis agglutination titre (n = 6).

I.4.3 Choice of animals

Tests performed to assure the safety and potency of diphtheria and tetanus vaccines should ideally be performed in guinea-pigs. However, these animals have become increasingly expensive and in some countries their supply and maintenance is problematic. There has been a trend towards substituting mice for these tests. While this presents problems of its own, this situation is often unavoidable. Where such tests are used, the national control authority should take the necessary steps to assure itself that the required validation has been performed.

For pertussis vaccine potency and safety tests, mice have to be used. The choice of strain and source of supply can have a critical effect on results. The user needs to ensure that the animals used are capable of producing valid results in tests with reference vaccine. In many laboratories the NIH strain is used for pertussis vaccine testing.

However, wide variations in sensitivity to pertussis toxin mice of NIH strain mice from different suppliers have been noted. Other, locally available strains e.g. ddy, may be found more suitable.

At least 1000 different strains of laboratory mice are recorded. In spite of their genetic diversity, most of these strains are albinos and at first sight appear to be uniform. Most of the strains used for quality control of vaccines are out-bred strains, and specific breeding methods are applied to ensure maximum genetic variation. This genetic diversity may be responsible for the variability observed in animal testing. For standardization and calibration of animals this may seem to be undesirable. Inbred strains will certainly lead to more uniform results. However, because of their limited genetic diversity they sometimes demonstrate higher sensitivity to environmental influences. Furthermore, extrapolation to humans is easier if test results are obtained with out-bred animals. On the other hand, bacterial vaccine potency control requirements are based on the lower 95% confidence limit of the estimated potency. The range of the 95% confidence interval depends to a large extent on the fluctuations in response from animal to animal. It is obvious that genetically identical animals will give a more uniform response and will facilitate comparison of data. The decision on whether to use an inbred or an outbred strain will be influenced by the nature of the test and the type of data that are sought.

Hendriksen *et al.* (*3*) investigated the effect of the use of inbred strains and F_1-hybrids (progeny of the mating of two different inbred strains, which are highly uniform) on the 95% confidence interval of estimated potency. A considerable improvement was achieved when inbred strains were used. The authors concluded that, although inbred strains may be more expensive and harder to breed than random-bred stocks, the use of inbred strains seems to be favourable for potency testing of vaccines containing diphtheria and tetanus toxoid.

In a study on the possible influence of mouse strain on the assayed potency of tetanus vaccines, Hardegree *et al.* (*4*) tested a batch of tetanus vaccine in parallel with the International Standard for Tetanus Toxoid in five different mouse strains. A significant difference in relative potency between the two vaccines was found for the individual strains. This is a recurrent problem and the difficulty of relating potency test data obtained in mice to results obtained by the traditional method in guinea-pigs has not been completely resolved.

I.4.4 Microbiological quality

Laboratory animals can suffer from a wide range of infections. There are many well documented examples showing interference (immunosuppression and immuno-enhancement) with experimental results. *Bordetella bronchiseptica* is very common in laboratory animal populations and can influence the results of pertussis potency tests, among others. In some cases infection can lead to sickness often followed by death, not only for the animals themselves but also for the animal handlers (Hantavirus, for example, can cause serious problems). Even more dangerous for animal populations as well as staff are latent infections that do not show any sign of illness e.g. lymphocytic choriomeningitis virus, Hantavirus.

Normally animals are free of pathogenic micro-organisms at birth. When they are housed under isolated conditions, introduction of pathogens can be prevented. However, for development of several biological functions, animals need the help of certain micro-organisms. Specific micro-organisms are therefore introduced. The result is an animal from which certain potentially harmful and pathogenic micro-organisms are known to be absent. This class of animals is called specific pathogen free (SPF); it is proven that these animals are free of specific pathogens. To maintain the SPF quality of an animal colony, certain hygienic and managerial measures should be taken to protect animals from contamination which may lead to unwanted infections.

Animal facilities should be suitably designed and personnel should receive appropriate training. An animal health monitoring system should be established to ensure the continuation of SPF status. It should include at least the clinical examination of the animals on a regular basis. Laboratory investigations of both sick and healthy animals are essential in the diagnosis of diseases. Post mortem examinations should be carried out on all diseased and dead animals in order to determine the responsible microorganism. Using bacteriological, virological, histopathological, mycoplasmatological and parasitological techniques, samples of healthy animals should be screened for the presence of micro-organisms. Such examinations should be carried out on a routine basis to prevent diseases from spreading.

Other managerial measures include:

- quarantine of incoming animals until they are screened;
- isolation of sick animals;
- separate breeding and experimentation facilities;
- restricted entrance of staff and visitors;
- prohibition of eating, drinking and smoking;
- an "all-in, all-out" system (finish experiment, clean and disinfect animal room and equipment, and start a new experiment);
- avoidance of overlap in experiments (by using multiple small animal rooms);
- separate routing of clean (entering) and dirty (waste) materials;
- employment of well trained staff;
- limited contact of personnel with other animals;
- monitoring of health of staff.

Hygienic measures include:

- decontamination of all materials (preferably chosen for easy cleaning and decontamination) entering the animal holding rooms;
- filtration of the air;
- sterilization of food (by gamma irradiation or possibly autoclave), water, bedding (autoclave) and cages;
- personal cleanliness among animal colony personnel (change of clothing, hand-washing and showering);
- making facilities proof against insects and wild rodents.

If adequate measures are taken, the SPF status of animals can be guaranteed. If no attempts are made to protect animals against pathogens misleading results could be obtained (*5*).

Whatever the disadvantages and problems associated with the use of animals as a "measuring device" in the quality control of vaccines, no test system is known which can replace animal models completely. The use of a reference can partly overcome the effect of external factors on test results. However, attention should be paid to the care and handling of laboratory animals to minimize effects of environment and nutrition and to maximize efficacy in their use, particularly in the quality control of bacterial vaccines. Animals should be bred and maintained in such a way that the maximum possible standardization and reproducibility are obtained.

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I.4.6 Selected further reading

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I.5. General laboratory procedures

I.5.1 The laboratory environment

Good supervision in the laboratory is essential in order to maintain appropriate operating procedures. These include a disciplined attitude, the use of correct techniques, the completion of approved protocols, and maintenance of a clean working environment.

The most important factor in guaranteeing quality is the avoidance of contamination in the operating system. Contamination may come from the original material, from careless manipulation of the materials, from the atmosphere or from the operator.

The list of what to do and what not to do is too long to include here in entirety; however, it is useful to record some of the most important instructions for avoiding contamination.

- **Avoid contamination from the atmosphere:** Do not permit the formation of air currents or turbulence in the working area. If working in laminar flow cabinets, avoid barriers that can interfere with the flow of air, such as too many objects inside the hood. Always work in an area that is protected, clean, decontaminated, and free from dust and draughts.
- **Avoid contamination by personnel:** Do not permit the entry of unnecessary personnel into the working area. When in the working area use sterile gloves and cover the mouth and nose with a sterile mask. Use manual pipetting devices for pipettes and never pipette by mouth.

I.6. Laboratory safety

The testing of diphtheria, tetanus and pertussis vaccines requires the use of pathogenic bacteria and highly potent toxins. These need to be handled under conditions of containment adequate to protect laboratory staff and the environment. *Bordetella pertussis, Clostridium tetani* and *Corynebacterium diphtheriae* are Risk Level 2 pathogens and require to be handled in facilities that provide containment at Biosafety Level 2 as a minimum. This implies certain requirements for the laboratory facilities. Basic guidance on this is given in the WHO Laboratory Biosafety Manual, Third Edition (2004).

While adequate facilities are essential, it should be emphasized that good laboratory technique is the best guarantee of laboratory safety. Moreover, staff who are adequately trained concerning the nature of their work and the possible hazards associated with it are better able to avoid unsafe practices. Laboratories should develop their own codes of safe laboratory practice, which must be uniformly enforced and regularly reviewed. Subjects to be included in such a code are immunization of laboratory workers, personal hygiene, proper attire and practices for laboratory workers, restriction of access, correct use of equipment to minimize hazards, decontamination and disposal procedures, and training. Good supervision is essential to laboratory safety.

I.6.1 Protection for staff

I.6.1.1 Physical barriers

Laboratory staff engaged in technical procedures should wear laboratory coats and/or gowns which are not worn outside the laboratory. Since larger particles and droplets released during microbiological manipulation settle rapidly on to bench surfaces and the hands of the operator, gloves should be worn, hands should be washed frequently, and working surfaces should be decontaminated after use. It is strongly advised that all procedures that may generate infectious or toxic aerosols should be performed in a bio-safety cabinet.

Laboratory techniques such as pipetting, centrifugation, pouring, and mixing can produce aerosols, which may put the operator and colleagues at risk. To avoid aerosols from centrifugation procedures, the centrifuge should always be operated according to the manufacturer's instructions. Tubes should be thick-walled and free of defects. They should not be overfilled, and should be sealed for centrifugation of infectious materials. Tubes should be filled and opened in a biological safety cabinet, as should all containers that have been subjected to shaking and homogenization. Where possible, plastic e.g. polycarbonate, centrifuge tubes and bottles with integral screw caps should be used in place of glass containers.

The biological safety cabinet is the best physical barrier to laboratory hazards if used properly. However, the equipment must be adequately maintained with regular replacement of filters and checks on airflows. It should be noted that laminar flow type cabinets are designed to protect the work rather than the operator and that flow-through or enclosed cabinets are more suitable for the latter purpose.

I.6.1.2 Restriction of access

Access to the laboratory must be denied to unauthorized personnel. Waste handlers and cleaners must be protected by proper disposal of infectious or toxic wastes, by autoclaving or disinfecting as indicated.

Oral infection can result from the use of such hazardous practices as mouth pipetting, failure to wash hands properly, and also from eating in work areas. Mouth pipetting should be prohibited and only manually operated pipettes used. Separate areas for eating, drinking, and applying cosmetics should be maintained, with access limited to personnel who have been appropriately decontaminated. Food and drink should not be stored or consumed in the laboratory, and smoking, gum chewing, or application of cosmetics should not be permitted.

I.6.1.3 Training and supervision

To avoid danger to personnel, strict attention to detail must be maintained, and laboratory procedures must be continually reviewed and upgraded and any changes recorded. The reasons for the rules and regulations of proper laboratory procedure should be explained to staff, who are then more willing to follow them scrupulously. Continuing education is important in protecting staff from laboratory hazards. It is especially important that lower grade staff who perform the more menial cleaning and disposal tasks are not neglected in this respect. They may be at high risk from the materials that they handle.

I.6.1.4 Immunization

All laboratory staff should be immunized against diphtheria, tetanus and pertussis. If childhood immunization has been performed, regular boosters with adult formulation diphtheria-tetanus –acellular pertussis vaccine should be given. If there is no record of primary immunization a full course should be given. Whole cell pertussis vaccine should not be given to adults.

I.6.2 Protection of the environment

I.6.2.1 Proper disposal

Improper disposal of infectious waste materials or toxic chemicals endangers the environment. Such materials must therefore be autoclaved or disinfected in the case of biological hazards, and detoxified in the case of toxic chemicals. Bio-safety cabinets vented to the outside must have HEPA filters that will prevent the release of hazardous agents into the air. They must be decontamination by formaldehyde or glutaraldehyde fumigation before cleaning or maintenance. Radioactive wastes must be properly disposed.

I.6.2.2 Containment

Laboratories that will be handling hazardous infectious agents must maintain a negative pressure relative to the outside environment in order to avoid unintentional release of infectious materials. In those laboratories rated P3, or handling biohazards rated higher than class 2, exit of infectious wastes and of personnel must be carefully controlled. In addition, air and water effluents should be decontaminated or filtered prior to release into the environment.

I.6.2.3 Laboratory clothing

Protective clothing and shoes worn by personnel within the laboratory should be reserved for use in that area only. Laboratory coats should be removed when workers go to a clean area for eating, drinking or office work, and should be decontaminated prior to laundering. Shoes reserved for laboratory use are a good practice. Hand washing should be scrupulously practiced on exiting the laboratory.

I.6.2.4 Protection of equipment and materials

Although some operators may feel that attention to detail to assure laboratory safety interferes with good working procedures, this is not the case. On the contrary, strict attention to good laboratory practice, which include safety practices, will ensure a higher quality of work. For example, proper disposal of wastes will reduce the possibility of contamination of equipment and materials by microorganisms or chemicals. Well thought-out standard operating procedures will minimize confusion and the possibility of inadvertently mixing up or cross-contaminating samples. Physical barriers, such as gloves and face masks, will reduce the likelihood of mycoplasma contamination of cell cultures from the operator.

Further information is available in the reference list.

I.6.3 Selected further reading

Laboratory Biosafety Manual, third edition, Geneva, World Health Organization, 2004. ISO Series 14000, Management of Environmental Quality Applied to Testing Laboratories, Geneva.

ISO 14001 Environmental Management System Manual, 2004. International Standards Organization, Geneva.

Contents

Chapter II.
Testing for Diphtheria Vaccines

II.1 Potency

II.1.1 General introduction

The purpose of the potency test is to assess in a suitable animal model the capacity of the product being tested to induce a protective response analogous to that shown to be efficacious in humans.

Diphtheria vaccines are among the most frequently used vaccines worldwide and have been remarkably successful products. Their use has resulted in a significant decrease in the incidence of these diseases in both the industrialized world and in developing countries. Nevertheless, some difficulties exist in the global harmonization of potency testing procedures, even when International Standards are used, and different approaches have been taken by different countries. Some follow WHO recommendations and *European Pharmacopoeia* procedures, whereas others follow the National Institutes of Health (NIH) procedures used in the USA, with or without modifications.

The approach taken by WHO [1] like that of *European Pharmacopoeia* [2], is based on the determination of the immunizing potency of each final bulk vaccine by comparison with an appropriate reference material calibrated in IU against the International Standard for Diphtheria Toxoid (adsorbed). The assigned activity of the current International Standard for Diphtheria Toxoid Adsorbed is based on calibration in guinea pig challenge assays, a gold standard assay method. Calibration of any secondary or product specific or working references should also be calibrated against the current WHO IS using the guinea pig challenge assay [3].

There has been much activity in recent years aimed at simplifying the current tests, by using serology, by reducing the number of animals used, refining the end-point used and by considering the possibility of using the same animals to test the potency of several antigens in serology assays. However, the use of methods other than the guinea pig challenge assay (the gold standard assay) should not be assumed to be transferable, without validation for a particular product [4].

The revised WHO Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (4) recommended that the potency of diphtheria vaccine may be determined using guinea pigs or mice. Where potency tests are carried out in mice instead of guinea pigs transferability should be demonstrated for the product under test. After immunization, guinea pigs may be challenged either by the subcutaneous or the intradermal route, or bled to obtain sera for measurement of the antitoxin or antibody response. When guinea pigs are bled, the antibody levels of the individual

animals may be titrated by means of toxin neutralization tests, such as the Vero cell assay, and information used for calculation of potency. The ELISA assay or another suitable in vitro method may also be used for titration of serum and potency calculation provided that these assays have been validated against the challenge assay or the toxin neutralization test using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be required for validation.

If mice are used for the potency assay, they should be bled and antibody levels of the individual animals titrated by means of toxin neutralization tests such as Vero cell assay. Because mice are not sensitive to diphtheria toxin, challenge with diphtheria toxin is not possible. When potency tests are carried out in mice instead of guinea pigs, transferability should be demonstrated to establish equivalent information with regards to expression of potency in International Units for a particular product. (Chapter V Statistical Analysis of Results provides further details on transferability of International Units from the guinea pig model to mice).

The potency of a diphtheria vaccine used for the primary immunization of children should significantly exceed 30 IU/SHD. For three-dilution assays, the potency is calculated based on demonstration that the lower 95% confidence limit of the estimated potency is not less than 30 IU/SHD. The results of all statistically valid tests should be combined in a weighted geometric mean estimate and the confidence limits calculated. These potencies may not apply to diphtheria vaccine for use in boosting of adolescents and adults.

Following licensing, and once consistency in production and quality control of the vaccine has been further confirmed on a continuous basis, determination of potency in routine lot release may, with the approval of the national regulatory authority (NRA), be based on the results of serological assays, or on a challenge assay, both involving a reduced number of animals and/or doses where appropriate.

To further confirm consistency on a continuous basis, the potency of about ten recent lots of vaccine should be tested using the full three-dilution assay. If potency expressed in International Units is relatively uniform and if the expectations of linearity and parallelism are consistently satisfied, then fewer doses may be used and the assumptions of linearity and parallelism need not be tested in each assay. When vaccine lots consistently give a lower limit of the 95% confidence intervals for the estimated potency well in excess of the minimum requirements per single human dose, one-dilution tests may offer advantages. An outline of the approach to be taken for single dilution assays is described in Chapter V. If one-dilution assays are not advantageous, a reduction in animal usage may, nevertheless, be achieved by use of two-dilution assays or another suitable design modification. (See further guidance in Statistical Analysis of Results, Chapter V).

Sections II.1.2, II.1.3 and II.1.4 of this chapter provide details of guinea pig challenge as well as guinea pig serology and mouse serology assays that have been used and confirmed suitable for potency testing of diphtheria vaccines.

The approach taken by the USA is based on the NIH assays [5] where the minimal acceptable potency is defined as the capacity of a test vaccine to induce a protective antibody response in guinea pigs that reaches or surpasses the threshold of 2 units of diphtheria antitoxin per ml. A suitable reference antitoxin, to which protective antitoxin "units/ml" have been assigned, is used to express antibody concentration in relative terms, as measured by an in vivo toxin neutralization assay in guinea pigs. This approach is therefore not dependent on a reference vaccine standard for expression of potency. The current International Standard for Diphtheria Antitoxin (DI), established in 1934, is made from hyperimmune horse serum and is suitable for use in toxin neutralization potency assays in vivo..

The NIH test procedure is not described in this manual, although an example of an in vivo toxin neutralization test (TNT) in guinea pigs is provided for information in section II.1.5 as an example of a method that could be used in support of validation studies.

References

1) Requirements for Diphtheria, Tetanus, pertussis and combined vaccines. WHO Expert Committee on Biological Standardization. Fortieth report, Geneva, World Health Organization, 1990 (WHO Technical Report Series, No. 800). Annex 2.

2) Assay of diphtheria vaccine (adsorbed), general chapter 2.7.6 version 01/2008:20706. European Pharmacopoeia 7th Edition. Strasbourg, France: Council of Europe; 2011.

3) WHO Manual for the Establishment of National and Other Secondary Standards for Vaccines. WHO/IVB/10.15; 2010.

4) Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2012 (WHO Technical Report Series) in press.

5) US Department of Health, education and Welfare Public health Service, National Institute of Health. Minimum requirements: Diphtheria toxoid. Forth Revision. Bethesda, MD, NIH, 1947.

II.1.2 Potency in guinea pigs by challenge

Introduction

The challenge potency test for diphtheria vaccine (adsorbed) is determined by comparing the dose of the vaccine to that of a reference preparation required to protect guinea pigs from either an erythrogenic toxic effect (toxin administered intradermally, i.d.) or a lethal toxic effect (toxin administered subcutaneously, s.c.) [1,2]. For this comparison, a reference preparation of diphtheria toxoid (adsorbed) calibrated in International Units (IU) and a suitable preparation of purified diphtheria toxin is required. The current International Standard for diphtheria toxoid with an assigned activity of 213 IU/ampoule (based on results obtained in guinea pig lethal and i.d. challenge assays, NIBSC code 07/216) was established in 2009 for determining the potency of vaccines containing diphtheria toxoid [3].

Materials

Critical reagents

- *Standard for Diphtheria vaccine (adsorbed):* The current established WHO International Standard (IS), with a defined activity in IU per ampoule. Other secondary reference preparations, including product-specific reference materials, may also be used provided they have been calibrated in terms of the WHO IS using challenge potency assays in guinea pigs.
- *Challenge toxin*: A purified preparation of diphtheria toxin of defined activity and stability should be used. A suitable challenge toxin will contain not less than 100 LD_{50} per millilitre (for lethal challenge method) and 25,000 to 50,000 minimal reacting doses for guinea pig skin in 1 Lf (for intradermal challenge method). An example of a toxin titration to confirm the minimal reacting dose is shown in Table 1 below. The challenge toxin should also have a purity of at least 1500 Lf per mg of protein (nondialysable) nitrogen. Where the challenge toxin preparation has been shown to be stable it is not necessary to verify the activity for every assay.

Table 1: Example of titration of challenge toxin to confirm the minimal reactive dose (MRD) or LD_{50}

Toxin Concentration Lf/dose	Guinea pig 1	Guinea pig 2	Guinea pig 3	Guinea pig 4	Guinea pig 5
0.00008	++	+	++	+	+
0.00004	+	+	+	+	+
0.00002	+	-	-	+	-
0.00001	-	-	-	-	-
0.000005	-	-	-	-	-

Each guinea pig is injected with 0.2 ml i.d. of toxin dilution and response (presence of erythema >5 mm) measured after 48 hrs. The MRD is defined as the lowest dose of toxin which, after 48 h, causes a specific erythema in more than 50% of the animals challenged with toxin. In this example, the MRD would be 0.00004 Lf. For systemic challenge, toxin activity is defined in LD_{50} (i.e. the smallest dose of toxin causing systemic effects of diphtheria toxin or death in 50% of the animals challenged with toxin).
++: >10 mm in diameter
+: 5-10 mm in diameter
-: less than 5 mm in diameter or no reaction

Animals

- *Animals*: Use guinea pigs from the same stock of the same sex or of equal numbers of males and females. For the intradermal challenge method, use a size suitable for the prescribed number of challenge sites (a start weight of 250 – 350 g is suitable for 6 challenge sites). Animals in the same size range can be used in the lethal challenge method. In both methods, the difference in body mass between the heaviest and lightest animal should not exceed 100 g. For both methods, use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed in Chapter V. If the challenge toxin to be used has not been shown to be stable include at least two, and up to five, guinea pigs as unvaccinated controls for the intradermal challenge method, and include four groups of 5 animals for the lethal challenge method.

Other reagents

- *Vaccine diluent:* A sterile sodium chloride solution (9 g/L) is suitable for preparation of reference and test vaccine dilutions.
- *Challenge toxin diluent:* Peptone buffer containing 7.5 g/L peptone, 1.45 g/L potassium dihydrogen orthophosphate (anhydrous), 7.6 g/L disodium hydrogen orthophosphate (dihydrate) and 14.8 g/L sodium chloride is suitable for use as a diluent for the diphtheria toxin. Other diluents may also be suitable.

Procedures

Intradermal Challenge Method (non-lethal)

A solution of sodium chloride (9 g/L), or other suitable diluent, is used to prepare dilutions of the reference and test vaccine. The dilution series should not be more than 2.5-fold. Allocate each dilution to a group of guinea pigs and inject each guinea pig with 1 ml subcutaneously. The dilution series used should be predetermined such that the intermediate dilution(s) induce a response that will result in an intradermal score of approximately 3 following intradermal challenge with diphtheria toxin (see below "Calculation of Results" for explanation of intradermal challenge scores).

After 28 days following immunization, animals are challenged intradermally with different dilutions of diphtheria toxin. Dilute the toxin with a suitable diluent to obtain a challenge toxin solution containing about 0.0512 Lf in 0.2 ml (0.256 Lf/ml). Using this toxin solution, prepare a series of 5 four-fold dilutions containing about 0.0128, 0.0032, 0.0008, 0.0002 and 0.00005 Lf in 0.2 ml. The toxin dilutions should be prepared immediately prior to use. Shave both flanks of each guinea pig and inject 0.2 ml of each toxin dilution intradermally into separate sites on each of the vaccinated guinea pigs in such a way as to minimise interference between adjacent sites. For 6 toxin dilutions, 3 intradermal injection sites per flank can be used.

Where the challenge toxin has not been shown to be stable inject the unvaccinated control animals with toxin dilutions containing 80, 40, 20, 10 and 5×10^{-6} Lf in 0.2 ml.

Lethal Challenge Method

A solution of sodium chloride (9 g/L), or other suitable diluent, is used to prepare dilutions of the reference and test vaccine. The dilution series should not be more than 2.5-fold. Allocate each dilution to a group of guinea pigs and inject each guinea pig with 1 ml subcutaneously. The dilutions used should be predetermined such that the intermediate dilution(s) induce a response that will result in protection of approximately 50% of the animals from a subcutaneous injection of diphtheria toxin prescribed for this method. After 28 days following immunization, animals are challenged subcutaneously with different dilutions of diphtheria toxin. Dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing approximately 100 LD_{50} per ml. Inject each animal subcutaneously with 1.0 ml of the challenge toxin solution (100 LD_{50}).

Only adequetly standardized toxin should be used for challenge.

The 4 groups of control animals should be challenged with different dilutions of diphtheria toxin to confirm the challenge dose. e.g. 100, 2, 1, and 0.5 LD_{50}.

Calculation of Results

Intradermal Challenge Method (non-lethal)

Following challenge with toxin, examine all injection sites after 48 h and record the incidence of specific diphtheria erythema. The results are positive if the weal of erythema is equal to or greater than 5 mm in diameter. Small localised red spots, often due to injection injury, should not be considered as positive. Sites free from reaction are recorded as negative and the number of negative sites is recorded as the intradermal challenge score for each animal. For example, an animal with positive erythema reactions at all six challenge sites (no protection) should be given a score of zero. An animal with three positive reactions and three negative reactions (moderate protection) should be given a score of three, and an animal with six negative reaction sites (full protection) should be given a score of six. Tabulate together the intradermal challenge scores for all animals receiving the same dilution of vaccine and apply a suitable transformation, such as $(score)^2$ or $\arcsin((score/6)^2)$, to obtain an estimate of the relative potency for each of the test preparations by parallel line quantitative analysis.

Lethal Challenge Method

Five days (Note: four days in some countries) following challenge with toxin, count the number of surviving guinea pigs. Calculate the potency of the test vaccine relative to the potency of the reference preparation on the basis of the proportion of animals surviving in each of the groups of vaccinated guinea pigs using appropriate statistical methods (see Chapter V).

An example of humane end points used for determining toxic effects of diphtheria toxin following subcutaneous challenge are as follows:

Stage 1: light pink skin colour at the injection site
Stage 2: dark purple/pink colour at the injection site and rough coat
Stage 3: black colour and tissue hardening at the injection site and rough coat

Table 2: Examples of additional parameters that could be monitored in guinea pigs during the lethal challenge assay method

Degree of severity	Coat and skin	Body condition	Behaviour	Belly skin
Normal	Normal	Normal	Bright/Alert	Normal
Mild	Ungroomed	Mild MA	Slow/Response	Light pink
Moderate	Piloerected	Moderate MA	Lethargic	Dark pink
Severe	Hair loss	Marked MA	Moribund	Black and pink
MA = muscle atrophy				

Validity of the Test

- for both intradermal and lethal challenge methods the test is valid if the statistical analysis shows no deviation from linearity or parallelism at the 5% significance level ($p<0.05$).

Additional validity criteria specific for each method are as follows:

Intradermal Challenge Method (non-lethal)

The test is not valid unless:

- for both the test and reference vaccine preparations, the mean score (before transformation) obtained at the lowest dose level is less than 3 and the mean score at the highest dose level is more than 3.
- if applicable, the toxin dilution that contains 40×10^{-6} Lf gives a positive erythema in at least 80 per cent of the control unvaccinated guinea pigs and the dilution containing 20×10^{-6} Lf gives a positive erythema in less than 80% of the guinea pigs.

Lethal Challenge Method

The test is not valid unless:

- for the vaccine to be examined and the reference preparation, the 50% protective dose lies between the largest and smallest doses of the preparations given to the guinea pigs.
- if applicable, the number of animals that die in the four groups of 5 injected with the challenge toxin solution and its dilutions indicates that the challenge dose was approximately 100 LD_{50}.

Retest

The test may be repeated, but when more than one test is performed the results of all statistically valid tests must be combined using the appropriate method described in Chapter V.

Single dilution test

When a one dilution assay is performed, the potency of the test vaccine should be demonstrated to be significantly greater than the minimum requirement per human dose for the product under test.

The single dilution assay requires previous demonstration of the following parameters:

- the potency of the test vaccine consistently and significantly exceeds minimum requirements.
- a significant regression has been demonstrated for the test vaccine over time
- parallelism between test and reference vaccine has been demonstrated over time.

Validation and suitability

For every new product verification of suitability of the method for the product must be confirmed. This is achieved by confirmation of positive regression to the dose response and defined maximum and minimum response for reference and test preparation so that estimates of suitable precision can be calculated. Furthermore, parallelism between the responses for the preparation and reference must be established.

Data monitoring should be performed for the reference vaccine by monitoring the ED_{50} or percent of animals responding at a minimum of one immunising dose. Acceptable limits for the dose response of the reference vaccine can be defined from historical data. Data monitoring should also be performed for the challenge toxin to confirm stability and confirm that the correct challenge dose of toxin is used.

References

1) Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2012 (WHO Technical Report Series) in press.

2) Assay of diphtheria vaccine (adsorbed), general chapter 2.7.6 version 01/2008:20706. Ph. Eur. 7th Edition. Strasbourg, France: Council of Europe; 2011.

3) Stickings P, Rigsby P, Coombes L, Malik K, Matejtschuk P, Sesardic D. Collaborative study for the calibration of a replacement international standard for diphtheria toxoid adsorbed. Biologicals 38 (2010): 529-538.

4) Sesardic D, Prior C, Daas A and Buchheit KH. Collaborative study for establishment of the European Pharmacopoeia BRP Batch 1 for diphtheria toxin. *Pharmeuropa Bio,* 2003-1: 7-21.

II.1.3 Potency in guinea pigs by serology

Introduction

To assess the potency of diphtheria vaccine by serology as an alternative procedure to the guinea pig challenge method (Section II.2), antibody responses to diphtheria toxoid induced in guinea pigs after 5 to 6 weeks are compared relative to the antibody response induced by the reference vaccine. For this comparison, a reference preparation of diphtheria toxoid calibrated in International Units (IU) and a suitable antibody detection method is required. The Vero cell toxin neutralisation test (TNT, Section II.1.3.2) and ELISA (Section II.1.3.3) are described in this chapter as suitable antibody detection methods [1-3].

II.1.3.1 Immunisation and bleeding of animals

Immunization

Use guinea pigs each weighing between 250-350 g, either of the same sex or with males and females equally distributed between the groups. Groups should contain a number of animals sufficient to obtain results that fulfil the requirements for a valid assay as described in separate method sections. Groups of up to 10 guinea pigs are used for each of the dilutions of reference and test vaccine. A further group of 2 non-immunised guinea pigs (non-injected or injected with diluent alone) may be used as negative controls. Alternatively, a control (negative) guinea pig serum may be used.

Reference and test vaccines are serially diluted (up to 4 dilutions) using an appropriate diluent (such as normal saline 0.9% NaCl). For most combined vaccines (for use in the primary immunisation of children), a series of 3-fold dilutions covering the range 1 in 5 to 1 in 45 has been found to be suitable for use with all methods detailed in sections II.1.3.2 and II.1.3.3 for testing both the diphtheria and tetanus components. However, the optimum dilution range will need to be confirmed by each laboratory and for each particular product.

- Divide the guinea pigs randomly into dose group cages.
- Leave the animals for up to a week to acclimatize.
- Under aseptic conditions, prepare dilutions of test and reference vaccines using a suitable diluent. Vaccine dilutions should be prepared immediately prior to use.
- Inject subcutaneously 1.0 ml of test vaccine dilution, reference vaccine dilution or diluent (where negative control animals are included). Possible sites for subcutaneous injection of guinea pigs include the nape or the abdomen.
- Bleed the animals under terminal anaesthesia after 5 or 6 weeks.

An example of a protocol to prepare dilutions of the test and reference vaccine:

The initial vaccine dilution is prepared from the pooled neat test vaccine or reference. Subsequent dilutions are made from the previous dilution in the series.

3 ml Vaccine	+	12 ml sterile diluent	DILUTION 1 (1 in 5)
5 ml Dilution 1	+	10 ml sterile diluent	DILUTION 2 (1 in 15)
5 ml Dilution 2	+	10 ml sterile diluent	DILUTION 3 (1 in 45)

Bleeding

After 5 to 6 weeks following immunization the guinea pigs are bled individually under general anaesthesia. Methods of bleeding guinea pigs by cardiac puncture and vena saphena are provided for information. If other methods are followed, which use different anaesthetics, care should be taken to ensure that anaesthetic residues in the serum do not affect the viability of Vero Cells, if titration of sera is to be done in cell culture.

The bleeding of guinea pigs should be performed in a clean and disinfected room to prevent contamination. Collect the blood in appropriately labelled glass tubes. Animals and blood samples should be clearly identified for example with a letter for the dilution group and a number for each of the animals within that group.

An example of bleeding guinea pigs by cardiac puncture:

- Anaesthetise an animal with a mixture of Ketamine/Xylazine/Atropine (Approximately 0.05 ml/100 g body weight, intramuscularly). The ratio of
  Ketamine : Xylazine : Atropine is 4 : 1.25 : 0.5 by intramuscular injection.
- When the animal is under deep anaesthesia lay it on its back and stretch the front legs in a cranial direction.
- Use a 10ml syringe with a 21G × 1.5" needle. The heart is reached by piercing the left ventricle through the chest wall at the sixth intercostal space, about one third of the ventral-dorsal distance. The puncture site can be confirmed manually, being the site at the chest with the strongest heart-beat. Attach the syringe and adjust the position of the needle up or down to achieve a good blood flow. Encourage blood flow by applying a gentle upward pressure on the syringe plunger.
- When a syringe if full empty the blood into the correct labelled glass container. Reattach the syringe to the needle to continue collecting blood. Once blood flow reduces the 10ml syringe may be replaced with a 5ml syringe.
- When enough blood is obtained, kill the guinea pig by dislocation of the neck.

An example of bleeding guinea pigs from the vena saphena

- Shave the thigh of the hind leg of the guinea pig 1 day before bleeding, paying particular attention to the hollow of the knee where the vena saphena is most easily observed.
- Approximately 15-20 minutes before bleeding inject Hypnorm® "Janssen" injection, (0.1 ml subcutaneously per 100 g body-weight) in the skin-fold in the axial region, using a 1 ml syringe fitted with a 23G × 1" needle.
- Hold the guinea pig to push the knee joint, to make the leg stretch out, and pinch or massage the musculature on the back of the thigh and around the knee, in order to let the vena saphena be filled with as much blood as possible.
- Grease the skin at the site of puncture with, for example, Dow Corning Valve Seal.
- Pierce the vein carefully with a 21G × 1 1/2 " needle.
- The blood then starts to drip and can be collected directly into centrifuge tubes. The leg must be held tight all the time in order to maintain stasis. Massage during the blood taking may be advantageous.
- A second vena saphena puncture of the hind leg thigh for blood sampling may be necessary. Alternatively, vena saphena puncture of the other hind leg thigh can be performed.
- When enough blood is obtained, kill the guinea pig by dislocation of the neck.

Separation of serum from blood

Serum from each individual animal is required. Serum should be handled under asceptic conditions for use in the Vero cell assay. Suitable aliquots can then be made and stored at -20°C until they are examined for antibody activity. Avoid frequent freezing and thawing of serum samples, ideally each sample should not be freeze-thawed more than once. Once thawed, samples may be stored at +4°C for a limited period of time (up to two days) but stability should be confirmed in-house.

The following procedure ensures a serum yield of approximately 40% of the total blood volume taken. Alternative in house procedures may be used.

An example of a protocol for separating serum from blood:

- Place the blood samples in closed containers (tubes containing gel with a clot activator in order to make a rapid separation of the blood cells may be used).
- Incubate for two hours at +37°C.
- Incubate for two hours at +4°C
- Centrifuge the tubes for 20 minutes at approximately 2000 rpm (800 g).
- Transfer the serum with a pipette to a new tube.
- Undertake a second centrifugation if blood cells are present.
- Store in suitable aliquots at -20°C

References

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II.1.3.2 Titration of immune sera by Vero cell assay

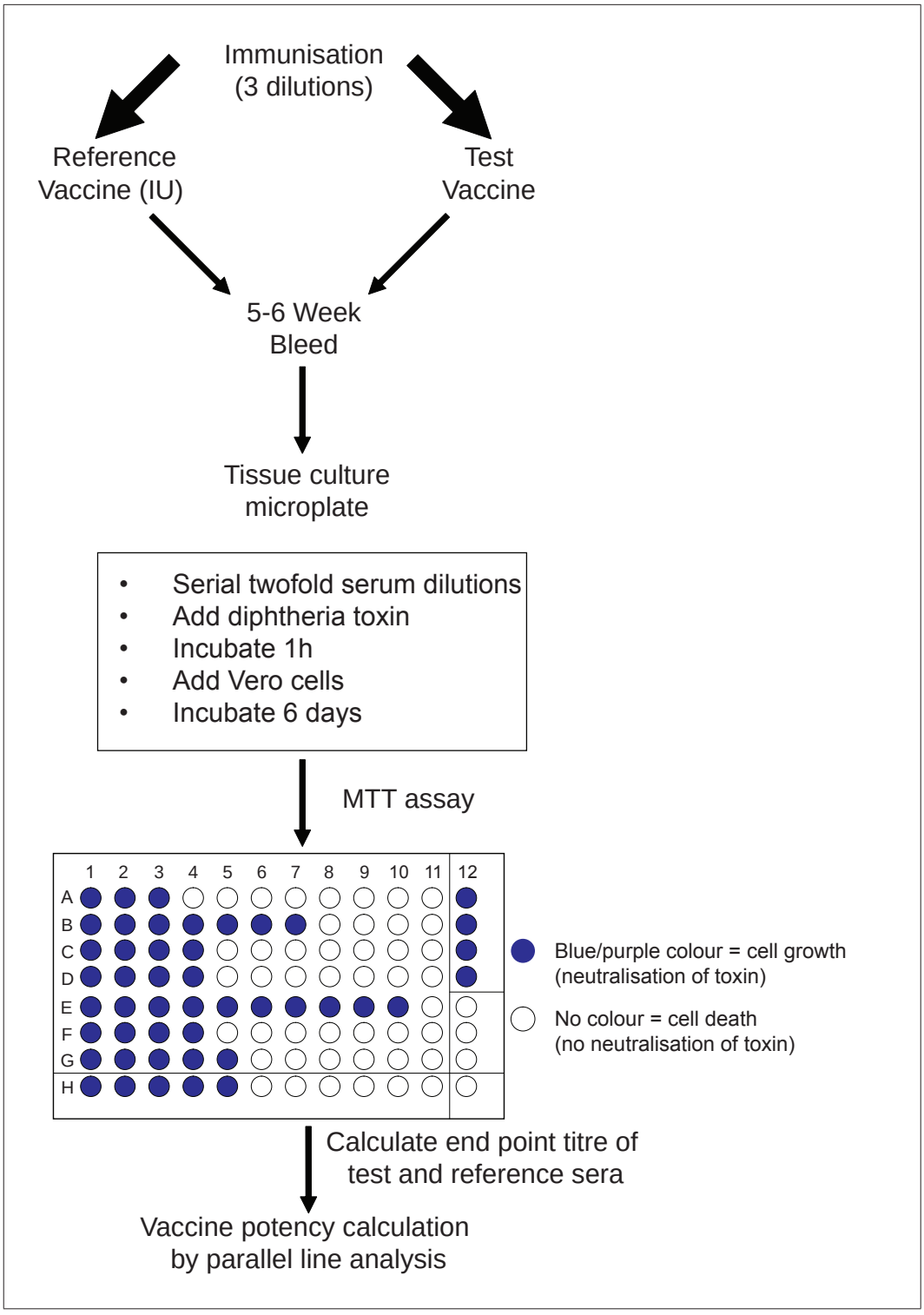
Introduction

WHO recommends that functional end-point assays such as serology using Vero cells can be used as an alternative procedure to the lethal and non-lethal (i.d.) challenge tests in guinea pigs for determination of diphtheria vaccine potency [1].

Vero cells have been identified as a suitable model for the specific detection of diphtheria toxin and have also been used as a toxin neutralisation test for determination of functional diphtheria antibodies [2-4]. Collaborative studies have compared potencies using serology with the Vero cell assay to those obtained in the guinea pig challenge assay [5, 6]. Sera of guinea pigs previously immunised with reference or test vaccine (section II.1.3.1) are diluted and incubated with diphtheria toxin. Non-neutralised toxin is detected by the addition of Vero cells which die in the presence of un-neutralised toxin in the incubation mixture. The more neutralising antibodies present in the serum the more dilutions will show positive cell growth with cells protected against the effects of diphtheria toxin. Information obtained in the Vero cell neutralisation test is used for calculation of vaccine potency in comparison to a suitable reference preparation calibrated in IU.

The Vero cell assay can be modified to increase sensitivity and accuracy of end-point determination by the inclusion of spectrophotometric analysis and incorporation of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to determine cytotoxic end points [7,8]. The method described here is based on the use of MTT dye to quantitatively measure metabolic activity of Vero cells and confirm cytopathic effects due to the presence of diphtheria toxin. Other aqueous dyes such as UptiBlue (Interchim) can also be used. Alternative methods are available for end-point determination such as those based on colour changes in the tissue culture medium due to metabolic inhibition [4]. End points determined by microscopic observation of individual wells may also be used. An overview of the assay procedure is shown in Figure 1. Note that modifications of certain assay parameters (such as incubation time of cells and total cell number) may be suitable and should be confirmed within individual laboratories.

Figure 1: Schematic diagram of the Vero cell assay for potency testing of diphtheria vaccine



Materials

Critical reagents

- *Control Diphtheria toxin*: A purified preparation of diphtheria toxin of defined activity and stability should be used in the Vero cell assay. The freeze-dried International Standard for Diphtheria Schick Test Toxin (STT) is suitable. Other toxin preparations of defined activity and stability can also be used.
- *Vero cells:* Vero cells are available on request from Coordinator, Quality, safety and Standards (QSS), World Health Organisation, Geneva, Switzerland. Cells may also be obtained from other sources provided that their sensitivity is verified.

Animals

See section II.1.3.1 for guinea pig immunisation and bleeding protocols.

Other reagents

- *Diphtheria antitoxin*: A positive control antitoxin can be used to confirm sensitivity of the assay and monitor variation between plates. Guinea pig serum (diphtheria and tetanus antitoxin, NIBSC code 98/572) or the WHO IS for Diphtheria antitoxin, DI, are suitable for use as the control antitoxin. Another suitable specific antitoxin may also be used.
- Flat bottomed 96 well tissue culture plates (commercially available)
- Multichannel pipettes 10 – 200 µl
- Micropipettes 50 – 200 µl
- Polyester pressure sensitive film (commercially available)
- Minimal Essential Media, MEM (commercially available)
- Foetal or newborn calf serum (commercially available)
- Antibiotic solution containing penicillin (10 000 U/ml) and streptomycin (10 mg/ml) (commercially available)
- 200 mM L-glutamine solution (commercially available)
- 10% D (+)-Glucose solution (commercially available)
- 1M HEPES buffer (commercially available)
- Hanks' balanced Salt solution (HBSS, commercially available)
- Tissue culture flasks, 75 cm^2 (or 150 cm^2)
- 0.25% trypsin/EDTA solution (commercially available)
- Trypan blue (0.4%) solution (commercially available)
- Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT (commercially available)
- Sodium lauryl sulphate, SDS (commercially available)
- *N*,N-dimethylformamide, DMF (commercially available)
- Haemocytometer (cell counting chamber) with Neubauer rulings

Preparation of Extraction Buffer for the Vero cell Assay

Prepare a solution of sodium lauryl sulphate (SDS, 10% w/v) in dimethylformamide (DMF, 50% v/v). Adjust pH to 4.7 and store at room temperature. Note that solvents such as dimethylsulfoxide (DMSO) are also suitable for extraction of formazan and solubilisation of cells in the MTT assay.

Preparation of Complete Medium for Vero cells

Prepare complete culture medium by supplementing minimal essential media (MEM) with calf serum (final concentration 5-10 % v/v), L-glutamine (2 mM), D-glucose (0.1% w/v), HEPES (0.015 M), penicillin (100 U/ml) and streptomycin (100 µg/ml). Other preparations of cell culture medium may also be suitable for use.

Culture and preparation of Vero cells

Established cultures of Vero cells can be maintained in 75 cm^2 tissue culture flasks in complete medium. Depending on the split ratio following passage and the percentage of serum in the medium, a confluent monolayer of cells is obtained after 4-6 days. The following procedure is suitable for routine passage of Vero cell cultures:

1) Remove the supernatant from a flask containing a confluent monolayer of Vero cells using a sterile pipette.
2) Add 1 ml of sterile HBSS (or PBS) solution to the flask rinse the cells and then remove using a sterile pipette.
3) Add 1 ml of sterile trypsin-EDTA solution to the flask and place in a 37°C incubator until the cells are detached from the flask (2-5 minutes).
4) Add approximately 5 ml of complete medium to the flask to neutralise the trypsin and resuspend the cell suspension using a sterile pipette to obtain a suspension of single cells for counting.
5) Prepare a 1/5 dilution of the cell suspension in 0.4% trypan blue solution and complete medium (e.g. 100 µl cells + 100 µl 0.4% trypan blue solution + 300 µl complete medium). Note that the final dilution of the cell suspension will depend on the total number of cells present. The cell suspension should be diluted such that the total number of cells counted exceeds 100 (minimum required for statistical significance).
6) Prepare the haemocytometer by placing the coverslip over the mirrored counting surface. It may be necessary to moisten the edges of the chamber (this can be done by breathing on the glass) such that Newton's rings (rainbow-like interference patterns) appear indicating that the coverslip is in the correct position to allow accurate cell counting (the depth of the counting chamber is 0.1 mm).
7) Using a pipette, introduce a small sample of the diluted cell suspension into the counting chamber such that the mirrored surface is just covered. The chamber fills by capillary action. Fill both sides of the chamber to allow for counting in duplicate.
8) The entire grid on a standard haemocytometer is comprised of nine large squares (bounded by 3 lines), each of which has a surface area of 1 mm^2. The total volume of each large square is 1×10^{-4} cm^3 (0.0001 ml).
9) Count the number of cells in one large square and calculate the cell concentration as follows: *cells/ml = total cell count in one large square x* 10^4.

10) For example, if 150 cells are counted in one large square (1mm^2), the concentration of the cell suspension = 150 × 10^4 cells/ml. If fewer than 100 cells are counted in 1 large square it may be necessary to count multiple large squares (for example the 4 corner squares plus the centre square) and divide the total cell count by the total number of large squares used for counting.

11) For the Vero cell assay, a cell suspension containing approximately 4 × 10^5 cells/ml in complete medium is required. Note that one 75 cm^2 tissue culture flask (~90% confluent) should contain enough cells to prepare 3 × 96 well tissue culture plates for the Vero cell assay).

12) To maintain the culture of Vero cells, seed approximately 1 × 10^6 cells into a new 75 cm^2 tissue culture flask (equivalent to a split ratio of approximately 1/10). This should provide a confluent monolayer after a further 4-6 days of culture.

Procedures

Part A - Determination of the test dose of diphtheria toxin

The toxin neutralisation assay described in this protocol is performed using a toxin dose of 4 x the minimum cytopathic dose (MCD). The MCD is defined as the lowest concentration of toxin (in Lf/ml) that is capable of causing cytotoxic effects on Vero cells after 6 days of culture. At this toxin dose level, it is possible to detect low levels of diphtheria antitoxin in test serum samples. The sensitivity of Vero cells to diphtheria toxin may vary when different batches of cells and/or serum are used. As a result, the MCD should be determined by each individual laboratory or whenever one of these variables is changed. The MCD is determined by titration of a stable, purified diphtheria toxin as follows:

1) Take a tissue culture microplate and mark the plate with the date and plate number.

2) Fill all the wells of columns 2–10 with 50 µl of complete medium using a multichannel micropipette.

3) Dilute the control diphtheria toxin in complete medium to give a starting concentration of approximately 0.001 Lf/ml.

4) Add 100 µl of the diluted diphtheria toxin solution to each well in column 1 using a micropipette.

5) Prepare serial two-fold dilutions in 50 µl volumes starting at column 1 through to column 10 using a multichannel micropipette. Discard 50 µl from column 10.

6) Add 50 µl of complete medium to all wells in columns 1-10 to bring the total volume to 100 µl.

7) Add 100 µl of complete medium to the "cell control" wells in column 11.

8) Add 150 µl of complete medium to the "blank control" wells in column 12.

9) Prepare a suspension of Vero cells in complete medium containing approximately 4 × 10^5 cells/ml.

10) Add 50 µl of the cell suspension to all wells of the microplate, except the "blank control" wells in column 12. The total volume in all wells is now 150 µl.

11) Shake the plates gently and cover with plate sealers to prevent the exchange of gas between medium and air. Note that the use of pressure film to seal plates is an important step for methods based on colour changes in the culture medium to determine assay end points.

12) Incubate for 6 days at 37°C in 5% CO_2 incubator.

13) Perform MTT assay as described below.

MTT Assay/Formazan Extraction

The yellow tetrazolium salt (MTT) is reduced in metabolically active, viable cells to form insoluble purple formazan crystals which are then solubilised buy the addition of detergent or solvent:

1) After 6 days of incubation at 37°C, remove the plate sealer and check the wells for microbial contamination.

2) Prepare a solution of MTT in PBS (5 mg/ml). Sterilise by passing through a 0.2 µM syringe filter. Add 10 µl of the sterile MTT solution to each well of the microplate using a multichannel micropipette.

3) Return the microplate to the 37°C incubator for 2–4 h to allow metabolism of the MTT by viable cells and formation of the blue formazan product.

4) Carefully remove the medium from all wells using a multichannel micropipette.

5) Add 100 µl of extraction buffer to all wells and return the microplate to the 37°C incubator and leave overnight to allow extraction and solubilisation of the intracellular formazan product. Once extraction and solubilisation is complete, the colour is extremely stable.

6) The plates are examined visually and the absorbance should be measured at 550-570 nm on a microplate reader.

The presence of dark blue colour indicates viable cells due to the ability of mitochondrial dehydrogenase in viable cells to reduce the MTT to the coloured formazan product. A light blue colour indicates partial toxicity while the absence of colour indicates complete toxicity and cell death.

Calculation of Minimum Cytopathic Dose of Diphtheria Toxin

Each tissue culture plate used in the Vero cell assay contains appropriate internal controls to allow for quantification of cell toxicity based on the absorbance measurement following MTT staining. The MCD can therefore be defined as the dilution and lowest toxin concentration at which the absorbance (and hence cell viability) is less than 50% of the geometric mean optical density obtained in the wells containing Vero cells alone ("cell control" wells). The MCD represents the sensitivity of the assay which can be confirmed in subsequent tests by titration of toxin. Note that if the Vero cells are killed at all dilutions of toxin, the assay to determine MCD should be repeated with a lower starting concentration of diphtheria toxin.

Part B - Titration of immune sera (Vero cell toxin neutralisation test)

For the toxin neutralisation assay, serial two-fold dilutions of test and reference vaccine serum obtained from individual animals are prepared in complete medium in a 96-well tissue culture microplate. After the addition of the test dose of diphtheria toxin (4 × MCD), the mixtures are incubated at room temperature for 1 h to allow toxin neutralisation to occur. Vero cells are then added and the plates incubated for 6 days. After 6 days of culture, the MTT assay is performed to determine assay end-points. As an internal control, a control antitoxin can be titrated on each plate and in order to avoid plate variation, test and reference sera samples should be randomised across the plates.

1) For every 7 test serum samples, take one tissue culture microplate and mark with the date and plate number. An example of the plate layout for this assay is shown in figure 2.

2) Fill all the wells of columns 2–11 with 50 µl of complete medium using a multichannel micropipette.

3) Fill the first four wells in column 12 (12A–12D) with 100 µl of complete medium using a multichannel micropipette ("cell control").

4) Fill the last four wells in column 12 (12E–12H) with 50 µl of complete medium using a multichannel micropipette ("toxin control").

5) Add 100 µl of each test serum sample into the appropriate well in column 1 (sera from all animals should be randomised across the plates).

6) Prepare a suitable dilution of the control antitoxin in complete medium. The control antitoxin should be diluted to give an end point at the midway point of the titration range – as a guide, for the NIBSC guinea pig serum 98/572, a starting dilution of 1/12.5 is suitable when a toxin dose of 4 × MCD is used. The dilution required for other antitoxins should be confirmed by titration against the control diphtheria toxin in house. Add 100 µl of the diluted control antitoxin to the appropriate well in column one in every plate.

7) Make a two-fold dilution series in 50 µl volumes starting at column 1 through to column 11 using a multichannel micropipette. Discard 50 µl from column 11 to equalise volumes.

8) Prepare a dilution of the toxin in complete medium with an antigen concentration of approximately 4 × the minimum cytopathic dose (4 × MCD).

9) Add 50 µl of the diluted diphtheria toxin solution to all wells in columns 1-11 using a multichannel micropipette. Add 50 µl of the diluted diphtheria toxin solution to the last four wells in column 12 (12E – 12H, "toxin control").

10) Mix antitoxin with toxin by gently shaking, and cover the plate with a lid.

11) Incubate at room temperature for one hour to allow toxin neutralisation to occur.

12) Meanwhile, prepare a Vero cell suspension in complete medium containing approximately 4×10^5 cells/ml.

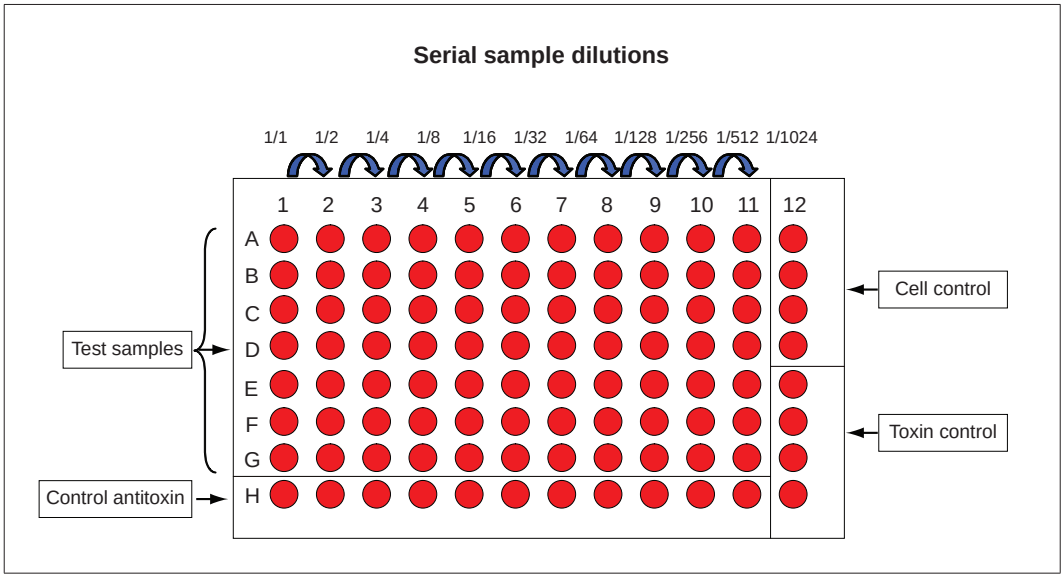
13) Add 50 µl of the cell suspension to all wells of the microplate. The total volume in all wells should now be 150 µl.

14) Shake the plates gently and cover with plate sealers to prevent the exchange of gas between medium and air. Note that the use of pressure film to seal plates is an important step for methods based on colour changes in the culture medium to determine assay end points.

15) Incubate for 6 days at 37°C in 5% CO_2 incubator.

16) Perform the MTT assay exactly as described in Part A.

Figure 2: Example arrangement of microtitre plate layout for titration of immune sera using the Vero cell assay



Calculation of antibody titres

The presence of dark blue colour indicates viable cells due to the ability of mitochondrial dehydrogenase in viable cells to reduce the MTT to the coloured formazan product. A light blue colour indicates partial toxicity while the absence of colour indicates complete toxicity and cell death.

The geometric mean optical density value (OD) of the wells containing Vero cells alone (cell control) should be calculated and divided by 2 to obtain the **50% control OD value** for each plate used in the assay. This value can be used to determine assay end-points. **The end-point of each test and reference serum sample is defined as the last well showing neutralisation of toxin which in turn can be defined as an OD value greater than the 50% control OD value.** The end-point is recorded as a score based on the dilution of the serum sample at the end-point (see figure 3).

Figure 3: Vero cell assay for potency testing of diphtheria vaccine: end point and calculation of results

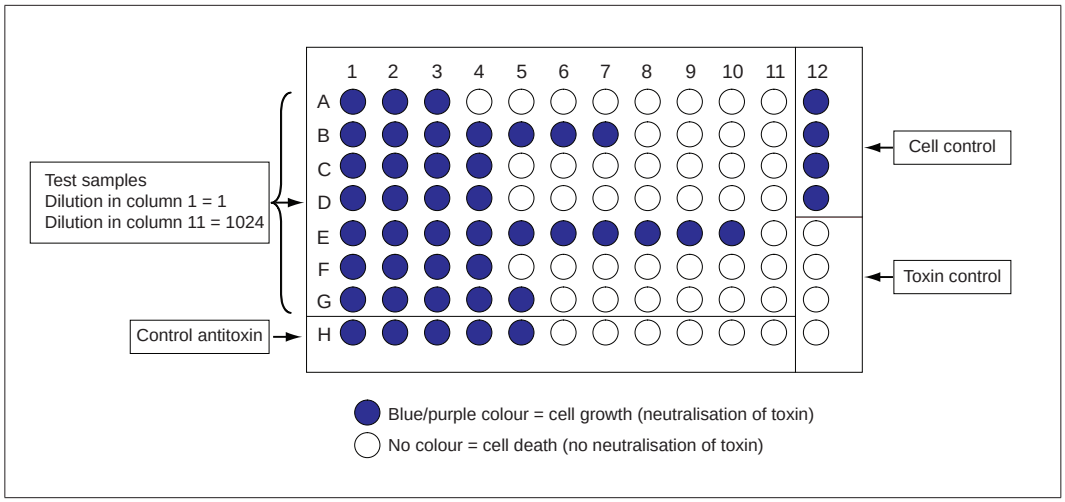


Figure 3 shows an example of the Vero cell assay following the MTT extraction. A sample with an end-point in the first well is assigned a score of 1. A sample with an end-point in well 6 is assigned a score of 32, and an end point in well 11 is assigned a score of 1024. Where the end-point is <1, a score of 0.5 should be applied and where the end-point >1024, a score of 2048 should be applied. Note that the presence of serum samples with "out of range" end-point scores may cause non-linearity and/or non-parallelism of the dose-response relationships and therefore affect validity of the test (see Chapter V), and should be avoided where possible.

Where a control antitoxin has been included, the end-point scores for individual test and reference serum samples should then be converted to relative titres by comparison with the end point of the control antitoxin on each plate. The end-point for the control antitoxin is determined as described for test serum samples (last well where the OD value is >50% control OD) and can be expressed as a titre based on the total dilution of the control serum at end point (e.g. if the control antitoxin is diluted 1/12.5 prior to titration and the end point in the assay occurs at dilution number 5 as shown in figure 3 then the titre for the control antitoxin is 12.5 × 16 = 200). The end-point titre in for individual serum samples is then calculated as follows:

Serum end-point score × control antitoxin end point titre = serum titre

Note that any pre-dilution of the serum samples prior to titration in the assay must be taken into account to obtain the final end-point titre.

Calculation of vaccine potency

The end-point titres for all serum samples titrated in the assay are used to calculate potency of the test vaccine relative to a reference preparation. Dose-response curves for reference and test vaccines are obtained by plotting the transformed (e.g. log or sqrt) antibody titres for individual serum samples against the log vaccine doses injected into the guinea pigs. Potency of the test vaccine is calculated in International Units relative to the reference vaccine preparation by comparing both dose-response curves by parallel line analysis using a suitable computer programme (see Chapter V).

Validity of the Test

Calculation of relative antibody titres (Vero cell end points)

- The test is invalid if no toxicity is observed in the wells containing Vero cells and diphtheria toxin ("toxin control").
- The test is invalid if the wells containing Vero cells alone (in complete medium, "cell control") do not show positive cell growth.
- The test is invalid if no end-point for the control antitoxin is observed.

Calculation of vaccine potency (parallel line analysis)

- For test and reference vaccine serum samples titrated in the Vero cell assay, the geometric mean score obtained at the lowest dose of vaccine should be less than 32 and the geometric mean score at the highest dose of vaccine should be more than 32, where score of 32 is defined as 50% of the dose response. (If relative antitoxin values are used, equivalent antitoxin IU/ml threshold can be calculated to confirm suitable dose response for potency calculation).
- The assay should meet any validity criteria for parallel-line analysis regarding linearity and parallelism of the dose-response relationships as detailed in Chapter V.
- The precision of the assay is determined by the 95% confidence limits. The confidence limits (P=0.95) of the estimated potency are recommended to be within 50-200% of the estimated potency.

Recommendation and specifications for 3-dilution and single-dilution methods remain the same as for the challenge assay.

Retest

Calculation of relative antibody titres

If the Vero cell assay does not meet one or more of the validity criteria of the test described above, the assay may be repeated.

If multiple end-point scores of >1024 (highest dilution tested in the example shown above, Figure. II.2) are obtained in the test or reference serum samples it is recommended that the Vero cell assay be repeated with the serum samples in question diluted (if previously used neat) or diluted further at higher dilutions (if previously diluted) in complete medium prior to titration in the assay. Similarly, if multiple end-point scores of <1 are obtained it may be necessary to repeat the immunisation of guinea pigs using a higher dose of vaccine (see calculation of potency below) – unless serum samples had been diluted prior to titration in the assay in which case the titration can be repeated using serum samples used at lower dilutions or neat if necessary.

Calculation of vaccine potency

Where the criteria for mean score at the highest and lowest vaccine doses is not met (for either the test or reference vaccine samples) leading to non-linearity and/or non-parallelism of the dose-response relationships the immunisation of guinea pigs will need to be repeated with suitable changes made to the doses of vaccine injected.

When more than one test is performed the results of all *valid* tests must be combined in the final estimate of potency, using the statistical methods described in Chapter V. Final potency estimates should be based on the results of all statistically valid experiments. The potency must be determined using the results of all valid tests combined using the methods described in Chapter V.

Exclusion of individual outlier samples from the data set (vaccine dilution) may be considered in order to improve precision of the potency estimates.

Validation and suitability

This method can be used provided that it is validated for a particular product as detailed in Chapter V.

Suitability of the method for particular product is achieved by confirmation of positive regression of the dose-response and parallelism between the responses for the test and reference vaccine, such that valid estimates of suitable precision are obtained.

Product-specific or in-house reference vaccine calibrated in International Units may be the most suitable reference in routine testing (see validation in Chapter V) particularly if sera are also used for potency testing of tetanus components in combined vaccines [5,6].

Data monitoring should be performed for the reference vaccine by monitoring scores or relative titres at a minimum of one immunising dose, or by calculating the ED_{50}. Acceptance limits of the dose response can be defined from historical data.

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II.1.3.3 Titration of immune sera by ELISA

Introduction

WHO recommends that Enzyme Linked Immunosorbent Assay (ELISA) can be used with serology assays to determine the potency of diphtheria vaccine for routine lot release provided that this assay is validated against the challenge assay or the toxin neutralization test, using the particular product [1]. Collaborative studies have compared potencies obtained using serology with ELISA to those obtained using guinea pig challenge assay and found good correlation for several combined vaccines [2-4].

Titrations of the guinea pig sera are made on ELISA plates coated with purified diphtheria toxoid. A positive control guinea pig antiserum, calibrated in antitoxin IU/ml, and a negative guinea pig serum control are titrated alongside the serum samples on each plate to monitor the assay performance. Anti-diphtheria antibodies bound to the toxoid are visualised by addition of a suitable detecting antibody directed against guinea pig IgG, followed by a suitable substrate. Optical density is measured and the antibody titre for each individual serum sample is calculated relative to the positive guinea pig antiserum. The potency of the test vaccine is then determined by comparing the antibody response in guinea pigs immunised with the test vaccine to the antibody response in guinea pigs immunised with the reference preparation, calibrated in IU.

Animals

See chapter II 1.3.1 for guinea pig immunisation and bleeding protocols.

Materials

Critical reagents

- Purified diphtheria toxoid (NIBSC 02/176) or equivalent, for coating ELISA plates.
- Diphtheria guinea pig antiserum, (NIBSC 98/572) is suitable as a positive control serum for monitoring performance of the ELISA assay and calculating relative antibody titres where appropriate. Another equivalent preparation may be used.
- Negative control guinea pig serum from non-immunised guinea pigs or guinea. pigs injected with diluent is suitable. Guinea pig serum NIBSC code 98/686 is also suitable for use as a negative control serum. Another equivalent preparation may also be used.

Other reagents

- Peroxidase-conjugated rabbit or goat antibody, directed against guinea pig IgG.
- Carbonate coating buffer pH 9.6.
- Phosphate buffered saline (PBS) pH 7.4.
- Wash buffer: PBS + 0.05% Tween 20 (PBST).
- Blocking buffer: PBST + 5% dried skimmed milk.
- Sample buffer: PBST + 1% dried skimmed milk.

- Citric acid substrate buffer: 0.05M Citric acid, pH 4.
- Substrate: 2,2'–Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tablets, 10 mg.
- Substrate solution: Shortly before use dissolve 10 mg ABTS in 20 ml citric acid solution. Immediately before used add 5 µl hydrogen peroxide solution.

Equipment

- ELISA plates: 96 well, Polyvinyl chloride or polystyrene.
- Multichannel pipettes 100 µl.
- Pipettes, 10-1000 µl.
- Plate sealant.
- Microplate reader.

Procedures

Coating of immunoassay plate

Plates are coated with purified diphtheria toxoid as follows:

- Dilute the diphtheria toxoid in carbonate coating buffer, pH 9.6. (For diphtheria toxoid NIBSC code 02/176 a suitable dilution is 1/1800).
- Add 100 µl to all wells.
- Seal plates and incubate at 4°C overnight in a humid container.

Plate washing

Plates are washed at the end of each incubation period before the addition of the next reagent. This can be done using a plate washer or by hand. The following describes one method for washing plates by hand.

- Prepare the PBST washing buffer.
- Discard the contents of the plates by inversion.
- Wash the plate by immersion into a container of PBST.
- Discard the contents of the plates.
- Repeat the washing procedure a further two times.
- Blot the plates dry against absorbent paper.

Blocking

Blocking is performed after the coating step once the initial reagent has bound to ensure that further reagents are not allowed to bind other than by immune adherence. Non-specific adhesion is reduced by inclusion in the buffer of an irrelevant protein at high concentration, such as bovine serum albumin (BSA) or casein. Detergents (usually Tween 20 at 0.5 ml/L) are also used to inhibit the adherence of hydrophobic molecules.

- Prepare the blocking buffer.
- Add 150 µl to all wells.
- Incubate at 37°C for 1 hour in a humid container.
- Wash the plates as described above.

Antibody dilutions

One ELISA plate is sufficient for the testing of up to 9 test sera with the positive diphtheria guinea pig antiserum in duplicate and a negative serum sample (see figure 1 for suggested plate layout). Test sera include those from guinea pigs immunized with either the test vaccine or the reference preparation. Care should be taken to ensure samples are randomly placed on the plate and that the positions are varied from plate to plate to avoid bias.

The positive control guinea pig antiserum, test sera and negative control are diluted in sample buffer to a concentration which produces a suitable dose response curve. The negative serum control should be diluted by no more than the lowest dilution of test sera. For NIBSC antiserum 98/572 a suitable initial dilution is 1/1000 . A dilution of 1/200 is suggested as a starting point for the test sera, but will vary depending on the dilution of vaccine used for immunization of guineas pigs. A series of two-fold dilutions are performed starting at row A through to row H.

- Add 100 µl of sample buffer to all wells except row A.
- Add 200 µl of diluted positive control serum to row A in duplicate columns and 200 µl of diluted test sample or negative serum control to row A of a single column. Prepare a series of doubling dilutions in 100 µl volumes to row H. Discard 100 µl from the last wells.
- Incubate the plates for 2 hours at 37°C in a humid container.
- Wash the plates as described in previous section.

Figure 1: Suitable plate layout
(samples and reference are randomized across the plate)

	Neg	Test1	Test2	GP	Test3	Test4	Test5	Test6	Test7	GP	Test8	Test9
	1	2	3	4	5	6	7	8	9	10	11	12
A	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*
B	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
C	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	¼
D	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8
E	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.
F												
G												
H												

Neg = Negative serum control
Test = Test sera
GP = Diphtheria guinea pig antiserum (positive control)
* Pre-diluted serum sample added to row A of a column

Conjugate

To detect antibody bound to the diphtheria toxoid an anti-guinea pig peroxidase conjugate IgG is added to all the wells.

- Dilute the conjugate to a suitable concentration in sample buffer (an optimum dilution should be established in-house or will be recommended by the supplier).
- Add 100 µl to all wells.
- Incubate at 37°C for 1 hour in a humid container.
- Wash the plates as described above.

Substrate

- Prepare the substrate solution immediately before use (as previously described)
- Add 100 µl of substrate to all wells.
- Incubate the plates at room temperature for 15-20 minutes.
- Following colour development, read the plates at OD 405 nm using a microplate reader.

Calculation of relative antibody titre

Development of colour in the wells is theoretically directly proportional to the concentration of diphtheria antibody in the sample.

The relative amount of diphtheria antibody in the test sera with respect to the positive control diphtheria guinea pig antiserum preparation may be obtained by parallel line regression analysis. Since dilutions are made on a log scale the most appropriate linear response is log optical density (OD) versus log dilution. Only those OD values which fall within the range of the linear part of the curve should be used to calculate titres. Analysis of variance is used to test the significance of departure of the dose-log response relationship from linearity and parallelism, using a suitable computer programe.

The final relative antibody titre for each test sample is obtained after taking into consideration differences in the dilution factors of the positive control diphtheria antiserum and test samples as necessary as shown below.

$$\text{Final relative antibody titre} = \frac{\text{(Relative estimate test sample} \times \text{dilution factor of sample)}}{\text{Dilution factor of positive control antiserum}}$$

Alternatively, individual antibody scores can be used, without expression relative to the positive control, serum. However, results expressed relative to the positive control serum may produce final potency estimates with better precision since any plate variation will be accounted for and inclusion of a positive control serum has been shown to help in assay standardization.

Calculation of vaccine potency

In order to use data to calculate potency of vaccine, dose-response curves for reference and test vaccines are obtained by plotting the transformed (e.g. log or sqrt) relative antibody titres (or scores) for individual serum samples (as calculated above) against the log vaccine doses injected into the guinea pigs. The potency of the test vaccine is calculated with respect to the reference vaccine preparation, calibrated in IU by comparing both dose-response curves by parallel line analysis using a suitable computer programme (see Chapter V).

Validity of test

Calculation of antibody titres

- The assay should meet the criteria set in the parallel line assay for linearity and parallelism of the dose-response relationship.
- Negative control sample must not show a positive response.
- The positive control diphtheria guinea pig antiserum must produce a dose response curve, overlapping with test sera, with the linear range covering at least 3 points.

Calculation of Vaccine Potency

- The analysis should show a significant common slope of the log dose-response lines, without significant deviations from parallelism or linearity at the 5% level ($p<0.05$).
- The precision of the assay is determined by the 95% confidence limits. The confidence limits ($P=0.95$) of the estimated potency are recommended to be within 50-200% of the estimated potency.

Retest

- Some test serum samples may need to be retested in the ELISA assay in order to obtain a valid result for the antibody titre (or score). This may require samples to be tested at a lower or higher dilution than in the original assay. In some cases, this may lead to statistical outliers which can be considered for exclusion from the data set. In some cases, non-responders (i.e. titre below the limit of detection) can also be considered for exclusion from the data set.
- If significant regression of dose response is not met, assay must be repeated with different immunising doses to guinea pigs.

The test may be repeated but when more than one test is performed the results of all valid tests must be combined in a weighted geometric mean for the final estimate of potency and confidence limits calculated.

Validation

This method can be used provided that it is validated for a particular product as detailed in Chapter V.

Suitability of the method for particular product is achieved by confirmation of positive regression of the dose-response and parallelism between the responses for the test and reference vaccine, such that valid estimates of suitable precision are obtained.

A product specific reference vaccine, calibrated in IU, may be the most suitable reference preparation for routine serological assays (see validation Chapter V) – particularly if the same animals are to be used for potency testing of tetanus components in combined vaccines.

For testing of both diphtheria and tetanus components in combined vaccines in the serology assay, the common immunization conditions must be shown to satisfy the requirements for validity described above for both components.

Data monitoring should be performed with the reference vaccine by monitoring the geometric mean titre at a minimum of one immunising dose or by calculating the ED_{50} of the dose response.

References

1) Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2012 (WHO Technical Report Series) in press.

2) Winsnes R, Sesardic D, Daas A and Behr-Gross M-E. (2004). Collaborative study for the validation of serological methods for potency testing of diphtheria toxoid vaccines. Phase I and II. *Pharmeuropa* BIO, 2003-2, 35-68.

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II.1.4 Potency in mouse by Vero cell assay

Principle

Because mice are not sensitive to diphtheria toxin, challenge with toxin is not possible. The mouse potency assay therefore involves the detection of functional antibodies against diphtheria toxoid induced in mice by observing neutralizing effect of sera on diphtheria toxin in a Vero cell culture model [2-4]. The potency of the test vaccine is calculated by the parallel line analysis comparing the scores obtained for test and reference vaccines at each dilution. A minimum of 3 serial dilutions of test and reference vaccine are injected in groups of mice. Experience confirmed that 8-12 animals per group are likely to be sufficient to enable a valid calculation of potency. Mice are bled after 4-5 weeks.

Immunisation

Immunisation doses will depend on type of vaccine tested but doses in the range of 13 to 2 IU/dose have shown to give reliable dose response [5]. Inject 0.5 ml of dilution subcutaneously into the right groin fold of the mouse. Starting with the highest dilution (lowest concentration), only one syringe is needed for a single vaccine. Use a new syringe for each vaccine.

Bleeding

After four to five weeks the animals are bled individually under narcosis from the retro-orbital plexus (right eye). Both ether and a mixture of halothane (1.8 %), nitrous oxide (N_2O) (9 L/min) and O_2 (3 L/min) may be used. When ether is used mice can be narcotized only one by one whilst halothane permits narcotization of e.g. a whole dose-group at the same time. In case other anaesthetics are used it should be checked whether anaesthetic residues in the serum do not affect the vitality of VERO-cells.

The bleeding procedure should be performed in a clean and disinfected workarea in order to prevent contamination. Blood is collected in glass tubes marked with an identification (e.g. numbered from 1 to 64).

An example of bleeding mice

1. Narcotise a mouse.
2. For right handlers, lay the anaesthetised mouse on its left side.
3. Secure the mouse in the left hand by holding the loose skin of the neck between thumb and index finger. Fix the skin from the neck back to the tail between the other fingers.
4. Strengthen the grip around the neck so that the right eye is bulging.
5. Take a small pair of tweezers and remove the right eye with a quick pull.
6. Hold the eye-socket above a small glass tube and collect all the blood.
7. Stimulate bleeding by brushing from the tail towards the neck with the thumb of the right hand.
8. When bleeding has stopped, euthanized the mouse by dislocation of the neck.

Alternatively bleeding of mice is obtained by cardiac puncture method after administration of suitable injectable anaesthetic given i.p. A 25G needle attached to a 1ml syringe is introduced through the skin and between the ribs until it intersects with the heart and blood is drawn up. With this method up to 1.0 ml of blood can be obtained per mouse. The mouse is then confirmed dead by performing a cervical dislocation.

Separation of serum from blood

To obtain the maximum amount of serum, the following procedure may be followed:

1. Put the blood samples in a closed container (such as eppendorf tube).
2. Incubate for two hours at 37°C (activation of the clotting system).
3. Incubate for two hours at 4°C (shrinking of the clot, maximum release of serum).
4. Centrifuge the tubes for 20 minutes at 2,000 rpm (800 *g*).
5. Carefully transfer the serum with a micropipette to a new tube.
6. A second centrifugation should be done if blood cells are still present in the serum.
7. Incubate the serum samples in a water-bath at 56°C for 30 minutes to inactivate complement.
8. Store the samples at -20°C until they are examined for antibody activity.

Alternative methods for separation can be used provided that sufficient volume of active serum is available for assay.

Performance of the assay is exactly the same as for guinea pig protocol described in section II.3.2. A positive control antitoxin may be used to monitor variation between plates and allow for expression of relative antibody titres. The same validity criteria will apply. In order to use data to calculate potency of vaccine, dose-response curves for reference and test vaccines are obtained by plotting the log scores for individual serum samples against the log vaccine doses injected into mice.

Potency of vaccine is calculated in International Units relative to the reference vaccine preparation by comparing both dose-response curves by parallel line analysis using a suitable computer programme (see Chapter V).

Validation

This method can be used provided that it is validated for a particular product as detailed in Chapter V.

Data monitoring should be performed for the reference vaccine by monitoring the geometric mean antibody titre at a minimum of one immunising dose or by calculating the ED_{50} of the dose response.

References

1) Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2012 (WHO Technical Report Series) in press.

2) Kreeftenberg JG et al., An investigation of a mouse model to estimate the potency of the diphtheria component in vaccines. Journal of Biological Standarationdization1985; 13: 229-223.

3) Kreeftenberg JG et al., A mouse model to investigate the potency of the diphtheria component in combined vaccines. Development of Biological Standardization, 1986: 64: 21-24.

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5) Iwaki M, Komiya T, Fukuda T, Arakawa Y, Takahashi M. Collaborative study. Standardization of Japanese reference diphtheria and tetanus (adsorbed Lot 2), for potency determination of diphtheria-tetanus-acellular pertussis combined vaccine. Japanese Journal of Infectious Diseases, 2003: 56: 183-183.

II.1.5 In vivo toxin neutralisation test

Introduction

The ability of diphtheria toxin to cause an erythematic reaction when injected intradermally into the skin of animals can also be used to determine the neutralising capacity of an antibody preparation. The *in vivo* toxin neutralisation test (TNT) can thus be performed on the depilated skin of guinea pigs using general principles described for potency testing of Diphtheria Antitoxin products [1]. Rabbit is also used for toxin neutralization test. Different dilutions of serum are mixed with fixed amounts of diphtheria toxin and injected intradermally into the depilated skin. The antitoxin protective concentration is estimated by ability to protect inflammatory erythema reaction of the fixed dose of toxin. The TNT test is able to confirm functional capacity of serum antibody and can thus be used to confirm relevance of *in vitro* serological methods, as part of the validation process [2].

TNT method can be performed at different sensitivity levels. With high toxin dose (Lr/100) the method is generally used for potency testing of antitoxin products which have potency in excess of 500 IU/ml [1]. The method described here is an example of a modified protocol at lower toxin dose (Lr/1000), designed to quantify protective capacity of antitoxin samples of low activity [2].

Many previous studies have focused on comparing in vivo TNT with in vitro Vero cell TNT methods [3,4]. However, these studies were primarily performed in support of validation of an alternative potency method for the NIH test, a method which is not described in this manual.

Materials

Critical reagents

- *Diphtheria antitoxin*: 1st WHO International Standard (IS) for Diphtheria antitoxin, equine (DI). The liquid standard preparation is distributed as a solution of the dried hyperimmune equine serum in 66% (v/v) glycerol in saline. Each ampoule contains 10 ml of diphtheria antitoxin solution with a defined activity of 10 IU/ml. A new liquid fill is prepared from the original freeze-dried formulation every two years at NIBSC. Another suitable material calibrated in IU by in vivo TNT may also be used.

Animals

- *Animals*: Use guinea pigs from the same stock of the same sex or of equal numbers of males and females. Use a size suitable for the prescribed number of challenge sites (a start weight of 350 – 450 g is suitable for 6 challenge sites). At least two and a maximum of three animals are used for the reference preparation and each antitoxin under test.

Other reagents

- *Diphtheria toxin*: A purified preparation of diphtheria toxin of defined activity and stability should be used. A suitable toxin will have a purity of at least 1500 Lf per mg of protein (nondialysable) nitrogen and should contain at least 200 minimal reacting doses in the Lr/100 dose. The minimal reacting dose and test dose of toxin is determined as described in section II.6 (*in vivo* specific toxicity).
- *Sterile saline solution*: A sterile saline solution containing 8.0 g/L sodium chloride, 0.4 g/L potassium chloride and 1.0 g/L glucose is suitable for use as a diluent in the *in vivo* toxin neutralisation test. Other diluent may also be suitable.

Procedures

The potency of diphtheria antitoxin is determined by comparing the dose necessary to protect guinea pigs against the effects of a fixed dose of diphtheria toxin, with the dose of the standard preparation of diphtheria antitoxin necessary to give the same protection. For this comparison, the standard preparation of antitoxin and a suitable preparation of toxin (for use as the challenge toxin) are required. The potency of the preparation being examined is then determined in relation to the standard preparation and expressed in IU/ml.

Part A Determination of test dose of diphtheria toxin (Lr/1000 dose)

The method described here is based on the procedure outlined in the Ph Eur monograph for Diphtheria Antitoxin [1] and is suitable for analysis of serum samples with low antibody titres. The dose of diphtheria toxin used in the assay is determined by titration against a reference diphtheria antitoxin. The Lr/1000 test dose refers to the smallest amount of toxin causing an erythematous lesion despite the presence of 0.001 IU reference antitoxin. To determine the [Lr/1000] test dose of diphtheria toxin, prepare a solution of the reference preparation of diphtheria antitoxin such that it contains 0.05 IU of antitoxin per ml. Prepare mixtures of the solution of the reference antitoxin and of the test toxin such that each contains 0.2 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient volume of a suitable diluent to bring the total volume to 2.0 ml (see example in Table 1). Add saline and toxin to tubes first followed by the fixed volume of antitoxin. Allow the mixtures to stand at room temperature, protected from light, for up to 15 min and inject to animals within the period of 15 to 60 min. Using two animals for each mixture, inject a dose of 0.2 ml intradermally into the shaven or depilated flanks of each animal (up to 6 doses can be injected into each animal – 3 per flank). Observe the animals for 48 h. **The test dose of toxin is the quantity in 0.2 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, a small but characteristic erythematous lesion at the site of injection**.

Table 1: Example: Preparation of toxin/antitoxin mixtures for the determination of the Lr/1000 test dose of diphtheria toxin. Based on the example erythema scores shown here the test dose (Lr/1000) of diphtheria toxin is 0.001 Lf.

	Volume (ml)					
	1	2	3	4	5	6
Reference Diphtheria Antitoxin (0.05 IU/ml)	0.2	0.2	0.2	0.2	0.2	0.2
Diphtheria Toxin (0.025 Lf ml)	0.05	0.1	0.2	0.4	0.6	0.8
Sterile saline	1.75	1.7	1.6	1.4	1.2	1.0
Total (ml)	2.0	2.0	2.0	2.0	2.0	2.0
Reference Diphtheria Antitoxin (IU/dose)	0.001	0.001	0.001	0.001	0.001	0.001
Toxin (Lf/dose)	0.000125	0.00025	0.0005	0.001	0.0015	0.002
Results (Example)						
Erythema Score	-	-	-	+	++	++

Part B Determination of potency of the antitoxin

To determine the potency of diphtheria antitoxin, prepare a solution of the reference preparation such that it contains 0.05 IU of antitoxin per ml. Prepare a solution of the test toxin such that it contains **10 test doses per ml**. Prepare mixtures of the solution of test toxin and the solution of the reference preparation such that each contains 1.0 ml of the toxin solution and one of a graded series of volumes of the reference antitoxin (0.05 IU/ml) centred on that volume (0.2 ml) containing 0.001 IU antitoxin. At this dilution there will be 0.001 IU of reference antitoxin in the 0.2 ml challenge dose. Using a suitable diluent, bring the total volume to 2.0 ml (see table 2 for an example of toxin/antitoxin dilutions). Add saline and antitoxin to tubes first followed by the fixed volume of diphtheria toxin. In addition, prepare mixtures of the solution of test toxin and of the test serum sample to be examined such that each contains 1.0 ml of the toxin solution, one of a graded series of volumes of the sample to be examined and a sufficient volume of diluent to bring the total volume to 2.0 ml. Add saline and antitoxin to tubes first followed by the fixed volume of diphtheria toxin. Allow the mixtures to stand at room temperature, protected from light, for up to 15 min and inject to animals within 15 to 60 min. Using two animals for each mixture, inject a dose of 0.2 ml intradermally into the shaven or depilated flanks of each animal (up to 6 doses can be injected into each animal – 3 per flank). Observe the animals for 48 h.

Table 2: Example of the preparation of toxin/antitoxin mixtures for the *in vivo* toxin neutralisation test. The dilutions are centred on the dilution (number 4) that contains 0.01 IU antitoxin (0.001 IU antitoxin/dose).

	Volume (ml)					
	1	2	3	4	5	6
Reference Diphtheria Antitoxin (0.05 IU/ml) (or test sample)	0.8	0.6	0.4	0.2	0.1	0.05
Diphtheria Toxin (10 × Lr/1000)	1.0	1.0	1.0	1.0	1.0	1.0
Sterile saline	0.2	0.4	0.6	0.8	0.9	0.95
Total (ml)	2.0	2.0	2.0	2.0	2.0	2.0
Reference Diphtheria Antitoxin (IU/ml)	0.02	0.015	0.01	0.005	0.0025	0.00125
Reference Diphtheria Antitoxin (IU/dose)	0.004	0.003	0.002	0.001	0.0005	0.00025
Toxin dose	Lr/1000	Lr/1000	Lr/1000	Lr/1000	Lr/1000	Lr/1000
Results (Example)						
Erythema score (reference preparation)	-	-	-	+	++	+++
Erythema score (test preparation)	-	+	++	++	+++	+++

Calculation of Results

The assay described here uses the test dose of toxin (Lr/1000) pre-determined by titration against the reference antitoxin. For the toxin neutralisation test, dilutions of the reference antitoxin are centred on that dilution containing in the 0.2 ml dose, the test dose of toxin and 0.001 IU antitoxin. **The end point of the assay is defined as the dilution containing the largest amount of antitoxin that fails to protect the guinea pigs from the erythematous effects of the toxin**. For the assay to be valid, the end point for the reference antitoxin should occur at the central dilution containing 0.001 IU antitoxin/dose. At this dilution there is 0.01 IU of antitoxin in the total 2.0 ml volume. By definition, the mixture (total volume 2.0 ml) that contains the largest volume of test sample that fails to protect the guinea pigs also contains 0.01 IU. The volume of test sample in this dilution is used to calculate the potency in International Units per millilitre as shown in the following example:

Volume of test sample (ml) in end point dilution = x
Potency of the test sample = **(1/*x*)** × **0.01 IU/ml**.

According to the example shown in table 2, if the end point of the test preparation occurs at dilution 2, the potency of the test preparation in IU/ml = **1/0.6** × **0.01** = **0.017 IU/ml**. Any initial dilution factors of the test sample must then be taken into account to obtain a final potency estimate expressed in IU/ml.

Validity of the Test

The toxin neutralisation test described here is performed using a series of dilutions of reference antitoxin that is centred on that dilution containing 0.001 IU/dose (Table 2). The test dose of diphtheria toxin is pre-determined by titration against the reference antitoxin fixed at 0.001 IU/dose (Table 1). Therefore, the centred dilution of the series contains (in the 0.2 ml injection dose) 0.001 IU antitoxin and the test dose of diphtheria toxin. For the test to be valid, all sites injected with 0.001 IU of the reference antitoxin/dose or less (dilutions 4, 5, 6 in table 2) should show positive erythema lesions and all sites injected with more than 0.001 IU of the reference antitoxin/dose (dilutions 1, 2, 3 in table 2) should be free from reaction.

Validation and suitability

TNT *in vivo* and *in vitro* TNT in Vero cells (section II.1.3.2) can show excellent correlation, but not necessarily equivalent value, which will be dependent on reference antitoxin and level of sensitivity at which the two assays are performed. For equivalent values in IU/ml both assays must be performed at same toxin/antitoxin ratios [2-4].

References

1) Diphtheria antitoxin, Monograph version 01/2008:0086. European Pharmacopoeia 7th Edition. Strasbourg, France: Council of Europe; 2011.

2) Sesardic D, Winsnes R, Rigsby P and Behr-Gross M-E. Collaborative Study for the Validation of Serological Methods for Potency Testing of Diphtheria Toxoid Vaccines Extended studies: Correlation of serology with in vivo toxin neutralisation. *Pharmeuropa Bio*, 2003-2, 69-75.

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II.2 Specific toxicity

II.2.1 In vivo test for absence of toxin and reversion to toxicity in guinea pigs

Introduction

The purpose of the specific toxicity test for diphtheria toxin is to confirm freedom from residual toxin and reversion to toxicity in final bulk vaccines and/or bulk purified toxoid. The *in vivo* assay remains the method of choice for routine testing or validation of production processes. The toxicity reversal test for diphtheria toxin is also suitable for the assessment of concentrated toxoid intermediate and is based on the measurement of specific toxicity following incubation of the test toxoid for a prolonged period of time at high temperature to ensure that no reversion of toxoid to toxin has occurred.

The WHO specifies the use of the specific toxicity test for the control of purified toxoid bulk and final bulk vaccine, whereas the toxicity reversal assay is only used for the control of purified toxoid bulk [1]. The *in vivo* tests for specific toxicity and toxicity reversal are usually performed in guinea pigs by subcutaneous injection. However, the induction of specific erythema following intradermal injections of at least 20 Lf of purified toxoid can also be used in rabbits and guinea pigs. [1].

The European Pharmacopoeia (Ph. Eur.) specifies the use of the *in vivo* specific toxicity test for validation during the production stage of the vaccine so that it may not be necessary to test the product at the final stage [2]. The Vero cell culture assay is established in the Ph. Eur. as the preferred method for specific toxicity and toxicity reversal testing for the control of bulk purified toxoid [2].

As an alternative to the animal model for toxicity testing of the purified toxoid bulk, the WHO also recommends the use of a cell culture test system, such as Vero cell assay, provided the sensitivity of the test is not less than the guinea pig model [1]. An example of the Vero cell assay method that could be considered for validation is described in Section II.2.2.

Materials

No standard reference material is required for the in vivo toxicity tests..

Animals

Adult, guinea pigs of either sex, each weighing approximately 250-350 g that have not been used for any other test are suitable. Groups of 5 guinea pigs are used per test sample. Ideally, the animals should be acclimatised for a week before starting the test.

Equipment

Class II safety cabinet.

Sterile syringes, 5.0 ml.

Sterile needles, 0.5 × 16 mm (25G × 5/8” are suitable).

Sterile glassware and plastic ware.

Volumes of less than 1 ml dispensing pipettes

Volumes of less than 1 ml dispensing pipettes

Buffers

The buffer solution used for dilution of the bulk purified toxoid should be comparable to that used in the final vaccine (except for the presence of adjuvant)

All reagents used in the preparation of solutions should be General Purpose Reagent Grade or equivalent.

Procedure

Test on bulk purified toxoid

Specific Toxicity Test

1) Groups of 5 guinea pigs are injected subcutaneously with 1.0 ml of the toxoid diluted to at least 500 Lf/ml in suitable diluent.
2) The animals are observed for 42 days for signs of ill health and diphtheria toxicity.

Reversion to toxicity

1) A solution of bulk purified toxoid, diluted in a suitable diluent to the same concentration as in the final vaccine (obtained from the manufacturer's documentation), is prepared.
2) The solution (at least 50 ml) is stored at +37°C for a period of 6 weeks (42 days). A duplicate aliquot may be stored at +4°C for the same period as a control.
3) Groups of 5 guinea pigs are injected subcutaneously with 10 single human doses (5 ml in 2 × 2.5 ml volume) with one of each of the test samples.
4) The animals are observed for 42 days for signs of ill health. It is advised to weigh the animals daily during the first few days and at regular intervals during thereafter, e.g. once a week.

Intradermal tests in guinea pigs or a cell culture based assay (vero cell assay, as described in section II 2.2) are preferred alternatives.

Test on final bulk vaccine for vaccines containing diphtheria component

Specific Toxicity Test

1) Groups of 5 guinea pigs are injected subcutaneously with five times the single human dose (SHD) stated on the vaccine label. The 2.5 ml volume of undiluted final bulk is injected at up to 3 different sites on the guinea pig (e.g. 2 × 1 ml and 1 × 0.5 ml).
2) The animals are observed for 42 days for signs of ill health and diphtheria toxicity.

Validity of test

The sample passes the test if none of the animals show symptoms of diphtheria toxaemia (progressive, symmetrical, ataxic paralysis; weakness of the trunk and all extremities) within 42 days. Animals that die shall be examined by autopsy for signs of diphtheria intoxication (red adrenal glands).

In the case of tests where adverse effects are noted, any clinical signs or deaths must be reported immediately, any reports from veterinarian should be included in the vaccine lot documentation.

Retest

If more than one animal dies from non-specific causes within the test period, the test must be repeated once. If more than one animal dies in the second test, the sample does not comply with the test.

References

1) Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2012 (WHO Technical Report Series) in press.

2) Diphtheria vaccine (adsorbed), Monograph version 01/2008:0443.European Pharmacopoeia 7th Edition. Strasbourg, France: Council of Europe; 2011.

II.2.2 Vero cell test for absence of toxin and reversion to toxicity

Introduction

Purified diphtheria toxin is detoxified with formaldehyde using a method that avoids destruction of the immunogenic potency of the toxoid and reversion of the toxoid to active toxin, particularly on exposure to heat. Each new lot of bulk purified toxoid must be tested to confirm absence of toxin and reversal to toxicity on exposure to heat. WHO, TRS 900 [1].

The purpose of the test is to detect residual toxin and/or reversion of the toxoid to toxin following prolonged storage at 37°C. The concentration of the toxoid tested should correspond to that of the final bulk vaccine. Therefore, the bulk purified toxoid concentrate is diluted prior to incubation at 37°C for six weeks. A preparation stored at 4°C serves as a control.

A Vero cell culture system may be used as an alternative to *in vivo* tests for specific toxicity and reversion to toxicity as long as sensitivity of the assay is shown to be comparable to the guinea pig test [1]. Vero cells have been identified as a suitable model for the specific detection of diphtheria toxin in toxoids and as an alternative to guinea pig test [2-6]. The manufacturing process currently used for preparation of toxoid vaccines does not necessarily produce proteins of high purity and bulk purified toxoid preparations may include components that can affect growth and viability of Vero cells. In view of the potential for interference or toxicity arising from chemicals routinely found in bulk purified toxoids, it is essential that any toxicity to Vero cells is shown to be specific and due to the presence of active diphtheria toxin. This is achieved with the use of a specific diphtheria antitoxin preparation. Dialysis may also need to be performed on the toxoid sample to remove components likely to cause non-specific toxicity in Vero cells.

The method described here is based on the use of MTT dye to quantitatively measure metabolic activity of Vero cells and confirm cytopathic effects due to the presence of diphtheria toxin. Other aqueous dyes such as UptiBlue (Interchim) can also be used. Alternative methods are available for end-point determination such as those based on colour changes in the tissue culture medium due to metabolic inhibitin or microscopic observation of individual wells to determine cytotoxic end points. Note that modifications of certain assay parameters (such as incubation time of cells and total cell number) may be suitable and should be confirmed within individual laboratories.

Materials

Critical Reagents

- *Reference Diphtheria toxin*. A purified preparation of diphtheria toxin of defined activity and stability should be used in the Vero cell assay. A suitable reference diphtheria toxin will contain not less than 100 LD_{50}/ml and 25 000 to 50 000 minimal reacting doses for guinea pig skin in 1 Lf and will have a purity of at least 1500 Lf per mg of protein (nondialysbale) nitrogen.

Other Reagents

- *Diphtheria Antitoxin*. A diphtheria antitoxin should be included in the Vero cell assay to confirm specificity of the Vero cell assay. The British Standard diphtheria antitoxin equine (NIBSC code 63/007), with a defined potency of 1300 IU/ampoule, is suitable for use in the Vero cell assay. Other antitoxin preparations with a potency equal to or greater than 1000 IU/ml may also be suitable. The antitoxin used must be shown to be non-toxic to Vero cells at the concentration used in the assay.
- *Control Diphtheria Toxoid*. A control diphtheria toxoid preparation is included in the assay as a diluent for the reference toxin and as a control for the test toxoid. A suitable reference toxoid should have an antigenic purity > 1500 Lf per mg protein nitrogen and a concentration > 2000 Lf/ml. In addition, the control toxoid must be shown to be non-toxic to Vero cells at 100 Lf/ml.
- *Vero cells.* Vero cells are available on request from Coordinator, Quality, Safety and Standards, World Health Organisation, Geneva, Switzerland. Vero cells obtained from other sources may also be suitable for use. The sensitivity of Vero cells to diphtheria toxin may be affected by different culture conditions and will also depend on the toxin used. As a result, it is important that each individual laboratory confirms the sensitivity of Vero cells to diphtheria toxin, and must be shown to be at least 200 times more sensitive than the lethal challenge method in guinea pigs and at least 5 times more sensitive than the intradermal challenge method. This ensures that the sensitivity of the Vero cell toxicity assay exceeds that of the *in vivo* methods. See procedures section for method to determine Vero cell assay sensitivity.
- Flat bottomed 96 well tissue culture plates
- Multichannel pipettes 50 – 200 µl
- Micropipettes 50 – 200 µ l - Polyester pressure sensitive film
- Minimal Essential Media, MEM - Foetal or newborn calf serum (commercially available)
- Antibiotic solution containing penicillin (10,000 U/ml) and streptomycin (10 mg/ml) (commercially available)
- 200 mM L-glutamine solution (commercially available)
- 10% D (+)-Glucose solution (commercially available)
- 1M HEPES buffer (commercially available)
- Hanks' Balanced Salt solution (HBSS)
- Diluent for test sample: the buffer used for dilution of the toxoid should be comparable to that used in the final vaccine (except for the presence of adjuvant and preservative)
- issue culture flasks, 75 cm^2 (or 150 cm^2)
- 0.25% trypsin/EDTA solution (commercially available)
- Trypan blue (0.4%) solution (commercially available)
- MTT (commercially available)
- Sodium lauryl sulphate, SDS (commercially available)

- *N*,N-dimethylformamide, DMF (commercially available)
- Haemocytometer (cell counting chamber) with Neubauer rulings
- Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT (commercially available).

Preparation of Extraction Buffer for the Vero Cell Assay

Prepare a solution of sodium lauryl sulphate (SDS, 10% w/v) in dimethylformamide (DMF, 50% v/v). Adjust pH to 4.7 and store at room temperature. Note that solvents such as dimethylsulfoxide (DMSO) are also suitable for extraction of formazan and solubilisation of cells in the MTT assay.

Preparation of Complete Medium for Vero Cells

Prepare complete culture medium by supplementing minimal essential media (MEM) with calf serum (final concentration 5-10 % v/v), L-glutamine (2 mM), D-glucose (0.1% w/v), HEPES (0.015 M), penicillin (100 U/ml) and streptomycin (100 µg/ml). Other preparations of cell culture medium may also be suitable for use.

Culture and preparation of Vero cells

Established cultures of Vero cells can be maintained in 75 cm^2 tissue culture flasks in complete medium. Depending on the split ratio following passage and the percentage of serum in the medium, a confluent monolayer of cells is obtained after 4-6 days. Refer to section II.1.3.2 for the procedure for maintaining Vero cells in culture.

Procedures

The Vero cell assay described here is the most sensitive assay reported to detect active diphtheria toxin. For toxins of high specific activity (>2000 Lf/mg protein N) this assay has the ability to detect approximately 3×10^{-6} Lf/ml diphtheria toxin. The method described here is based on the method described in the Ph. Eur. Monograph for Diphtheria vaccine (adsorbed) [3]. This method uses a purified diphtheria toxin established as the Ph. Eur. Diphtheria Toxin Biological Reference Preparation Batch 1 [7]. The assay monograph includes a validity criteria based on the concentration of this toxin (5×10^{-5} Lf/ml) at which cytotoxic effects must be observed in order for the assay to be considered valid. It should be noted that different toxin preparations may show different levels of sensitivity and suitable validity criteria should therefore be established in house where necessary.

The diphtheria toxin selected for the assay should be titrated by each individual laboratory in a preliminary Vero cell test in order to determine the sensitivity of the assay and the minimum cytopathic dose (MCD) of toxin. The reference toxin should then be included on each plate in the Vero cell assay to confirm sensitivity of the test. Note that the MCD should be determined using toxin diluted in a control diphtheria toxoid that is non-toxic to Vero cells at 100 Lf/ml. Dilute the control toxoid in a suitable buffer (PBS is suitable) to give a final concentration of 100 Lf/ml.

Part A - Vero Cell Assay to Determine MCD

To be performed for each new batch of diphtheria toxin or Vero cells to confirm sensitivity of the assay. The assay described here to determine the MCD uses all 8 rows of a 96-well plate to give an n=8. However, a minimum of n=4 is sufficient to determine the MCD for the test toxin. Note that this is a modified procedure of that described in section II.1.3.2 because of different controls and buffers that are used in the toxicity assay.

1) Take a tissue culture microplate and mark the plate with the date and plate number.
2) Fill all the wells of columns 2–10 with 50 µl of diluted control diphtheria toxoid (100 Lf/ml) using a multichannel micropipette.
3) Dilute the reference diphtheria toxin in diluted diphtheria toxoid to give a starting concentration of approximately 0.001 Lf/ml.
4) Add 100 µl of the diluted diphtheria toxin solution to each well in column 1 using a micropipette.
5) Prepare serial two-fold dilutions in 50 µl volumes starting at column 1 through to column 10 using a multichannel micropipette. Discard 50 µl from column 10.
6) Add 50 µl of complete medium to all wells in columns 1-10 to bring the total volume to 100 µl.
7) Add 50 µl of diluted diphtheria toxoid (100 Lf/ml) and 50 µl of complete medium to the "cell control" wells in column 11.
8) Add 50 µl of diluted diphtheria toxoid (100 Lf/ml) and100 µl of complete medium to the "blank control" wells in column 12.
9) Prepare a suspension of Vero cells in complete medium containing approximately 4×10^5 cells/ml as described previously (section II.1.3.2).
10) Add 50 µl of the cell suspension to all wells of the microplate, except the "blank control" wells in column 12. The total volume in all wells is now 150 µl.
11) Shake the plates gently and cover with plate sealers to prevent the exchange of gas between medium and air. Note that the use of pressure film to seal plates is an important step for methods based on colour changes in the culture medium to determine assay end points.
12) Incubate for 5-6 days at 37°C in 5% CO_2 incubator.
13) Perform MTT assay as described in section II.1.3.2 or use another suitable detection methods.

MTT Assay / Formazan Extraction

The yellow tetrazolium salt (MTT) is reduced in metabolically active, viable cells to form insoluble purple formazan crystals which are then solubilised buy the addition of detergent or solvent:

1) After 5-6 days of incubation at 37°C, remove the plate sealer and check the wells for microbial contamination.

2) Prepare a solution of MTT in PBS (5 mg/ml). Add 10 µl of this MTT solution to each well of the microplate using a multichannel micropipette.

3) Return the microplate to the 37°C incubator for 2–4 h to allow metabolism of the MTT by viable cells and formation of the blue formazan product.

4) Carefully remove the medium from all wells using a multichannel micropipette.

5) Add 100 µl of extraction buffer to all wells and return the microplate to the 37°C incubator and leave overnight to allow extraction and solubilisation of the formazan product. Once extraction and solubilisation is complete, the colour is extremely stable.

6) The plates are examined visually and the absorbance should be measured at 550-570 nm on a microplate reader.

The presence of dark blue colour indicates viable cells due to the ability of mitochondrial dehydrogenase in viable cells to reduce the MTT to the coloured formazan product. A light blue colour indicates partial toxicity while the absence of colour indicates complete toxicity and cell death.

Minimum Cytopathic Dose of Diphtheria Toxin

The MCD is defined as the lowest concentration of diphtheria toxin (in Lf/ml) that causes cytotoxic effects on Vero cells after 6 days of culture. Each tissue culture plate used in the Vero cell assay contains appropriate internal controls to allow for quantification of cell toxicity based on the absorbance measurement following MTT staining. The MCD can therefore be defined as the dilution and lowest toxin concentration at which the absorbance (and hence cell viability) is less than 50% of the geometric mean optical density obtained in the wells containing Vero cells alone and the control toxoid ("cell control" wells). The MCD represents the sensitivity of the assay which can be confirmed in subsequent tests by titration of toxin. Note that if the Vero cells are killed at all dilutions of toxin, the assay to determine MCD should be repeated with a lower starting concentration of diphtheria toxin.

Part B - Vero Cell Assay for Specific Toxicity and Reversion to Toxicity

Preparation of Test Diphtheria Toxoid

Prepare a solution of the bulk purified toxoid at 100 Lf/ml using a suitable diluent. The buffer used for dilution of the toxoid should be comparable to that used in the final vaccine (except for the presence of adjuvant and preservative). Divide the solution into two equal parts. Maintain one part at 5°C ± 3°C and the other at 37°C for 6 weeks. On the day of the Vero cell assay, prepare a fresh solution of the test diphtheria toxoid at 100 Lf/ml using the same buffer solution that was used for the incubated samples. Note that, depending on the nature of excipients, some toxoids may need to be dialysed against the same buffer which is used as diluent for diphtheria toxoid prior to use in the Vero cell assay to remove components likely to cause non-specific toxicity.

Preparation of Control Diphtheria Toxoid

Using the same buffer solution as for the test toxoid, prepare a solution of the control diphtheria toxoid at 100 Lf/ml.

Preparation of Reference Diphtheria Toxin

Dilute the toxin in the control diphtheria toxoid (containing 100 Lf/ml). The concentration of diphtheria toxin to use in the assay should contain at least 4 × the minimum cytopathic dose that has been previously determined in-house.

Preparation of Diphtheria Antitoxin

The antitoxin stock solution should be diluted to a working concentration of at least 100 IU/ml in complete medium for use in the Vero cell assay. The antitoxin used must be shown to be non toxic to Vero cells and must be shown to neutralise the diphtheria toxin diluted in the control toxoid (containing 100 Lf/ml).

Dilution of Samples and Incubation of Vero Cells

Serial dilutions of the diluted test toxoid are prepared in a tissue culture microplate followed by addition of Vero cells. Where active toxin is present in the test toxoid sample, the cells will die during the 6 day incubation period. To confirm that any toxicity observed is specific for diphtheria toxin, duplicate samples are incubated with diphtheria antitoxin. As a positive control, a reference diphtheria toxin (diluted in a control diphtheria toxoid) is also included (with and without antitoxin) to confirm assay sensitivity and specificity. The procedure described here is based on the plate layout shown in Figure 1.

1) Take a tissue culture microplate and mark the plate with the date and plate number.
2) Fill all the wells of columns 2–10 with 50 µl of PBS (or buffer used for dilution of toxoid samples) using a multichannel micropipette. Note: for the toxin control in row D and H, wells should be filled with 50 µl of the control diphtheria toxoid at 100 Lf/ml.
3) Add 100 µl of the appropriate test toxoid sample or reference diphtheria toxin (e.g. at 4 × MCD in control toxoid at 100 Lf/ml) to the appropriate wells in column 1 using a micropipette (Figure 1).

4) Prepare serial two-fold dilutions (in 50 µl volumes) starting at column 1 through to column 10 using a multichannel micropipette. Discard 50 µl from column 10.

5) Add 50 µl of PBS (or buffer used for dilution of test toxoid samples) and 50 µl of complete medium to the "cell control" wells (A-B 11-12).

6) Add 50µl of PBS (or buffer used for dilution of test toxoid samples) and 100 µl of complete medium to the "blank control" wells (C-D 11-12).

7) Add 50µl of PBS (or buffer used for dilution of test toxoid samples) and 50 µl of diluted diphtheria antitoxin (100 IU/ml) to "antitoxin control" wells (E-F 11-12).

8) Add 50 µl of diluted control diphtheria toxoid (100 Lf/ml) and 50 µl of complete medium to "toxoid control" wells (G-H 11-12).

9) Add 50 µl of complete medium to all wells in rows A-D up to and including column 10.

10) Add 50 µl of diphtheria antitoxin (100 IU/ml) to all wells in rows E-H up to and including column 10.

11) Mix antitoxin with toxin by gently shaking, and cover the plate with a lid.

12) Incubate at room temperature (20–25 °C) for one hour to allow toxin neutralisation to occur.

13) Prepare a Vero cell suspension in complete medium containing approximately 4×10^5 cells/ml as described previously.

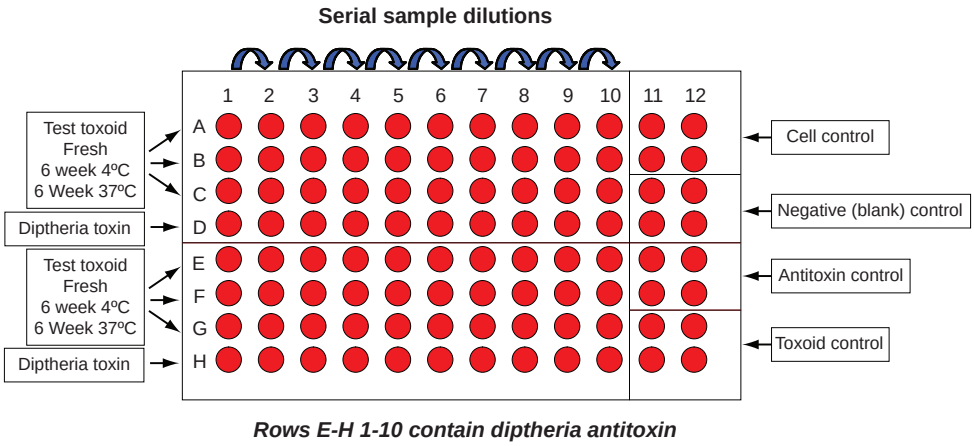
14) Add 50 µl of the cell suspension to all wells of the microplate, except the "blank control" wells (C-D 11-12). The total volume in all wells should be 150 µl.

15) Shake the plates gently and cover with plate sealers to prevent the exchange of gas between medium and air.

16) Incubate for 5-6 days at 37 °C in 5% CO_2 incubator.

17) Perform MTT assay as described in section II.1.3.2 or use another suitable detection method.

Figure 1: Example of the plate layout for Vero cell toxicity assay



Calculation and Interpretation of Results

The presence of dark blue colour indicates viable cells due to the ability of mitochondrial dehydrogenase in viable cells to reduce the MTT to the coloured formazan product. A light blue colour indicates partial toxicity while the absence of colour indicates complete toxicity and cell death. See figure 2 for an example of the results obtained in the Vero cell test following staining with MTT.

Figure 2: Example of the results obtained in the Vero cell toxicity test

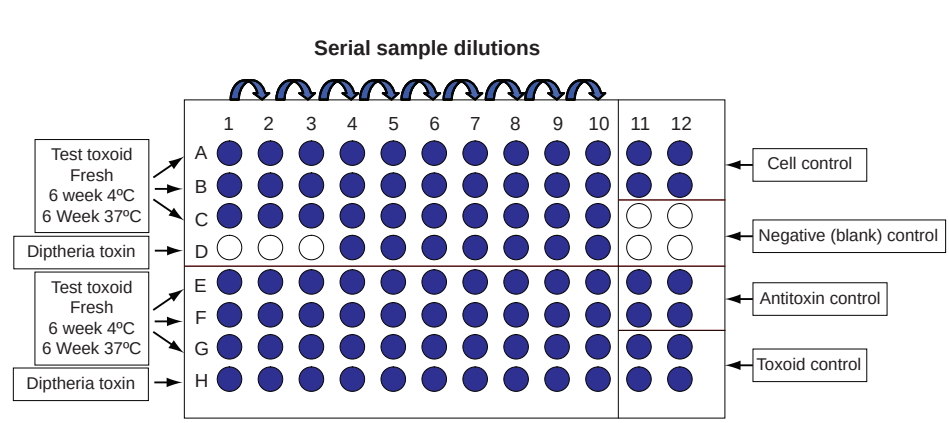


Figure 2 shows an example of the results obtained in a Vero cell toxicity test. In this example, there is no toxicity observed in wells containing the test toxoid sample freshly diluted or following prolonged exposure to heat. In this example, a toxin dilution of 4 × MCD was used and this dilution is toxic in the first 3 wells which contain 4 × MCD, 2 × MCD and 1 × MCD respectively. This confirms the sensitivity of the assay. The toxic effect is neutralised by the diphtheria antitoxin present in row H which confirms the specificity of the assay. See below for details of all validity criteria.

Determination of Toxic End Points Based on Absorbance Readings

Each tissue culture plate used in the Vero cell assay contains appropriate internal controls to allow for definition of cell toxicity based on the absorbance measurement following MTT staining. The 50% optical density (OD) value in the 4 wells containing Vero cells and the control toxoid can be used to quantify any cytopathic effects observed following incubation with bulk purified toxoid samples.

The geometric mean optical density value (OD) of the wells containing Vero cells together with the control toxoid (G-H 11-12, Figure 2) should be calculated and divided by 2 to obtain the 50% OD value. This 50% OD value (plate specific) is used as a threshold value for the test toxoid samples. If the test sample OD is greater than the 50% threshold value the toxoid passes the test. If, on the other hand, the sample OD is below the 50% threshold value, it is necessary to examine the duplicate sample in the presence of diphtheria antitoxin. If, in the presence of antitoxin, the sample OD is restored to a level above the 50% control threshold, the toxic effect of the test sample is specific to diphtheria toxin and the toxoid fails the test. If, in the presence of antitoxin the sample OD remains below the threshold value, the cytopathic effects of the toxoid are likely to be non-specific and the test may need to be repeated using samples that have been dialysed.

Requirements

No specific toxicity should be observed (i.e. toxicity that is neutralised in duplicate wells containing diphtheria antitoxin).

Validity of the Test

- The assay is valid if the sensitivity and specificity of the assay are confirmed: i.e. the control toxin is toxic to Vero cells and this toxic effect is neutralised in duplicate wells containing toxin and antitoxin.
- The test is valid if no toxicity is seen following the addition of diphtheria antitoxin or control toxoid. In this case, the threshold for toxicity is based on the 50% OD value of the 4 wells containing Vero cells alone ("cell control", A-B 11-12, Figure 2).

Retest

If non-specific toxicity is observed (i.e. toxic effects that are not neutralised in the presence of antitoxin) the test sample should be dialysed against the toxoid diluent prior to use in a repeat assay.

Validation

WHO recommends the use of Vero cell culture assay provided that the test is validated against the guinea pig test. During such validation studies recommendations regarding pass/fail requirements can be made.

References

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4) Aggerbeck H, Heron I. Improvement of a Vero cell assay to determine diphtheria antitoxin content in sera. *Biologicals*, 1991: 19: 71-76.

5) Hoy CS and Sesardic D. *In vitro* Assays for Detection of Diphtheria Toxin. *Toxicology. In vitro,* 1994: 8 (4): 693-695

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II.3 Lf and identity

II.3.1 Flocculation test (Ramon and laser light scattering)

Introduction

The antigenic strength and purity of diphtheria toxoid as well as antigen content can be expressed in flocculation (Lf) units. By definition, 1 Lf unit is the quantity of toxoid (or toxin) that flocculates in the shortest time with 1 Lf-equivalent of specific antitoxin. The current IS for diphtheria toxoid for use in flocculation test has an assigned value of 1100 Lf/ampoule, established in a collaborative study [1].

The current WHO minimum requirements for antigenic purity of diphtheria toxoids have been set as not less than 1500 Lf units per milligram of protein nitrogen for use in production of vaccines for human use [2].

The Ramon version of the flocculation test method [3] is generally used by vaccine manufacturers as an in-process control test and is applicable for use with purified diphtheria toxoids of high concentration in Lf/ml. Unlike other immunological binding assays, the reaction in solution between the antigen and the antibody provides additional information on antigenic quality due to a correlation existing between time factor and quality of the antigen. The optimum concentration range of the assay is between 10 to 100 Lf/ml. When the expected antigen concentration is very low, i.e. below 5 Lf/ml, the measurement of toxoid concentration can be carried out using blend flocculation method involving comparison of the Lf value of a known toxoid and that of a mixture of the sample with the known toxoid. However other suitable methods can be more appropriate for detection of samples with low Lf/ml range (as detailed in Sections II.8.2 and II.8.3 of this Chapter).

The flocculation test can also be used to confirm Lf content in non-adsorbed and adsorbed vaccine [2]. However other suitable methods can be more appropriate and provide increased sensitivity (as detailed in Sections II.3.2 and II.3.3).

Principle

This immunological binding assay in solution consists of the detection of a complex formed between the antigen and the antibody [3]. This assay, known as **Ramon assay,** is based on the observation by naked eye of a macroscopic flocculation complex. The time required for the formation of this complex depends on the ratio of toxoid and specific antitoxin. The time in minutes for the first flocculation to occur, is called the Kf value. Kf depends on both antigen and antitoxin concentration and it is assumed that the quality of the antigen also affects the Kf. At low concentrations or when poor-quality antigen is used the Kf value is high. The 1st tube in which flocculation appears is used to determine the Lf value of the sample. The "Limit of Flocculation" is defined as the antigen content forming 1:1 ratio against 1 unit of antitoxin [3,4].

A new method has been developed in Japan involving **laser light-scattering** for a more accurate and objective detection of antigen-antibody complexes [5]. Particles in suspension are subjected to aggregometry. A laser beam is passed through the suspension. Scattered light intensity and signal counts are recorded. These values reflect size and number of detected particles, respectively.

Materials

Critical reference materials

Diphtheria toxoid

Diphtheria toxoid NIBSC 02/176 (2nd WHO International Standard of diphtheria toxoid for use in flocculation test with an assigned value of 1100 Lf/ampoule [1]), or in-house equivalent preparation, calibrated in terms of WHO IS, can be used. Each ampoule is reconstituted in PBS, NaCl or a suitable diluent then diluted from 30 to 50 Lf/ml for Ramon flocculation test and from 25 to 200 Lf/ml for laser light-scattering method.

Diphtheria antitoxin

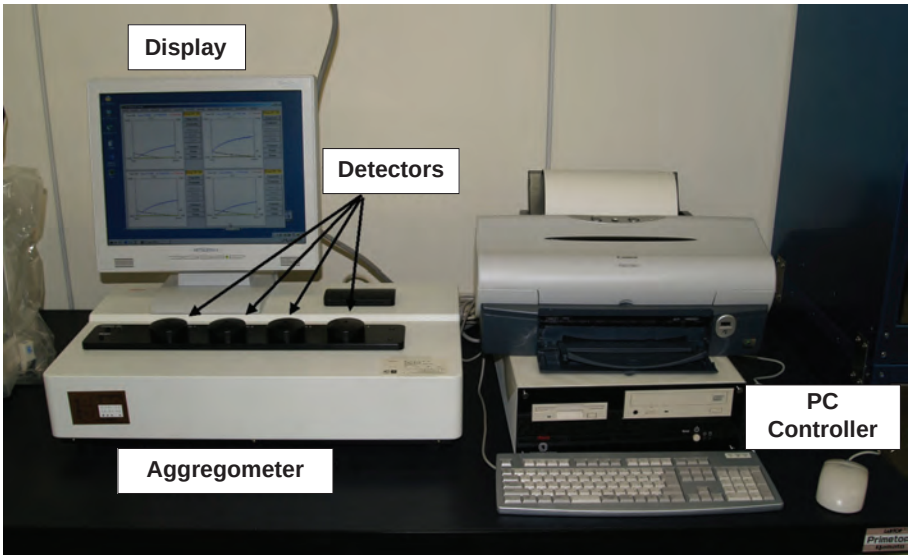
Reference diphtheria antitoxin calibrated in IU/ml, NIBSC, 63/007 at 1300 IU/ampoule or in-house equivalent can be used. Each ampoule of 63/007 is reconstituted in PBS, NaCl or a suitable diluent to give 100 IU/ml. When used in the flocculation assay, the Lf equivalent (Lf/eq.) value must be determined in-house.

Equipment for Ramon flocculation test

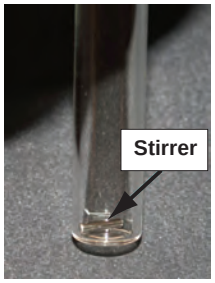
- micropipettes, 0.2-20µl
- micropipettes, 20-200µl
- micropipettes, 200-1000µl
- glass tubes
- water bath
- clock

Equipment for laser light-scattering method

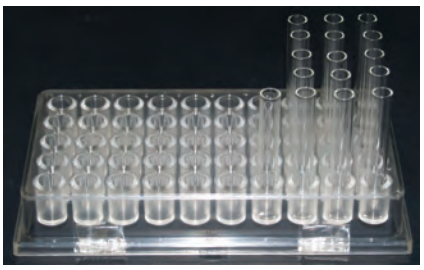
Figure 3: Apparatus: Laser light-scattering platelet aggregometer (Kowa model PA-20 or PA-200)



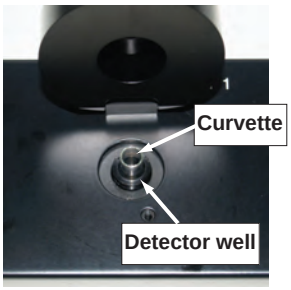
Cuvette Kowa 7 x 50 mm



Rack for Incubation



Cuvette and detector well



Procedures

Ramon method

Antitoxin Calibration

The results of the calibration of antitoxins in International Units (IU) against international antitoxin standards depend on the immunochemical method used. For this reason, antitoxins used for the Ramon assay must be directly calibrated against the current WHO IS for diphtheria toxoid for flocculation tests [1], using the methods described below. The concentration thus determined may be indicated in Lfeq. per millilitre (Lfeq./ml).

Reaction

Antitoxin is added in increasing concentrations to a series of glass tubes. The volumes in each tube at this stage should be the same, for example, 1 ml. Test sample is diluted to give an expected concentration of approximately 30 to 50 Lf/ml, and, for example, 1 ml aliquots of this dilution is dispensed into each tube containing antitoxin. Final volume in each tube must be the same. Each tube is mixed then incubated between +30°C and +50°C in the water bath. Tubes are observed at regular intervals in order to observe the flocculation reaction. The 3 first tubes to show flocculation are reported as well as the time (Kf) to develop the reaction.

Lf value of the test diphtheria toxoid is then calculated from the concentration of diphtheria antitoxin present in the 1st tube to show flocculation (see the example of a calculation below).

Calculation

Table 1: Example: Preparation of tubes containing an increasing amount of antitoxin with a fixed amount of diphtheria toxoid test sample. The first three tubes to show flocculation are indicated (F1-F3).

Reagents	Tubes							
	A	B	C	D	E	F	G	H
Antitoxin (Lfeq.)	30	40	50	60	70	80	90	100
Antitoxin (ml)	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
PBS or NaCl (ml)	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
Toxoid (ml)	1	1	1	1	1	1	1	1
Flocculation sequence		F3	F1	F2				

The first tube to flocculate is the one that contains the amount of antitoxin closest in equivalence to the amount of antigen in the sample. The antitoxin content of this tube can be used to calculate the Lf of the sample. In the example above, tube C is the first tube where flocculation is observed, and therefore it can be assumed that the Lf value of the diluted test sample is 50 Lf/ml. In order to obtain the Lf value of the undiluted product tested, dilution factor must be taken into account. In this example, if the toxoid has been diluted 1/10 prior to the reaction, its final Lf value is 500 Lf/ml. When two tubes flocculate simultaneously, the mean from the results obtained for the two tubes is given as result.

If a more precise result is desired, flocculation test can be repeated using a smaller range of concentration of the diphtheria antitoxin. For example: 30, 35, 40, 45, 50, 55, 60 and 65 Lfeq. range can be used. Concentration can be narrowed to 2 or 3 Lfeq. increase from tube to tube. Additionally, more precision can be obtained by making allowances for the sequence of flocculation after the first tube. In the example quoted, the second tube to flocculate is tube D, and therefore the final value for the diluted sample would be 55 Lf/ml. If the second tube to flocculate had been tube B then the final value would be 45 Lf/ml.

Time taken for the first tube to flocculate (Kf) is a useful indicator of the quality of the antigen and should be reported.

Determination of diphtheria toxoids with very low concentration - blend flocculation:

The Lf value for diphtheria toxoids with very low concentration is assessed using the blend flocculation method. It involves the comparison of the Lf value of a reference toxoid with the Lf value of a mixture composed by the same reference toxoid and the sample to be tested.

Two series of tubes are prepared in parallel.

Lf value of the reference diphtheria toxoid (Lf_{ref})

A first series of tubes is prepared.

Diphtheria antitoxin is added in increasing concentrations to a series of tubes containing a constant amount of the reference diphtheria toxoid.

The Lf value of the reference diphtheria toxoid (Lf_{ref}) is then calculated from the concentration of diphtheria antitoxin present in the 1st tube to show flocculation.

Lf value of the mixture containing the reference and unknown diphtheria toxoids (Lf_{ref+s})

A second series of tubes is prepared.

Diphtheria antitoxin is added in increasing concentrations to a series of tubes containing a constant amount of the reference diphtheria toxoid at 50 Lf/ml and 1.0 ml of the unknown diphtheria toxoid to test. Final volume is 3.0 ml.

Lf value of the solution containing both the reference and the unknown diphtheria toxoid (Lf_{ref+s}) is then calculated from the concentration of diphtheria antitoxin present in the 1st tube to show flocculation.

Lf value of the unknown diphtheria toxoids (Lf_s)

When a reference diphtheria toxoid and an unknown diphtheria toxoid are tested together, the mixture will flocculate as the sum of their values.

To calculate the Lf value of the unknown diphtheria toxoids (Lf_s):

$$Lf_s = Lf_{ref+s} - Lf_{ref}$$

Laser light-scattering method

Antitoxin Calibration

Antitoxins used for this assay must be directly calibrated against the current WHO IS for diphtheria toxoid for flocculation tests [1]. The concentration thus determined may be indicated in Lf-equivalents per millilitre (Lfeq./ml).

Computer set up:

Measurement settings should be chosen and confirmed before starting the assays.

Samples preparation

Increasing volumes of antitoxin solution are added to a series of aggregometry cuvettes and final volume adjusted to 200 µl. Just before starting the measurement, 200 µl of diphtheria toxoid solution is added. Final reaction volume should be 400µl (see table 2 below as an example). The tubes are immediately transferred and submitted to the measurement by laser light-scattering platelet aggregometer.

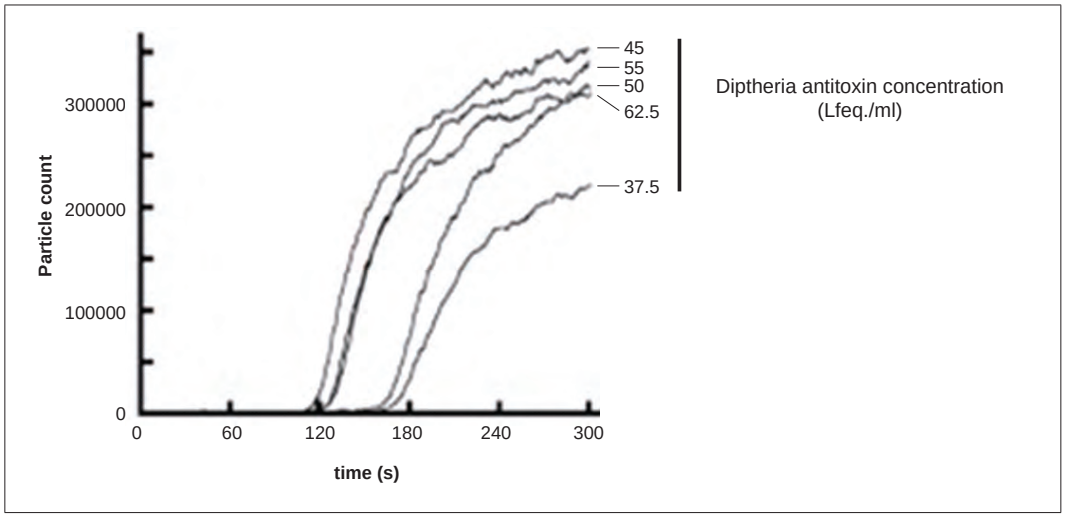
Table 2: Example: Preparation of samples for laser light scattering. Cuvettes contain an increasing amount of antitoxin, with a fixed amount of the diphtheria toxoid test sample.

Reagents	Tubes				
	A	B	C	D	E
Final antitoxin concentration (Lfeq./ml)	37.5	45	50	55	62.5
Added vol (µl) of antitoxin at 200 Lfeq./ml	75	90	100	110	125
PBS (µl)	125	110	100	90	75
Approx. 100 Lf/ml toxoid (µl)	200	200	200	200	200

Extraction of cut-off time

Data saved in PA-200/Version 3 binary format are converted to Excel csv format. Time must be recorded when total particle count reaches 50000. This will constitute the time point expressed in seconds.

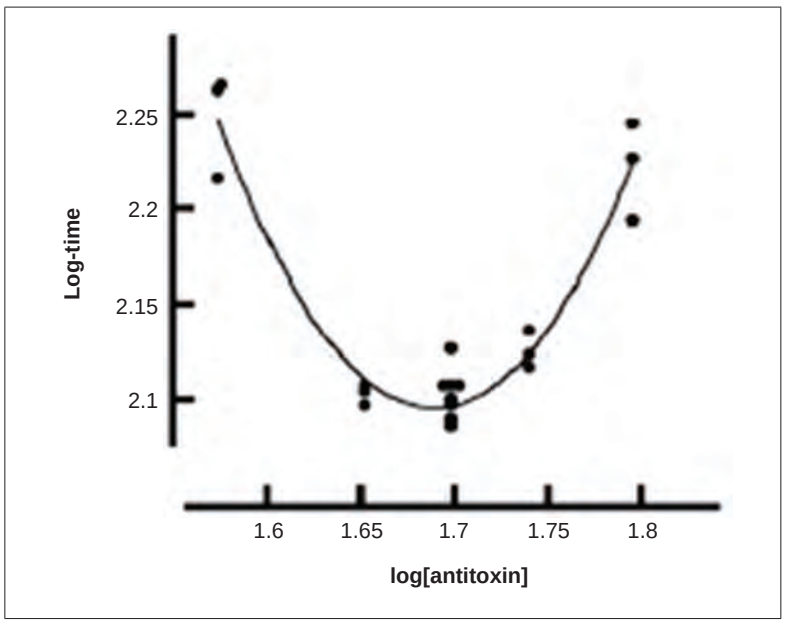
Figure 1: Example of the detection of particle formation by laser light-scattering platelet aggregometer. Diphtheria antitoxin concentration varies from 37.5 to 62.5 Lfeq./ml and diphtheria toxoid fixed concentration is approximately 50 Lf/ml. (Reprinted from [5] with permission from Elsevier).



Plotting and Lf estimation

Log cut-off time and log diphtheria antitoxin concentration are plotted on a scatter graph. A parabolic regression curve is obtained and the equation allows antitoxin concentration giving the minimum cut-off time to be calculated. This concentration directly corresponds to the Lf value of the tested diphtheria toxoid.

Figure 2: Example of a parabolic regression curve. Time required to reach a particle count of 50,000 was extracted from recorded data for diphtheria toxoid at the 50 Lf/ml level and plotted. (Reprinted from [5] with permission from Elsevier).



Validity

Ramon flocculation method

Each test should be performed on at least 2 independent occasions. For each experiment, 2 series of tubes should be prepared and run in parallel. If results from the 2 independent experiments are not consistent with each other, flocculation test should be performed on a 3rd occasion with 2 series of tube run in parallel.

Retest

If flocculation first appears in the tube with highest or smallest diphtheria antitoxin concentration the assay must be repeated with either a different range of reference antitoxin or different dilution of tested toxoid.

Laser light-scattering method

Flocculation should be clearly recordable. Particle count should reach plateau at 150,000-200,000 counts.

Retest

If the earliest cut-off time is obtained in the tube with highest or smallest diphtheria antitoxin concentration, the assay must be performed again with either a different range of reference antitoxin or different dilution of tested toxoid.

References

1) Preneta-Blanc R, Rigsby P, Sloth Wilhelmsen E, Tierney R, Brierley M and Sesardic D. Collaborative study: Calibration of replacement international standards of diphtheria toxoid for use in flocculation test. WHO ECBS 2007. WHO/BS/07.2062.

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5) Iwaki, M., Horiuchi, Y., Komiya, T., Fukuda, T., Arakawa, Y. and Takahashi, M. Toxoid flocculation assay by laser light-scattering. Journal of Immunological Methods, 2007, 318; 136-148.

II.3.2 Radial Immunodiffusion

Introduction

Single radial immunodiffusion (SRD) method is used as an identity test for adsorbed and combined vaccines containing diphtheria components. WHO and Ph. Eur stipulate that each final lot of vaccine must be tested to confirm the presence of diphtheria antigen [1, 2].

Vaccines adsorbed on aluminium phosphate or on aluminium hydroxide have to be desorbed (eluted) from the adsorbent with either sodium citrate or sodium EDTA prior to test. The identity test can also be performed by Double Diffusion method (DD) [3].

The detection limit and linear dose response range for diphtheria toxoid in this assay is between 4 Lf/ml and 100 Lf/ml. SRD imunoprecipitation method is a highly robust and easy test to confirm identity of antigen in final product requiring only a small amount of vaccine [4]. SRD method can also be used to confirm consistency of production process through the determination of antigen content in Lf/ml.

- SRD can be used for quantitative measurement of Lf/ml on non-adsorbed, purified diphtheria toxoid intermediate as an alternative to the flocculation test, as the difference between results from the two methods does not exceed 10% [5]

- SRD could also be applicable for semi-quantitative measurement of Lf/ml on final adsorbed product, after desorption.

Principle

Single radial diffusion is a simple qualitative and quantitative immunodiffusion technique based on methods previously published by Melville-Smith [4]. A concentration gradient is established for the antigen diffusing from a well that has been cut into the gel medium containing the antitoxin at a comparatively low concentration. When the equilibrium between the antigen and antitoxin has been established, the circular precipitation area, originating from the well into which antigen has been added, is directly proportional to the concentration of the antigen in the gel.

For DD assay [3], diphtheria antitoxin and the test antigen sample are loaded in opposing wells. Precipitation lines indicating presence of antigen can be read before and after staining confirming the identity of the sample.

Materials

Critical reference materials

Diphtheria toxoid

Diphtheria toxoid NIBSC 02/176 (2nd WHO IS of diphtheria toxoid for use in flocculation test, with an assigned value of 1100 Lf/ampoule [6]), or in-house equivalent, can be used. Each ampoule is reconstituted in a suitable diluent to give 100 Lf/ml.

Diphtheria antitoxin

Diphtheria antitoxin calibrated in IU/ml (NIBSC, 63/007, 1300 IU/ampoule), or any in-house equivalent can be used. Each ampoule is reconstituted in PBS to give 100 IU/ml.

Tetanus toxoid (used as a negative control)

Tetanus toxoid, NIBSC 04/150 (2nd WHO IS of tetanus toxoid for use in flocculation test, with an assigned value of 690 Lf/ampoule [7]) or an in-house equivalent, can be used. Each ampoule is reconstituted in a suitable diluent to give 100 Lf/ml.

Buffers

Gel: 1% Seakem Agarose in Phosphate Buffer Saline 1X pH7.4.

Staining solution: Coomassie brilliant blue (0.2% w/v) in a solution of methanol (50%) and acetic acid (10%).

Destain solution: methanol (50%); acetic acid (10%).

Equipment

- micropipettes, 0.2-20µl
- micropipettes, 20-200µl
- micropipettes, 200-1000µl
- disposable 5 ml plastic pipettes
- plastic immunodiffusion plates
- single well cutter
- glass plate
- weight (>1.5Kg)
- filter paper
- paper towels
- glass bijoux

Procedures

Desorption of adsorbed vaccines

Vaccines are desorbed to remove antigen from adsorbant before test. Desorption with 5-10% of sodium citrate is suitable for most vaccines adsorbed onto aluminium hydroxide or aluminium phosphate adjuvants. 0.1% EDTA may be used as an alternative desorbent, particularly for vaccines containing reduced diphtheria antigen contents. The examples of appropriate methods are provided but other methods may be used if shown to be suitable.

Desorption with sodium citrate:

To 1.0 ml of vaccine, sufficient sodium citrate (solution or powder) is added to make the final concentration of sodium citrate of 5-10%. The mixture is incubated for 16-20 hours at +37°C, followed by centrifugation until a clear supernatant, free from adsorbant, is observed. Supernatant is removed and stored for up to 1 week at +4°C until required for test.

Desorption with EDTA:

1.5 ml of vaccine is centrifuged. The supernatant is removed and the pellet is resuspended in 0.5 ml of a freshly prepared solution of EDTA (1.12 g/L EDTA, 88.2 g/L Na_2HPO_4). (1 volume of 56 g/L solution of EDTA and 49 volumes of a 90 g/L solution of sodium hydrogen phosphate).

The mixture is maintained at +37°C for not less than 6 hours and centrifuged. The clear supernatant is removed and stored for up to 1 week at +4°C until required for test.

Preparation of Controls

The test has a requirement for both positive and negative controls.

When testing the diphtheria component of the vaccine, a positive control of diphtheria toxoid (100 Lf/ml) and a negative control of tetanus toxoid (100 Lf/ml) are required.

Gel Preparation

Molten seakem agarose (3.0 ml) is mixed with 100 µl of diphtheria antitoxin (N.B: agarose should be allowed to cool down to 56°C before adding the antitoxin). The preparation is poured immediately onto a clean plastic immunodiffusion plate and allowed to set for 15-30 minutes. Suitable wells are cut into the gel to allow the addition of toxoid controls and test samples.

Identity test

Positive control diphtheria toxoid (5 µl), negative control tetanus toxoid (5 µl) and 5 µl of test sample are added to wells. For vaccines with low-dose diphtheria content (suitable for adults and adolescents), it may be necessary to add 20 µl of toxoid (4 × 5 µl, allowing each 5µl addition to absorb into the gel before addition of the next) to generate an adequate ring of precipitation.

Quantitative and Semi-quantitative test

The reference diphtheria toxoid is diluted to 100, 40, 30, 20 and 10 Lf/ml, and 5 µl of each dilution are added to wells. Then 5 µl of test samples are added in other wells (see pattern 1).

Staining of precipitates

The plates are covered with lids and incubated for 16 to 48 hours in a humidified box at room temperature. Gels are then maintained on plates with elastic bands before being soaked in sterile PBS for 4 to 24 hours. The gels are removed from the plates and placed onto an alcohol-cleaned glass plate. Wells are filled with sterile PBS and covered with a sheet of filter paper and a thick layer of paper towels. A heavy weight is applied to flatten the gels onto the glass sheet for 20 minutes. The paper towels are changed and the weight is reapplied for a further 20 minutes. The filter paper and glass are then dried at +37°C for up to 10 minutes. After removing the filter paper, the glass plate and gels are allowed to air-dry for up to 60 minutes. Gels are stained by immersion in Coomassie blue for 3 minutes, and then immersed in destain solution until the background is clear. A blue precipitate ring for diphtheria antigen should be clearly visible to the naked eye.

Validity and limit of detection

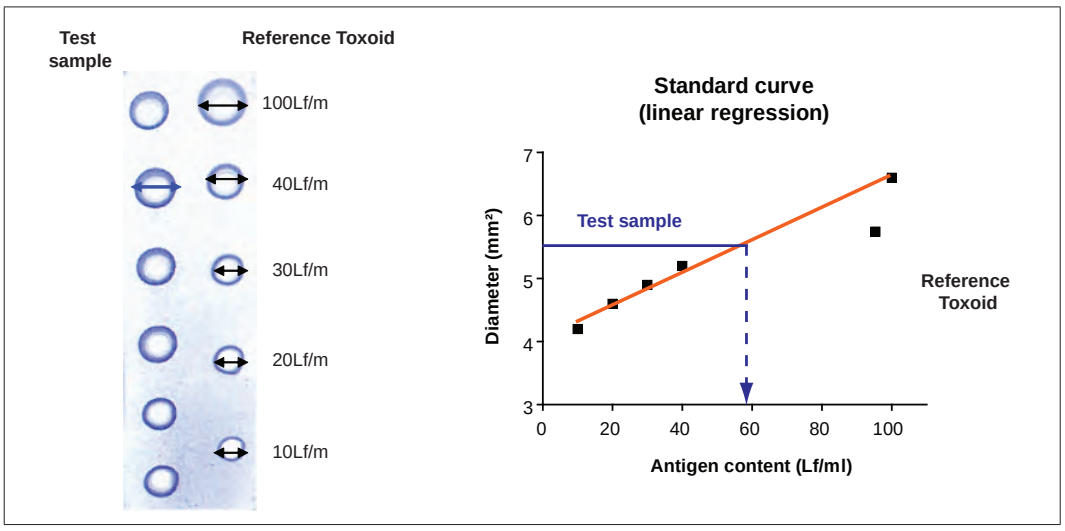
Identity test

For the identity test to be valid, the positive control toxoid should demonstrate a blue precipitation line or ring of identity. The negative control toxoid should show no blue precipitation line or ring of identity. If the test is invalid a retest should be performed.

Quantitative and semi-quantitative test

The standard curve is obtained by plotting the square diameter of the ring of precipitation against the corresponding concentration of the reference diphtheria toxoid. The square diameter of the sample to test is then plotted on the graph and its concentration can be determined.

Figure 1: Pattern 1



The limit of detection for the assay is approximately 4 Lf/ml for diphtheria toxoid.

SRD is a robust and easy method to confirm the presence of toxoids in final products, and provides a suitable alternative to the flocculation test. However, the test is not sufficiently sensitive to detect low levels of non-adsorbed antigen in vaccines (see section II.4.1, Capture and direct ELISA).

References

1) Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2012 (WHO Technical Report Series) in press.

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II.3.3 Rocket immunoelectrophoresis

Introduction

Rocket immunoelectrophoresis can be used as an identity test and antigen quantification test for diphtheria antigen in final vaccine products [1,2].

Vaccines adsorbed onto aluminium phosphate or on aluminium hydroxide have to be desorbed or eluted from the adsorbent with either sodium citrate or sodium EDTA prior to the test.

The detection limit and linear dose response range for diphtheria toxoid in this assay is between 2.5 Lf/ml and 30 Lf/ml. This immunoprecipitation method is a robust and easy test to confirm identity in final product. This method can also be used to confirm consistency of the production process through the determination of antigen content in Lf/ml.

Rocket immuno-electrophoresis can be used for quantitative measurement of Lf/ml of non-adsorbed purified diphtheria toxoid as an alternative to flocculation test as the difference between results from the two methods does not usually exceed 10% [3].

Principle

This method allows determination of antigen content due to the difference of charge existing between toxoid and antitoxin [4, 5, 6]. The electrophoresis of the antigen to be determined is carried out in a gel containing a comparatively lower concentration of the corresponding antitoxin. The test material and dilution of a reference toxoid used for calibration are loaded into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear [4].

Materials

Critical reference material

Diphtheria toxoid

Diphtheria toxoid NIBSC 02/176 (2nd WHO IS of diphtheria toxoid for use in flocculation test with an assigned value of 1100 Lf/ampoule [7]), or any in-house equivalent, can be used.

Diphtheria antitoxin

Diphtheria antitoxin calibrated in IU/ml (NIBSC, 63/007 1300 IU/ampoule), or any in-house equivalent, can be used.

Buffers

Gel: 1% Seakem Agarose in PBS pH 7.2 (Optimization of pH may be required depending on the standard used).

Staining solution: Coomassie brilliant blue (0.3% w/v) in a solution of methanol (50%) and acetic acid (10%).

Destaining solution: methanol (50%); acetic acid (10%).

Equipment

- micropipettes, 0.2-20µl
- micropipettes, 20-200µl
- micropipettes, 200-1000µl
- disposable 20ml plastic pipettes
- immunodiffusion tray & apparatus
- well cutter
- glass plate
- filter paper
- paper towels
- electrophoresis power or apparatus

Procedures

Desorption of adsorbed vaccines

Vaccines are desorbed to remove antigen from adsorbant before test. Desorption with 5-10% of sodium citrate is suitable for most vaccines adsorbed onto aluminium hydroxide or aluminium phosphate adjuvants. 0.1% EDTA may be used as an alternative desorbent, particularly for vaccines containing reduced diphtheria antigen contents. The examples of appropriate methods are provided but other methods may be used if shown to be suitable.

Desorption with sodium citrate:

To 1.0 ml of vaccine sufficient sodium citrate (solution or powder) is added to make the final concentration of sodium citrate of 5-10%. The mixture is incubated for 16-20 hours at +37°C, followed by centrifugation until a clear supernatant, free from adsorbant, is observed. Supernatant is removed and stored for up to 1 week at +4°C until required for test.

Desorption with EDTA:

1.5 ml of vaccine is centrifuged. The supernatant is removed and the pellet is resuspended in 0.5 ml of a freshly prepared solution of EDTA (1.12 g/L EDTA, 88.2 g/L Na_2HPO_4). (1 volume of 56 g/L solution of EDTA and 49 volumes of a 90 g/L solution of sodium hydrogen phosphate).

The mixture is maintained at +37°C for not less than 6 hours and centrifuged. The clear supernatant is removed and stored for up to 1 week at +4°C until required for test.

Gel Preparation

Molten seakem agarose (20 ml) is mixed with 200 µl of diluted diphtheria antitoxin. (N.B: agarose should be allowed to cool down to +56°C before adding the antitoxin). The preparation is poured immediately onto a clean immunodiffusion glass plate and allowed to set for 15-30 minutes. A row of wells is cut in the gel about 25 mm from the edge. Those wells will further be loaded with standard toxoid and sample to test. A control plate should be prepared without antitoxin in the gel as a negative control.

N.B: Optimization of the gel pH is important (pH 7.2 is recommended). The migration will depend on the charge of the molecule. For migration to take place, toxoid and antitoxin must have a different charge.

Addition of Samples and Controls

Each concentration of the standard diphtheria toxoid (10 µl) is loaded into different wells. Test samples (10µl) are loaded onto separate wells. The tray is then placed in the apparatus. Current is adjusted to 5mA/cm and electrophoresis is conducted for about 45 minutes.

Reaction

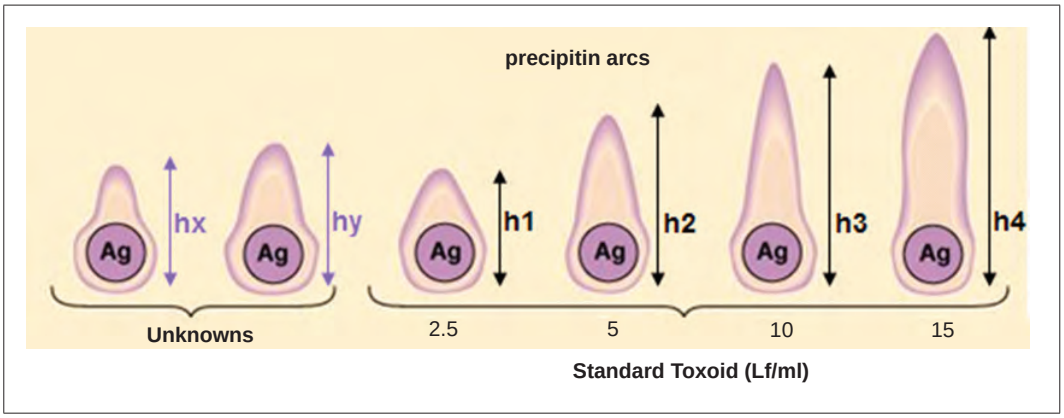
The reaction should not occur more than 10-15 minutes after the samples have been loaded in order to reduce diffusion.

The tray is connected to the buffer tank using filter paper as bridges from the buffer to the gel. The tray is positioned to have diphtheria toxoid samples close to the anode in order to observe migration with the current. An electric potential difference of 180V is applied to the gel at +4°C for 18 hours. A precipitin rocket arc will form when the reaction has occurred between the antitoxin and the antigen component present in the sample to test.

Staining

Gels are stained by immersion in Coomassie blue for 3 minutes. The gels are then immersed in the destaining solution until the background is clear and the blue precipitate ring for diphtheria antigens is clearly visible to the naked eye. The height of the rocket shape is measured as indicated in pattern 1.

Figure 1: Pattern 1



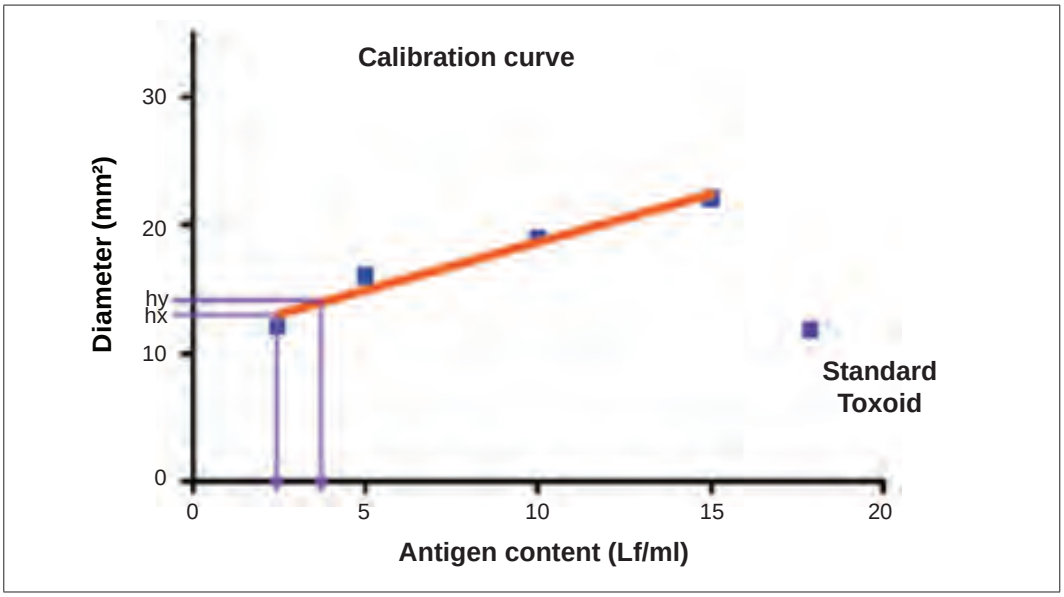
(Figure adapted from http://www.lib.mcg.edu/edu/esimmuno/ch4/rocket.htm)

Validation and limit of detection

The diffusion zone diameter should be between 5 and 40 mm for the test to be valid.

Diphtheria concentration for the test sample is calculated from the linear relationship existing between the height of the rocket and the concentration of standard diphtheria toxoid, calibrated in Lf.

Figure 2:



The limit of detection for the assay is approximately 2.5 Lf/ml for diphtheria toxoids.

References

1) Recommendations to assure the quality, safety and efficacy of diphtheria vaccines. WHO Expert Committee on Biological Standardization. Sixty-third report. Geneva, World Health Organization, 2012 (WHO Technical Report Series) in press.

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II.4 Antigen content and degree of adsorption

II.4.1 Capture and direct ELISA

Introduction

In vitro methods, such as Enzyme Linked Immunosorbent Assay (ELISA), can be used for monitoring lot to lot consistency of vaccines and are an essential tool for quality control [1]. ELISA can be used to measure diphtheria toxoid (antigen) in adsorbed vaccine final products with a limit of quantification of approximately 0.005 Lf/ml (dependent on the reference and other reagents used in the assay, particularly coating antibody). The high level of sensitivity makes these assays the only suitable methods for monitoring the degree of adsorption in adsorbed diphtheria vaccines.

Adsorbed diphtheria vaccine consists of diphtheria formol toxoid adsorbed onto a mineral carrier (adjuvant), and other antigens are present in combined vaccines. In order to measure the total diphtheria antigen content, vaccines must first be desorbed to remove the adjuvant from the diphtheria toxoid. The assay is also applied to the supernatant of the adsorbed vaccine sample in order to determine the non-adsorbed antigen content, thus allowing the degree of adsorption in vaccine preparations to be monitored. The degree of adsorption is product-specific, dependent on the type of antigen and adjuvant and other components present in the vaccine, and may be dependent on age of formulation.

Principle

In general, this immunoassay can be performed either as a direct or capture ELISA. For direct ELISA, microplates are coated with test samples in titration along with a diphtheria toxoid reference, calibrated in Lf/ml. The amount of antigen bound to the plate is visualised by successive incubations with purified polyclonal antibody against diphtheria toxoid, detection-labelled antibody, and suitable substrate. For capture ELISA, microplates are initially coated with purified monoclonal antibody against diphtheria toxin/toxoid, and then test samples are added in titration along with the diphtheria toxoid reference. The amount of antigen bound to the monoclonal antibody is visualized by successive incubations with purified polyclonal antibody against diphtheria toxoid, detection-labelled antibody, and suitable substrate. The method is dependent on critical, well characterized, reagents. The method presented is provided as an example and in house variations are possible.

Although both direct and capture formats have been confirmed to be highly sensitive and reproducible, in some multi-component products, interference is observed in the dose-response curve when measuring non-adsorbed antigens, causing deviations from parallelism when compared to the monovalent reference diphtheria toxoid. Therefore, the capture method is considered preferable for routine monitoring of the diphtheria toxoid antigen and degree of adsorption, particularly for combined vaccine products that are not fully adsorbed.

Capture ELISA format using polyclonal, instead of well-characterised monoclonal antibody, can be used, as the principle of the assay is the same. However, the quality of information obtained will be dependent on antibody preparation and may not be comparable. Studies have confirmed superiority of monoclonal antibodies in testing of combined vaccines and in detecting critical changes not observed with polyclonal formulations (unpublished work).

Materials

Critical reagents

- Reference Diphtheria toxoid NIBSC 02/176 (2nd WHO IS of diphtheria toxoid for use in flocculation test with an assigned value of 1100 Lf/ampoule [2]), or an in-house equivalent, can be used. Each ampoule of 02/176 is reconstituted in 1 ml of a suitable diluent to give 1100 Lf/ml.
- Purified anti-diphtheria monoclonal antibody* against diphtheria toxin/toxoid (for capture ELISA only): NIBSC monoclonal antibody 10/130 (each ampoule reconstituted with 0.5 ml sterile water), or in-house equivalent. (*NB: polyclonal antibodies can be used for capture ELISA, but quality of information obtained will be antibody-dependent).
- Purified anti-diphtheria IgG, prepared from serum of guinea pigs immunized with diphtheria vaccine : NIBSC polyclonal antibody 10/128 (each ampoule reconstituted in 0.5 ml sterile water), or in-house equivalent.
- In-house positive control: Non-adsorbed diphtheria toxoid with known antigen content (Lf/ml).

Other reagents (Commercially available)

- Peroxidase-conjugated rabbit or goat antibody, directed against guinea pig IgG.
- Carbonate coating buffer, pH 9.6.
- Phosphate buffered saline, pH 7.4 (PBS).
- Wash solution: PBS + 0.05% Tween 20 (PBST).
- Blocking buffer and sample buffer: PBST + dried skimmed milk (2.5%).
- 0.05M Citric acid substrate buffer, pH 4.
- Substrate: 2,2'–Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tablets, 10 mg.
- Substrate solution: Shortly before use, dissolve 10 mg ABTS in 20 ml citric acid solution. Immediately before use, add 5 µl hydrogen peroxide solution.
- Vaccine desorbent: 10% w/v tri-sodium citrate (final concentration in vaccine sample) or 0.1% w/v disodium edetate (EDTA) (a solution of EDTA 1.12 g/L and disodium hydrogen phosphate (Na_2HPO_4) 88.2 g/L).

Equipment

- Microcentrifuge.
- ELISA plates: 96 well, Polyvinyl chloride or polystyrene.
- Multichannel pipettes, 100 µl.
- Pipettes, 10- 1000 µl.
- Microplate sealant film.
- Microplate reader.

Procedures

Preparation of vaccine samples

Desorption of antigen using 10% w/v sodium citrate is suitable for most vaccines adsorbed onto aluminium hydroxide or aluminium phosphate adjuvants. 0.1% EDTA may be used as an alternative desorbent, particularly for diphtheria vaccine with reduced antigen content. The following are examples of appropriate methods to use, but other methods may be used if shown to be suitable.

Desorption with 10% w/v sodium citrate

1 ml of homogenized vaccine is mixed with an equal volume of a 20% w/v solution of tri-sodium citrate. The sample is incubated for 16-20 hours at +37°C and then centrifuged. The supernatant is collected and retained at +4°C for testing the total antigen content. It is important to note that the sample is diluted 2-fold during the desorption process, which needs to be taken into consideration when calculating antigen content.

To measure the non-adsorbed antigen content, a duplicate sample of homogenized vaccine is centrifuged and the supernatant collected and retained at +4°C.

Supernatants can be kept at +4°C for up to 24 hours prior to the assay, but any prolonged length of storage has to be confirmed as suitable. Ideally, supernatants are tested on the same day.

Desorption with 0.1% w/v EDTA

1.5 ml of homogenized vaccine is centrifuged and the supernatant is retained (as above) for testing the non-adsorbed antigen content. After removing all of the supernatant, the pellet is resuspended in 0.5 ml of a freshly prepared solution of EDTA (1.12 g/L EDTA, 88.2 g/L Na_2HPO_4). The samples are incubated for 16-20 hours at +37°C, centrifuged, and the supernatant is retained (as above) for testing the adsorbed antigen content. It is important to note that the sample is re-suspended in 1/3 of its original volume which needs to be taken into consideration when calculating antigen content.

> For the EDTA desorption method, total antigen content is obtained by adding the adsorbed value to the non-adsorbed value.

Direct ELISA protocol

Coating of immunoassay plates

One ELISA plate is sufficient for the testing of up to 4 vaccine samples in duplicate, along with an internal positive control and a reference preparation (see suggested plate layout in Figure 1). The reference diphtheria toxoid, internal positive control and vaccine samples are diluted in coating buffer to a concentration which produces a suitable dose response curve. For the internal positive control and the reference diphtheria toxoid (NIBSC 02/176), a suitable initial concentration is 0.25 Lf/ml. A dilution of 1/100 is suggested as a starting point for testing the desorbed antigen samples, and a 1/10 dilution for non-adsorbed antigen content samples. These dilutions are product-dependant.

In some multi-component vaccines, non-adsorbed antigen content samples will need to be diluted more than 1/10 if interference from other components and matrix effect is observed. Interference will produce deviations from linearity and parallelism between the sample and the reference dose response curves. In such situations the capture ELISA may be a more suitable protocol.

- Add 100 µl of carbonate coating buffer to all wells except row A.
- Add 200 µl of diluted reference, diluted positive control, or diluted test sample to row A in duplicate columns. Prepare a series of two-fold dilutions in 100 µl volumes to row H and discard 100 µl from the last well. For columns with the positive control, stop the two-fold dilution in row G to set blank, negative control wells.
- Seal the plates and incubate for 2 hours at +37°C or overnight at +4°C in a humid container.

Figure 1: Suitable ELISA plate layout (The positive control is fixed in the outside columns and the duplicates for the reference and samples are spaced equally apart to minimise plate effects).

	PC	S1	S2	Ref	S3	S4	S1	S2	Ref	S3	S4	PC
	1	2	3	4	5	6	7	8	9	10	11	12
A	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*
B	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
C	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4
D	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8
E	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.
F												
G												
H	BL											BL

Ref = Reference diphtheria toxoid

S = Vaccine sample (Non-adsorbed antigen, adsorbed antigen or total antigen)

PC = Positive control diphtheria toxoid

BL = Blank (Negative control)

* Pre-diluted reference toxoid, vaccine sample or positive control toxoid added to row A of a column.

Plate washing

Plates are washed at the end of each incubation period before the addition of the next reagent. This can be done using a plate washer or by hand. The following describes one method for washing plates by hand:

- Prepare the PBST washing buffer.
- Discard the contents of the plates by inversion.

- Wash the plate by immersion into a container of PBST.
- Discard the contents of the plates.
- Repeat the washing procedure a further two times.
- Blot the plates dry against absorbent paper.

Blocking

Blocking is performed after the initial reagent has bound to the plate to ensure that further reagents are not allowed to bind other than by immune adherence. Non-specific adhesion is reduced by including in the buffer an irrelevant protein at high concentration, such as bovine serum albumin (BSA) or casein. Detergent (usually Tween 20 at 0.5 ml/L) is also used to inhibit the adherence of hydrophobic molecules.

- Prepare blocking buffer.
- Add 150 µl to all the wells.
- Incubate at +37 °C for 1 hour in a humid container.
- Wash the plates as described in the previous section.

Diphtheria toxoid detecting antibody

Guinea pig anti-diphtheria polyclonal IgG is used as the first detecting antibody in the assay, diluted in sample buffer prior to use.

- Prepare a dilution of guinea pig anti-diphtheria IgG. For NIBSC 10/128 a suitable dilution is 1/200. For other antibody preparations the optimum dilution should be determined in-house.
- Add 100 µl to all the wells.
- Seal the plates and incubate at +37°C for 2 hours in a humid container.
- Wash the plates as described in the previous section.

Detecting conjugate antibody

To detect the polyclonal antibody bound to the diphtheria toxoid, an anti-guinea pig peroxidase conjugate IgG is added to all the wells.

- Dilute the conjugate in sample buffer (an optimum dilution should be established in-house).
- Add 100 µl to all wells.
- Seal the plates and incubate at +37 C for 1 hour in a humid container.
- Wash the plates as described in the previous section.

Substrate

- Prepare the substrate solution immediately before use (see materials section).
- Add 100 µl of substrate to all the wells.
- Incubate the plates at room temperature for 15-20 minutes.
- Following colour development, read the plates at OD 405 nm using a microplate reader.

Capture ELISA protocol

Coating of immunoassay plates

Plates are coated with purified monoclonal antibody, diluted to a suitable concentration in coating buffer.

- Dilute the monoclonal antibody in coating buffer. For NIBSC 10/130 a suitable dilution is 1/200. For other antibody preparations the optimum dilution should be determined in-house.
- Add 100 µl to all the wells.
- Seal the plates and incubate at +37°C for 2 hours or overnight at +4°C in a humid container.
- Wash the plates as described in the previous section.

Blocking

Block the plates as described in the previous section.

Antigen dilutions

Reference diphtheria toxoid, the internal positive control and vaccine samples are diluted in sample buffer to a concentration which produces a suitable dose response curve. For the internal positive control and reference diphtheria toxoid (NIBSC 02/176), a suitable initial concentration is 0.055 Lf/ml. A dilution of 1/1000 is suggested as a starting point for desorbed antigen samples, and a 1/50 dilution for non-adsorbed antigen content samples. These dilutions are product-dependant. See Figure 1 in the previous section for a suitable plate layout.

- Add 100 µl of sample buffer to all wells except row A.
- Add 200 µl of diluted reference, diluted positive control, or diluted test sample to row A in duplicate columns. Prepare a series of two-fold dilutions in 100 µl volumes to row H and discard 100 µl from the last well. For columns with the positive control, stop the two-fold dilution in row G to set blank, negative control wells.
- Incubate the plates for 2 hours at +37°C in a humid container.
- Wash the plates as described in the previous section.

Proceed with method as for direct ELISA.

Calculation

The development of colour in the wells indicates the presence of diphtheria toxoid. The relative diphtheria antigen content of vaccine samples with respect to the reference diphtheria toxoid preparation may be obtained by parallel line regression analysis. Since dilutions are made on a log scale, the most appropriate linear response is log optical density versus log dilution. Only those optical densities which fall within the range of the linear part of the curve should be used to calculate the titres. Analysis of variance is used to test the significance of departure of the dose-log response relationship from linearity and parallelism.

Relative antigen content estimates are then converted to Lf/ml values by multiplying the estimates by the concentration of reference diphtheria toxoid in the stock solution, taking into consideration differences in the dilution factors of the reference and samples as necessary. For samples desorbed using EDTA antigen content, estimates have to be divided by 3 prior to calculation, due to the fact that the samples are concentrated 3-fold in the desorption step. For total antigen content samples, desorbed with a solution of 20% sodium citrate, antigen content estimates have to be multiplied by 2 prior to calculation due to the fact samples are diluted 2-fold in the desorption method.

$$\text{Sample Lf/ml} = \frac{(\text{Antigen content estimate} \times \text{dilution factor of sample})}{\text{Dilution factor of reference}} \times [\text{Reference Lf /ml}]$$

Once Lf values have been determined for all vaccine samples, the degree of adsorption may be calculated using the following formula:

$$\text{Percent adsorbed (\%)} = \frac{\text{Total antigen Lf/ml} - \text{non adsorbed antigen Lf/ml}}{\text{Total antigen Lf/ml}} \times 100$$

NB if using EDTA desorption method, total antigen is obtained by adding adsorbed and non adsorbed antigen values.

Validity of test

The value of the internal positive control must be within the established limits (product-specific).

The negative control must not show a positive response higher than the last dilution of the reference diphtheria toxoid.

The reference diphtheria toxoid standard curve and each test sample must show suitable dose-response, overlapping with samples, and with the linear range covering at least 3 points.

The assay should meet the criteria set in the parallel line assay for linearity and parallelism of the dose-response relationship.

Retest

If duplicates from the same sample show significant deviation from the mean, the sample is re-tested.

References

1) Coombes L, Stickings P, Tierney R, Rigsby P, Sesardic D (2009). Development and use of a novel *in vitro* assay for testing of diphtheria toxoid in combination vaccines. *Journal of Immunological Methods* 350, 142-149.

2) Preneta-Blanc R, Rigsby P, Sloth Wilhelmsen E, Tierney R, Brierley M and Sesardic D (2008). Calibration of replacement international standards of diphtheria and tetanus toxoids for use in flocculation test. Biologicals 36, 315 – 326.

Contents

Chapter III:
Testing for Tetanus vaccines

III.1 Potency:

III.1.1 General introduction

The purpose of the potency test is to assess in a suitable animal model the capacity of the product being tested to induce a protective response analogous to that shown to be efficacious in humans.

Tetanus vaccines are among the most frequently used vaccines worldwide and have been remarkably successful products. Their use has resulted in a significant decrease in the incidence of these diseases in both the industrialised world and in developing countries. Nevertheless, some difficulties exist in the global harmonisation of potency testing procedures, even when International Standards are used, as different approaches have been taken by different countries. Some follow World Health Organisation (WHO) recommendations and *European Pharmacopoeia* (Ph Eur) procedures, whereas others follow the National Institutes of Health (NIH) procedures used in the USA, with or without modifications.

The approach taken by WHO, [1] like that of the Ph Eur [2] is based on the determination of the immunising potency of each final bulk vaccine by comparison with an appropriate reference material calibrated in International Units (IU) against the International Standard (IS) for Tetanus Toxoid (adsorbed). The assigned activity of the current IS for Tetanus Toxoid (adsorbed) is based on calibration in guinea pig or mouse protection by challenge assays. Calibration of any secondary, product-specific, or working references should also be calibrated against the current WHO IS using challenge assay in guinea pigs or mice. WHO and Ph Eur has established separate units when challenge assays are performed in mice [1, 3].

There has been much activity in recent years aimed at simplifying the current tests - by using serology, by reducing the number of animals used, refining the end-point used, and by considering the possibility of using the same animals to test the potency of several antigens in serology assays. However, use of surrogate methods other than functional assay, should not be assumed to be transferable without validation [1, 4].

Revised recommendations to assure the quality, safety and efficacy of tetanus vaccines [1] stipulates that as part of pre-licensing characterization, to evaluate consistency of production as well as to calibrate reference preparations potency assays consisting of multiple dilutions with functional end points should be performed using guinea pigs or mice. Multiple-dilution assays should also be used to test product stability for the purpose of establishing shelf-life.

Potency for routine lot release should be determined by the inoculation of guinea pigs or mice with appropriate doses or dilutions of both the tested product and the reference material. After immunisation, animals may be challenged either with a lethal or paralytic dose of toxin, or bled to obtain sera for measurement of the antitoxin or antibody response. When animals are bled, the antibody levels of the individual animals may be titrated by means of toxin neutralisation tests *in vivo*. The ELISA assay, or another suitable *in vitro* method, such as ToBI, may also be used for titration of serum and subsequent potency calculation provided that these assays have been validated against the challenge assay or the toxin neutralisation test, using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be desirable for validation.

The minimum requirement for potency of tetanus vaccine, tested according to the specified methods described in this manual was amended as part of revised recommendations, such that specification now applies to the lower 95% confidence limit. Such amendment was felt justified to promote global harmonization together with demonstrating that the vaccine potency significantly exceed defined minimum specification. Because the minimum potency requirement now applies to the lower limit of the 95% confidence interval there is no requirement to achieve a 95%confidence interval narrower than 50-200%. Thus minimum potency specifications of tetanus vaccine used for the primary immunisation of children not be less than 40 IU per single human dose (SHD) now applies to the lower 95% confidence limit of the estimated potency. When potency of tetanus vaccine is in DTwP combinations and is performed in mice, the minimum potency of not less that 60 IU per SHD for the lower limit is specified. The minimum potency specification for tetanus vaccine intended for booster immunization of older children and adults may be lower and should be approved by the National Regulatory Authority (NRA). Product specific minimum requirements for tetanus potency are acceptable provided they are justified and based on potency values obtained in practice for the vaccine in question and be approved by the NRA.

Following licensing, and once consistency in production and quality control of the vaccine has been further confirmed on a continuous basis, determination of potency in routine lot release may, with the approval of the national regulatory authority (NRA), be based on the results of serological or challenge assays, both involving a reduced number of animals and/or doses where appropriate.

To further confirm consistency on a continuous basis, the potency of approximately ten recent lots of vaccine should be tested using the full three-dilution assay. If potency expressed in IU is relatively uniform, and if the expectations of linearity and parallelism are consistently satisfied, then fewer doses may be used and the assumptions of linearity and parallelism need not be tested in each assay. When vaccine lots consistently give a lower limit of the 95% confidence intervals for the estimated potency well in excess of the minimum requirements per SHD, one-dilution tests may offer advantages. An outline of the approach to be taken for single dilution assays is described in Chapter V. If one-dilution assays are not advantageous, a reduction in animal usage may nevertheless be achieved by use of two-dilution assays, or another suitable design modification. (see further guidance in Statistical Analysis of Results, Chapter V).

Sections III.1.2, III.1.3 and 1.4 of this chapter provide details of guinea pig and mouse challenge assays as well as guinea pig and mouse serology assays that have been used and confirmed suitable for potency testing of tetanus vaccines.

The NIH test procedure is not described in this manual, although an example of *in vivo* toxin neutralisation test (TNT) is provided for information in section III.1.5 as an example of a method that could be used in support of validation studies to confirm presence of functional antibodies.

References

1) Recommendation to Assure the Quality, Safety and Efficacy of Tetanus Vaccines. WHO Expert Committee on Biological Standardisation. Sixty-third report, Geneva, World Health Organisation, 2012 (WHO Technical Report Series, in press).

2) Assay of tetanus vaccine (adsorbed), general chapter 2.7.8 version 01/2008:20708. European Pharmacopoeia 7th Edition. Strasbourg, France: Council of Europe; 2011.

3) Sesardic D, Winsnes R, Rigsby P, Tierney R and Gaines-Das R. (2002). Collaborative study: Calibration of replacement International Standard and European Pharmacopoeia Biological Reference Preparation for tetanus toxoid, adsorbed. Biologicals. 30: 49-68.

4) Recommendations to Assure the Quality, Safety and Efficacy of DT-based Combined Vaccines. WHO Expert Committee on Biological Standardisation, Sixty-third report, Geneva, World Health Organisation, 2012 (WHO Technical Report Series, in press).

III.1.2 Potency in guinea pigs and mice by challenge (lethal and paralysis)

Introduction

The challenge potency test for tetanus vaccine (adsorbed) is determined by comparing the dose of the vaccine to that of a reference preparation required to protect guinea pigs or mice from either a lethal or paralytic toxic effect following subcutaneous (s.c.) challenge with tetanus toxin [1,2]. For this comparison, a reference preparation of tetanus toxoid (adsorbed) calibrated in International Units (IU) and a suitable preparation of tetanus toxin of known activity and stability is required.

Materials

Critical Reagents

Standard for Tetanus vaccine (adsorbed): The current established 4th WHO IS for Tetanus Toxoid (adsorbed), established by WHO ECBS in 2010 has assigned value of 490 IU/ampoule on the basis of challenge assays in guinea pigs and 260 IU/ampoule on the basis of challenge assays in mice. [3]. Other secondary reference preparations, including product-specific reference material, may be used provided they are calibrated in terms of the WHO IS using challenge assays in guinea pigs or mice.

Challenge toxin: Tetanus toxin containing not less than 50 times the ED_{50} or LD_{50} dose is selected for use in the assay. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the effective dose for every assay.

Animals

Guinea pigs

Use guinea pigs of the same sex (or use males and females equally distributed between groups) from a strain shown to be suitable, each weighing approximately 250-350g. Use groups containing a number of animals sufficient to obtain results that fulfill the requirements for a valid assay. Equal groups of guinea pigs are allocated for the standard vaccine and for each of the test vaccines. If the activity of the challenge toxin has to be determined, include 3 further groups of 5 guinea pigs as unvaccinated controls.

Mice

Use 5 week old mice of the same sex (or use males and females equally distributed between groups), from a strain shown to be suitable (e.g. DDY, NIH or CD1). Use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay. Equal groups of mice are allocated for the standard vaccine and for each of the test vaccines. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include at least 3 further groups of not fewer than 5 mice to serve as unvaccinated controls.

Equipment

Class II safety cabinet.

Sterile syringes 2.0 ml.

Sterile needles, e.g. 22 G × 1 ½ inch for guinea pig and 26 G × 1 inch for mice

0.4 × 12 mm (27G × 1/2").

Sterile glassware and plastic ware.

Volumes of less than 1.0 ml are dispensed using pipettes.

Buffers

Vaccine diluent: 0.9% Sodium Chloride is suitable for preparation of reference and test vaccine dilutions.

Toxin diluent: Gelatine Phosphate Buffered Saline, pH 7.4 (GPBS) or 0.9% sodium chloride is suitable for use as a diluent for the challenge toxin.

All salts used in the preparation of buffers should be of minimum General Purpose Reagent grade, unless otherwise stated.

Procedures

Determination of the challenge dose of tetanus toxin (tetanus toxin titration)

If it is necessary to assess the activity of the challenge tetanus toxin in order to standardise it for subsequent vaccine potency assays, the toxin is titrated to determine the LD_{50} dose. LD_{50} dose is the quantity of tetanus toxin which, when injected subcutaneously into guinea pigs or mice, will cause death in approximate 50% of the animals challenged. When paralysis is used as the end-point, the PD_{50} of the toxin is the smallest quantity of tetanus toxin which, when injected subcutaneously into guinea pigs or mice, will cause paralysis in approximate 50% of the animals challenged.

Guinea pigs

Preparation of the test and reference preparations and immunisation

Using 0.9% sodium chloride, prepare 3 to 4 dilutions of the vaccine to be examined and of the reference preparation, such that for each the dilutions form a series differing by not more than 2.5-fold steps, and in which the intermediate dilutions, when injected subcutaneously with a dose of 1.0 ml per guinea pig, protect approximately 50 per cent of the animals from death or paralysis following subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

Allocate the dilutions, one to each of the groups of guinea pigs, and inject subcutaneously 1.0 ml of each dilution into each guinea pig in the group to which that dilution is allocated.

Preparation of the challenge toxin solution and challenge

After 28 days (in some laboratories 4-6 weeks) following immunization, and immediately before use, dilute the challenge toxin with a suitable diluent (for example, GPBS or 0.9% sodium chloride) to obtain a stable challenge toxin solution containing approximately 50 × LD_{50}/dose. If control groups are included, challenge the unvaccinated groups of guinea pigs with suitable dilutions of the challenge toxin solution (e.g. 1/16, 1/50 and 1/160) that will indicate that the challenge toxin dose was approximately 50 × LD_{50}/dose.

In guinea pigs, injection of toxin should be made mid-ventrally directly behind the sternum with the needle pointing toward the forelimbs so that the tetanus toxin will produce paralysis in the forelimbs which can be easily recognised at an early stage.

Inject subcutaneously into each animal 1.0 ml of the challenge toxin solution (containing 50 × LD_{50}/dose). If it is necessary to determine the activity of the challenge toxin, allocate the 3 dilutions made from the challenge toxin solution, one to each of the groups of 5 guinea pigs, and inject subcutaneously 1.0 ml of each solution into each guinea pig in the group to which that solution is allocated. The guinea pigs are examined over the following 96 hours (3 times on first 3 days and twice on last day), and the number of deaths is monitored and recorded. Alternatively, and for those countries where paralytic challenge is obligatory, the tetanus grades in guinea pigs are characterised by the specific signs which are used for interpretation of results and determination of an end-point in the assay.

This scale gives typical signs when subcutaneous injection of the challenge toxin is made mid-ventrally, directly behind the sternum with the needle pointing towards the forelimbs. Grade T3 is taken as end-point, but with experience grade T2 can be used instead. Tetanus toxin produces, in at least one of the forelimbs, paralysis that can be recognised at an early stage.

T1: slight stiffness of one forelimb, but difficult to observe;

T2: paresis of one forelimb which can still function;

T3: paralysis of one forelimb. The animal moves reluctantly, the body is often slightly banana-shaped owing to scoliosis;

T4: the forelimb is completely stiff and the toes are immovable. The muscular contraction of the forelimb is very pronounced and usually scoliosis is observed;

T5: tetanus seizures, continuous tonic spasm of muscles;

D: death.

Mice

Preparation of the test and reference vaccines and immunisation

Using 0.9% sodium chloride, prepare 3 to 4 dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps, and in which the intermediate dilutions, when injected subcutaneously at a dose of 0.5 ml per mouse, protect approximately 50 per cent of the animals from the paralytic (or lethal) effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

Allocate the dilutions, one to each of the groups of mice, and inject subcutaneously 0.5 ml of each dilution into each mouse in the group to which that dilution is allocated.

Preparation of the challenge toxin solution and challenge

After 28 days (in some laboratories 4-6 weeks) following immunisation, and immediately before use, dilute the challenge toxin with a suitable diluent (for example, GPBS) to obtain a stable challenge toxin solution containing approximately 50 × PD_{50}/dose or LD_{50}/dose in 0.5 ml. If control groups are included, challenge the unvaccinated groups of mice with suitable dilutions of the challenge toxin solution (e.g. 1/16, 1/50 and 1/160) that will indicate that the challenge was approximately 50 × PD_{50}/dose or 50 × LD_{50}/dose.

When paralysis is used as an end-point, mice should be challenged subcutaneously over the lumbar region of the spine to produce a distinctive hind limb paralysis which can be recognised at an early stage. Inject into each animal 0.5 ml of the challenge toxin solution (containing 50 × PD_{50}/dose). If it is necessary to determine the activity of the challenge toxin, allocate the 3 dilutions made from the challenge toxin solution, one to each of the groups of not fewer than 5 mice, and inject subcutaneously 0.5 ml of each solution into each mouse in the group to which that solution is allocated. The mice are examined over the following 96 hours (3 times on first 3 days and twice on last day), and when they show the earliest sign of paralysis of the left hind limb they are culled.

When lethal end-point is used, mice should be challenged subcutaneously with 0.5 ml of the challenge toxin solution (containing 50 × LD_{50}/dose). If it is necessary to determine the activity of the challenge toxin, allocate the 3 dilutions made from the challenge toxin solution, one to each of the groups of not fewer than 5 mice, and inject subcutaneously 0.5 ml of each solution into each mouse in the group to which that solution is allocated. The mice are examined over the following 96 hours (3 times on first 3 days and twice on last day), and the number of deaths is monitored and recorded.

Reading and interpretation of results

In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as those shown in the following scale:

This scale gives typical signs when injection of the challenge toxin is made in the dorsal region, close to one of the hind legs of mice. Grade T3 is taken as end-point, but with experience grade T2 can be used instead. Tetanus toxin produces, in the toxin-injected hind leg, paresis, followed by paralysis, that can be recognised at an early stage.

T1: slight stiffness of toxin-injected hind leg, only observed when the mouse is lifted by the tail;

T2: paresis of the toxin-injected hind leg, which still can function for walking;

T3: paralysis of the toxin-injected hind leg, which does not function for walking;

T4: the toxin-injected hind leg is completely stiff with immovable toes;

T5: tetanus seizures, continuous tonic spasm of muscles;

D: death.

Calculation of results

Appropriate statistical methods should be used to calculate the potency of the final bulk (see Chapter V). The NRA should approve the method and the interpretation of the results.

Data analysis for vaccine potency

Calculate the potency of the test vaccine relative to the potency of the reference preparation on the basis of the proportion of challenged animals surviving or without paralysis in each group of vaccinated animals, using a probit program for parallel line assay (see Chapter V).

If two successive doses for the same preparation each give extreme responses of 100%, the larger of the two doses is deleted from the analysis. Similarly, if two successive doses for the same preparation each give extreme responses of 0%, the smaller of the two doses is deleted from the analysis.

Validity of the test

In order for the test to be valid, all animals in the control group given the least dilute dose of challenge toxin must be effected (i.e. show local paralysis or death), whereas all control animals given the most dilute dose of challenge toxin must be unaffected. Additionally, for both the test vaccines examined and the reference preparation, the 50% protective dose should lie between the largest and the smallest dose of the preparations.

The test is valid if statistical analysis shows significant common slope of the log dose-response lines, without significant deviation from parallelism and linearity (Chapter V. describes possible alternatives if significant deviations are observed).

Where applicable, the number of affected animals in the unvaccinated control groups injected with the dilutions of the challenge toxin solution must indicate that the challenge was approximately $50 \times PD_{50}$/dose or $50 \times LD_{50}$/dose.

Retest

The test may be repeated, but, when more than one test is performed, the results of all valid tests must be combined in a weighted geometric mean estimate of potency, and the confidence limits calculated, using the appropriate method described in Chapter V.

Single dilution test

When a single dilution test is performed, the potency of the test vaccine should be determined to be significantly greater than the minimum requirement per human dose.

The single dilution assay requires previous demonstration of the following parameters:

- the potency of the test vaccine consistently and significantly exceeds the minimum requirements (lower 95% limit >40 IU/human dose).
- a significant regression has been demonstrated for the test vaccine over time.
- parallelism between test and reference vaccine has been demonstrated over time.

Validation and suitability

For every new product, verification of suitability of the method for the product must be confirmed. This is achieved by confirmation of positive regression to the dose-response and defined maximum and minimum responses for reference and test preparation so that estimates of suitable precision can be calculated. Furthermore, parallelism between the responses for the test preparation and reference must be established.

Data monitoring should be performed for the reference vaccine by monitoring the ED_{50} or LD_{50} or percent of animals responding at each immunising dose. Acceptable limits for the dose-response of the reference vaccine can be defined from historical data. Data monitoring should also be performed for the challenge toxin to confirm stability and confirm that the correct challenge dose of toxin is used.

References

1) Recommendations to Assure the Quality, Safety and Efficacy of Tetanus Vaccines. WHO Expert Committee on Biological Standardisation, Sixty-third report, Geneva, World Health Organisation, 2012 (WHO Technical Report Series, in press).

2) Assay of tetanus vaccine (adsorbed), general chapter 2.7.8 version 01/2008:20708. European Pharmacopoeia 7th Edition. Strasbourg, France: Council of Europe; 2011.

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III.1.3 Potency in guinea pigs by serology

Introduction

To assess the potency of tetanus vaccine by serology as an alternative to the guinea pig challenge method (Section III.1.2), antibody responses to tetanus toxoid induced in guinea pigs after 5 to 6 weeks are compared relative to the antibody response induced by a reference vaccine. For this comparison, a reference preparation of tetanus toxoid, calibrated in International Units (IU), and a suitable antibody detection method is required. ELISA (Section III.1.3.2) and ToBI (section III.1.3.3) are described in this chapter as suitable antibody detection methods [1-3].

III.1.3.1 Immunisation and bleeding of animals

Immunisation

Use guinea pigs, each weighing approximately 250-350 g, either of the same sex or with males and females equally distributed between groups. Groups should contain a number of animals sufficient to obtain results that fulfil the requirements for a valid assay as described in separate method sections. A further group of 2 non-immunised guinea pigs (non-injected or injected with diluent alone) may be used as negative controls. Alternatively, a control (negative) guinea pig serum may be used.

For each reference or test vaccine a series of up to 4 dilutions are made in a suitable diluent (such as 0.9% NaCl). For most combined vaccines, a series of 3-fold dilutions in the range of 1:1.67, 1:5, 1:15 and 1:45 has been found to be suitable for use with all methods detailed in sections III.1.3.2 and III.1.3.3 for testing both the diphtheria and tetanus components. However, the optimum dilution range will need to be confirmed in-house for a particular product.

- Divide the guinea pigs randomly into dose group cages.
- Leave the animals for up to a week to acclimatise.
- Under sterile conditions, prepare dilutions of test vaccine and reference in sterile diluent. Vaccine dilutions should be prepared immediately prior to use.

An example of a protocol to prepare dilutions of the test and reference vaccine:

The initial vaccine dilution is prepared from the pooled neat test vaccine or reference. Subsequent dilutions are made from the previous dilution in the series.

3 ml Vaccine	+	12 ml sterile diluent	DILUTION 1 (1 in 5)
5 ml Dilution 1	+	10 ml sterile diluent	DILUTION 2 (1 in 15)
5 ml Dilution 2	+	10 ml sterile diluent	DILUTION 3 (1 in 45)

- Inject subcutaneously 1.0 ml of vaccine dilution, reference vaccine dilution, or diluent (where negative control animals are included). Possible sites of subcutaneous injection of guinea pigs include the nape or the abdomen. Starting with the highest dilution (lowest concentration) only one syringe is needed for a single vaccine. Use a new syringe for each vaccine

Bleeding

After 5 to 6 weeks following immunisation the guinea pigs are bled individually under general anaesthesia. Methods of bleeding guinea pigs by cardiac puncture and vena saphena are provided for information. If other methods are followed, which use different anaesthetics, care should be taken to ensure that anaesthetic residues in the serum do not affect the viability of antibody detection method.

The bleeding should be performed following a suitable method in a clean and disinfected room to prevent contamination. Collect the blood in numbered glass tubes. Animals and blood samples should be clearly identified, for example with a letter for the dilution group and a number for each of the animals within that group.

An example of bleeding guinea pigs by cardiac puncture:

- Anaesthetise an animal with a mixture of Ketamine/Xylazine/Atropine. (Approximately 0.05 ml/100 g body weight, intramuscularly). The ratio of Ketamine : Xylazine : Atropine is 4 : 1.25 : 0.5 by intramuscular injection.
- When the animal is under deep anaesthesia lay it on its back and stretch the front legs in a cranial direction.
- Use a 10ml syringe with a 21G × 1.5 needle. The heart is reached by piercing the left ventricle through the chest wall at the sixth intercostal space, about one third of the ventral-dorsal distance. The puncture site can be confirmed manually, being the site at the chest with the strongest heartbeat. Attach the syringe and adjust the position of the needle up or down to achieve a good blood flow. Encourage blood flow by applying a gentle upward pressure on the syringe plunger.
- When a syringe if full empty the blood into the correct glass container. Reattach the syringe to the needle to continue collecting blood. Once blood flow reduces the 10ml syringe may be replaced with a 5ml syringe.
- When as much blood as needed has been obtained, animals are terminated by approved procedure.

An example of bleeding guinea pigs from the vena saphena

- Shave the thigh of the hind leg of the guinea pig 1 day before bleeding, paying particular attention to the hollow of the knee where the vena saphena is most easily observed.
- Approximately 15-20 minutes before bleeding inject Hypnorm® "Janssen" injection, (0.1 ml subcutaneously per 100 g body-weight) in the skin-fold in the axially region, using a 1 ml syringe fitted with a 23G × 1" needle.
- Hold the guinea pig to push the knee joint, to make the leg stretch out, and to pinch or massage the musculature on the back of the thigh and around the knee, in order to let the vena saphena be filled with as much blood as possible.
- Grease the skin at the site of puncture with, for example, Dow Corning Valve Seal.
- Pierce the vein carefully with a 21G × 1 1/2 " needle.
- The blood then starts to drip and can be collected directly into centrifuge tubes. The leg must be held tight all the time in order to maintain stasis. Massage during the blood taking may be advantageous.
- A second vena saphena puncture of the hind leg thigh for blood sampling may be necessary. Alternatively, vena saphena puncture of the other hind leg thigh can be performed.
- When as much blood as needed has been obtained, animals are terminated by approved procedure..

Separation of serum from blood

Serum from each individual animal is required. Suitable aliquots can then be made and stored at -20°C until they are examined for antibody activity. Avoid frequent freezing and thawing of serum samples - ideally each sample should not be freeze-thawed more than once. Once thawed, samples may be stored at +4°C for a limited period of time (up to two days), but stability should be confirmed in-house.

The following procedure ensures a serum yield of approximately 40% of the total blood volume taken. Alternative in-house procedures may be used.

References

1) Recommendation to Assure the Quality, Safety and Efficacy of Tetanus Vaccines. WHO Expert Committee on Biological Standardisation. Sixty-third report, Geneva, World Health Organisation, 2012(WHO Technical Report Series, in press).

2) Winsnes R and Hendriksen C. Collaborative study for the validation of serological methods for potency testing of tetanus toxoid vaccines for human use – summary of all three phases. Pharmeuropa Spec issue Biol 2002 (2) 1-92.

3) Assay of tetanus vaccine (adsorbed), general chapter 2.7.8 version 01/2008:20708. European Pharmacopoeia 7th Edition. Strasbourg, France: Council of Europe; 2011.

III.1.3.2 Titration of immune sera by ELISA

Introduction

WHO recommends that Enzyme Linked Immunosorbent Assay (ELISA) can be used with serology assays to determine the potency of tetanus vaccine for routine lot release provided that this assay is validated against the challenge assay or the toxin neutralisation test, using the particular product [1].

The level of anti-tetanus antibodies in the serum of immunised guinea pigs is directly proportional to the potency of the vaccine. Titrations of the guinea pig sera are made on ELISA plates coated with purified tetanus toxoid. A positive control guinea pig antiserum (calibrated in antitoxin IU/ml), and a negative control guinea pig serum are titrated alongside the serum samples on each plate to monitor the assay performance. Anti-tetanus antibodies bound to the toxoid are visualised by addition of a suitable detecting antibody directed against guinea pig IgG, followed by a suitable substrate. Optical density is measured and the antibody titre for each individual serum sample is calculated relative to the positive guinea pig antiserum. The potency of the test vaccine is then determined by comparing the antibody response in guinea pigs immunised with the test vaccine to the antibody response in guinea pigs immunised with the reference preparation, calibrated in IU [2-6].

Materials

Critical reagents

- Purified tetanus toxoid (NIBSC 02/126, or equivalent) for coating ELISA plates.
- Tetanus guinea pig antiserum, (NIBSC 98/572) is suitable as a positive control serum for monitoring performance of the ELISA assay and calculating relative antibody titres where appropriate. Another equivalent preparation may be used.
- Negative control guinea pig serum from non-immunised guinea pigs or guinea pigs injected with diluent is suitable. Guinea pig serum, NIBSC code 98/686 is also suitable for use as a negative control serum. Another equivalent preparation may also be used.

Animals

See Chapter II 1.3.1 for guinea pig immunisation and bleeding protocols.

Other reagents

- Peroxidase conjugate: peroxidase-conjugated rabbit or goat antibody, directed against guinea pig IgG.
- Carbonate coating buffer pH 9.6.
- Phosphate buffered saline (PBS) pH 7.4.
- Wash buffer: PBS + 0.05% Tween 20 (PBST).
- Blocking buffer: PBST + 5% dried skimmed milk.
- Sample buffer: PBST + 1% dried skimmed milk.

- Citric acid solution: 0.05M Citric acid, pH 4.
- Substrate: 2,2'–Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tablets, 10 mg.
- Substrate solution: Shortly before use dissolve 10 mg ABTS in 20 ml citric acid solution. Immediately before use add 5 µl hydrogen peroxide solution.

Equipment

- ELISA plates: 96 well, polvinyl chloride or polystyrene.
- Multichannel pipettes 100 µl.
- Pipettes, 10-1000 µl.
- Plate sealent.
- Microplate reader.

Procedures

Coating of immunoassay plate

Plates are coated with purified tetanus toxoid at a concentration of 0.5 Lf/ml as follows:

- Dilute the tetanus toxoid in carbonate coating buffer, pH 9.6.
- Add 150 µl to all wells.
- Seal plates and incubate at 4°C overnight in a humid container.

Plate washing

Plates are washed at the end of each incubation period before the addition of the next reagent. This can be done using a plate washer or by hand. The following describes one method for washing plates by hand:

- Prepare the PBST washing buffer.
- Discard the contents of the plates by inversion.
- Wash the plate by immersion into a container of PBST.
- Discard the contents of the plates.
- Repeat the washing procedure a further two times.
- Blot the plates dry against absorbent paper.

Blocking

Blocking is performed after the coating step once the initial reagent has bound to ensure that further reagents are not allowed to bind other than by immune adherence. Non-specific adhesion is reduced by inclusion in the buffer of an irrelevant protein at high concentration, such as bovine serum albumin (BSA) or casein. Detergents (usually Tween 20 at 0.5 ml/L) are also used to inhibit the adherence of hydrophobic molecules.

- Prepare the blocking buffer.
- Add 100 µl to all wells.
- Incubate at 37°C for 1 hour in a humid container.
- Wash the plates as described previously.

Antibody dilutions

One ELISA plate is sufficient for the testing of up to 9 test sera along with the positive control tetanus guinea pig antiserum in duplicate and a negative serum sample (see figure 1 for suggested plate layout). Test sera include those from guinea pigs immunised with either the test vaccine or the reference vaccine. Care should be taken to ensure samples are randomly placed on the plate and that the positions are varied from plate-to-plate to avoid bias.

The positive control guinea pig antiserum, test sera and negative control are diluted in sample buffer to a concentration which produces a suitable dose-response curve. The negative serum control should be diluted by no more than the lowest dilution of test sera. For NIBSC antiserum, 98/572, a suitable initial concentration is 0.007 U/ml. A dilution of 1/200 is suggested as a starting point for the test sera, but will vary depending on the concentration of vaccine used for immunisation of guinea pigs. A series of two-fold dilutions are performed starting at row A through to row H.

- Add 100 µl of sample buffer to all wells except row A.
- Add 200 µl of diluted positive control serum to row A in duplicate columns and 200 µl of diluted test sample or negative serum control to row A of a single column. Prepare a series of doubling dilutions in 100 µl volumes to row H. Discard 100 µl from the last wells.
- Incubate the plates for 2 hours at 37°C in a humid container.
- Wash the plates as described previously.

Figure 1: Suitable plate layout
(samples and reference are randomised across the plate)

	Neg	Test1	Test2	GP	Test3	Test4	Test5	Test6	Test7	GP	Test8	Test9
	1	2	3	4	5	6	7	8	9	10	11	12
A	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*
B	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
C	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4
D	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8
E	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.
F												
G												
H												

Neg = Negative guinea pig serum control
Test 1-9 = Test guinea pig sera
GP = Tetanus guinea pig control antiserum

* Pre-diluted serum sample added to row A of a column

Conjugate

To detect antibody bound to the tetanus toxoid an anti-guinea pig peroxidase conjugate IgG is added to all the wells.

- Dilute the conjugate to a suitable concentration in sample buffer (an optimum dilution should be established in-house or will be recommended by the supplier).
- Add 100 µl to all wells.
- Incubate at 37°C for 1 hour in a humid container.
- Wash the plates as described previously.

Substrate

- Prepare the substrate solution immediately before use as described in the materials section.
- Add 100 µl of substrate to all wells.
- Incubate the plates at room temperature for 15-20 minutes until colour develops.
- Following colour development read the plates at 405 nm using an ELISA plate reader

Calculation

Calculation of a relative antibody titre

Development of colour in the wells is theoretically directly proportional to the concentration of tetanus antibody in the sample.

The relative amount of tetanus antibody in the test sera with respect to the positive control tetanus guinea pig antiserum preparation may be obtained by parallel line regression analysis. Since dilutions are made on a log scale the most appropriate linear response is log optical density (OD) versus log dilution. Only those OD values which fall within the range of the linear part of the curve should be used to calculate titres. Analysis of variance is used to test the significance of departure of the dose-log response relationship from linearity and parallelism, using a suitable computer program.

The final relative antibody titre for each test sample is obtained after taking into consideration differences in the dilution factors of the positive control tetanus antiserum and test samples as necessary, as shown below.

$$\text{Sample U/ml} = \frac{(\text{Relative antibody titre estimate} \times \text{dilution factor of sample})}{\text{Dilution factor of antiserum}} \times [\text{Antiserum U/ml}]$$

Alternatively, individual antibody scores can be used, without expression relative to the positive control serum. However, results expressed relative to the positive control serum may produce final potency estimates with better precision since any plate variation will be accounted for and inclusion of a positive control serum has been shown to help in assay standardisation.

Calculation of vaccine potency

In order to use data to calculate the potency of the vaccine, dose-response curves for the reference and test vaccines are obtained by plotting the transformed (e.g. log or sqrt) antibody titres for individual serum samples against the log vaccine doses injected into the guinea pigs.

The potency of the test vaccine is calculated with respect to the reference vaccine preparation calibrated in IU by comparing both dose-response curves by parallel line analysis using a suitable computer programme (see Chapter V).

Validity of test

Calculation of antibody titres

- The positive control tetanus guinea pig antiserum must produce a dose-response curve, overlapping with the test sera, with the linear range covering at least 3 points.
- The assay should meet the criteria set in the parallel line assay for linearity and parallelism of the dose-response relationship.
- The negative control must not show a positive response.

Calculation of vaccine potency

- The analysis should show a significant common slope of the log dose-response lines, without significant deviation from parallelism or linearity at the 1% level ($p<0.01$).
- The precision of the assay is determined by the 95% confidence limits. The confidence limits (P=0.95) of the estimated potency are recommended to be within 50-200% of the estimated potency.
- Recommendations and specifications for 3 dilution and single dilution assays remain the same as for challenge assays.

Retest

- Some test serum samples may need to be retested in the ELISA in order to obtain a valid result for the antibody titre (or score). This may require samples to be tested at a lower or higher dilution than in the original assay. In some cases, this may lead to statistical outliers which can be considered for exclusion from the data set. In some cases, non-responders (i.e. titre below the limit of detection) can also be considered for exclusion from the data set.
- If significant regression of dose-response is not met, the assay must be repeated with different immunising doses.
- The test may be repeated, but when more than one test is performed the results of all valid tests must be combined in a weighted geometric mean for the final estimate of potency and the confidence limits calculated.

Validation

This method can be used for routine lot release provided that it is validated for a particular product against the functional assays [1] and as detailed in Chapter **V**.

Suitability of the method for a particular product is achieved by confirmation of positive regression to the dose-response and parallelism between the responses for the test and reference vaccine, such that valid estimates of suitable precision are obtained. It is recommended that the reference vaccine for use in this potency assay should be product-specific and calibrated against the WHO IS using the guinea pig challenge test [2-4]. A minimum of 5 and up to 10 individual potency tests should be used in assigning the value to the reference vaccine. Data from more than one laboratory may be combined to assign a value to the reference for use in ELISA potency test. The NRA or other competent authority should approve the reference and value assigned to the reference vaccine.

For testing of both tetanus and diphtheria components in combined vaccines in the serology assay, the common immunisation conditions must be shown to satisfy the requirements for validity described above for both components.

Data monitoring should be performed with the reference vaccine by monitoring the geometric mean titre at each immunising dose or by calculating the ED_{50} of the dose-response.

Stability of the reference vaccine must be established and data obtained must be used in support of establishing shelf-life and any replacement strategy.

References

1) Recommendation to Assure the Quality, Safety and Efficacy of Tetanus Vaccines. WHO Expert Committee on Biological Standardisation. Sixty-third report, Geneva, World Health Organisation, 2012(WHO Technical Report Series, in press).

2) Winsnes R and Hendricksen C. Collaborative study for the validation of serological methods for potency testing of tetanus vaccines for human use. Pharmeuropa Spec Issue Biol 2001: 2: 1-92.

3) Winsnes R, Sesardic D, Daas A and Behr-Gross M-E. (2004). Collaborative study for the validation of serological methods for potency testing of diphtheria and tetanus toxoid vaccines. Pharmeuropa BIO, 2003-2, 35-68.

4) Sesardic D, Winsnes R, Rigsby P and Behr-Gross M-E. (2003). Collaborative Study for the Validation of Serological Methods for Potency Testing of Vaccines Extended studies: Correlation of serology with *in vivo* toxin neutralisation. Pharmeuropa Bio, 2003-2, 69-75.

5) Assay of tetanus vaccine (adsorbed), general chapter 2.7.8 version 01/2008:20708. European Pharmacopoeia 7th Edition. Strasbourg, France: Council of Europe; 2011.

6) Rob Tierney, Jason Hockley, Peter Rigsby and Dorothea Sesardic (2010). International Collaborative Study: Calibration of Replacement WHO International Standard for Tetanus Toxoid Adsorbed. WHO/BS/10.2150.

III.1.3.3 Titration of immune sera by ToBI

Introduction

WHO recommends that Toxin Binding Assay (ToBI) can be used with serology assays to determine the potency of tetanus vaccine for routine lot release provided that this assay is validated against the challenge assay or the toxin neutralisation test, using the particular product [1].

The level of anti-tetanus antibodies in the serum of immunised guinea pigs is directly proportional to the potency of the vaccine. The ToBI test is based on the titration of immune guinea pig sera by incubation with a fixed amount of tetanus toxin or toxoid. The free toxin/toxoid is then measured by ELISA using an anti-tetanus antiserum for capture [2-6].

Titrations of the guinea pig sera are prepared in PBS and they are incubated with a known amount of tetanus toxin or toxoid in a round-bottomed microtitre plate. The next day, free or unbound toxin or toxoid is detected in an ELISA system using tetanus antitoxin bound to an ELISA plate as the capturing antibody. For this purpose the antitoxin/toxin reaction mixtures are transferred to the tetanus antitoxin-coated ELISA plates. The amount of toxin or toxoid bound is visualised by successive incubation with detecting agents, such as peroxidase-labelled, equine anti-tetanus IgG conjugate and tetramethylbenzidine substrate. Absorbance values are measured by means of an automatic plate reader and antibody scores determined for individual serum samples. The potency of the test vaccine is then determined by comparing the antibody scores in guinea pigs immunised with the test vaccine to the antibody scores in guinea pigs immunised with the reference preparation, calibrated in IU. A suitable reference preparation is the WHO IS for tetanus toxoid, or a product-specific reference calibrated in IU against the WHO IS in challenge assay.

Materials

Critical reagents

- Purified tetanus toxin or toxoid, with a defined value (Lf/ml).
- Equine anti-tetanus IgG (e.g. RIVM lot GTL34 at 200 IU/ml or equivalent), for coating ELISA plates.
- Peroxidase-conjugated equine anti-tetanus IgG (e.g. RIVM lot 32-33 or equivalent).
- 4 control guinea pig sera with known score (e.g. 0, 2, 4, 7), or positive control guinea pig serum (e.g. NIBSC 98/572) may also be used.
- Negative control guinea pig serum from non-immunised guinea pigs, or guinea pigs injected with diluent is suitable. Guinea pig serum, NIBSC code 98/686 is also suitable for use as a negative control serum. Another equivalent preparation may also be used.

Animals

See Chapter II 1.3.1 for guinea pig immunisation and bleeding protocols.

Other reagents

- Carbonate coating buffer, pH 9.6, 0.04 mol/L.
- Phosphate buffered saline (PBS), pH 7.2 - 7.4, 0.01 mol/L.
- Blocking buffer: PBS containing 0.5 % bovine serum albumin (BSA).
- Wash buffer: Tap water containing 0.05% Tween 80.
- Diluent: PBS containing 0.5% BSA and 0.05% Tween 80.
- Tetramethylbenzidine (TMB) solution in ethanol 6 mg/ml.
- Pehydrol 30% hydrogen peroxide solution.
- Sodium acetate substrate buffer, pH 5.5, 1.1 mol/L.
- TMB substrate solution: 90 ml distilled water, 10 ml of 1.1 mol/L sodium acetate buffer, 1.67 ml of TMB solution in ethanol, 20 µl of a 30% solution of hydrogen peroxide
- Stop solution: Sulphuric acid (H_2SO_4), 2 M.

Equipment

- Micro-titration plates: polystyrene round-bottomed plates, rigid.
- ELISA plates: polyvinyl chloride or polystyrene.
- Pipettes, 50–200 µl.
- Multichannel pipettes.
- Multiwell plate washer.
- Multiwell plate reader.

Procedures

Blocking of plates used for the pre-incubation step

Round-bottomed polystyrene "pre-incubation plates" should be blocked with an inert protein, such as BSA, to prevent non-specific binding of toxin and/or antibodies to the plate.

- Prepare a blocking buffer of 0.5% BSA in PBS, pH 7.2–7.4, using 16 ml/plate.
- Introduce 150 µl of the blocking buffer to each well.
- Incubate for one hour at 37°C in a humid atmosphere.
- Wash the plates using the plate washer.

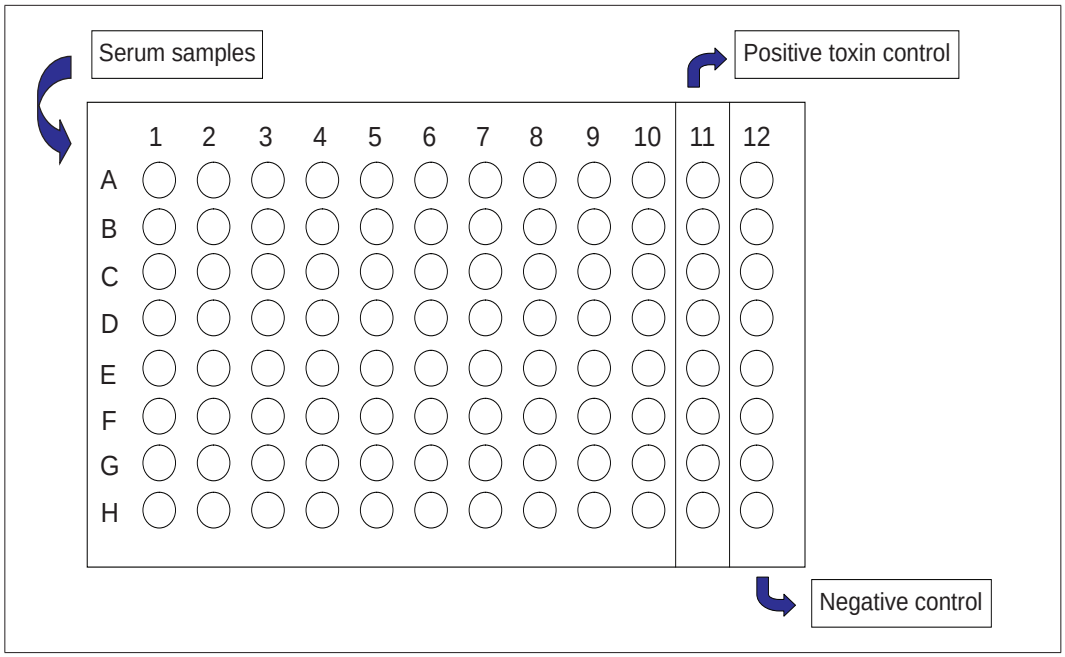
Preparation of serum-toxin/toxoid mixtures (Figure 2)

Using PBS, prepare a serial two-fold dilutions of each test and control serum in a microtitre plate. Care should be taken to ensure samples are randomly placed on the plate and that the positions are varied from plate-to-plate to avoid bias.

The dilutions chosen should contain a concentration of antibody that allows the determination of a score for the tetanus antitoxin titre (section III.1.3.1). Serum scores should range from 0 to 10. The accuracy of the test is improved if only a few 0 and 10 scores are registered as they represent scores of < 0 and > 10 respectively. Initially, serum samples should be tested neat. If the scores tend to be lower, one can decrease the amount of toxin (0.02 Lf/ml) in combination with a signal amplification system like biotin/streptavidin. If scores tend to be higher, serum samples can be prediluted 1/10 in PBS.

- Fill all the wells of the round-bottomed preincubation plates with 100 µl of PBS.
- Add another 50 µl of PBS to the wells of columns 1 and 12.
- Introduce 50 µl of test serum or control serum into the wells of column 1 using a micropipette and mix PBS and serum.
- Transfer 100 µl of the 1/4 diluted serum in the wells of column 1 to the adjacent wells of column 2 and mix.
- Transfer 100 µl of the 1/8 diluted serum in the wells of column 2 to the adjacent wells of column 3 and mix.
- Continue the transferring and mixing process through to column 10.
- Discard 100 µl of the contents of the wells of column 10 to equalise the volumes.
- Prepare a toxin solution containing the test dose of 0.1 Lf/ml in PBS, 3 ml/plate.
- Except for the wells of column 12 (negative control) introduce 40 µl of the toxin solution into all wells using a multichannel pipette.
- Shake the plates gently by tapping at the sides.
- Cover the plates with lids.
- Incubate overnight at 37°C in a humid atmosphere to prevent evaporation of the liquids in the wells.

Figure 2: Suggested plate lay out for preparation of serum-toxin/toxoid mixtures



Coating of ELISA plates

For the detection of free or unbound toxin or toxoid, ELISA plates should be coated with tetanus antitoxin.

- Prepare a coating solution of equine anti-tetanus IgG at a concentration of 1.0 IU/ml in carbonate buffer, pH 9.6.
- Introduce 100 µl of the coating solution into each well of the plate.
- Cover the plates with lids.
- Incubate overnight at 37°C in a humid atmosphere. Carry out the remaining procedures on the following day.

Blocking of ELISA plates

The coated ELISA plates should be blocked with an inert protein to prevent non-specific binding to the plate.

- Prepare a blocking buffer of 0.5% BSA in PBS, pH 7.2–7.4, 16 ml/plate.
- Wash the plates.
- Introduce 125 µl of blocking buffer into each well.
- Incubate for one hour at 37°C in a humid atmosphere.

Transfer of pre-incubation mixtures

- Volumes of 100 µl of the pre-incubation mixture are transferred from the pre-incubation plates to the corresponding wells of the prepared ELISA plates.
- Wash the ELISA plates with the plate washer.

- Using a multichannel pipette, transfer 100 µl of the contents of the wells in column 12 of the pre-incubation plate to the wells of column 12 of the ELISA plate.
- Using the same tips, transfer 100 µl from the wells of column 1 of the pre-incubation plate to the corresponding wells of the ELISA plate.
- Continue the transferring process through to column 11.
- Incubate for 2 hours at 37°C in a humid atmosphere.

Conjugate

- Prepare the diluent: PBS containing 0.5% BSA and 0.05% Tween 80 (blocking buffer plus Tween).
- Prepare a dilution of 1/4000 of the peroxidase-conjugated, equine anti-tetanus IgG in diluent.
- Wash the ELISA plates with the plate washer.
- Add 100 µl of the diluted conjugate to all wells.
- Incubate for 1.5 hours at 37°C in a humid atmosphere.

Substrate

Tetanus toxin bound to the antitoxin-coated ELISA plate is visualised by adding 100 µl of a (freshly) prepared TMB/ethanol substrate to all wells.

- Prepare the substrate as described under materials.
- Wash the plates with the plate washer.
- Add 100-µl quantities of the substrate to all wells.
- Incubate for 10 minutes at room temperature (20–25 °C).
- Stop the enzyme reaction by the addition of 100 µl of 2 M H_2SO_4 to each well. The colour will change from blue to yellow.
- Measure the absorbance at 450 nm using an automatic plate reader.

Calculation

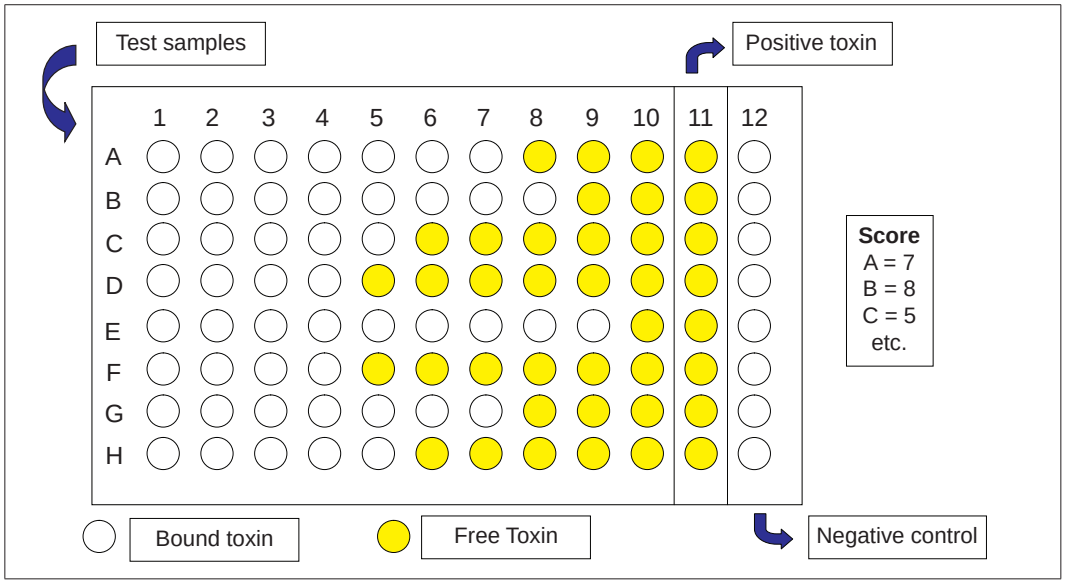
Determination of the score

A yellow colour indicates the presence of free toxin. The tetanus antitoxin titre in each serum sample is expressed as a score. For each serum, the score is the number of the last well having an optical density below the value representing 50% of the sum optical density (OD_{50}). The sum optical density is defined as the sum of the arithmetic mean OD of the positive control wells (A11–H11) and the arithmetic mean OD of the negative control wells (A12–H12). In this scoring system, which uses ten serum dilution steps, serum antitoxin scores range from 0 to 10. Calculate the OD_{50} value for each plate, applying the following formula:

[mean absorbance value of column 11 + mean absorbance value of column 12)] ÷ 2

Determine the score for each serum sample, which is the number of the last well with an absorbance value below the OD_{50} (Figure 3).

Figure 3: Example of results and determination of scores



Interpretation of controls:

- Columns 11 and 12 (positive and negative control) can be considered as indicators of an accurate performance of the test. Fluctuations of the OD values within these columns reflect inaccurate pipetting or washing procedures.
- The OD value of column 11 reflects the dose of toxin used. In general this value (mean of eight values) should be constant for each test performed.
- OD values in the negative control (wells in column 12) that are too high can be caused by non-specific binding (insufficient blocking or Tween), impurity of toxin or toxoid, incubation periods that were too long, inappropriate dilution of conjugate, or wrong composition of substrate.

Calculation of vaccine potency

The potency of the test vaccine is calculated with respect to the reference preparation, calibrated in IU, using parallel line regression analysis (see Chapter V). Analysis of variance is used to test the significance of departure of the dose-log response relationship from linearity and parallelism.

Validity

Calculation of antibody scores

- The estimated score obtained for each control serum in each assay should not deviate by more than one from the usually estimated score.
- Mean OD values of positive and negative control wells in each assay should be monitored using a Shewhart control chart. Plates with a mean positive or negative OD value outside 2 SD should be retested (Positive control wells are expected to have maximum OD levels 0.5-1.3).

- The assay should meet the criteria set in the parallel line assay for linearity and parallelism of the dose–response relationships.
- The Yg value of the reference vaccine (=mean score of all dilutions of the reference vaccine) should be within cumulative mean ± 2 SD of all previously performed tests.

Calculation of vaccine potency

- For test and reference vaccine serum samples there should be significant regression of dose-response.
- The assay should meet any validity criteria for parallel line analysis regarding linearity and parallelism of the dose-response relationships.
- Exclusion of individual outlier samples from the data set (vaccine dilution) may be considered in order to improve precision of the potency estimates.

Retest

- Some test serum samples may need to be retested in the ToBI in order to obtain a valid result for the antibody titre (or score). This may require samples to be tested at a lower or higher dilution than in the original assay. In some cases, this may lead to statistical outliers which can be considered for exclusion from the data set. In some cases, non-responders (i.e. titre below the limit of detection) can also be considered for exclusion from the data set.
- If significant regression of dose-response is not met, the assay must be repeated with different immunising doses in order to improve regression.
- The test may be repeated, but when more than one test is performed the results of all valid tests must be combined in a weighted geometric mean for the final estimate of potency and the confidence limits calculated.

Validation

This method can be used for routine lot release provided that it is validated for a particular product against the functional assays [1] and as detailed in Chapter **V**.

A minimum of three assays, with a suitable dose-response range, is likely to be required for validation. Suitable dose-response range and positive regression to the dose-response must be confirmed for reference vaccine and test preparation.

It is recommended that the reference vaccine for use in this potency assay should be product-specific and calibrated against the WHO IS in the guinea pig challenge test. A minimum of 5 and up to 10 individual potency tests should be used in assigning the value to the reference vaccine. Data from more than one laboratory may be combined to assign the value in reference for use in ToBI potency test. The National Control Laboratory or competent authority should approve the reference and value assigned to the reference vaccine.

Data monitoring should be performed with the reference vaccine by monitoring the geometric mean titre at each immunising dose, or by calculating the ED_{50} of the dose-response. Stability of the reference vaccine must be established and data obtained must be used in support of establishing shelf-life and any replacement strategy.

For testing of both tetanus and diphtheria components in combined vaccines in the serology assay, the common immunisation conditions must be shown to satisfy the requirements for validity described above for both components.

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III.1.4 Potency in mice by serology (ToBI)

Introduction

According to the latest revision of WHO recommendations, potency test for routine lot release can be performed by immunising mice as well as guinea pigs with appropriate dilutions of the calibrated reference preparation and the test vaccine. Titration of immune sera may be performed in vitro by ToBI test provided that product induces antibody response in comparison to the reference preparation and that assay has been validated against the functional test [1].

The current International Standard for Tetanus Toxoid adsorbed established in 2010 was assigned activity of 490 IU/ampoule based on calibration in guinea pig challenge assays and 260 IU/ampoule based on calibration in mice challenge assays[2]. It is known that qualitative differences between the standard and the test vaccine can be responsible for apparent differences in potencies when comparing results from tests performed in mice and guinea pigs.

Principle

The level of antitoxin/toxoid antibodies in the serum of immunised mice is directly proportional to the potency of the vaccine. The ToBI test is based on the titration of immune mouse sera by incubation with a fixed amount of tetanus toxin or toxoid. The free toxin/toxoid is then measured by ELISA using an antitoxin antiserum for capture [3, 4].

The potency of the test vaccine is then determined by comparing the antibody scores in mice immunised with the test vaccine to the antibody scores in mice immunised with the reference preparation, calibrated in IU. A suitable reference preparation is the WHO IS [2], or a product-specific reference calibrated in IU against the WHO IS in a challenge assay.

Materials

Critical reagents

- Purified tetanus toxin or toxoid, with a defined value (Lf/ml).
- Equine anti-tetanus IgG (e.g. RIVM lot GTL34 at 200 IU/ml or equivalent), for coating ELISA plates.
- Peroxidase-conjugated equine anti-tetanus IgG (e.g. RIVM lot 32-33 or equivalent).
- 4 control mouse sera with known score (e.g. 0, 2, 4, 7) or suitable mouse antiserum.
- Negative control serum, from non-immunised mice, or mice injected with diluent is suitable.

Other reagents

- Carbonate coating buffer, pH 9.6, 0.04 mol/L.
- Phosphate buffered saline (PBS), pH 7.2 - 7.4, 0.01 mol/L.
- Blocking buffer: PBS containing 0.5 % bovine serum albumin (BSA).
- Wash solution: Tap water containing 0.05% Tween 80.
- Diluent: PBS containing 0.5% BSA and 0.05% Tween 80.
- Tetramethylbenzidine (TMB) solution in ethanol 6 mg/ml.
- Pehydrol 30% hydrogen peroxide solution.
- Sodium acetate substrate buffer, pH 5.5, 1.1 mol/L.
- TMB substrate solution: 90 ml distilled water, 10 ml of 1.1 mol/L sodium acetate buffer, 1.67 ml of TMB solution in ethanol, 20 µl of a 30% solution of hydrogen peroxide.
- Stop solution: Sulphuric acid (H_2SO_4), 2 M.

Equipment

- Micro-titration plates: polystyrene round-bottomed plates, rigid.
- ELISA plates: polyvinyl chloride or polystyrene.
- Pipettes, 50–200 µl.
- Multichannel pipettes.
- Multiwell plate washer.
- Multiwell plate reader.

Procedure

Immunisation and bleeding of animals

Immunisation

A minimum of 3 serial dilutions of test and reference are injected into 3 groups of 10 mice. Mice are bled after 4-5 weeks. Immunisation doses will depend on the type of vaccine, but doses in the range of 13-2 IU/dose have shown to give reliable dose-response. Inject 0.5 ml of dilution subcutaneously into the right groin fold of the mouse. If starting with the highest dilution (lowest concentration), only one syringe is needed for a single vaccine. Use a new syringe for each vaccine.

Bleeding

4 to 5 week post immunization the animals are bled individually under narcosis from the retro-orbital plexus (right eye). Both ether and a mixture of halothane (1.8 %), nitrous oxide (N_2O) (9 L/min) and O_2 (3 L/min) may be used. When ether is used mice can be narcotized only one by one whilst halothane permits narcotization of e.g. a whole dose-group at the same time. In case other anaesthetics are used it should be checked whether anaesthetic residues in the serum do not affect the assay.

The bleeding procedure should be performed in a clean and disinfected workarea in order to prevent contamination. Blood is collected in glass tubes marked with an identification (e.g. numbered from 1 to 64).

An example of bleeding mice

1. Narcotise a mouse.
2. For right handers, lay the anaesthetised mouse on its left side.
3. Secure the mouse in the left hand by holding the loose skin of the neck between thumb and index finger. Fix the skin from the neck back to the tail between the other fingers.
4. Strengthen the grip around the neck so that the right eye is bulging.
5. Take a small pair of tweezers and remove the right eye with a quick pull.
6. Hold the eye-socket above a small glass tube and collect all the blood.
7. Stimulate bleeding by brushing from the tail towards the neck with the thumb of the right hand.
8. When bleeding has stopped, euthanise the mouse by dislocation of the neck.

Alternatively bleeding of mice is obtained by cardiac puncture method after administration of suitable injectable anaesthetic given i.p. A 25G needle attached to a 1ml syringe is introduced through the skin and between the ribs until it intersects with the heart and blood is drawn up. With this method up to 1.0 ml of blood can be obtained per mouse. The mouse is then confirmed dead by performing a cervical dislocation.

Separation of serum from blood

To obtain the maximum amount of serum, the following procedure may be followed:

1) Put the blood samples in a closed container.
2) Incubate for two hours at 37°C (activation of the clotting system).
3) Incubate for two hours at 4°C (shrinking of the clot, maximum release of serum).
4) Centrifuge the tubes for 20 minutes at 2 000 rpm (800 *g*).
5) Transfer the serum with a micropipette to a new tube.
6) A second centrifugation should be done if blood cells are still present in the serum.
7) Incubate the serum samples in a water-bath at 56°C for 30 minutes to inactivate complement.
8) Store the samples at -20°C until they are examined for antibody activity.

Blocking of plates use for the pre-incubation step

Round-bottomed polystyrene "pre-incubation plates" should be blocked with an inert protein, such as BSA, to prevent non-specific binding of toxin and/or antibodies to the plate.

- Prepare a blocking buffer of 0.5% BSA in PBS, pH 7.2–7.4, using 16 ml/plate.
- Introduce 150 µl of the blocking buffer to each well.
- Incubate for one hour at 37°C in a humid atmosphere.
- Wash the plates using the plate washer or other suitable methods.

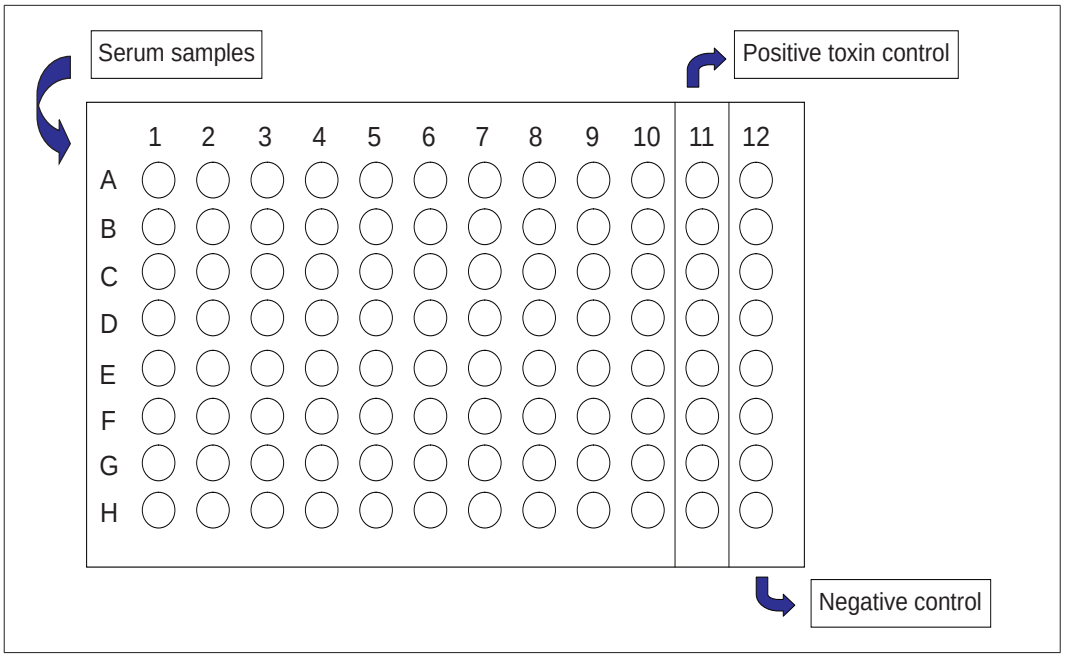
Preparation of serum-toxin/toxoid mixtures (Figure 4)

Using PBS, prepare a serial two-fold dilutions of each test and control serum in a microtitre plate. Care should be taken to ensure samples are randomly placed on the plate and that the positions are varied from plate-to-plate to avoid bias.

The dilutions chosen should contain a concentration of antibody that allows the determination of a score for the tetanus antitoxin titre (section III, 1.3.3). Serum scores should range from 0 to 10. The accuracy of the test is improved if only a few 0 and 10 scores are registered as they represent scores of < 0 and > 10 respectively. Initially, serum samples should be tested neat. If the scores tend to be lower, one can decrease the amount of toxin (0.02 Lf/ml) in combination with a signal amplification system, such as biotin/streptavidin. If scores tend to be higher, serum samples can be prediluted 1/10 in PBS.

- Fill all the wells of the round-bottomed preincubation plates with 100 µl of PBS.
- Add another 50 µl of PBS to the wells of columns 1 and 12.
- Introduce 50 µl of test serum or control serum into the wells of column 1 using a micropipette and mix PBS and serum.
- Transfer 100 µl of the 1/4 diluted serum in the wells of column 1 to the adjacent wells of column 2 and mix.
- Transfer 100 µl of the 1/8 diluted serum in the wells of column 2 to the adjacent wells of column 3 and mix.
- Continue the transferring and mixing process through to column 10.
- Discard 100 µl of the contents of the wells of column 10 to equalise the volumes.
- Prepare a toxin solution containing the test dose of 0.1 Lf/ml in PBS, 3 ml/plate.
- Except for the wells of column 12 (negative control) introduce 40 µl of the toxin solution into all wells using a multichannel pipette.
- Shake the plates gently by tapping at the sides.
- Cover the plates with lids.
- Incubate overnight at 37°C in a humid atmosphere to prevent evaporation of the liquids in the wells.

Figure 4: Suggested plate lay out for preparation of serum-toxin/toxoid mixtures



Coating of ELISA plates

For the detection of free toxin/toxoid, ELISA plates should be coated with tetanus antitoxin.

- Prepare a coating solution of equine anti-tetanus IgG at a concentration of 1.0 IU/ml in carbonate buffer, pH 9.6.
- Introduce 100 µl of the coating solution into each well of the plate.
- Cover the plates with lids.
- Incubate overnight at 37°C in a humid atmosphere. Carry out the remaining procedures on the following day.

Blocking of ELISA plates

The coated ELISA plates should be blocked with an inert protein to prevent non-specific binding to the plate.

- Prepare a blocking buffer of 0.5% BSA in PBS, pH 7.2–7.4, 16 ml/plate.
- Wash the plates.
- Introduce 125 µl of blocking buffer into each well.
- Incubate for one hour at 37°C in a humid atmosphere.

Transfer of pre-incubation mixtures

- Volumes of 100 µl of the pre-incubation mixture are transferred from the pre-incubation plates to the corresponding wells of the prepared ELISA plates.
- Wash the ELISA plates with the plate washer.

- Using a multichannel pipette, transfer 100 µl of the contents of the wells in column 12 of the pre-incubation plate to the wells of column 12 of the ELISA plate.
- Using the same tips, transfer 100 µl from the wells of column 1 of the pre-incubation plate to the corresponding wells of the ELISA plate.
- Continue the transferring process through to column 11.
- Incubate for 2 hours at 37°C in a humid atmosphere.

Conjugate

- Prepare the diluent: PBS containing 0.5% BSA and 0.05% Tween 80 (blocking buffer plus Tween).
- Prepare a dilution of 1/4 000 of the peroxidase-conjugated, equine anti-tetanus IgG in diluent.
- Wash the ELISA plates with the plate washer.
- Add 100 µl of the diluted conjugate to all wells.
- Incubate for 1.5 hours at 37°C in a humid atmosphere.

Substrate

Tetanus toxin or toxoid bound to the antitoxin-coated ELISA plate is visualised by adding 100 µl of a (freshly) prepared TMB/ethanol substrate to all wells.

- Prepare the TMB substrate as described in Materials: Other reagents.
- Wash the plates with the plate washer or other suitable methods.
- Add 100 µl quantities of the substrate to all wells.
- Incubate for 10 minutes at room temperature (20–25°C).
- Stop the enzyme reaction by the addition of 100 µl of 2 M H_2SO_4 to each well. The colour will change from blue to yellow.
- Measure the absorbance at 450 nm using an automatic plate reader.

Calculation

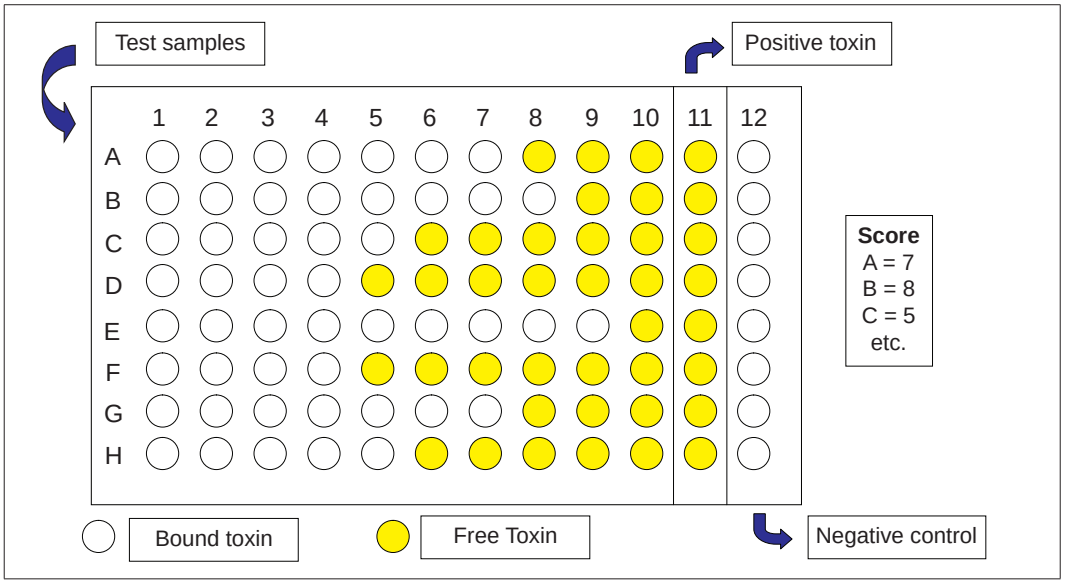
Determination of the score

A yellow colour indicates the presence of free or unbound toxin. The tetanus antitoxin titre in each serum sample is expressed as a score. For each serum the score is the number of the last well having an optical density below the value representing 50% of the sum optical density (OD_{50}). The sum optical density is defined as the sum of the arithmetic mean OD of the positive control wells (A11–H11) and the arithmetic mean OD of the negative control wells (A12–H12). In this scoring system which uses ten serum dilution steps, serum antitoxin scores range from 0 to 10. Calculate the OD_{50} value for each plate, applying the following formula:

[mean absorbance value of column 11 + mean absorbance value of column 12)] ÷ 2

Determine the score for each serum sample, which is the number of the last well with an absorbance value below the OD_{50} (Figure 5).

Figure 5: Example of results and determination of scores



Calculation of vaccine potency

The potency of the test vaccine is calculated with respect to the reference preparation, calibrated in IU, using parallel line regression analysis. Analysis of variance is used to test the significance of departure of the dose-log response relationship from linearity and parallelism.

Validity

Calculation of antibody scores

- The estimated score obtained for each control serum in each assay should not deviate by more than one from the usually estimated score.
- Mean OD values of positive and negative control wells in each assay should be monitored using a Shewhart control chart. Plates with a mean positive or negative OD value outside 2 SD should be retested (Positive control wells are expected to have maximum OD levels 0.5-1.3).
- The assay should meet the criteria set in the parallel line assay for linearity and parallelism of the dose–response relationships.
- The Yg value of the reference vaccine (=mean score of all dilutions of the reference vaccine) should be within cumulative mean ± 2 SD of all previously performed tests.

Calculation of vaccine potency

- For test and reference vaccine serum samples there should be significant regression of dose-response.
- The assay should meet any validity criteria for parallel-line analysis regarding linearity and parallelism of the dose-response relationships.
- Exclusion of individual outlier samples from the data set (vaccine dilution) may be considered in order to improve precision of the potency estimates.

Retest

- Some test serum samples may need to be retested in the ToBI assay in order to obtain a valid result for the antibody titre (or score). This may require samples to be tested at a lower or higher dilution than in the original assay. In some cases, this may lead to statistical outliers which can be considered for exclusion from the data set. In some cases, non-responders (i.e. titre below the limit of detection) can also be considered for exclusion from the data set.
- If significant regression of dose-response is not met, the assay must be repeated with different immunising doses in order to improve regression.
- The test may be repeated, but when more than one test is performed the results of all valid tests must be combined in a weighted geometric mean for the final estimate of potency and the confidence limits calculated.

Validation

This method can be used provided that it is validated for a particular product against the functional test [1] and as detailed in the Chapter V.

It is essential that the reference vaccine for use in this potency assay should be product-specific and calibrated against the WHO IS. A minimum of 5 and up to 10 individual potency tests should be used in assigning the value to the reference vaccine. Data from more than one laboratory may be combined to assign the value to the reference for use in ToBI potency test. The National Control Laboratory or competent authority should approve the reference and value assigned to the reference vaccine.

Data monitoring should be performed with the reference vaccine by monitoring the geometric mean titre at each immunising dose, or by calculating the ED50 of the dose-response. Stability of the reference vaccine must be established and data obtained must be used in support of establishing shelf-life and any replacement strategy.

For testing of both tetanus and diphtheria components in combined vaccines in the serology assay, the common immunisation conditions must be shown to satisfy the requirements for validity described above for both components.

References

1) Recommendation to Assure the Quality, Safety and Efficacy of Tetanus Vaccines. WHO Expert Committee on Biological Standardisation. Sixty-third report, Geneva, World Health Organisation, 2012(WHO Technical Report Series, in press).

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III.1.5 In vivo toxin neutralisation test in mice

Introduction

The purpose of the tetanus toxin neutralisation test (TNT) is to determine the functional potency of tetanus antitoxin by comparing the dose required to protect mice against the paralytic effects of a fixed dose of tetanus toxin, with the quantity of reference tetanus antitoxin required to give the same protection. For this comparison, a standard preparation of tetanus antitoxin, calibrated in International Units, and a suitable preparation of tetanus toxin are necessary. The TNT can be performed in mice using general principles described for potency testing of Tetanus Immunoglobulin products [1].

The assay is performed at different levels of sensitivity depending on the amount of antitoxin estimated to be present in the samples to be tested.

TNT can be used to confirm neutralising (functional) antibodies in serum in support of validation of *in vitro* methods (such as ELISA and ToBI) used for potency testing of tetanus immunoglobulins [2], and in support of validation of serological methods (such as ELISA and ToBI) used for potency testing of tetanus vaccines [3].

Materials

Critical reagents

First International Standard for Tetanus Antitoxin (TE-3), Human, containing 120 IU/ampoule [4,5] or a working reference standard calibrated against international or national standard.

Tetanus toxin of defined activity is used. The toxin is diluted to a concentration previously determined to be suitable, depending on the sensitivity of the assay used.

Animals

Adult, female mice, each weighing approximately 16-20 g, that have not previously been treated with any material that will interfere with the test, are suitable. Groups of up to 6 mice are used for each dilution of reference and test antitoxin.

Equipment

Class II safety cabinet.

Sterile syringes, 2.0 ml.

Sterile needles, e.g. 0.4 × 12 mm (27G × 1/2”).

Sterile glassware and plastic ware.

Volumes of less than 1 ml are dispensed using pipettes.

Buffers

Gelatine Phosphate Buffered Saline, pH 7.4 (GPBS) or Peptone PBS buffer, pH 7.4 or 0.9% sodium chloride are suitable.

All salts used in the preparation of buffers should be of minimum General Purpose Reagent grade, unless otherwise stated.

Procedures

The test serum samples are first screened by ELISA to determine the likely levels of antitoxin present prior to testing by toxin neutralisation assay *in vivo*. For testing of tetanus human immunoglobulin products, standard antitoxin is used at a sensitivity of 0.5 IU/ml in the toxin neutralisation assay, with the toxin used at a test dose of Lp/10. Usually, for other serum samples with antitoxin titres of >0.1 IU/ml, standard antitoxin is used at a sensitivity of 0.025 IU/ml in the toxin neutralisation assay, and for samples with <0.1 IU/ml by ELISA, standard antitoxin is used at a sensitivity of 0.0025 IU/ml. In each case, the test dose of toxin to be used has to be determined for each sensitivity of antitoxin used.

Determination of the test dose of toxin

The dose of tetanus toxin used in the assay is determined by titration against a reference tetanus antitoxin. For example, the Lp/10 test dose is used when performing the assay at a standard antitoxin sensitivity of 0.5 IU/ml. Lp/10 refers to the smallest amount of toxin causing paralysis when mixed with 0.1 IU of reference antitoxin. To determine the test dose of tetanus toxin [Lp/10 or L+/10, if lethal method is used], prepare a solution of the reference preparation of tetanus antitoxin such that it contains 0.5 IU of antitoxin per ml. Prepare mixtures of the solution of the reference antitoxin and of the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin, and sufficient volume of a suitable diluent to bring the total volume to 5.0 ml (see example in Table 1). Add diluent and toxin to tubes first, followed by the fixed volume of antitoxin. Allow the mixtures to stand at room temperature, protected from light, for up to 60 minutes. Using up to 6 mice for each mixture, inject a dose of 0.5 ml subcutaneously into the left thigh of each mouse. Observe the animals for 96 hours for paralysis. **The test dose of toxin is the quantity in 0.5 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, paralysis (or death if lethal method is used) in all of the mice injected with the mixture.**

Table 1. Example: Preparation of toxin/antitoxin mixtures for the determination of the Lp/10 test dose of tetanus toxin. Based on the example paralysis scores shown here the test dose (Lp/10) of tetanus toxin is 1/80.

	Volume (ml)					
	1	2	3	4	5	6
Reference Tetanus Antitoxin (0.5 IU/ml)	2.0	2.0	2.0	2.0	2.0	2.0
Tetanus Toxin	1.54	1.43	1.33	1.25	1.18	1.11
GPBS	1.46	1.57	1.67	1.75	1.82	1.89
Total (ml)	5.0	5.0	5.0	5.0	5.0	5.0
Reference Tetanus Antitoxin (IU/ml)	0.2	0.2	0.2	0.2	0.2	0.2
Reference Tetanus Antitoxin (IU/dose)	0.1	0.1	0.1	0.1	0.1	0.1
Toxin (dilution/dose)	1/65	1/70	1/75	1/80	1/85	1/90
Results (Example)						
Paralysis Score	+	+	+	+	-	-

Determination of potency of the antitoxin

To determine the potency of tetanus antitoxin when performing the assay at a standard antitoxin sensitivity of 0.5 IU/ml, prepare a solution of the reference preparation such that it contains 0.5 IU of antitoxin per ml. Prepare a solution of the test toxin such that it contains **5 test doses per ml**. Prepare mixtures of the solution of test toxin and the solution of the reference preparation such that each contains 2.0 ml of the toxin solution and one of a graded series of volumes of the reference antitoxin centred on that volume (2.0 ml) containing 1 IU antitoxin. Using a suitable diluent, bring the total volume to 5.0 ml (see table 2 for an example of toxin/antitoxin dilutions). Add diluent and antitoxin to tubes first followed by the fixed volume of tetanus toxin. In addition, prepare mixtures of the solution of test toxin and of the test serum sample to be examined such that each contains 2.0 ml of the toxin solution, one of a graded series of volumes of the sample to be examined and a sufficient volume of diluent to bring the total volume to 5.0 ml. Add diluent and antitoxin to tubes first followed by the fixed volume of tetanus toxin. Allow the mixtures to stand at room temperature, protected from light, for 60 minutes. Using up to 6 mice for each mixture, inject a dose of 0.5 ml subcutaneously into the left thigh of each animal. Observe the animals for 96 hours for paralysis.

Table 2. Example of the preparation of toxin/antitoxin mixtures for the in vivo toxin neutralisation test. The dilutions are centred on the dilution (number 4) that contains 1 IU antitoxin (0.2 IU antitoxin/dose).

	Volume (ml)					
	1	2	3	4	5	6
Reference Tetanus Antitoxin (0.5 IU/ml) (or test sample)	1.7	1.8	1.9	2.0	2.1	2.2
Tetanus Toxin (5 × Lp/10)	2.0	2.0	2.0	2.0	2.0	2.0
GPBS	1.3	1.2	1.1	1.0	0.9	0.8
Total (ml)	5.0	5.0	5.0	5.0	5.0	5.0
Reference Tetanus Antitoxin (IU/ml)	0.17	0.18	0.19	0.20	0.21	0.22
Reference Tetanus Antitoxin (IU/dose)	0.085	0.09	0.095	0.10	0.105	0.11
Toxin dose	Lp/10	Lp/10	Lp/10	Lp/10	Lp/10	Lp/10
Results (Example)						
Paralysis score (reference preparation)	+	+	+	+	-	-
Paralysis score (test preparation)	+	+	+	-	-	-

In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale describes the typical signs observed when the injection of the challenge toxin is made in the dorsal region, close to one of the hind legs. Grade T3 is taken as the end-point, but, with experience, grade T2 can be used instead. Tetanus toxin produces paresis in the toxin-injected hind leg, followed by paralysis that can be recognised at an early stage. The tetanus grades in mice are characterised by the following signs:

T1: slight stiffness of the toxin-injected hind leg, only observed when the mouse is lifted by the tail;

T2: paresis of the toxin-injected hind leg, which can still function for walking;

T3: paralysis of the toxin-injected hind leg, which does not function for walking;

T4: the toxin-injected hind leg is completely stiff with immovable toes;

T5: tetanus seizures, continuous tonic spasm of muscles;

D: death.

Calculation of Results

Test antitoxin that contains the largest volume of immunoglobulin that fails to protect the mice from paralysis contains 1 IU/ml of tetanus antitoxin.

In the example shown in Table 2, the dilution of the test antitoxin that contains the largest volume of immunoglobulin that fails to protect the mice from paralysis is dilution number 3, which contains 1.9 ml of test sample. This mixture therefore contains 0.53 IU/ml of tetanus antitoxin. Any initial dilution factors of the test sample must then be taken into account to obtain a final potency estimate in IU/ml.

Validity of the Test

The test is not valid unless all of the mice injected with mixtures containing 2.0ml or less of the solution of the reference preparation show paralysis, and all those injected with mixtures containing more do not.

References

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III.2 Specific toxicity

III.2.1 In vivo *test for absence of toxin and reversion to toxicity in guinea pigs*

Introduction

The purpose of the specific toxicity test for tetanus is to confirm freedom from residual toxin and reversion to toxicity in final bulk vaccines and/or bulk purified toxoid. The toxicity reversal test for tetanus is also suitable for the assessment of concentrated toxoid intermediate product, and is based on the measurement of specific toxicity following incubation of the test toxoid for a prolonged period of time at high temperature to ensure that no reversion of toxoid to toxin has occurred. The tests for specific toxicity and toxicity reversal are usually performed in guinea pigs by subcutaneous injection. Although mice are not as sensitive to tetanus toxin as guinea pigs, WHO allows the use of mice for the toxicity reversal test, subject to approval by the National Regulatory Authority.

The WHO specifies the use of the specific toxicity test for the control of bulk purified toxoid and final bulk vaccine, whereas the toxicity reversal assay is only used for the control of bulk purified toxoid (Revised Recommendation to Assure the Quality, Safety and Efficacy of Tetanus Vaccines. WHO Expert Committee on Biological Standardisation. Sixty-third report, Geneva, World Health Organisation, 2012(WHO Technical Report Series, in press) [1].

The European Pharmacopoeia (Ph Eur) also specifies the use of the specific toxicity test and reversal to toxicity for testing of purified toxoid whereas testing on final bulk can be omitted as part of routine lot release subject to validation [1, 2].

Materials

No standard reference material is available or necessary for specific toxicity and toxicity reversal tests.

Animals

Adult, guinea pigs of either sex, each weighing approximately 250-350g, that have not previously been treated with any material that will interfere with the test, are suitable. Groups of not less than 5 guinea pigs are used per test sample. The animals need preferably one week of acclimatisation before starting the test.

Equipment

Class II safety cabinet.

Sterile syringes, 5.0 ml.

Sterile needles, e.g. 0.5 × 16 mm (25G × 5/8”).

Sterile glassware and plastic ware.

Volumes of less than 1 ml are dispensed using pipettes.

Buffers

The buffer solution used for dilution of the bulk purified toxoid should be comparable to that used in the final vaccine (except for the presence of adjuvant).

All reagents used in the preparation of solutions should be General Purpose Reagent Grade, unless otherwise stated.

Procedure

Test on bulk purified toxoid

Specific toxicity test

1) Groups of not less than 5 guinea pigs are injected subcutaneously with 1 ml of the purified toxoid diluted to at least 500 Lf/ml in a suitable diluent.
2) Animals are observed for 21 days for signs of ill health and tetanus toxicity.

Reversion to toxicity

1) A solution of bulk purified toxoid, diluted in suitable diluent (e.g. PBS or physiological saline) to the same concentration as in the final vaccine (obtained from the manufacturer's documentation), is prepared.
2) The solution of 50 ml is incubated at +34°C to37°C for a period of 6 weeks (42 days). Similar dilution of toxoid held at +2-8°C for the same period of time may be tested as controls.
3) Groups of 5 guinea pigs are injected subcutaneously with one of each of the test samples (2 × 2.5 ml, equivalent to 10 single human doses using multiple injection sites).
4) The animals are observed for 21 days for signs of ill health.

Test on final bulk vaccine for vaccines containing tetanus component

Specific Toxicity Test

1) Groups of not less than 5 guinea pigs are injected subcutaneously with five times the single human dose (SHD) stated on the vaccine label. The 2.5 ml volume of undiluted final bulk is injected at 3 different sites on the guinea pig (2 × 1 ml and 1 × 0.5 ml).
2) Animals are observed for 21 days for signs of ill health and tetanus toxicity.

Validity of the test

The sample passes the specific toxicity test if none of the animals show specific signs or dies from, tetanus toxicity within 21 days of injection and if at least 80% (i.e. 4/5) of the animals survive the test period. Animals that die from whatever causes will need to be examined by autopsy.

The sample passes the reversal toxicity test if no toxicity is detected and if no animals show specific signs of tetanus within 21 days of injection.

In the case of tests where adverse effects are noted, any clinical signs or deaths must be fully reported at the time that they occur, and any reports from a veterinarian should be included in the vaccine lot documentation.

Retest

If more than one animal dies from non-specific causes within the test period, the test must be repeated once. If more than one animal dies in the second test, the sample does not comply with the test.

References

1) Recommendation to Assure the Quality, Safety and Efficacy of Tetanus Vaccines. WHO Expert Committee on Biological Standardisation. Sixty-third report, Geneva, World Health Organisation, 2012(WHO Technical Report Series, in press).

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III.3 Lf and identity

III.3.1 Flocculation test (Ramon and laser light scattering)

Introduction

Antigenic strength and purity of tetanus toxoid as well as content of toxoid (and toxin) in a sample can be expressed in flocculation units (Lf units). By definition, 1 Lf unit is the quantity of toxoid (or toxin) that flocculates in the shortest time with 1 Lf-equivalent of specific antitoxin. Lf unit of toxoid is defined by the WHO and current IS for tetanus toxoid for use in flocculation test has an assigned value of 690 Lf/ampoule, established in a collaborative study [1].

The current WHO minimum requirements for antigenic purity of tetanus toxoids have been set as not less than 1000 Lf units per milligram of protein (non-dialyzable) nitrogen for use in production of vaccines for human use [2]. Purified toxoid of equivalent or higher purity is recommended for use in the conjugation process during production of the conjugate vaccines.

The Ramon version of the flocculation test method [3] is generally used by vaccine manufacturers as an in-process control test and is applicable for use with purified tetanus toxoids of high concentration in Lf/ml. Unlike other immunological binding assays, the reaction in solution between the antigen and the antibody provides additional information on antigenic quality due to a correlation existing between time factor and quality of the antigen. The optimum concentration range of the assay is between 20 to 100 Lf/ml. When the expected antigen concentration is very low, i.e. below 5 Lf/ml, the measurement of toxoid concentration can be carried out using blend flocculation method involving comparison of the Lf value of a known toxoid and that of a mixture of the sample with the known toxoid. However other suitable methods can be more appropriate for detection of samples with low Lf/ml range (as detailed in sections III.3.2 and III.3.3).

Flocculation test can also be used to confirm Lf content in non-adsorbed and adsorbed vaccine [2]. However other suitable methods can be more appropriate and provide increased sensitivity (as detailed in sections III.3.2 and III.3.3).

Principle

This immunological binding assay in solution consists of the detection of a complex formed between the antigen and the antibody [3]. This assay, known as **Ramon assay,** is based on the observation by naked eye of a macroscopic flocculation complex. The time required for the formation of this complex depends on the ratio of toxoid and specific antitoxin. The time in minutes for the first flocculation to occur, is called the Kf value. Kf depends on both antigen and antitoxin concentration and it is assumed that the quality of the antigen also affects the Kf. At low concentrations or when poor-quality antigen is used the Kf value is high. The 1st tube in which flocculation appears is used to determine the Lf value of the sample. The "Limit of Flocculation" is defined as the antigen content forming 1:1 ratio against 1 unit of antitoxin [3, 4].

A new method has been developed in Japan involving **laser light-scattering** for a more accurate and objective detection of antigen-antibody complexes [5]. Particles in suspension are subjected to aggregometry. A laser beam is passed through the suspension. Scattered light intensity and signal counts are recorded. These values reflect size and number of detected particles, respectively.

Materials

Critical reagents

Tetanus toxoid: NIBSC 04/150 (2nd WHO International Standard of tetanus toxoid for use in flocculation test, with an assigned value of 690 Lf/ampoule [1]), or in-house equivalent preparation calibrated in terms of WHO IS, can be used. Each ampoule is reconstituted in PBS or NaCl then diluted from 30 to 50Lf/ml for Ramon flocculation test and from 25 to 200Lf/ml for laser light-scattering method.

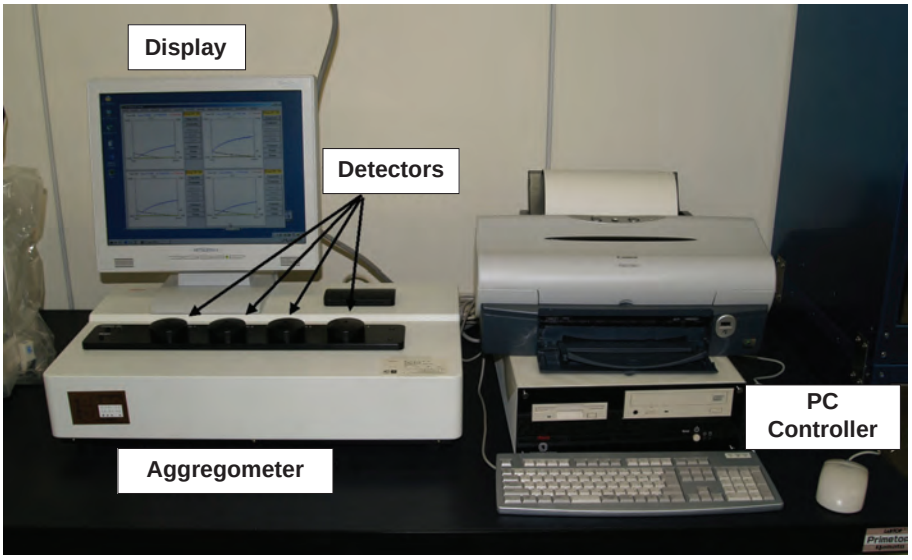
Tetanus antitoxin: reference tetanus antitoxin calibrated in IU/ml, NIBSC 66/021 (with an assigned value of 1400 IU/ampoule), or in-house equivalent, can be used. Each ampoule is reconstituted in PBS or NaCl to give 100 IU/ml. When used in the flocculation assay, the Lf equivalent (Lfeq.) value must be determined in-house.

Equipment for Ramon flocculation test

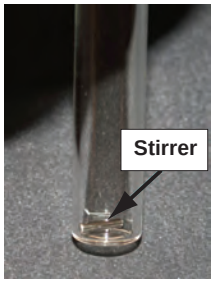
- micropipettes, 0.2-20µl
- micropipettes, 20-200µl
- micropipettes, 200-1000µl
- glass tube
- water bath
- clock

Equipment for laser light-scattering method

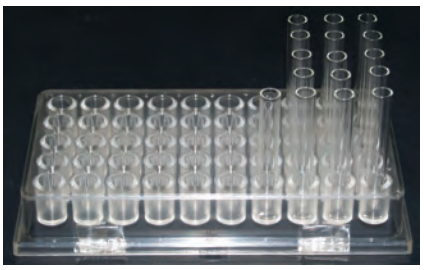
Figure 6: Apparatus: Laser light-scattering platelet aggregometer (Kowa model PA-20 or PA-200)



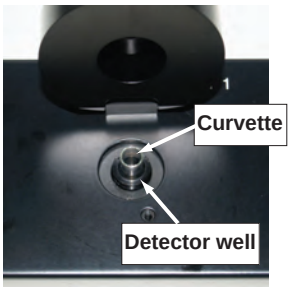
Cuvette Kowa 7 x 50 mm



Rack for Incubation



Cuvette and detector well



Procedures

Ramon method

Antitoxin Calibration

The results of the calibration of antitoxins in International Units (IU) against international antitoxin standards depend on the immunochemical method used. For this reason, antitoxins used for the Ramon assay must be directly calibrated against the current WHO IS [1] or national standard for tetanus toxoid for flocculation tests [1], using the principles described below. The concentration thus determined may be indicated in Lfeq./ml.

Reaction

Antitoxin is added in increasing concentrations to a series of glass tubes. The volumes in each tube at this stage should be the same, for example, 1 ml. The test sample is diluted to give an expected concentration of approximately 30 to 50 Lf/ml, and, for example, 1 ml aliquots of this dilution are dispensed into each tubes containing antitoxin. The final volume in tube must be the same. Each tube is mixed and then incubated between +30°C and +50°C in a water bath. Tubes are observed at regular intervals in order to see the flocculation reaction. The 3 first tubes to show flocculation are reported as well as the time (Kf) to develop the reaction.

Lf value of the test tetanus toxoid is then calculated from the concentration of tetanus antitoxin present in the 1st tube to show flocculation (see the example of a calculation below).

Calculation

Table 3. Example: Preparation of tubes containing an increasing amount of antitoxin with a fixed amount of tetanus toxoid test sample. The first three tubes to show flocculation are indicated (F1-F3).

Reagents	Tubes							
	A	B	C	D	E	F	G	H
Antitoxin (Lf eq.)	30	40	50	60	70	80	90	100
Antitoxin (ml)	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
PBS or NaCl (ml)	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
Toxoid (ml)	1	1	1	1	1	1	1	1
Flocculation sequence		F3	F1	F2				

The first tube to flocculate is the one that contains the amount of antitoxin closest in equivalence to the amount of antigen in the sample. The antitoxin content of this tube can be used to calculate the Lf of the sample. In the above example, tube C is the first tube where flocculation is observed and therefore it can be assumed that the Lf value of the diluted test sample is 50 Lf/ml. In order to obtain the Lf value of the undiluted product tested, dilution factor must be taken into account. In the example, if the toxoid has been diluted 1/10 prior to the reaction, its final Lf value is 500 Lf/ml. When two tubes flocculate simultaneously, the mean from the results obtained for the two tubes is given as the result.

If a more precise result is desired, the flocculation test can be repeated using a smaller range of concentration of the tetanus antitoxin. For example: 30, 35, 40, 45, 50, 55, 60 and 65 Lfeq. range can be used. Concentration can be narrowed to 2 or 3 Lfeq. increase from tube to tube. Additionally, more precision can be obtained by making allowances for the sequence of flocculation after the first tube. In the example shown, the second tube to flocculate is tube D, and therefore the final value for the diluted sample would be 55 Lf/ml. If the second tube to flocculate had been tube B then the final value would be 45 Lf/ml.

The time taken for the first tube to flocculate (Kf) is a useful indicator of the quality of the antigen and should be reported. Indeed, comparison can be made between two mixtures where the same concentration of antitoxin and toxoid is present but where the nature of the toxoid differs. If the flocculation time is higher for one of the mixtures, it indicates that the antigen present in that mixture has been damaged and is less likely to produce good antibody response.

Determination of tetanus toxoids with very low concentration - blend flocculation:

The Lf value for tetanus toxoids with very low concentration is assessed using the blend flocculation method. This involves the comparison of the Lf value of a reference toxoid with the Lf value of a mixture composed by the same reference toxoid and the sample to be tested.

Two series of tubes are prepared in parallel.

Lf value of the reference tetanus toxoid (Lf_{ref})

A first series of tubes is prepared.

Tetanus antitoxin is added in increasing concentrations to a series of tubes containing a constant amount of the reference tetanus toxoid.

Lf value of the reference tetanus toxoid (Lf_{ref}) is then calculated from the concentration of tetanus antitoxin present in the 1st tube to show flocculation.

Lf value of the mixture containing the reference and unknown tetanus toxoids (Lf_{ref+s})

A second series of tubes is prepared.

Tetanus antitoxin is added in increasing concentrations to a series of tubes containing a constant amount of the reference tetanus toxoid at 50 Lf/ml and 1.0 ml of the unknown tetanus toxoid to test. Final volume is 3.0 ml.

Lf value of the solution containing both the reference and the unknown tetanus toxoid (Lf_{ref+s}) is then calculated from the concentration of tetanus antitoxin present in the 1st tube to show flocculation.

Lf value of the unknown tetanus toxoids (Lf_s)

When a reference tetanus toxoid and an unknown tetanus toxoid are tested together, the mixture will flocculate as the sum of their values.

To calculate the Lf value of the unknown tetanus toxoids (Lf_s):

$$Lf_s = Lf_{ref+s} - Lf_{ref}$$

Laser light-scattering method

Antitoxin calibration

Antitoxins used for this assay must be directly calibrated against the current WHO IS for tetanus toxoid for flocculation tests [1]. The concentration thus determined may be indicated in Lf-equivalents per millilitre (Lfeq./ml).

Computer set up:

Measurement settings should be chosen and confirmed before starting the assays.

Sample preparation

Increasing volumes of antitoxin solution are added to a series of aggregometry cuvettes and final volume adjusted to 200 µl. Just before starting the measurement, 200 µl of tetanus toxoid solution is added. Final reaction volume should be 400µl (see table 2 below as an example). The tubes are immediately transferred and submitted to the measurement by laser light-scattering platelet aggregometer.

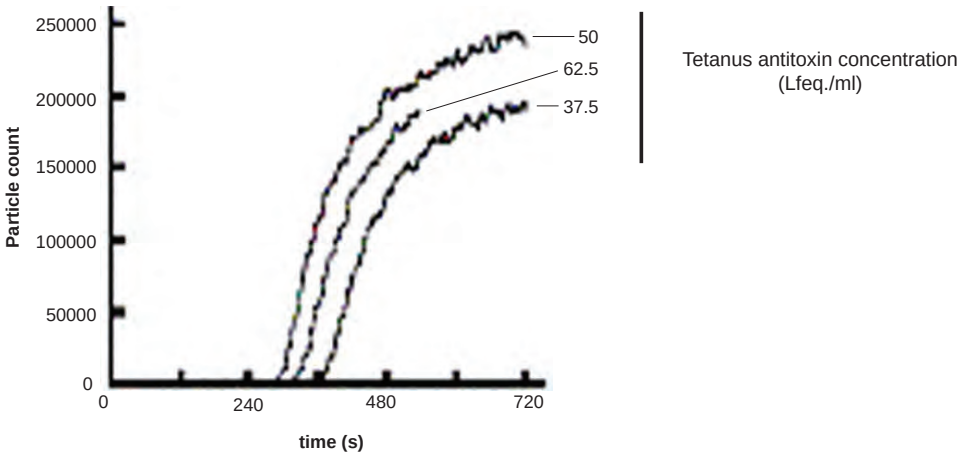
Table 4. Example: preparation of samples for laser light scattering. Cuvettes contain an increasing amount of antitoxin, with a fixed amount of the tetanus toxoid test sample.

Reagents	Tubes				
	A	B	C	D	E
Final antitoxin concentration (Lf eq./ml)	37.5	45	50	55	62.5
Added vol (µl) of antitoxin at 200 Lfeq./ml	75	90	100	110	125
PBS (µl)	125	110	100	90	75
Approx. 100 Lf/ml toxoid (µl)	200	200	200	200	200

Extraction of cut-off time

Data saved in PA-200/Version 3 binary format are converted to Excel csv format. Time must be recorded when total particle count reaches 50000. This will constitute the time point expressed in seconds

Figure 7: Example of the detection of particle formation by laser light-scattering platelet aggregometer. Tetanus antitoxin concentration varies from 37.5 to 62.5 Lfeq./ml and tetanus toxoid fixed concentration is approximately 50Lf/ml (reprinted from [5].with permission from Elsevier).



Plotting and Lf estimation

Log cut-off time and log tetanus antitoxin concentration are plotted on a scatter graph. A parabolic regression curve is obtained and the equation allows antitoxin concentration giving the minimum cut-off time to be calculated. This concentration directly corresponds to the Lf value of the tested tetanus toxoid.

Validity

Ramon flocculation method

Flocculation should be clearly visible.

Each test should be performed on at least 2 independent occasions. For each experiment, 2 series of tubes should be prepared and run in parallel. If results from the 2 independent experiments are not consistent with each other, flocculation test should be performed on a 3rd occasion with 2 series of tubes run in parallel.

Calibration of the antitoxin should be performed periodically against the current WHO International Standard for flocculation test [1].

Regarding blend flocculation, the mixture of reference and unknown tetanus toxoid must be homogeneous. If toxoids are not homogeneous, different zones of flocculation can be involved producing a confused pattern with two flocculation maxima.

Retest

If flocculation first appears in the tube with the highest or smallest tetanus antitoxin concentration, the assay must be repeated with either a different range of reference antitoxin or different dilution of tested toxoid.

Laser light-scattering method

Flocculation should be clearly recordable. Particle count should reach plateau at 150,000-200,000 counts.

Each test should include at least three antitoxin doses, in duplicate.

Calibration of the antitoxin should be performed periodically against the current WHO International Standard for flocculation test [1]. Using the model PA-200, four samples can be measured at one time (two samples for the model PA-20). To obtain enough data (6 data points), the combination of results from two to three series of assays is required.

Retest

If the earliest cut-off time is obtained in the tube with highest or smallest tetanus antitoxin concentration, the assay must be performed again with either a different range of reference antitoxin or different dilution of tested toxoid.

References

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2) Recommendation to Assure the Quality, Safety and Efficacy of Tetanus Vaccines. WHO Expert Committee on Biological Standardisation. Sixty-third report, Geneva, World Health Organisation, 2012(WHO Technical Report Series, in press).

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4) Lyng, J. and Bentzon, M.W. The quantitative estimation of diphtheria and tetanus toxoids. 1. The flocculation test and the Lf-unit. Journal of Biological Standardisation, 1987: 15: 27-37

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III.3.2 Radial immunodiffusion

Introduction

Single radial immunodiffusion (SRD) method is used as an identity test for adsorbed and combined vaccines containing tetanus components. The WHO and Ph Eur stipulate that each final lot of vaccine must be tested to confirm the presence of tetanus antigen [1, 2].

Vaccines adsorbed onto aluminium phosphate or on aluminium hydroxide have to be desorbed (eluted) from the adsorbent with either sodium citrate or sodium EDTA prior to test. The identity test can also be performed by Double Diffusion method (DD) [3].

The detection limit and linear dose-response range for tetanus toxoid in this assay is between 4 Lf/ml and 100 Lf/ml. SRD immunoprecipitation method is a highly robust and easy test to confirm identity of antigen in final product requiring only a small amount of vaccine [4]. SRD method can also be used to confirm consistency of the production process through the determination of antigen content in Lf/ml.

- SRD can be used for quantitative measurement of Lf/ml on non-adsorbed, purified tetanus toxoid intermediate as an alternative to flocculation test, as the difference between results from both methods does not exceed 10% [5].
- SRD could also be applicable for semi-quantitative measurement of Lf/ml on final adsorbed product, after desorption.

Principle

Single radial diffusion is a simple qualitative and quantitative immunodiffusion technique [1, 2] based on methods previously published by Melville-Smith [4]. A concentration gradient is established for the antigen diffusing from a well that has been cut into the gel medium containing the antitoxin at a comparatively low concentration. When the equilibrium between the antigen and antitoxin has been established, the circular precipitation area, originating from the well into which antigen has been added, is directly proportional to the concentration of the antigen in the gel.

For DD assay [3], tetanus antitoxin and the test antigen sample are loaded in opposing wells. Precipitation lines indicating presence of the antigen can be read before and after staining and thus confirming the identity of the sample.

Materials

Critical reagents

Tetanus toxoid: NIBSC 04/150 (2nd WHO IS of tetanus toxoid for use in flocculation test, with an assigned value of 690 Lf/ampoule [6]), or in-house equivalent, can be used. Each ampoule is reconstituted in a suitable diluent to give 100Lf/ml.

Tetanus antitoxin: NIBSC 66/021 (with an assigned value of 1400 IU/ampoule), or in-house equivalent, can be used. Each ampoule is reconstituted in PBS to give 100 IU/ml.

Diphtheria toxoid (used as a negative control): NIBSC 02/176 (2nd WHO IS of diphtheria toxoid for use in flocculation test, with an assigned value of 1100 Lf/ampoule [7]), or in-house equivalent, can be used. Each ampoule is reconstituted in a suitable diluent to give 100 Lf/ml.

Buffers

Gel: 1% Seakem Agarose in Phosphate Buffered Saline pH7.4.

Staining solution: Coomassie brilliant blue (0.2% w/v) in a solution of methanol (50%) and acetic acid (10%).

Destaining solution: methanol (50%); acetic acid (10%).

Equipment

- micropipettes, 0.2-20µl
- micropipettes, 20-200µl
- micropipettes, 200-1000µl
- disposable 5 ml plastic pipettes
- plastic immunodiffusion plates
- single well cutter
- glass plate
- weight (>1.5 Kg)
- filter paper
- paper towels
- glass bijoux

Procedures

Desorption of adsorbed vaccines

Vaccines are desorbed to remove the antigen from adsorbent before test. Desorption with 5-10% sodium citrate is suitable for most vaccines adsorbed onto aluminium hydroxide or aluminium phosphate adjuvants. 0.1% EDTA may be used as an alternative desorbent, particularly for vaccines containing reduced tetanus antigen contents. The examples of appropriate methods are provided but other methods may be used if shown to be suitable.

Desorption with sodium citrate

To 1.0 ml of vaccine, sufficient sodium citrate (solution or powder) is added to make final concentration of sodium citrate of 5-10%. The mixture is incubated for 16-20 hours at +37°C, followed by centrifugation until a clear supernatant, free from adsorbent, is observed. Supernatant is removed and stored for up to 1 week at +4°C until required for test.

Desorption with EDTA

1.5 ml of vaccine is centrifuged. The supernatant is removed and the pellet is resuspended in 0.5 ml of a freshly prepared solution of EDTA (1.12g/l EDTA, 88.2 g/l Na2HPO4). (1 volume of a 56 g/l solution of EDTA and 49 volumes of a 90 g/l solution of sodium hydrogen phosphate).

The mixture is maintained at +37°C for not less than 6 h and centrifuged. The clear supernatant is removed and stored for up to 1 week at +4°C until required for test.

Preparation of Controls

The test has a requirement for both positive and negative controls.

When testing the tetanus component of the vaccine, a positive control of tetanus toxoid (100 Lf/ml) and a negative control of diphtheria toxoid (100 Lf/ml) are required.

Gel Preparation

Molten seakem agarose (3.0 ml) is mixed with 100 µl of tetanus antitoxin (N.B: agarose should be allowed to cool down to 56°C before adding the antitoxin). The preparation is poured immediately onto a clean plastic immunodiffusion plate and allowed to set for 15-30 minutes. Suitable wells are cut into the gel to allow the addition of toxoid controls and test samples.

Identity test

Positive control tetanus toxoid (5 µl), negative control diphtheria toxoid (5 µl) and 5 µl of test sample are added to the wells. For some tetanus vaccines it may be necessary to add 20 µl of toxoid sample to generate an adequate ring of precipitation. The 20 µl should be added as 4 × 5 µl, allowing each 5 µl addition to absorb into the gel before addition of the next.

Quantitative and semi-quantitative test

The reference tetanus toxoid is diluted to, for example, 100, 40, 30, 20 and 10 Lf/ml, and 5µl of each dilution are added to wells. Then 5µl of test samples are added in other wells (see pattern 1).

Staining of precipitates

The plates are covered with lids and incubated for 16 to 48 hours in a humidified box at room temperature. Gels are then maintained on plates with elastic bands before being soaked in sterile PBS for 4 to 24 hours. The gels are removed from the plates and placed onto an alcohol-cleaned glass plate. Wells are filled with sterile PBS and covered with a sheet of filter paper and a thick layer of paper towels. A heavy weight is applied to flatten the gels onto the glass sheet for 20 minutes. The paper towels are changed and the weight is reapplied for a further 20 minutes. The filter paper and glass are then dried at +37°C for up to 10 minutes. After removing the filter paper, the glass plate and gels are allowed to air-dry for up to 60 minutes. Gels are stained by immersion in Coomassie blue for 3 minutes and then immersed in destaining solution until the background is clear. A blue precipitate ring for tetanus antigens should be clearly visible to the naked eye.

Validity and limit of detection

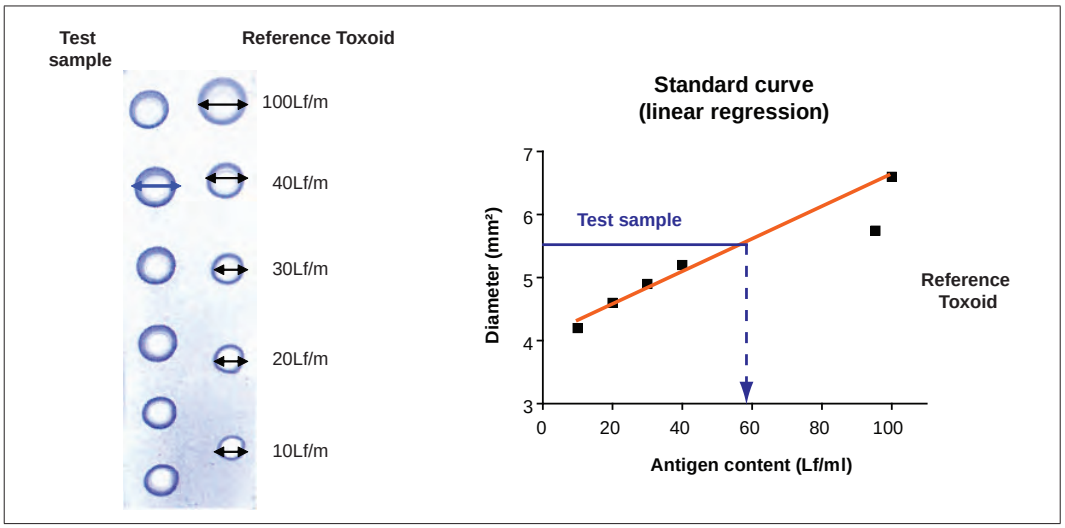
Identity test

For the identity test to be valid, the positive control toxoid should demonstrate a blue precipitation line or ring of identity. The negative control toxoid should show no blue precipitation line or ring of identity. If the test is invalid a retest should be performed.

Quantitative and semi-quantitative test

The standard curve is obtained by plotting the square diameter of the ring of precipitation against the corresponding concentration of the reference tetanus toxoid. The square diameter of the test sample is then plotted on the graph and its concentration can be determined.

Figure 8: Example of SRD results and plotted standard curve



The limit of detection for the assay is approximately 4 Lf/ml for tetanus toxoid.

SRD is a robust and easy method to confirm the presence of toxoids in final products, and provides a suitable alternative to the flocculation test. However, the test is not sufficiently sensitive to detect low levels of non-adsorbed antigen in vaccines (see section III.4.1, Capture ELISA).

References

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III.3.3 Rocket immunoelectrophoresis

Introduction

This electro-immunoassay method, often referred to as rocket immuno-electrophoresis, is used as an identity test for every batch of finished product of all combined vaccines containing tetanus components. The WHO and Ph Eur stipulate that each final lot of vaccine must be tested to confirm the presence of tetanus antigen in the final product [1, 2].

Vaccines adsorbed onto aluminium phosphate or on aluminium hydroxide have to be desorbed (eluted) from the adsorbent with either sodium citrate or sodium EDTA prior to the test.

The detection limit and linear dose-response range for tetanus toxoid in this assay is between 2.5 Lf/ml and 30 Lf/ml. This immunoprecipitation method is a robust and easy test to confirm identity in final product.

This method can also be used to confirm consistency of the production process through the determination of antigen content (Lf/ml).

- Rocket immuno-electrophoresis can be used for quantitative measurement of Lf/ml of non-adsorbed purified tetanus toxoid as an alternative to flocculation test as the difference between results from the two methods does not usually exceed 10% [3].
- Rocket immuno-electrophoresis could also be applicable for semi-quantitative measurement of Lf/ml on final adsorbed product.

Principle

This method allows determination of antigen content due to the difference of charge existing between toxoid and antitoxin [4, 5, 6]. The electrophoresis of the antigen to be determined is carried out in a gel containing a comparatively lower concentration of the corresponding antitoxin. The test material and dilution of a reference toxoid used for calibration are loaded into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear [4].

Materials

Critical reagents

Tetanus toxoid: NIBSC 04/150 (2nd WHO IS of tetanus toxoid for use in flocculation test, with an assigned value of 690 Lf/ampoule [7]) or in-house equivalent, can be used. Each ampoule is reconstituted in a suitable diluent to give a range of concentrations from 2.5 Lf/ml to 30 Lf/ml.

Tetanus antitoxin: NIBSC 66/021 (with an assigned value of 1400 IU/ampoule), or in-house equivalent, can be used. Each ampoule is reconstituted in PBS to give 100 IU/ml.

Buffers

Gel: 1% Seakem Agarose in PBS, pH 7.2 (Optimisation of pH may be required depending on the standard used).

Staining solution: Coomassie brilliant blue (0.3% w/v) in a solution of methanol (50%) and acetic acid (10%).

Destaining solution: methanol (50%); acetic acid (10%).

Equipment

- micropipettes, 0.2-20 µl
- micropipettes, 20-200 µl
- micropipettes, 200-1000 µl
- disposable 20 ml plastic pipettes
- immunodiffusion tray & apparatus
- well cutter
- glass plate
- filter paper
- paper towels
- electrophoresis power or apparatus

Procedures

Desorption of adsorbed vaccines

Vaccines are desorbed to remove antigen from adsorbent before test. Desorption with 5-10% of sodium citrate is suitable for most vaccines adsorbed onto aluminium hydroxide or aluminium phosphate adjuvants. 0.1% EDTA may be used as an alternative desorbent, particularly for vaccines containing reduced tetanus antigen contents. The examples of appropriate methods are provided but other methods may be used if shown to be suitable.

Desorption with sodium citrate

Add sufficient sodium citrate (solution or powder) to 1.0 ml of vaccine to make the final concentration of sodium citrate of 5-10%. The mixture is incubated for 16-20 hours at +37°C, followed by centrifugation until a clear supernatant, free from adsorbant, is observed. Supernatant is removed and stored for up to 1 week at +4°C until required for test.

Desportion with EDTA

1.5 ml of vaccine is centrifuged. The supernatant is removed and the pellet is resuspended in 0.5 ml of a freshly prepared solution of EDTA (1.12 g/l EDTA, 88.2 g/l Na_2HPO_4). (1 volume of 56 g/l solution of EDTA and 49 volumes of a 90 g/l solution of sodium hydrogen phosphate).

The mixture is maintained at +37°C for not less than 6 hours and centrifuged. The clear supernatant is removed and stored for up to 1 week at +4°C until required for test.

Gel Preparation

Molten seakem agarose (20 ml) is mixed with 200µl of diluted tetanus antitoxin. (N.B: agarose should be allowed to cool down to 56°C before adding the antitoxin). The preparation is poured immediately onto a clean immunodiffusion glass plate and allowed to set for 15-30 minutes. A row of wells is cut in the gel about 25mm from the edge. Those wells will further be loaded with standard toxoid and test sample. A control plate should be prepared without antitoxin in the gel as a negative control.

N.B: Optimisation of the gel pH is important (pH 7.2 is recommended). The migration will depend on the charge of the molecule. For migration to take place, toxoid and antitoxin must have a different charge.

Addition of Samples and Controls

Each concentration of the standard tetanus toxoid (10 µl) is loaded into different wells.

Test samples (10 µl) are loaded onto separate wells. The tray is then placed in the apparatus. Current is adjusted to 5 mA/cm and electrophoresis is conducted for about 45 minutes.

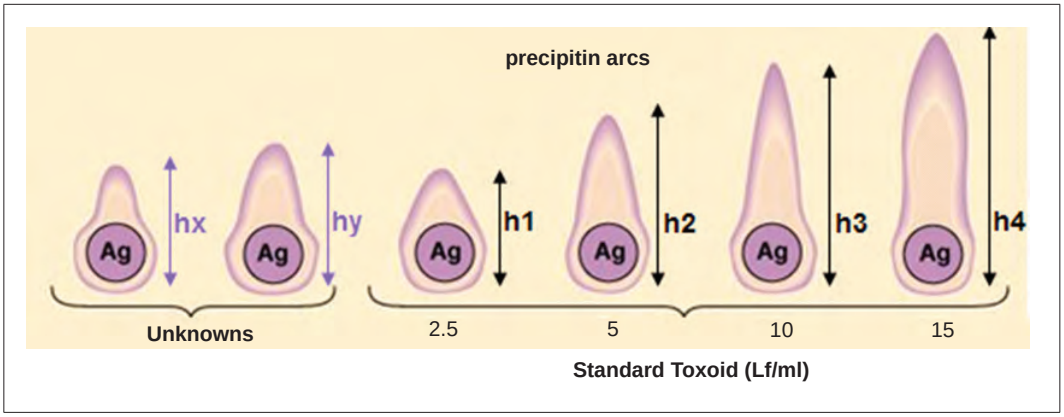
Reaction

The reaction should not occur more than 10-15 minutes after the samples have been loaded in order to reduce diffusion. The tray is connected to the buffer tank using filter paper as bridges from the buffer to the gel. The tray is positioned to have tetanus toxoid samples close to the anode in order to observe migration with the current. An electric potential difference of 180 V is applied to the gel at +4°C for 18 hours. A precipitin rocket arc will form when the reaction has occurred between the antitoxin and the antigen component present in the test sample.

Staining

Gels are stained by immersion in Coomassie blue for 3 minutes. The gels are then immersed in the destaining solution until the background is clear and the blue precipitate ring for tetanus antigens is clearly visible to the naked eye. The height of the rocket shape is measured as indicated in Figure 9.

Figure 9: Example of typical precipitin arcs in rocket immunoelectrophoresis test



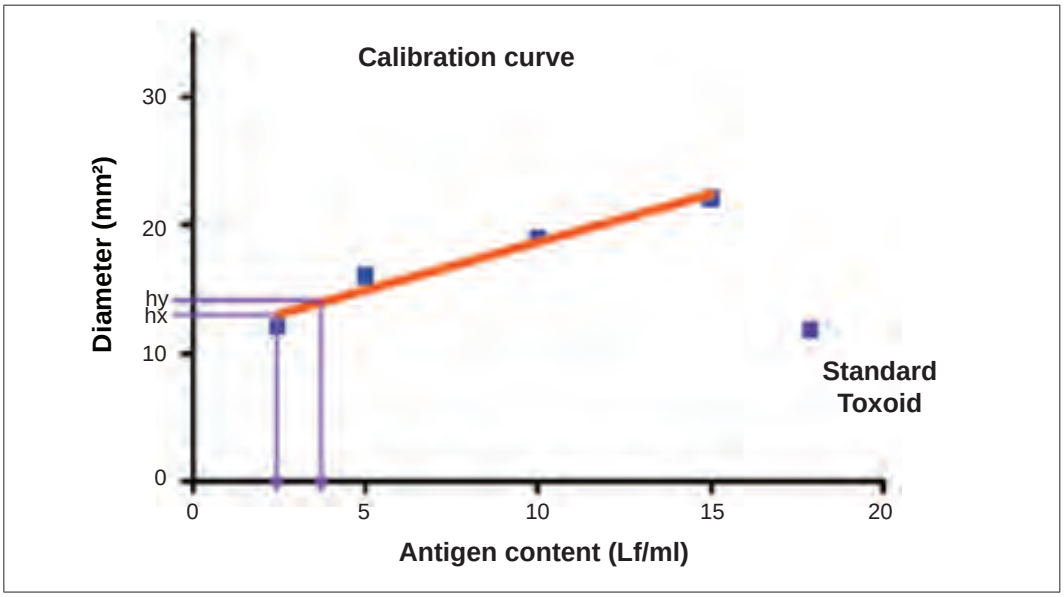
(Adapted from http:// lib.mcg.edu/edu/esimmuno/ch4/rocket.htm)

Validity and limit of detection

The diffusion zone diameter should be between 5 and 40 mm for the test to be valid.

Tetanus concentration for the test sample is calculated from the linear relationship existing between the height of the rocket and the concentration of standard tetanus toxoid, calibrated in Lf (Figure 10).

Figure 10: Typical standard curve for rocket immunoelectrophoresis



The limit of detection for the assay is approximately 2.5 Lf/ml for tetanus toxoids.

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III.4 Antigen content and degree of adsorption

III.4.1 Capture ELISA

Introduction

In vitro methods, such as Enzyme Linked Immunosorbent Assay (ELISA), can be used for monitoring batch-to-batch consistency of vaccines and are an essential tool for quality control [1]. Capture ELISA can be used to measure tetanus toxoid (antigen) in adsorbed vaccine final products with a limit of quantification of approximately 0.002 Lf/ml (dependant on the reference and other reagents used in the assay, particularly coating antibody). The high level of sensitivity makes this assay the only suitable method for monitoring the degree of adsorption in adsorbed tetanus vaccines.

Adsorbed tetanus vaccine consists of tetanus formol toxoid adsorbed onto a mineral carrier (adjuvant), and other antigens are present in combined vaccines. In order to measure the total tetanus antigen content, vaccines must first be desorbed to remove the adjuvant from the tetanus toxoid. The assay is also applied to the supernatant of the adsorbed vaccine sample in order to determine the non-adsorbed antigen content, thus allowing the degree of adsorption in vaccine preparations to be monitored. The degree of adsorption is product-specific, and is dependent on the type of antigen and adjuvant and other components present in the vaccine, and may be dependent on the age of formulation.

Principle

Capture ELISA is considered to be more specific than direct ELISA as it can be designed to detect functionally important epitopes on the tetanus toxoid. In this capture ELISA procedure, a monoclonal antibody directed against the Hc fragment of the tetanus toxoid is used to coat microplates. Test samples are then added in titration along with a tetanus toxoid reference, calibrated in Lf/ml. The amount of antigen bound to the monoclonal antibody is visualised by successive incubations with purified polyclonal antibody against tetanus toxoid, peroxidase-labeled antibody, and substrate [2]. Other capture ELISA formats, in which the polyclonal antibody against tetanus toxoid is used to capture the antigen, and the monoclonal antibody is used for detection, have been described [3]. Suitable protocols for both formats are provided here as examples.

The majority of vaccines are adsorbed and the assay requires any antigen to be eluted from the adsorbent before being tested in the ELISA.

Materials

Critical reagents

- Reference Tetanus toxoid, NIBSC 04/150 (2nd WHO IS of tetanus toxoid for use in flocculation test, with an assigned value of 690 Lf/ampoule [4]), or in-house equivalent, can be used. Each ampoule of 04/150 is reconstituted in 1.0 ml of a suitable diluent to give 690 Lf/ml.
- In-house positive control: Non-adsorbed tetanus toxoid with known antigen content (Lf/ml).

Capture or detecting antibodies

When monoclonal antibody is used as a capture antibody:

- Capture antibody: Purified rat anti-tetanus monoclonal antibody directed against Hc fragment: NIBSC monoclonal antibody NIBSC code no 10/134 (reconstituted in 0.5 ml water) is a suitable example.
- Tetanus toxoid detecting antibody: anti-tetanus IgG, prepared from serum of guinea pigs immunised with tetanus vaccine: NIBSC code 10/132 (reconstituted in 0.5 ml water) is a suitable example.

When monoclonal antibody is used as a detecting antibody [3]:

- Capture antibody: Horse hyperimmune serum used for tetanus prophylaxis in human.
- Tetanus toxoid detecting antibody: Purified anti-Hc fragment monoclonal antibody, prepared in mice.

Other reagents (commercially available)

- Peroxidase-conjugated rabbit or goat antibody, directed against guinea pig IgG or mouse IgG as required (see above).
- Carbonate coating buffer, pH 9.6.
- Phosphate buffered saline, pH 7.4 (PBS).
- Washing solution: PBS + 0.05% Tween 20 (PBST).
- Blocking buffer and sample buffer: PBST + 2.5% dried skimmed milk.
- 0.05M Citric acid substrate buffer, pH 4.
- Substrate: 2,2'–Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tablets, 10 mg.
- Substrate solution: Shortly before use, dissolve 10 mg ABTS in 20 ml citric acid solution. Immediately before use add 5 µl of hydrogen peroxide to the solution.

Or alternative substrate:

- Tetramethilbenzidine (TMB) substrate solution ready to use.
- TMB stop solution: 0.5 M sulphuric acid (H_2SO_4).
- Vaccine desorbent: 10% w/v tri-sodium citrate (final concentration in vaccine sample), or 0.1% w/v Disodium edetate (EDTA) (a solution of EDTA 1.12 g/L and disodium hydrogen phosphate (Na_2HPO_4) 88.2 g/L).

Equipment

- Microcentrifuge.
- ELISA plates: 96 well, Polyvinyl chloride or polystyrene.
- Multichannel pipettes, 100 µl.
- Pipettes, 10-1000 µl.
- Microplate sealant film.
- Microplate reader.

Procedures

Preparation of vaccine samples

Desorption of antigen using 10% w/v sodium citrate is suitable for most vaccines adsorbed onto aluminium hydroxide or aluminium phosphate adjuvants. 0.1% w/v EDTA may be used as an alternative desorbent. The following are examples of appropriate methods to use, but other methods may be used if shown to be suitable.

Desorption with 10% w/v sodium citrate

1 ml of homogenised vaccine is mixed with an equal volume of a 20% w/v solution of tri-sodium citrate. The sample is incubated for 16-20 hours at +37°C and then centrifuged. The supernatant is collected and retained at +4°C for testing the total antigen content. It is important to note that the sample is diluted 2-fold during the desorption process, which needs to be taken into consideration when calculating antigen content.

To measure the non-adsorbed antigen content, a duplicate sample of homogenised vaccine is centrifuged and the supernatant collected and retained at +4°C.

Supernatants can be kept at +4°C for up to 24 hours prior to the assay, but any prolonged length of storage has to be confirmed as suitable. Ideally, supernatants are tested on the same day.

Desorption with 0.1% w/v EDTA

1.5 ml of homogenised vaccine is centrifuged and the supernatant is retained (as above) for testing the non-adsorbed antigen content. After removing all of the supernatant, the pellet is re-suspended in 0.5 ml of a freshly prepared solution of EDTA (1.12 g/L EDTA, 88.2 g/L Na_2HPO_4). The sample is incubated for 16-20 hours at +37°C, centrifuged and the supernatant is retained (as above) for testing the adsorbed antigen content. It is important to note that the sample is re-suspended in 1/3 of its original volume which needs to be taken into consideration when calculating antigen content.

> For the EDTA desorption method, the total antigen content is obtained by adding the adsorbed value to the non-adsorbed value.

Capture ELISA protocol using a monoclonal antibody as the capturing antibody

Coating of immunoassay plates

The plates are coated with purified monoclonal antibody diluted to a suitable concentration in coating buffer.

- Dilute the monoclonal antibody in carbonate coating buffer, pH 9.6 for NIBSC code no 10/134 a suitable dilution is 1:200. For other preparations an optimum dilution should be established in house.
- Add 100 µl to all the wells.
- Seal the plates and incubate at +37°C for 2 hours or overnight at +4°C in a humid container.

Plate washing

Plates are washed at the end of each incubation period before the addition of the next reagent. This can be done using a plate washer or by hand. The following describes one method for washing plates by hand:

- Prepare the PBST washing buffer.
- Discard the contents of the plates by inversion.
- Wash the plate by immersion into a container of PBST.
- Discard the contents of the plates.
- Repeat the washing procedure a further two times.
- Blot the plates dry against absorbent paper.

Blocking the plates

Blocking is performed after the initial reagent has bound to the plate to ensure that further reagents are not allowed to bind other than by immune adherence. Non-specific adhesion is reduced by including in the buffer an irrelevant protein at high concentration, such as bovine serum albumin (BSA) or casein. Detergent (usually Tween 20 at 0.5 ml/L) is also used to inhibit the adherence of hydrophobic molecules.

- Prepare blocking buffer.
- Add 150 µl to all the wells.
- Incubate at +37°C for 1 hour in a humid container.
- Wash the plates as described in the previous section.

Antigen dilutions

One ELISA plate is sufficient for the testing of up to 4 vaccine samples in duplicate, along with an internal positive control and a reference preparation (see suggested plate layout in Figure 11). Vaccine samples are prepared as described above. The reference tetanus toxoid, internal positive control, and vaccine samples are diluted in sample buffer to a concentration which produces a suitable dose-response curve. For the internal positive control and reference tetanus toxoid (NIBSC, 04/150), a suitable initial concentration is 0.069 Lf/ml. A dilution of 1/200 is suggested as a starting point for desorbed antigen samples, and 1/10 for non-adsorbed antigen samples. These dilutions are product-dependant.

- Add 100 µl of sample buffer to all wells except row A.
- Add 200 µl of diluted reference, diluted positive control, or diluted test sample to row A in duplicate columns. Prepare a series of two-fold dilutions in 100 µl volumes to row H and discard 100 µl from the last well. For columns with the positive control, stop the two-fold dilution in row G to set blank, negative control wells.
- Seal the plates and incubate for 2 hours at +37°C in a humid container.
- Wash the plates as described in the previous section.

Figure 11: Suitable ELISA plate layout (The positive control is fixed in the outside columns and the duplicates for the reference and samples are spaced equally apart to minimise plate effects).

	PC	S1	S2	Ref	S3	S4	S1	S2	Ref	S3	S4	PC
	1	2	3	4	5	6	7	8	9	10	11	12
A	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*	1/1*
B	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
C	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4	1/4
D	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8
E	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.
F												
G												
H	BL											BL

Ref = Reference diphtheria toxoid

S = Vaccine sample (Non-adsorbed antigen, adsorbed antigen or total antigen)

PC = Positive control diphtheria toxoid

BL = Blank (Negative control)

* Pre-diluted reference toxoid, vaccine sample or positive control toxoid added to row A of a column.

Tetanus toxoid detecting antibody

Guinea pig anti-tetanus polyclonal IgG is used as the first detecting antibody in the assay, diluted in sample buffer prior to use.

- Prepare the dilution of guinea pig anti-tetanus IgG in sample buffer (for NIBSC code no 10/132 a suitable dilution is 1:200. For other preparations an optimum dilution should be established in-house).
- Add 100 µl to all the wells.
- Seal the plates and incubate at +37°C for 2 hours in a humid container.
- Wash the plates as described in the previous section.

Detecting conjugate antibody

To detect the polyclonal antibody bound to the tetanus toxoid, an anti-guinea pig peroxidase conjugate IgG is added to all the wells.

- Dilute the conjugate in sample buffer (an optimum dilution should be established in-house).
- Add 100 µl to all the wells.
- Seal the plates and incubate at +37°C for 1 hour in a humid container.
- Wash the plates as described in the previous section.

Substrate

- Prepare the citric acid substrate solution immediately before use (see materials section).
- Add 100 µl of the substrate to all the wells.
- Incubate the plates at room temperature for 15-20 minutes until colour develops.
- Following colour development, read the plates at OD 405 nm using a microplate plate reader.

Capture ELISA protocol using a monoclonal antibody as the detecting antibody.

Coating of immunoassay plates

Plates are coated with a hyperimmune anti-tetanus serum (calibrated in IU/ml), at an optimum concentration established in-house.

- Dilute the hyperimmune anti-tetanus serum in carbonate coating buffer, pH 9.6.
- Add 200 µl to all the wells.
- Incubate overnight at room temperature with agitation.

Plate washing

Plates are washed as described in the previous section.

Plate blocking

Plates are blocked as described in the previous section, using 200 µl blocking agent instead of 150 µl, and incubating at room temperature with agitation.

Antigen dilutions

Reference tetanus toxoid, the internal positive control, and vaccine samples are diluted in sample buffer to a concentration which produces a suitable dose-response curve. For the internal positive control and reference tetanus toxoid (NIBSC, 04/150), a suitable initial concentration is 0.25 Lf/ml. A dilution of 1/64 is suggested as a starting point for desorbed antigen samples, and 1/4 for non-adsorbed antigen samples. These concentrations are product-dependant. See Figure 1, for a suitable plate layout.

- Add 150 µl of sample buffer to all wells except row A.
- Add 300 µl of diluted reference, diluted positive control, or diluted test sample to row A in duplicate columns. Prepare a series of two-fold dilutions in 150 µl volumes to row H and discard 150 µl from the last well. For columns with the positive control, stop the two-fold dilution in row G to set blank, negative control wells.
- Incubate for 1 hour at room temperature with agitation.
- Wash the plates as described in the previous section.

Tetanus toxoid detecting antibody

The anti-Hc fragment monoclonal antibody is used as the primary detecting antibody in the assay, diluted to an optimum concentration established in-house.

- Prepare the dilution of anti-Hc fragment monoclonal antibody in sample buffer.
- Add 150 µl to all the wells.
- Incubate at room temperature for 1.5 hours with agitation.
- Wash the plates as described in the previous section.

Detecting conjugate antibody

To detect the anti-Hc monoclonal antibody bound to the tetanus toxoid, an anti-mouse Fc specific peroxidise conjugate IgG is added to all the wells.

- Dilute the conjugate in PBS to an optimum concentration established in-house.
- Add 150 µl to all the wells.
- Incubate at room temperature for 1 hour.
- Wash the plates as described in the previous section.

Substrate

- Add 150 µl of the TMB substrate solution to all the wells.
- Stop the enzymatic reaction after 5-10 minutes at room temperature, when an intensive blue colour appears, by adding 50 µl H_2SO_4 stop solution.
- Following a yellow colour development, read the plates at OD 405 nm using a microplate plate reader.

Calculation

The development of colour in the wells indicates the presence of tetanus toxoid. The relative tetanus antigen content of vaccine samples with respect to the reference tetanus toxoid preparation may be obtained by parallel line regression analysis. Since dilutions are made on a log scale, the most appropriate linear response is log optical density (OD) versus log dilution. Only those OD values which fall within the range of the linear part of the dose-response curve should be used to calculate the titres. Analysis of variance is used to test the significance of departure of the dose-log response relationship from linearity and parallelism.

Relative antigen content estimates are then converted to Lf/ml values by multiplying the estimates by the concentration of the reference tetanus toxoid in the stock solution, taking into consideration differences in the dilution factors of the reference and samples as necessary. For samples desorbed using EDTA, antigen content estimates have to be divided by 3 prior to calculation due to the fact that samples are concentrated 3-fold in the desorption step. For total antigen content samples, desorbed with a solution of 20% sodium citrate, antigen content estimates have to be multiplied by 2 prior to calculation due to the fact samples are diluted 2-fold in the desorption method.

$$\text{Sample Lf/ml} = \frac{(\text{Antigen content estimate} \times \text{dilution factor of sample})}{\text{Dilution factor of reference}} \times [\text{Reference Lf /ml}]$$

Once Lf values have been determined for all vaccine samples, the degree of adsorption may be calculated using the following formula:

$$\text{Percent adsorbed (\%)} = \frac{\text{Total antigen Lf/ml} - \text{non adsorbed antigen Lf/ml}}{\text{Total antigen Lf/ml}} \times 100$$

NB. if using EDTA desorption method, total antigen is obtained by adding adsorbed and non adsorbed antigen values.

Validity of test

The value of the internal positive control must be within the established limits (product-specific).

The negative control (blank) must not show a positive response higher than the last dilution of the reference tetanus toxoid.

The reference tetanus toxoid standard curve and each test sample must show a suitable dose-response, with an overlapping linear range covering at least 3 points.

The assay should meet the criteria set in the parallel line assay for linearity and parallelism of the dose-response relationship.

Retest

If duplicates from the same sample show significant deviation from the mean, the sample is re-tested.

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Contents

Chapter IV: Testing for whole cell pertussis vaccine

IV.1 Kendrick test

Principles:

The current pertussis potency test for whole-cell vaccine is based on the intracerebral mouse protection test as described by Kendrick (1947). The predictive value of this test for the protective activity of the vaccine in man was investigated by the Medical Research Council (1956). The Kendrick test is an assay designed to estimate the potency of pertussis containing vaccines on the basis of their ability to protect mice against intra-cerebral challenge with virulent *Bordetella pertussis*. The potency is expressed in International Units (IU) calculated by comparing the effective dose of the test vaccine to the reference vaccine.

The general guidance on how to perform the test is given below as an example. Individual laboratories may adopt these procedures according to their own conditions.

Materials:

Animals:

Healthy male or female mice from a strain, all of the same sex, 3–4 weeks old and weighing not less than 10 g and not more than 18 g, but all within a weight range of 4 g (this weight range should not vary from assay to assay). If there is a need to use mice of both sexes and different weight described above, they should be distributed equally throughout the test and the sexes segregated. Validation needs to be carried out and should give appropriate results.

Media and reagents:

Currently two types of agar media are used to grow *Bordetella pertussis.*

Bordet Gengou Agar (Copenhagen)

Ingredients

Distilled water	4000 ml
NaCl	24 g
Glycerol	160 ml
Potatoes	2000 g
Beef broth	6000 ml
Bacto Agar	210 g
Norit (Carbon)	40 g
Fresh sheep blood (or other animal blood e.g. horse)	120 ml

Peel the potatoes, wash and slice. Dissolve and boil the potatoes in this solution until the slices fall apart. Adjust the volume for evaporated water. Filter through to remove coarse materials. Dissolve the potato extract. Adjust pH to 7.0-7.5 with about 10 ml 1 M NaOH. Sterilise for 15 min. at 120°C. Put cotton wool plugs on stock bottles. Let them cool to about 50° C and add aseptically 120 ml of fresh sheep blood or any other suitable animal blood and mix it thoroughly.

Alternatively, commercial B.G. agar base are also used widely.

Bordet - Gengou medium (B.G. Medium)

Proteose peptone	10 g
Potatoes, infusion form	125 g
Glycerol	10 g
NaCl	5.6 g
Bacto agar	15 g
Distilled water to make	800 ml
Defribrinated sheep blood	200 ml

Procedure:

1) Dissolve proteose peptone, potatoes and glycerol in distilled water.
2) Adjust the volume with distilled water to 800 ml.
3) Add sodium chloride and stir vigorously to dissolve it.
4) Add agar, stir vigorously and boil to dissolve it.
5) Adjust the pH to 7.2 by using 1 N NaOH solution
6) Autoclave at 121°C for 20 minutes and cools it to 45°C
7) Aseptically add 200 ml of defibrinated sheep blood to the basal medium and mix (preheat the blood before mixing with the medium and keep at 45-50°C in water bath with stirring while dispensing).
8) Dispense 15-18 ml into sterile petri dishes.
9) Stand for overnight at room temperature.
10) Incubate at 36°C for 24 hours in inverted position.
11) Check for any contamination.
12) Store in cold room (2-8°C).

Charcoal Agar (CA)

CA Base	12.75 g
Distilled Water	250 ml
Defibrinated horse blood	25 ml

Sterilise by autoclaving at approximately 121°C for 15 minutes and allow to cool to about 50°C. Add, aseptically, 25 ml sterile defibrinated sheep blood or any other suitable animal blood and pour 15-20 ml aliquots into 9 cm Petri dishes and store at +2 to +8°C for up to 6 weeks.

Measure the final pH, this should be 7.4±0.2. The ability to support growth of the challenge strain 18.323, and the sterility of the plates, should also be checked. This will be carried out for each batch.

Phosphate buffered saline (PBS) pH 7.2 – 7.4:

NaCl	0.75 g
KCl	1.44 g
$Na_2HPO_4.12H_2O$	0.125 g
KH_2PO_4 (anhydrous)	10 g

Dissolve to a final volume of 1 litre in distilled water. Decant 100 ml into 150 ml glass bottles and sterilize by autoclaving at approximately 121°C for at 15 minutes. Store at +2 to +8°C for up to 2 years.

Check the post autoclave pH (7.2-7.4).

1% Casein:

NaCl	6 g
Casamino acid (Acid-hydrolyzed Casein)	10 g
Distilled water	1000 ml

Dissolve in 1 litre of distilled water and adjust pH to 7.1±0.2 with 1 M NaOH. Decant 100 ml into 150 ml glass bottle and sterilize by autoclaving at approximately 121°C for 15 minutes. Store at +2 to +8°C for up to 1 year.

Vaccine Reference preparation:

International standard for whole cell pertussis vaccine, or other national/or in-house reference preparation calibrated in terms of International Standard should be used as the working reference. Experience indicates that for calibration of a secondary reference, a weighted geometric mean of at least five independent valid results of the calibration should be calculated for establishing the IU of the working reference.

Challenge strain:

Bordetella pertussis strain 18.323, stored in lyophilized state is suitable to be used for animal challenge. Examples of preparation of lyophilized stock and frozen aliquots are given in Appendix.

Experimental Procedures:

Distribution of mice:

To minimise the effect of stress associated with shipping, mice should be allowed to acclimatise for a period of time before immunisation. Mice should be either all of the same sex or equally distributed between male and female into groups by using the computer program for randomization of animals over cages as following [1]:

1) At least three groups of not less than 16 mice each for immunization with reference preparation
2) At least three groups of not less than 16 mice each for immunization with test vaccine sample
3) Four groups of 10 mice each are unimmunized control.

Immunisation:

The choice of appropriate doses for immunisation is critical for the test and it should be cautiously adjusted to keep the median dose of both test vaccine and reference preparations as close to the ED_{50} as possible under each laboratory conditions. Since the response to the vaccine differs between mouse strains, it is recommended that each laboratory establish the optimal dose range during the validation stage (see "Validation/establishment of the assay"), therefore the following immunisation doses (dilutions) serves only as an example.

1) Reconstitute an ampoule or ampoules of reference vaccine in sterile PBS or 0.9% sodium chloride to give a stock suspension containing 2.5 IU/ml.
2) Two further five-fold serial dilutions are made, also in sterile saline or PBS, to generate suspensions containing 0.5 IU/ml and 0.1 IU/ml. All dilutions are of sufficient volume to inject the appropriate group of mice with 0.5 ml each. The dilutions are made in, and kept in, sterile labelled glass containers.
3) The test vaccine is initially diluted one in four, in sterile saline or PBS, and then two further five-fold serial dilutions are made, all to final volumes sufficient to inject the appropriate groups of mice with 0.5 ml each. All dilutions are made in sterile labelled glass containers. Any unused PBS remaining is discarded.
4) The stock suspension and dilutions are then kept at +2 to +8°C and must be used within 4 hours.
5) Inject 0.5 ml of the appropriate dilution of the standard or test vaccines intraperitoneally into each animal.
6) The four control groups remain untreated.

Care should be taken to ensure that cage position and injection sequences are randomly allocated, since failure to do so can result in invalid or misleading estimates of potency. This can be achieved by allocating vaccine dilutions to cages of mice according to a random number sequence by using the computer program for randomization of dilutions over cages [1].

Animals are observed for 14 days post-immunisation. During this period mice should show good health and normal growth. No more than 6% of the animals per dilution group may die before challenge.

Preparation of challenge suspension:

A challenge suspension can be prepared directly from a freeze-dried ampoule of *B. pertussis* strain 18.323 or a *B. pertussis* strain 18.323 suspension previously prepared, aliquot and stored in liquid nitrogen or -70°C (please see appendix I).

Five days before the intracerebral challenge, prepare the challenge suspension by re-suspension of one ampoule of freeze-dried *B. pertussis* strain 18.323 into 0.5 ml sterile distilled water. If a pre-frozen suspension is used, allowed to thaw at room temperature.

Two to four pre-prepared BG agar plates are removed from the refrigerator and labelled with assay number, culture name and date. Any condensation is removed by leaving the plates open in the cabinet until all the condensation has evaporated.

Using a sterile glass Pasteur pipette, the bacterial suspension is dropped equally onto 2 to 4 BG plates. The plates are incubated for 2-3 days at +33°C to +39°C (the exact time needed to obtain a well grown culture under local conditions should be determined by each laboratory).

These plates are examined for contamination and only non-contaminated cultures are used for the next step.

The culture is then inoculated by spread/or streak on to 3 to 8 fresh BG plates.

These plates are then incubated at +33°C to +39°C for 18 to 24 hours before they are used to prepare the challenge suspension.

In some laboratories, a frozen challenge suspension made from a 18 hours culture, (e.g. 5% Glycerol in 1% sterile Casein as described in Appendix I), and pre-calibrated in terms of c.f.u/ml and LD_{50} was used to give better consistency of the challenge suspension (see Appendix I).

Calculation of bacterial concentration in the challenge suspension:

Two common methods described in **Appendix II** are used by laboratories for calculating bacterial concentration in the challenge suspension, one uses opacity units (OU) calculation, and another one is the calculation based on a calibration curve. Different laboratories may use a different method/or different type of spectrophotometer to prepare the suspension. Therefore the methods in **Appendix II** serve only as an example.

Once the challenge **suspension A** (**Appendix II**) has been prepared, make at least further three dilutions in 1% casamino-acids as indicated in the table to obtain challenge suspensions B-D for control groups.

Table 1:

Dilution with 1%casamino-acids	Suspension
	Challenge suspension A
1 ml of suspension A +9 ml	B
1 ml of suspension B +9 ml	C
1 ml of suspension C +9 ml	D

The viable count of the challenge dose is monitored by seeding the challenge suspension at a dilution of 1/1,000 (suspension D) on a BG agar plate at the beginning and at the end of challenge. This dilution should contain a total of approximately 100-300 organisms per challenge dose volume (0.02 – 0.03 ml).

If the mean post-challenge count is consistently found to be less than half of the pre-challenge count, the time between the resuspension of the challenge preparation and the completion of the challenge procedure should be reduced. It may also be necessary to keep the challenge suspension in melting ice for the whole duration of the challenge procedure.

As an alternative approach to above method, some laboratories use a calibration curve prepared according to the extinction as described in Appendix II, Method Two.

Challenge administration:

Fourteen to seventeen days after the immunisation, the three groups of mice receiving the dilutions of each test vaccine and the three groups of mice receiving the dilutions of the reference vaccine are challenged intracerebrally with 10-30 µl of the suspension A through the frontal bone of the cranium, 2 mm behind the eye and 2 mm from the mid-line. The injections are performed intracerebrally using a 27 gauge, three-eighth inch needle fitted to a 1-ml syringe or 0.25 ml glass syringe. Alternatively, a more consistent and accurate challenge dose may be achieved by using a racheted 1-ml syringe, such as a Hamilton, which delivers 0.010 -0.020 ml at each stroke, with a 3-mm stopped needle or syringe suitable for multiple injections to deliver the challenge dose.

In some laboratories, animals are under light halothane narcosis (a mixture of halothane (1.8%), nitrous oxide (9 L/min) and oxygen (3 L/min)) before the challenge. The whole procedure from the moment of preparing the challenge culture to the injection of the last mouse shall not last more than 4 hours.

The four unimmunized control groups are used for the titration of the challenge dose (LD_{50}). Each animal is inoculated intracerebrally with 10-30µl of the challenge suspension (either dilution A, B, C or D).

After finishing the challenge procedure, inoculate again 0.1 ml of suspension D on 2 BG plates.

Observation:

Observe the animals daily post challenge for fourteen days and record the number of dead animals. Mice in which pertussis infection has progressed to the point where they no longer can reach food and water should be killed and recorded as died a day later. The deaths are recorded for 14 days after challenge.

Mice dying within 72 hours should be excluded from the results as considered to be caused by the technical inoculation error.

Calculation of results:

Mice dying in the first three days after challenge are not included in the calculation of vaccine potency.

The data from the control groups are used to estimate the LD_{50} of the challenge suspension using probit analysis or any other suitable statistical method.

The potency of vaccine test sample should be calculated by comparing the ED_{50} of the standard and vaccine sample using parallel probit analysis program (See Chapter V Statistical Analysis of Results) using a statistical computer programme. In addition the 95% fiducial limits are also calculated.

Assay validity criteria:

1) The ED_{50} of each vaccine is between the largest and smallest immunising doses.
2) The challenge dose has been shown to contain between 100-1000 LD_{50}.
3) The LD_{50} should contain no more than 300 colony forming units (CFU).
4) The dose-response curves of the test and reference vaccine do not deviate significantly ($p < 0.05$) from parallelism and linearity. This means that the assay should meet the criteria set in the probit analysis programme for linearity and parallelism of the dose–response relationships

Pass criteria:

The vaccine passes the test if it has a potency of not less than 4.0 International Units (IU) per single human dose and the lower fiducial limit ($P = 0.95$) of the estimated potency is not less than 2.0 IU.

Fail to pass:

If the vaccine fails to pass, the test may be repeated but when more than one test is performed the results of all valid tests must be combined and the weighted geometric mean estimate and its lower fiducial limit should be calculated. The vaccine fails to pass if the potency is less than 4.0 IU per single human dose and the lower fiducial limit ($P = 0.95$) of the estimated potency is less than 2.0 IU.

Data monitoring:

Performance of standard vaccine:

In order to assure the quality of the test, each individual ED_{50} value of the reference vaccine should be monitored. Trending analysis should be carried out and values should be within the range of the cumulative mean ± 2SD.

(In some laboratories, mice in the group immunized with the highest dose of reference standard vaccine are monitored for signs of abnormalities in gait, posture and general condition. Less than 50% of animals in this group should show the clinical symptoms).

Performance of challenge strain:

The challenge dose for each assay is checked by monitoring the LD_{50} which must fall between 100-1000 LD_{50}.

Validation/establishment of the assay:

As the Kendrick test has already been standardized, the full validation programme is not required for the first implementation of this test. However, since each laboratory may use different experimental conditions e.g. mouse strain and challenge strain etc, test samples can be differed in their potencies, it is necessary to carry out validation before establishing of the experimental conditions under their own environment.

1) For a laboratory that wishes to set up the assay for the first time, it is important to determine the appropriate dilutions for both the reference preparation and test vaccine for immunisation for a selected mouse strain. For example, if no previous data exist, the assay can be started with dilutions of reference preparation containing 5 IU, 1.0 IU and 0.2 IU per ml and dilutions of 1/2, 1/10 and 1/50 of the test vaccine. Further justification can be made if the results are not satisfactory. The dose response relationship should be assessed using the five-fold dilution steps which should be appropriate for most strains of mice. If significant regression is not obtained with five-fold dilution steps, the selection of mice from an alternative source may be considered (for some inbred mouse strains, four-fold or even less may be more suitable).
2) Select suitable number of mice (not less than 16) for each dilution and the time required to challenge the animals. The optimisation of dilutions of test vaccine and reference standard and the other testing conditions mentioned above, should provide the optimal conditions to get results that meet all assay validity criteria.
3) The precision of the test should also be determined.
4) The culture conditions for growing the challenge bacterial strain and the challenge dose should be also carried out by monitoring the LD_{50} in conjunction with the information described in Appendix II.

Reference:

1) Recommendation for Whole Cell Pertussis Vaccine. WHO Expert Committee on Biological Standardization, Fifty-sixth Report, Geneva, World Health Organization, 2007 (WHO Technical Report Series, No. 941). Annex 6.

Appendix I: Preparation of frozen aliquots for storing the challenge bacterial culture

An important tool in achieving better reproducible results in the Kendrick test is the use of a more standardised challenge suspension.

1) Remove one freeze dried vial of *B. pertussis* 18.323 and re-suspend in 1.5ml sterile 1% casamino-acids.

2) Place ~5 drops onto each of the 10 BG agar plate using a Pasteur pipette. Spread the drops with a spreader, use new spreader for each plate.

3) Incubate plates at 35-37ºC for 72 h (some laboratories use 18-24 hours culture, or perform a second cultivation and freeze the 24 hours harvest).

4) Prepare freezing media: 5% Glycerol in 1% sterile Casein (filter sterilise using 0.2µm filter).

5) Scrape bacteria off the BG agar plates and re-suspend without clumps into approximately 5ml freezing media (pay attention to possible contamination). (In some laboratories, the bacteria were re-suspended in casamino acids and the suspension diluted to a standardized opacity before adding the Glycerol or DMSO. In this way the content per nunc tube can be standardized for one test per tube so far as stability of the diluted bacteria was ensured).

6) Place 200-500 µl aliquots into Cryotubes and store at -70°C/or put the tubes in a polystyrene box in the gas-phase of the liquid nitrogen container.

7) Place few drops of the suspension onto 2 fresh BG agar plates and gram stain to check for contamination. Further examination of contamination can be carried out by removing 2 new vials from -70°C and checked for contamination by spreading the contents of each onto a BG agar plate and incubating at 37°C.

8) Virulent phase (Phase 1) of the bacteria can be confirmed by plating out a frozen aliquot on a nutrient agar (NA) plate.

   Virulent Phase (Phase 1) = No growth on NA plate

For each new batch of frozen-aliquot of the bacteria, the LD_{50} is checked in a virulence control before it's use for routine purpose. The challenge dose should show to contain between 100-1000 LD_{50} and the LD_{50} is no more than 300 CFU. The frozen challenge suspension is kept at –70°C or in liquid nitrogen for up to 1 year or upto a validated period. However, its viability and virulence should be monitored from time to time.

Appendix II: Preparation and calculation of bacterial concentration in the challenge suspension

Two methods are currently used for calculating bacterial concentration in the challenge suspension. Different laboratories may adopt the procedure to fit in with their own conditions. Since the virulence of bacteria and sensitivity of mice may vary between laboratories, the bacterial concentration in challenge dose calculated by either method described below only serve as example. Individual laboratories need to establish their own acceptable concentration for challenge suspension (see *Validation/establishment of the assay*).

Method one: calculation based on opacity units (OU):

1) Opacity should be calibrated with WHO 5th International Standard for Opacity. Scrape enough bacterial culture from the plates (pay attention to possible contaminations, in case of doubt, do a check for purity by microscope) and **suspend without clumps** in to 3-6 ml of 1% casamino-acids solution to give a suspension with an optical density of about 10 OU (approximately 10 x 10^9 organisms per ml).

2) Measure and calculate bacterial concentration in the challenge suspension.

   a) Prepare a suspension with an extinction (at 590 nm) between 0.3-0.4 equivalent to approximately 6-8 OU. Write down the dilution factor used to obtain this extinction.

   b) Read from the following correlation table the amount of bacteria per ml, which corresponds with the extinction.

Table 2:

Correlation between extinction at 590 nm and the opacity of Bordetella pertussis bacteria (Vitatron extinctionmeter type Vitalab 10, 590 nm, cuvette diameter 10 mm)

Extinction	Bordetella pertussis concentration OU
0.300	6.00
0.310	6.20
0.320	6.40
0.330	6.60
0.340	6.80
0.350	7.00
0.360	7.20
0.370	7.40
0.380	7.60
0.390	7.80
0.400	8.00

Correlation between extinction and bacterial concentration is applicable for a Vitatron extinctionmeter type Vitalab 10, 590 nm, cuvette diameter 10 mm. This photometer was calibrated on the International Reference Preparation of Opacity by using a freshly prepared pertussis suspension, which was diluted until the opacity was identical with the Opacity Reference when compared by eye. The opacity of such a suspension is 10 IOU. Each laboratory should calibrate the photometer used for this purpose.

c) Calculate the pertussis concentration in the original undiluted suspension.

Example:

dilution factor to obtain an extinction between 0.3-0.4 is 1/10.

extinction 0.360 corresponds with 7.2 IOU.

original suspension 7.2 × 10 = 72 IOU.

Dilute the original suspension with 1% solution of casamino-acids to a concentration of 0.01 OU bacteria/ml to obtain a suspension containing approximately 2-5 x10^6 CFU/ml (**suspension A**). (the LD_{50}* of the challenge suspension should contain 100-1000 CFU per dose and different laboratory may verify the challenge suspension according to their own conditions.)

**LD*$_{50}$ *is the number of B. pertussis organisms that, when injected intracerebrally into a group of mice, causes deaths within 14 days of one half of mice injected excluding the initial 3 days non- specific deaths.*

Method two: Calculate according to a calibration curve from new frozen aliquots:

Preparation of calibration curve:

A calibration curve is prepared each year from a new batch of frozen aliquot (Appendix I).

1) Remove one new aliquot of *B. pertussis* challenge strain from freezer and allow it to thaw. Distribute evenly the contents of the vial onto two charcoal agar plates. Incubate at +33°C to +39°C for 48h.

2) Examine the plates for contamination after 48 h. Use a sterile loop to remove a visible amount of culture and streak onto four fresh charcoal agar plates. Incubate plates at +33°C to +39°C for 18-24h.

3) Again, examine the plates for contamination and only non-contaminated plates are used. Scrape off the bacteria from the plates and re-suspend in 6 ml sterile 1% casamino acids using a sterile loop. Obtain a solid free suspension. Measure the absorbance of the stock solution using 3 ml suspension at 625 nm. Dilute the stock solution to obtain a minimum of 5 or more absorbance values ranging from 0.05-1.5 with even intervals (A-E).

4) Further dilute each starting suspension in sterile 1% casamino acids solution as follows (i.e. dilute each starting dilution by three 100 fold dilutions and two 10 fold dilutions):

Table 3:

Dilution No.	Optical Density					Dilution factor
1	A	B	C	D	E	10^0
2	0.1ml of 1A+ 9.9ml of diluent	0.1ml of 1B+ 9.9ml of diluent	0.1ml of 1C + 9.9ml of diluent	0.1ml of 1D+ 9.9ml of diluent	0.1ml of 1E + 9.9ml of diluent	10^{-2}
3	0.1ml of 2A+ 9.9ml of diluent	0.1ml of 2B + 9.9ml of diluent	0.1ml of 2C + 9.9ml of diluent	0.1ml of 2D + 9.9ml of diluent	0.1ml of 2E + 9.9ml of diluent	10^{-4}
4	0.1ml of 3A + 9.9ml of diluent	0.1ml of 3B + 9.9ml of diluent	0.1ml of 3C + 9.9ml of diluent	0.1ml of 3D + 9.9ml of diluent	0.1ml of 3E + 9.9ml of diluent	10^{-6}
5	1ml of 4A + 9ml of diluent	1ml of 4B + 9ml of diluent	1ml of 4C + 9ml of diluent	1ml of 4D + 9ml of diluent	1ml of 4E + 9ml of diluent	10^{-7}
6	1ml of 5A + 9ml of diluent	1ml of 5B + 9ml of diluent	1ml of 5C + 9ml of diluent	1ml of 5D + 9ml of diluent	1ml of 5E + 9ml of diluent	10^{-8}

Above dilution protocol will result in suspensions with 5 different absorbance and six dilutions for each absorbance. This will result in a total of 30 suspensions.

5) Place 4 × 20 µl drops on a charcoal (or BG) agar plate for each suspension and incubate the plates at +33°C to +39°C for 4-5 days.

After the incubation the colonies on each plate are counted and recorded as follows:

Table 4:

OD	Average number of colonies on each drop					
	10^0	10^2	10^4	10^6	10^7	10^8
A						
B						
C						
D						
E						

6) Multiply the average number of colonies on each plate by 5 to get bacterial cells/0.1 ml. Multiply this again by 10 to get bacterial cells/ml for each OD taking into consideration the dilution factors. Plot a graph of OD against bacterial cells/ml for one of the dilution factor that has colonies for all 5 optical densities. Draw a best-fit line. Keep a record of values used to plot the graph in the file.

Calculation of bacterial counts in the challenge suspension:

For routine testing, the bacterial counts in the challenge suspension can be calculated using its optical density against the calibration curve and justified to approximately 10×10^5 to 10×10^6 /ml (**suspension A**) depending on the virulence of challenge strain and sensitivity of the mice.

IV.2 Specific toxicity tests

IV.2.1 Mouse weight gain test (MWGT)

Principles:

The MWGT is considered as a general, non-specific test measuring overall toxicity of pertussis whole cell vaccine, since a number of *B. pertussis* toxins may induce weight loss in mice. Correlation of the results of the MWGT with adverse reactions in children has been reported [3-6]. It is a test used to assess the toxicity of whole cell pertussis containing vaccines, and it is based on the ability of certain toxins or components from *B. pertussis* to cause weight loss in young mice.

The general guidance on how to perform the test is given below. Each individual laboratory may modify these procedures according to their own laboratory conditions.

Materials:

Animals:

Healthy mice* each weighting 14-16 grams. Use mice of the same sex or distribute segregated males and females equally between all groups. Mice should have access to food and water for at least 2 h before injection, and continuously after injection for the duration of the test. A reference preparation for B. *pertussis* whole-cell vaccine can be used as control (optional)

* *Mice used in this test may differ between laboratories (e.g. strain, feeding conditions etc)*

Solution/buffer:

0.85% NaCl aqueous solution (Saline) or 0.85% NaCl aqueous solution (Saline) with the preservative concentration equivalent to that of the vaccine.

Alternatively, Phosphate Buffered Saline (PBS) pH 7.2 –7.4 may be used instead of Saline.

NaCl	10 g
KCl	0.75 g
$Na_2HPO_4.12H_2O$	1.44 g
KH_2PO_4 (anhydrous)	0.125 g

Dissolve to a final volume of 1 litre in distilled water. Decant 100 ml into 150 ml glass bottles and sterilise by autoclaving at approximately 121°C for 15 minutes. Store at +2 to +8°C for up to 2 years. Check the pH (7.2-7.4) after sterilization.

Reference vaccine (optional):

In some laboratories, a reference vaccine is included in the assay as a positive control to standardise the assay and to determine the reproducibility between assays.

Experimental procedures:

Preparation of dilution of test vaccine:

The test vaccine (one single human dose (SHD) = 0.5 ml) is diluted (1:2) with sterile saline.

Therefore; 4 ml (neat vaccine) +4 ml saline = ~1 SHD/ml

Preparation of dilutions of reference vaccine (optional):

Reconstitute an ampoule of reference vaccine with sterile saline to give a suspension containing 4 units per ml (if the unitage of the reference vaccine is known) /or prepare a dilution containing 1 SHD/ml for reference vaccine with, in a sterile labelled container.

Reference and test vaccine dilutions are kept at +2 to +8°C and must be used within 4 hours.

Inoculation:

Just before inoculation, (day 0) mice are weighed individually and randomly distributed into groups of 10. The groups are allocated for control, reference vaccine and test vaccines. All mice are caged and the cages labelled with the unique identifier of the preparation code. The total weight (normally to 2 decimal places) of each group is recorded on the assay sheet (Appendix I) together with the time and number of mice.

Mice are injected, intraperitoneally using a 5 ml syringe, with 0.5 ml, of either sterile saline (controls), reference vaccine suspension or test vaccine.

Mice are checked daily over the next seven days and each group weighed (to 2 decimal places) on days 1, 3 and 7 at the same time of day as on day 0*. The weights and number of surviving mice in each group are recorded on the assay sheet (Appendix I).

**In some countries, weight change at 16-24 h is considered to reflect the presence of lipo-oligosaccharide in the vaccine and the number of leukocytes at day 7 to reflect the presence of pertussis Toxin.*

Calculation of results:

The results on the assay sheet (Appendix I) are then calculated to give average weight gain per mouse in each group at seven days, expressed as a percentage of the weight gained by the control group.

Assay validity criteria:

The test is valid if:

The control group shows no weight loss, at any point, over the seven days.

Pass criteria:

1) At the end of day 3 the average weight of the group of vaccinated mice is not less than that preceding the injection
2) At the end of 7 days the average weight gain per mouse is not less than 60% of that per control mouse.
3) No deaths occur when 10 mice are used and not more than one death occurs when 20 mice are used.

Fail to pass:

If test vaccine fails to meet the pass requirements in a first test, it can be retested once, and the data of the two valid tests should be combined and final result calculated. The vaccine fails to pass if the above criteria are not met.

Data monitoring:

The growth, monitored in the MWGT may also be affected by other factors not related to pertussis vaccine, such as strain of mice used, housing conditions and microbiological status. For that reason, control mice have to be included in the test.

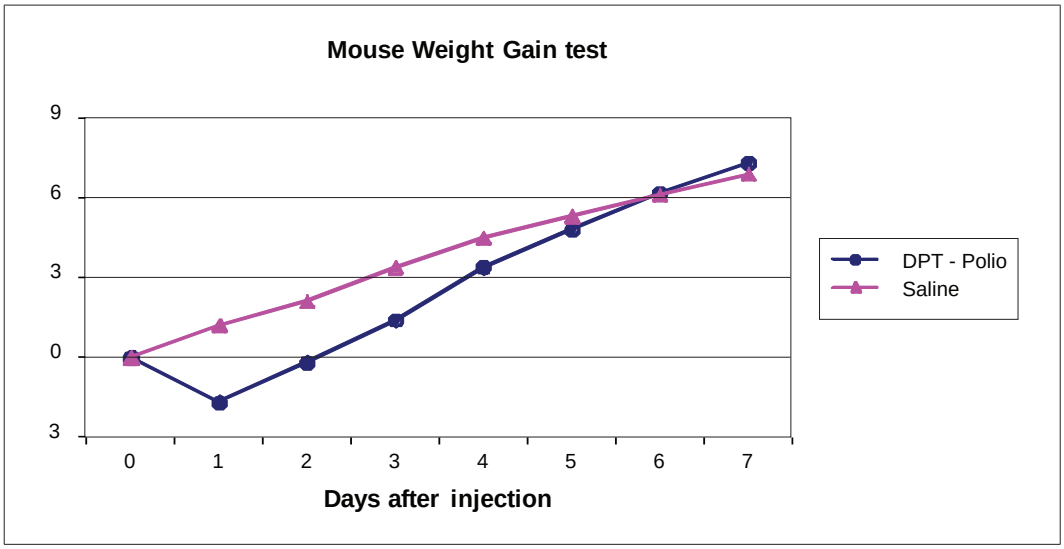
1) Control group gains weight
2) If a reference vaccine is used, the animals receiving the reference vaccine are monitored and they must have at least regained their initial weight by day three and at day seven the average weight gain per mouse must not be less than 60% that of the mice in the control group.

Validation/establishment of the assay:

Selection of suitable mouse strain and reproducibility of the tests by assessing the weight gain in control group and reference vaccine group from at least three independent assays.

Note: Some vaccine preparations may produce ascitis in mice. This accumulation of fluid in the peritoneal cavity is not normal but will not be reflected as weight loss but rather as weight gain, in some cases more weight gain than control mice e.g. greater than 150% of that per control mouse. However, such vaccine lots should not be considered as passing the MWGT.

Figure1: Example for results of a typical test



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Appendix I: Example for MWGT Assay Sheet

Test identification number: _____

Date of test: _____ Operator: _____

Time of weighing on day 0: _____ Reference vaccine: _____

Time of final injection: _____

Prep code	Weight	Total weight* (2 decimal places) Days after vaccination				Av. weight gain/mouse At day 7 (2 Decimal places)	Percent weight gain# At day 7	Pass / fail
	Number	0	1	3	7			
Control	W							-
(PBS)	N							
REF	W							
	N							
	W							
	N							
	W							
	N							
	W							
	N							
	W							
	N							
	W							
	N							
	W							
	N							
Serial number of balance used								
OPERATOR								

* average weights are calculated by dividing the total weight (W) with the total number of mice (N) in that group.
\# weight gain expressed as a percentage of the weight gained by the control group.

DATA ANALYSIS

Assay Valid: _____ YES / NO**

Analysis performed by: _____

Signature: _____ Date: _____

Calculations checked by: _____

Signature: _____ Date: _____

** Please delete as appropriate

IV.2.2 Other tests for monitoring pertussis toxicity

The tests described in this section (IV.2.2) are **not regulatory tests** for lot release of the whole cell pertussis vaccine. However, they may be used for in-house monitoring of the products consistency or for validation of the inactivation procedures.

IV. 2.2.1 Chinese Hamster Ovary (CHO) cells assay for pertussis toxin

Principles:

The CHO cell assay is an *in vitro* assay to assess the active pertussis toxin (PT) content of pertussis containing vaccines on the basis of the morphological changes to Chinese Hamster Ovary (CHO) cells in the presence of active PT [1]. In this test, the CHO cells are treated with different dilutions of PT reference /or test vaccine. After incubation, the degree of clustering of the cells is observed and scored under an inverted microscope. The highest dilution of the test vaccine showing total cell clustering represents the titre. The amount of active PT in the test sample can be semi-quantified against a reference preparation of known concentration.

The general guidance on how to perform the test is given below. However, each individual laboratory may adopt this procedure according to their own laboratory conditions.

Materials and methods:

Cells:

CHO K1 cells, ICN Flow (Cat no. 03-402-83 (ECACC No. 8505/005)). Stored in gas phase of liquid nitrogen (it may be necessary to assess the stability and introduce a 'shelf life' by recording cell viability every year).

Complete RPMI 1640 medium:

Foetal bovine serum (FBS) heat inactivated (Life Technologies 10108-066 or other commercial sources). Stored frozen in 10 ml aliquots.

Penicillin and streptomycin solution (10000 units penicillin, 10000 µg streptomycin/ml) (Life Technologies 15140-114 or other commercial sources). Stored frozen in 1 ml aliquots.

Make up to 100 ml with RPMI 1640 medium with glutamine (Life Technologies 21875-034 or other commercial sources) and add 10 ml of FBS and 1 ml of penicillin and streptomycin. Stored at +2 to +8°C for up to 1 week.

Reference pertussis toxin preparation:

Reference pertussis toxin preparation used in the assay should be calibrated against IS.

Test vaccine: DTP, DTP combination vaccines

Phosphate buffered saline (PBS) pH 7.4

NaCl	10 g
KCl	0.75 g
$Na_2HPO_4.12H_2O$	1.44 g
KH_2PO_4 (anhydrous)	0.125 g

Dissolve to a final volume of 1 litre in distilled water. Decant 100 ml into 150 ml glass bottles and sterilise by autoclaving at a minimum of 15 lbs (121°C) for at least 15 minutes. Store at +2 to +8°C for up to 2 years.

Trypsin solution (0.25%):

(Life Technologies 25050-022, or other commercial sources). Stored frozen in 5 ml aliquots.

10% Formaldehyde

Crystal violet solution:

Crystal violet	7.5 g
96% ethanol	36.5 ml
40% Formaldehyde	250 ml

Make upto 1000 ml with distilled water.

Freezing media:

Foetal Bovine Serum	2 ml
DMSO	1 ml

Make up to 10 ml with RPMI 1640. Store at +2 to +8°C for up to 1 week.

or

Foetal Bovine Serum	9 ml
DMSO	1 ml

Store at +2 to +8°C for upto 1 week.

Flat bottomed 96 well plate (Falcon 3072 or other commercial sources)

Sterile plastics

Experimental procedures:

All tissue culture procedures, up to the point of cell staining, must be performed in a Class II Microbiological Safety Cabinet and sterile disposable materials should be used throughout. The staining of the cells must be performed in a fume hood.

Recovery of cells from liquid nitrogen storage:

1) Cryovial is removed from liquid nitrogen and allowed to thaw at 37°C until just thawed.
2) The contents are poured immediately into 20 ml complete RPMI 1640 medium in a 50 ml centrifuge tube and spun at 1000 rpm, +2 to +8°C, for 6 minutes. The supernatant is discarded.
3) Resuspend cells in 50 ml complete medium and transfer to a 75 cm^3 culture flask.
4) Cells are placed in 37°C 5% CO_2 incubator until confluent (usually 3 days).

Titration of reference toxin and test vaccines:

1) One ampoule of freeze-dried pertussis toxin reference is dissolved in PBS to give a concentration of 105 IU/ml and frozen in aliquots of 0.1 ml. ***Aliquots are stored at -20°C for upto 6 months.***
2) One aliquot is thawed and 64 µl of this diluted in 1 ml PBS to give a starting concentration of 6.72 IU/ml.
3) Whole cell vaccines are diluted 10 fold with sterile PBS prior to dilution across the plate.
4) 25 µl of RPMI medium is placed in all wells in columns 2 - 12 of a sterile 96 well plate.
5) 25 µl of reference (at 6.72 IU/ml) is placed in wells in columns 1 and 2 of rows A and E.
6) 25 µl of test samples* are then placed in columns 1 and 2 in the remaining rows, in duplicate.
7) The reference and test samples are then diluted across the plate using a multichannel pipette set at 25 µl.
8) The last two wells of row A are left containing only medium as negative controls.
9) The location of the test samples is recorded on form (Appendix I).

Preparation of CHO cell suspension:

1) Once cells are confluent the medium is poured off the cells
2) The monolayer is rinsed twice with sterile PBS.
3) 5 ml 0.25% trypsin is added and flask is left at room temperature for 3 minutes.
4) Trypsin is removed and flask incubated at 37°C for 5 minutes.
5) 5 ml of complete medium is added to the flask and the cells gently washed off.
6) Cells in the suspension are counted using a haemocytometer (or a cell-counter) and diluted to a concentration of 2×10^4 cells/ml with complete medium.
7) Cell suspension is added to the above prepared plate at 200 µl per well.
8) Plate is incubated at 37°C 5% CO_2 until sufficient level of growth to observe morphological changes using an inverted microscope (usually 2 days).

* For vaccines containing preservative, a control containing preservative may be included.

Staining of cells:

1) The plate is shaken to remove media and immersed in 10% formaldehyde for 5 minutes.

2) Excess formaldehyde is removed by shaking and the plate washed in tap water for 5 minutes.

3) Once excess water is removed the plate is placed in crystal violet solution for 5 minutes followed by several rinses in tap water.

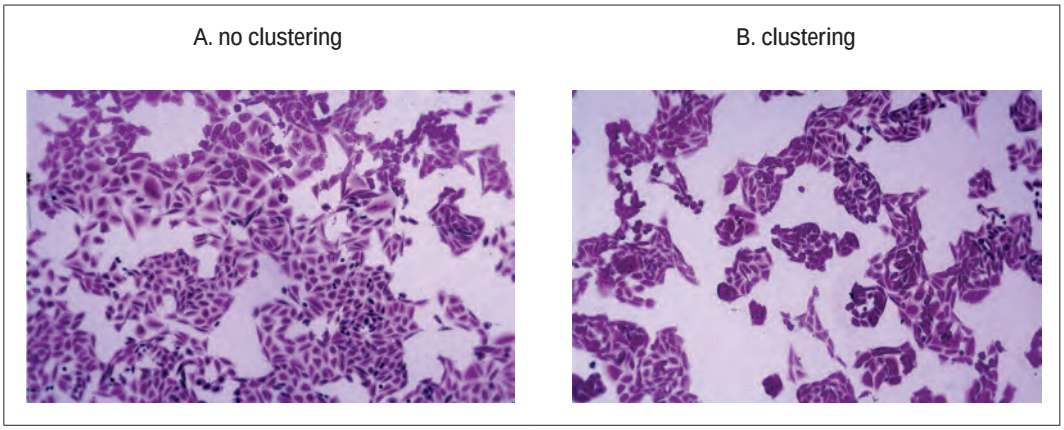
4) Plate is left to dry at 37°C for 1-4 hours.

View plate:

Plate is viewed inverted on a normal microscope at 4 or 10 fold magnification. Each well is examined for presence of clustering and the results are filled into the CHO cell Assay Sheet (Appendix I), using the following symbols:

+ = clustering
- = no clustering
O = cell death.

Figure 2: Example of CHO cell morphology



Calculation of results:

The highest dilution of the reference which caused cell clustering, is the reference endpoint. The IU/ml of the endpoint is calculated by multiplying the starting concentration of 6.72 IU/ml by the dilution factor. The average endpoint of the duplicate results is calculated.

The active PT content of the test samples is calculated by multiplying the reciprocal of the test endpoint dilution factor (i.e. highest titre at which clustering was observed) by the reference endpoint (IU/ml) and then by the initial dilution factor (i.e. by 10 for whole cell vaccines).

Table 5: Example for calculation:

CHO Cell Assay Sheet

Date: _____Operator: _____

Dilution Factor	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	-	-	-	-	-	-
B	+	+	+	+	+	-	-	-	-	-	-	-
C	+	+	+	-	-	-	-	-	-	-	-	-
D	+	+	+	+	+	+	+	+	-	-	-	-
E	+	+	+	+	+	+	-	-	-	-	-	-
F	+	+	+	+	+	-	-	-	-	-	-	-
G	+	+	+	-	-	-	-	-	-	-	-	-
H	+	+	+	+	+	+	+	+	-	-	-	-

Samples : A – ref. C – test 2 E – ref. G – test 2

B - +ve control D – test 3 F -+ve control H – test

Results:

REF toxin Endpoint dilution =1/32
Endpoint dilution =1/32 } 6.72 IU = 0.21 IU/ml is positive
32

Test 1(+ve) Endpoint dilution =1/16
Endpoint dilution =1/16 } 0.21 IU × 16 = 3.36 IU/ml

Test 2 Endpoint dilution =1/4
Endpoint dilution =1/4 } 0.21IU × 4 × 10 = 8.4 IU/ml = 4.2 IU/SHD

Test 3 Endpoint dilution =1/128
Endpoint dilution =1/128 } 0.21 IU × 128 × 10 = 268.8IU/ml =134.4 IU/SHD

Assay validity criteria:

- no clustering in negative control wells
- clustering in positive vaccine wells (first few dilutions)
- The difference between the duplicates ≤ 2 folds

Pass criteria: ≤ 105IU active PT per 0.5 ml vaccine*

Data monitoring:

1) Negative control
2) End point of the positive control for reproducibility assessment.

Validation/establishment of the assay:

1) Suitability of CHO-cell culture to be used for the assay needs to be carefully evaluated using negative and positive control.
2) The presence of preservative(s) e.g. thiomersal and adjuvant e.g. aluminium may influence the test. During the stage of validation/establishment of the assay, individual laboratory is recommended to set up appropriate dilution for both the reference and test vaccine /or select appropriate control.
3) Since there are no internationally accepted criterions for this test, in-house limits should be set up for a given product.

Preparation of CHO cells stock aliquots for storage in liquid nitrogen

CHO cells are grown in 75 cm^3 flask until confluent as above. Following trypsinisation they are washed off the flask with 5 ml complete medium and counted. The cell suspension is centrifuged at 1000 rpm, +2 to +8°C, for 6 min, the supernatant removed and the pellet resuspended in freezing medium to give 2×10^6cells/ml. 1 ml aliquots are placed in cryovials for storage. After 2-3 hours at -20°C they are transferred to -70°C overnight before being finally stored in liquid nitrogen.

References

1) Gillenius P, Jaatmaa E, Askelof P, Granstrom M, and Tiru M. The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. *J Biol. Stand*. 13, 61-66 (1985).
2) D. Xing, R. Gaines Das, P. Newland and M. Corbel. Comparison of the bioactivity of reference preparation for assaying Bordetella pertussis toxin activity in vaccines by the histamine sensitisation and Chinese hamster ovary-cell tests: assessment of validity of expression of activity in terms of protein concentration. Vaccine, 20:3535-3542 (2002).

* In-house criteria. This is not a regulatory test. There is no international acceptable limit for CHO-cell assay for whole cell pertussis vaccines.

IV.2.2.2 Histamine sensitization assay

There are two methods that are currently used for histamine sensitization assay. One is based on temperature measurement (Temperature method) and another is based on histamine-sensitizing death.

IV.2.2.2.1 Histamine sensitization assay (Temperature method)

Principles:

Mice inoculated with pertussis toxin become highly sensitive to a histamine challenge. The effects include reduction in body temperature and in the severe cases death. The reduction in body temperature occurs within 30 minutes after histamine challenge, but in the non-lethal situations it returns to normal levels after 30 minutes. Therefore, reduction in body temperature 30 minutes following histamine challenge is directly proportional to the dose of active PT present in the vaccine. This method is highly sensitive, it can detect levels of PT activity that do not induce lethal effects following histamine challenge.

Body temperature in mice can be assessed by measuring rectal or dermal temperature using either an electric thermometer with a probe specific for mice or an infrared thermometer, respectively. Results are obtained as continuous variables so as to allow calculating mean and variance for each group. Rectal temperature method has been used in Japan since 1981 [2, 3]. Assessment of body temperature by both methods (rectal and dermal) correlates with PT toxicity in animals. However, for practical reasons dermal measurements are preferred in some countries.

The general guidance on how to perform the test is given below. Individual laboratories may adopt the procedure according to their own laboratory conditions.

Materials:

Reagents:

Histamine dihydrochloride

Diluents:

Phosphate buffered saline containing 0.2 w/v % gelatine should be used for the assay using PT reference calibrated against the IS.

When PT reference is not used, phosphate buffered saline or physiological saline would be adequate.

Animals:

Mice of appropriate strain of one sex or, if not feasible, equal number of the both sexes with adequate sensitivity to pertussis toxin activity and to histamine

References:

Standard PT or adequately stabilized and calibrated reference material.

To use PT reference, reproducibility of measurements should be ensured in several independent assays by comparing with the reproducibility for a vaccine sample.

Equipments:

Syringes for not more than 3 mL particularly for adsorbed products and appropriate needles (smaller in size than 24 gauge)

For rectal temperature method; Electric thermometer with a probe for mouse (eg.KN-90, Natume Seisakusyo Co. Ltd., Tokyo)

For dermal temperature method; Infrared thermometer with resolution of ±0.1°C (This may be depending on manufacturer and thermometer. There are thermometers having a resolution of ±0.1°C such as http://www.ambientweather.com/ex42foteinth.html)

Experimental procedures:

Dilution:

Dilute reconstituted reference preparation and sample vaccine according to the selected dilution procedure.

Mice:

Mice should be randomly grouped into five or ten mice per cage. Ten mice each should be allocated to each treatment.

Sensitization:

0.5 mL of a dilution of the reference preparation or sample vaccines should be injected intraperitoneally into a group of mice.

Challenge:

On the fourth day of sensitization, the mice should be intraperitoneally injected with 4 mg of histamine dihydrochloride in 0.5 mL of saline and the time of challenge injection should be recorded.

Temperature measurement:

Rectal temperature or dermal temperature should be measured 30 min after challenge to record the temperature of each mouse.

Calculation of results:

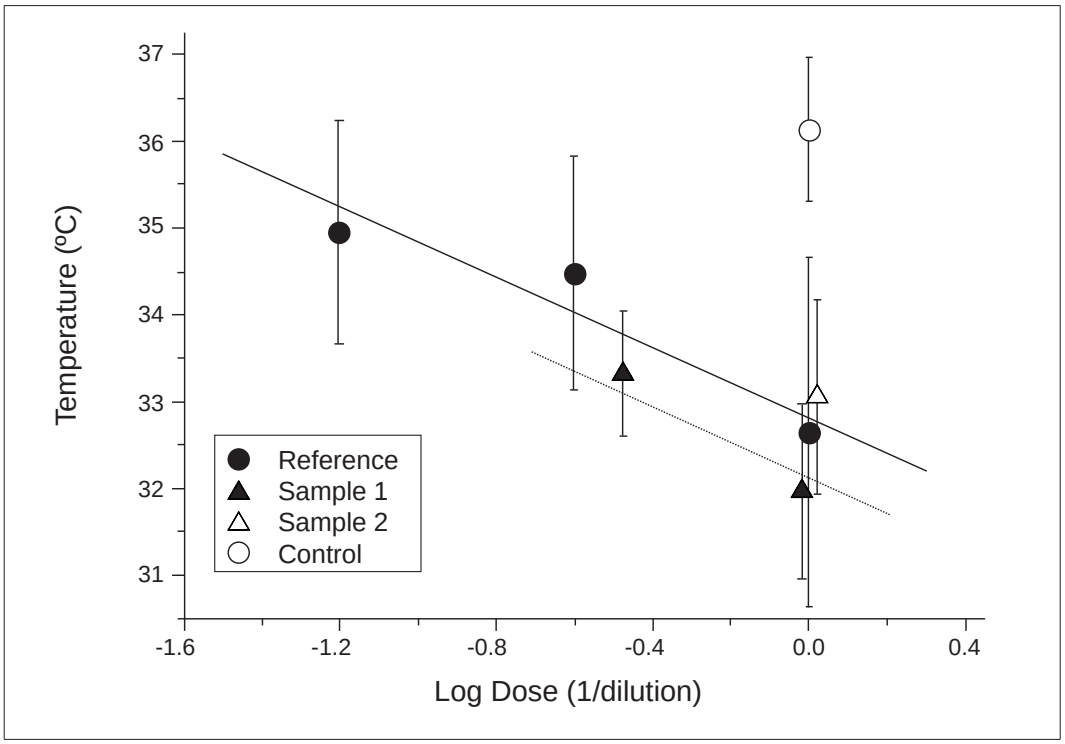
Data as follows would be obtained in an assay. The data can be analysed according to the parallel line assay method. (See Statistical Analysis of Results in Chapter V for the calculation).

Table 6: Data 1

Exp.	Source	Sample	Sample ID	Dose	1	2	3	4	5	6	7	8	9	10
Exp 1	NCL	Referece	Referece L2	0.0625	37.0	34.2	31.6	36.4	34.9	32.9	36.4	36.6	35.7	33.8
				0.25	36.1	35.3	33.0	32.0	31.8	37.7	35.2	33.8	34.2	35.7
				1	29.7	31.1	36.0	31.8	31.1	30.7	31.0	36.4	37.5	31.3
Exp 1	Man. A	DTaP	sample 1	0.33	33.4	35.2	32.6	33.0	31.4	33.9	33.8	33.1	32.8	34.0
				1	35.1	30.6	30.7	31.4	32.9	31.6	30.5	31.5	32.4	32.9
Exp 1	Man B	DTaP	Sample 2	1	31.0	34.1	33.9	34.2	30.7	33.0	33.7	31.0	34.3	34.7
Exp 1	Cont	Control	Saline	1	37.0	36.0	38.2	35.9	35.8	34.0	35.8	35.1	36.7	36.9

Transform the dose into log and carry out analysis of variance.

Figure 2:
Estimate dose-response regression lines by parallel line assay method.



Calculate relative potency and Unit value as well as 95% confidence interval.

Table 7:

Preparation	Relative potency	95% C.I	
Reference	5		
Sample 1	10.472	3.223	199.374
Sample 2	3.755	0.845	82.929
Control	0.114	0.001	0.546

Assay validity criteria:

Homogeneity of variance: Homogeneity of variance of all groups in an assay is essential for applying parallel line assay method. If alternative validity criteria were specified regarding the homogeneity, it should be validated at least for reproducibility of relative potency or toxicity.

Linearity: The dose-response regression line for the reference preparation should not be significantly deviated from straight linearity.

Regression coefficient: This validation would be possible after population value of regression coefficient was estimated by sufficient data accumulation. The regression coefficient should not be significantly deviated from the population value.

Parallelism; If multiple dilutions were employed for sample vaccines, the dose-response regression should not be significantly deviated from parallelism with that of the reference preparation.

Pass criteria:

This is not a regulatory test. No international acceptable limit has been set for this test yet. It is possible to specify an appropriate limit value for this test. However, it is recommended to assay lots suspected to have caused adverse events in comparison to those that did not for setting the limit value. If no correlation between the activity and probability of adverse events has been identified yet, a limit value could be set to approximately the highest value of the vaccines that have ever been used nationally without any problem.

If relative toxicity value did not significantly exceed the limit value, the lot could pass the test.

Fail to pass:

If relative toxicity value was suspected to have exceeded the limit value, the test should be repeated.

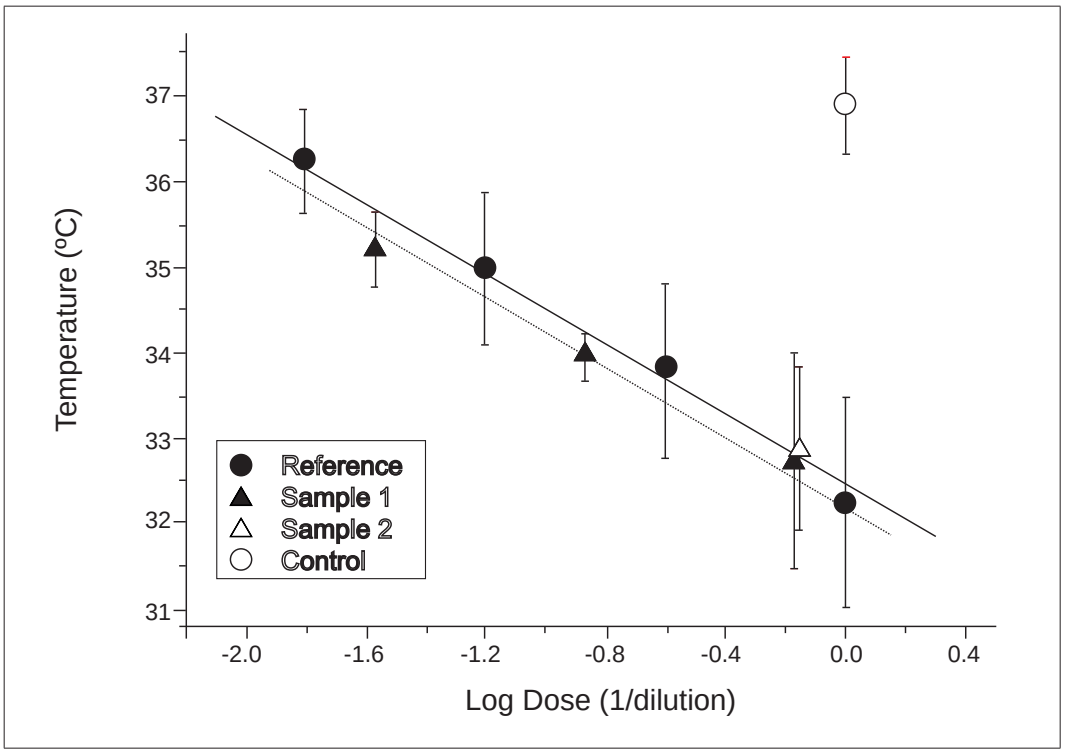
Data such as the following should be obtained in a repeated assay.

Table 8: Data 2 (data of a retest)

Exp.	Source	Sample	Sample ID	Dose	1	2	3	4	5	6	7	8	9	10
Exp 2	NCL	Referece	Reference L2	0.016	35.1	35.5	37.5	37.1	36.7	36.0	37.1	35.6	36.4	35.5
				0.063	33.8	32.7	33.9	35.7	36.2	34.9	36.0	34.5	36.5	35.6
				0.25	35.2	34.7	32.5	32.5	35.3	33.7	34.9	34.4	30.9	33.7
Exp 2	Man. A	DTaP	Sample 1	1	31.9	33.2	33.1	30.8	30.5	31.6	32.2	31.5	36.4	31.3
				0.027	34.8	34.5	35.9	35.0	34.2	35.2	35.9	35.9	35.2	35.5
				0.134	34.7	33.8	34.2	33.9	34.3	33.7	34.0	33.5	33.4	34.3
				0.67	31.7	33.5	36.7	31.8	33.5	33.7	33.0	31.0	30.7	31.7
Exp 2	Man B	DTaP	Sample 2	0.67	32.2	34.6	34.0	31.8	34.2	32.2	31.0	34.0	31.3	33.5
Exp 2	Cont	Control	Saline	1	38.6	37.0	37.1	36.7	35.7	37.5	36.7	36.8	36.0	36.8

Transform the dose into log and carry out analysis of variance.

Figure 3:
Estimate dose-response regression lines by the parallel line assay method.



Calculate relative potencies and their 95% confidence intervals

Table 9:

Preparation	Relative potency	95% C.I	
Reference	5		
Sample 1	6.851	3.552	13.757
Sample 2	4.690	1.751	16.520
Control	0.034	0.007	0.103

Calculation of combined data

Calculate weighted mean and 95% confidence interval as explained in Chapter V

Table 10: Statistical Analysis of Results

Sample	$\overline{M}$	Relative toxicity	Lower limit	Upper limit
Sample 1	0.852	7.108	4.377	11.542
Sample 2	0.549	3.541	1.708	7.345
Saline	-1.323	0.048	0.020	0.114
$\overline{M}$	: Mean log relative toxicity			

If weighted mean toxicity significantly exceeded the limit value, the lot fails to pass.

Data monitoring:

Regression coefficient (slope), error variance, log relative toxicity (M) and its variance estimate are recommended to monitor (See Chapter V Statistical Analysis of Results for details).

Validation/establishment of the assay:

Mice: Mice should be ensured to have adequate sensitivity to PT and histamine so that all can be fatally sensitized by the highest dose of reference preparation.

Dilution: Three or four fold dilution intervals are assumed adequate for an accurate estimation of a dose-response regression for the assay. Appropriate dilutions for the reference preparation and vaccine samples should be selected in preparatory studies as indicated below.

For the reference preparation, the dose range giving a linear dose-response that covers from the least to the maximum responses of mice should be selected. The maximum response should be at least slightly higher than the strongest response possibly seen for sample vaccines. Ordinarily, the dilution could be started with a vaccine concentration that was prepared without any special detoxification process. But if adequate, one can start with a lower concentration to avoid sensitizing deaths of mice.

For sample vaccines, appropriate multiple dilutions starting with the vaccine concentration or a single dilution that induces nearly the similar level of response to the median dose of the reference preparation can be selected.

It would be useful to remember that the larger the number of dilutions and the wider the dilution intervals, the better the accuracy.

Reference preparation: Standard PT or an adequately stabilized and calibrated reference preparation.

References

1) Kind, L. S.(1954): Antagonism of cortisone to body temperature reducing effect of histamine, Proceedings of the Society of Experimental Biology and Medicine 85, 371-372.

2) Ishida S. et al. (1979): A sensitive assay method for the histamine-sensitizing factor using change in rectal temperature of mice after histamine challenge as a response. J Biol Stand, 7,21-29.

3) Kurokawa M. (1984): Toxicity and Toxicity Testing of pertussis vaccine. Jap J med Sci Biol, 37, 41-81.

4) Ochiai M, Yamamoto A, Kataoka M, Toyoizumi, H, Arakawa, Y, Horiuchi Y (2007): Highly sensitive histamine-sensitization test for residual activity of pertussis toxin in acellular pertussis vaccine. Biologicals 35,259-264.

IV.2.2.2.2 Histamine sensitization assay (Lethal end-point method)

Principles:

An assay to assess the active pertussis toxin (PT) content of pertussis containing vaccines on the basis of the histamine sensitising effect of active PT on mice. Pertussis toxin increases the sensitivity of mice to histamine. The exact mode of action is not yet fully understood. Even when small amounts of active PT are present in a vaccine, mice will become vulnerable to challenge with histamine, resulting in anaphylactic shock and inevitable death. The amount of histamine sensitisation factor (HSF) activity in a vaccine can be quantified in a parallel-line assay in comparison with a reference vaccine. In this assay the reference and test vaccine doses, which induce a histamine sensitisation in 50% of the animals, as measured by death after challenge with histamine, are compared and a relative HSF activity is calculated for the vaccine. Different mouse strains may show different sensitivity to the test, laboratories are recommended to set up their own experimental conditions. An example of the test is given below.

Materials and animals:

Animals:

Mice of appropriate strain of one sex or, if not feasible, equal numbers of both sexes with adequate sensitivity to pertussis toxin activity and to histamine

Reference preparation:

Standard pertussis toxin (IS) or adequately stabilized and calibrated reference material e.g. against IS for *B. pertussis* whole-cell vaccine toxicity. If a pertussis toxin is used as reference, phosphate buffered saline containing 0.2 w/v % gelatine should be used for the assay using PT reference.

Phosphate buffered saline (PBS) pH 7.4

NaCl	10 g
KCl	0.75 g
$Na_2HPO_4.12H_2O$	1.44 g
KH_2PO_4 (anhydrous)	0.125 g

Dissolve to a final volume of 1 litre in distilled water. Decant 100 ml into 150 ml glass bottles and sterilize by autoclaving at a minimum of 15 lbs (121°C) for at least 15 minutes. Store at 2-8°C for up to 2 years.

Histamine (diphosphate salt)

Store at -20°C for manufacturers shelf life

Other materials:

Sterile 5 ml syringes
Sterile needles 27G × ½
Sterile glass or plastics
0.2 µm disposable syringe filter

Experimental procedures:

Preparation of vaccine dilution:

All whole cell vaccines are diluted as follows:

1.5 ml of 1 SHD/0.5 ml + 6 ml PBS = 0.2 SHD/0.5 ml
2 ml of 0.2 SHD/0.5 ml + 8 ml PBS = 0.04 SHD/0.5 ml
2 ml of 0.04 SHD/0.5 ml + 8 ml PBS = 0.008 SHD/0.5 ml.
Whole cell vaccines are given at 0.2, 0.04 & 0.008 SHD/0.5 ml.

Inoculation:

The mice are randomly distributed into cages (10 per cage), one cage for each vaccine dilution and a further group for PBS control. Mice are inoculated intraperitoneally, using a 5 ml syringe, with a nominal 0.5 ml of the appropriate vaccine dilution or PBS according to their cage label.

Challenge:

Mice are challenged with histamine 4 days after inoculation of test vaccine or reference preparation. .

The histamine solution is prepared at 5.52 mg/ml histamine diphosphate in PBS to give 1 mg histamine base per dose (0.5 ml). It is prepared in sufficient quantity to allow 0.5 ml per mouse plus at least 10 ml excess. The suspension is stirred until dissolved and filter sterilized through a 0.2 µm syringe filter.

Mice challenged by intra-peritoneal injection of 0.5 ml histamine solution. Deaths are recorded on the histamine sensitization assay sheet after 2 hours.

Calculation of results: (example, statistic method and possible software)

Based on the number of surviving animals calculate the HSF activity of the preparation by probit analysis as a relative activity in comparison with the reference, or calculate HSD_{50} of the reference preparation and test sample.

Assay validity criteria:

1) None of the mice must die in the negative control group before and after the challenge.
2) The dose-response regression should not be significantly deviated from parallelism with that of the reference preparation if Parallel line assay is used for the calculation.

Pass criteria:

This is not a regulatory test. There are no international acceptable criteria for HIST assay for whole cell vaccines. However, it is possible to specify an appropriate limit value for this test by assessing the highest value of the vaccines that have ever been used without any problem in the clinic, or by comparison to a reference vaccine which has shown no problem in clinical use. In this case, the test vaccine must contain less active PT than the reference vaccine or the SHD_{50} in the test sample must show less or equivalent to the reference preparation.

Fail to pass:

If the first test failed to meet the pass criteria, a repeat test can be carried out and calculation should be carried with combined data. The vaccine fails if cannot meet the in-house pass specification.

Data monitoring:

Consistency of performance of the reference vaccine should be monitored

Validation/establishment of the assay:

1) Select a specific mouse strain and age to ensure adequate sensitivity to this test (i.e. lethal effect using the highest dose of reference preparation).
2) Dilution: Three to five fold dilution intervals have been found to be adequate for an accurate estimation of a dose-response regression for this assay. However, appropriate dilutions for the reference preparation and vaccine samples should be selected in preparatory studies.
3) For the reference preparation, the dose range giving a linear dose-response that covers the least and the maximum responses of mice should be selected. The maximum response should be at least slightly higher than the strongest response possibly seen for sample vaccines.
4) For vaccine samples, appropriate multiple dilutions that induce nearly the similar level of response to the median dose of the reference preparation can be selected.
5) References: Standard PT (IS) or an adequately stabilized and calibrated in-house reference preparation.

References

1) Munoz J, Bergman RK. (1968) Histamine-sensitizing factors from microbial agents, with special reference to *Bordetella pertussis.* Bacteriological Review. 32:103-126.
2) Bernard D. Geller and Margaret Pittman (1973) Immunoglobulin and histamine-sensitivity response of mice to live *Bordetella pertussis.* Infect. Immun. 8:83-90.

IV.2.2.3 Heat-Labile Toxin (HLT) test (Dermonecrotic toxin test)

Principle

Heat-labile toxin (HLT) is a heat labile protein toxin of *B. pertussis* which can be inactivated in 10 minutes at 56°C. HLT is dermonecrotizing, lethal, and causes spleen atrophy in experimental animals. Since its discovery by Bordet and Gengou (1909), the toxin has been considered to play an important role in pathogenicity, most probably in the initial stage of whooping cough. The toxin is produced by all phase I *B. pertussis* strains. Pertussis vaccine should not contain biologically active HLT. Absence of HLT is not considered to be a product release criterion but validation of the manufacturing process should demonstrate the absence of HLT in the pertussis bulk after inactivation.

Suckling mice are most responsive to the lethal or dermonecrotizing activity of HLT after subcutaneous injection into the nuchal area. Both lethality and dermonecrocity, can be used as parameters. As a negative control saline may be used or heat inactivated sample (56°C, 10 minutes). Usually one to three dilutions per sample are tested.

Materials and animals

NIH mice, 4 days old.
4 mice + one lactating mother animal for each sample dilution.
10 ml of each sample to be tested.
Negative control: sterile saline or heat inactivated sample (56°C, 10 minutes).

Experimental procedure

1) Distribute the animals over the cages
2) When necessary, dilute the sample to a concentration of 5 OU/ml
3) Inject, subcutaneously into the nuchal area of four mice, 0.1 ml of each sample or dilution.
4) Observe the animals daily for four days and register purple-black lesions and death for each individual animal according to the following grades:

-	no symptoms
+	mild necrosis
++	severe necrosis
†	dead

Requirements for test validity

No toxic reactions may occur in the negative control group.

Requirements for the result

No animal injected by the diluted sample shows any toxic reaction related to the active heat-labile toxin.

Note:

When the test is used for validation of the heat inactivation process, samples taken at various stages during the process can be taken e.g. at 4°C, 40°C, 56°C, 2 min. 56°C, 4 min. 56°C, 6 min 56°C etc.

References

1) van Straaten, Dev Biol 2002;111:47-55

2) NVI Manual Quality Control of DTP Vaccines, NVI RIVM/BIS/VTTC 1998

V.2.2.4 Leukocytosis Promotion (LP) Test

Principles:

Pertussis vaccine contains pertussis toxin, formerly Leukocytosis Promoting Factor (LPF) that enhances the number of circulating leukocytes. Although most methods applied for determining the pertussis toxin-induced leukocytosis do not discriminate between leukocytes and lymphocytes some people persist in calling it lymphocytosis promoting factor. The LP activity of a pertussis vaccine is estimated by counting the number of circulating leukocytes 7 days after injection of mice with the test vaccine. Counting the number of circulating leukocytes can be done either by haemocytometer or electronic cell counter. In some laboratories, this is done in combination with MWGT. The procedure described below is an example of the test.

Materials:

Mice:

Male N:NIH/RIVM outbred mice (14-17 g) were bred and housed under specific pathogen free conditions. 10 Mice are used for each test vaccine as well as the saline control and reference vaccine.

Reference vaccine:

A lyophilised in house working reference pertussis vaccine, containing 200 OU per ampoule is dissolved in 12.5 ml saline to 16 OU/ml, equivalent to one single human dose (SHD).

Standard pertussis toxin (IS) or adequately stabilized and calibrated reference PT material can be also used.

Test Vaccines:

DTP, DTP-IPV and concentrated pertussis bulk vaccine

Experimental procedure:

1) For each vaccine, bulk product, reference and saline group 10 male mice are used.
2) Before immunisation the mice are distributed randomly in groups of 5 mice per cage, stained and weighed individually.
3) Each mouse is given an intraperitoneal injection of 0.5 ml of 0.85% NaCl aqueous solution containing half of the recommended single human dose.
4) The control group of mice is inoculated with 0.5 ml of physiological saline, preferably containing the same amount of preservative as the vaccine injected into the test mice.
5) If a MWGT is combined with the LP test, mice are weighed individually, (16-20 hours after inoculation), as well as 3 and 7 days after inoculation.
6) During the test period the animals are examined daily for abnormalities.

7) On day 7 after weighing, 10 µl blood sample is taken from the tail vein, using an EDTA-impregnated capillary, diluted in 1.6 ml Isoton (Coulter) and the number of leukocytes is counted with a Coulter Counter.

8) Per group of 10 mice, the average number of leukocytes in the test vaccine, reference vaccine or the saline control at day 7, are calculated.

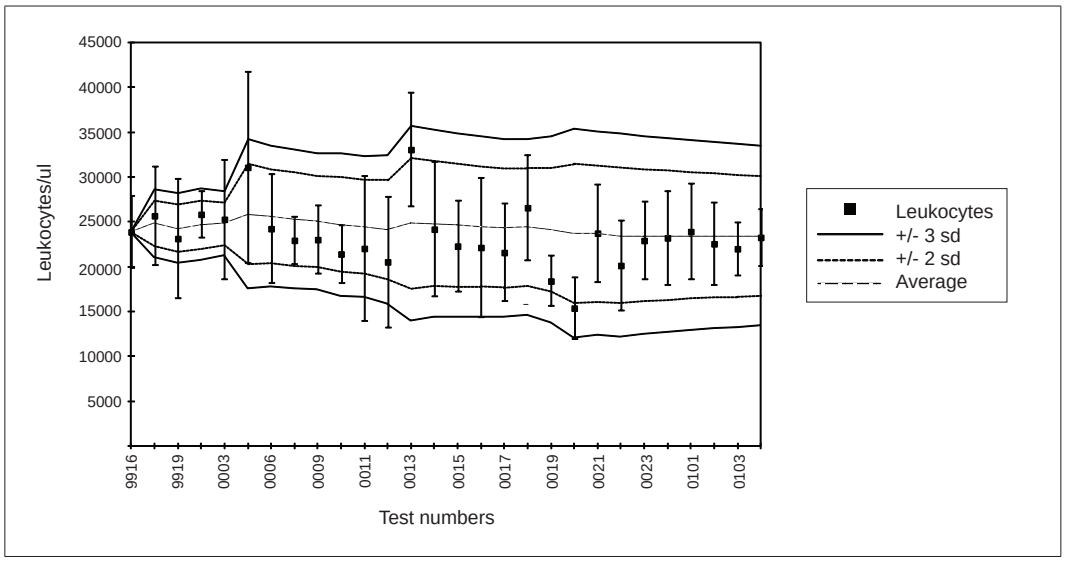
Assay validity:

The number of leukocytes at day 7 in the reference vaccine or saline control group are recorded in Shewhart Control Charts to establish the consistency and validity of the test. The values, should fall within the 3 SD range. In case the test does not meet this criterion, the test is considered to be invalid and should be repeated. An example of a Control Chart of the leukocytes induced by the reference is given in figure below.

Vaccine results:

Leukocyte counts at day 7 after inoculation are recorded in Shewhart Control Charts to monitor the consistency of the product**.**

Figure 4:



Pass criteria:

No international acceptable criteria are set for the number of leukocytes induced by the vaccine.

References

1) van Straaten, Dev Biol 2002;111:47-55

2) NVI Manual Quality Control of DTP Vaccines, NVI RIVM/BIS/VTTC 1998

IV.2.2.5 Endotoxin assays (LAL):

Principles:

The endotoxin assay is a test to detect or quantify bacterial endotoxins using a reagent prepared from lysate extract of blood of horse shoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). The test is based on highly sensitive clotting of horse shoe crab blood in the presence of endotoxin. There are two types of techniques for the test; gel clot and photometric techniques. The gel clot techniques are based on gel formation of the lysate by the reaction with endotoxin [1, 2]. The photometric techniques are based on optical change due to the reaction of the lysate reagents with endotoxin. The photometric techniques include turbidimetric techniques, which are based on the change in turbidity during gel formation [3, 4], and chromogenic techniques, which are based on the colour development due to cleavage of a synthetic peptide-chromogen complex [5].

The general guidance on how to perform the test is given below. Individual laboratories may adapt these procedures according to their own conditions.

Materials:

Apparatus:

All glassware and heat-stable materials should be depyrogenated by heating in a hot-air oven by a validated process. Commonly used time and temperature settings are 30 min at 250°C. Plastic apparatus such as tips for micropipettes and multi-well plates should be those ensured free of detectable endotoxin, not to interfere with the test and also do not adsorb endotoxin.

Standard Endotoxin Solution: Dissolve Reference Standard Endotoxin (RSE) in water for bacterial endotoxin test (water LAL) to prepare Standard Endotoxin Stock Solution (RSE stock solution). After mixing thoroughly, prepare appropriate serial dilutions of RSE Stock Solution using water LAL.

Sample solutions:

Sample solutions should be prepared by dissolving or diluting with water LAL to store in containers free of detectable endotoxin. If necessary, pH of sample solutions should be adjusted so that it falls within the rage specified for the lysate reagent to be used.

Experimental procedures:

The dilution process is one of the biggest variation factors for the test. Appropriate measurements, therefore, should be done on dilutions of independent series rather than repeated measurements on a single dilution series.

Gel-clot techniques:

Dilute RSE stock solution and sample solutions with water LAL to prepare appropriate dilutions. The range of RSE dilutions should include the labelled sensitivity of the lysate reagent. Spike an appropriate dilution of RSE to sample dilutions for testing interfering effect of samples on the test. Transfer aliquots of the dilutions to receptacles such as tubes or slides to add to an equal volume of the lysate reagent. Water for LAL should also be tested as the control. The order of addition of the dilutions and the reagent could be according to a validated procedure. Then mix gently the reaction mixture to incubate at 37±1°C without vibration, avoiding loss of water by evaporation for a predefined period of time.

Turbidimetric and chromogenic techniques:

Dilute RSE stock solution and sample solutions with water LAL to prepare appropriate serial dilutions. The range of RSE dilutions should include the labelled sensitivity of the lysate reagent and also the possible highest endotoxin concentration in sample dilutions. Spike an appropriate dilution of RSE to a sample dilution that is further diluted similarly to RSE for testing interfering effect of samples on the test. Transfer aliquots of the dilutions to tubes to add with an equal volume of the lysate reagent. Water LAL should also be tested as the control. The order of addition of the dilutions and the reagent could be according to a validated procedure. Then mix gently the reaction mixture to incubate at 37±1°C for a predefined period of time. Vibration should be avoided during incubation for turbidimetric techniques. Measurements for turbidimetric techniques should be done on the turbidity at a specified reaction time, the rate of turbidity development or time needed to reach a predefined turbidity. For chromogenic techniques, measurements should be done on the absorbance at specified reaction time, the rate of colour development or time needed to reach a predefined absorbance.

Calculation of results:

Gel-clot techniques:

A positive results is indicated by the formation of a firm gel that does not disintegrate when receptacle is gently inverted. If no such gel is formed, the result is negative.

Turbidimetric techniques:

A set of example data of a turbidimetric kinetic assay that measures the time in minutes to reach a predefined turbidity is given below.

Table 11:

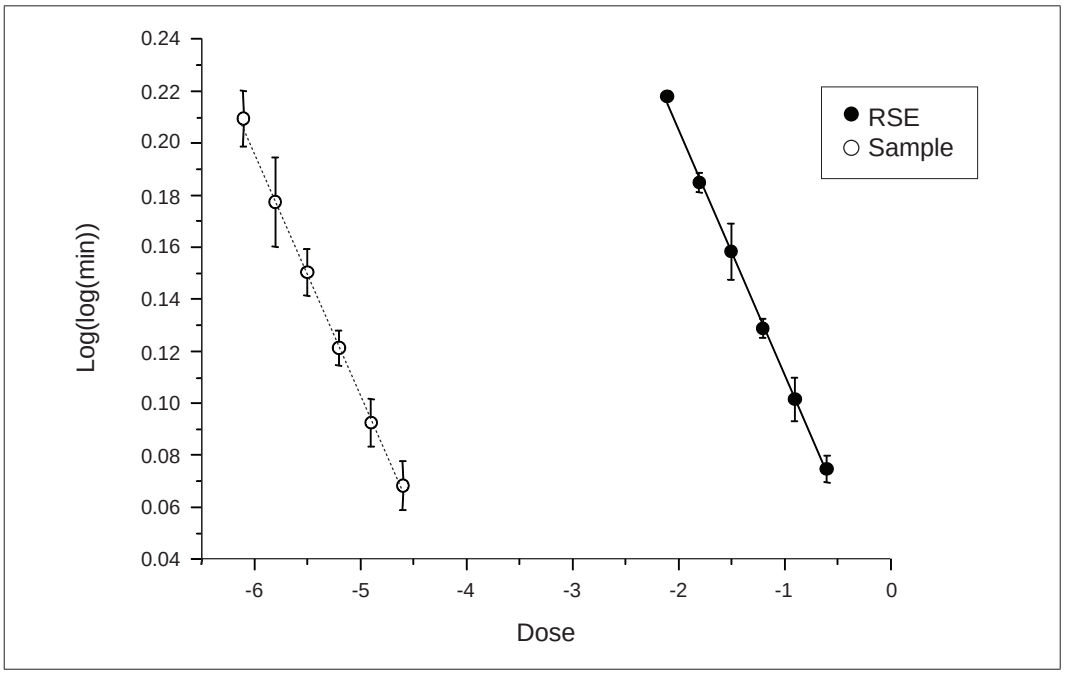
	Concentration	Gelation time(min)		
		1	2	3
RSE	0.25 IU/mL	15.4	15.6	15.2
	0.125 IU/mL	18.2	18.8	18.0
	0.0625 IU/mL	22.0	22.4	22.0
	0.03125 IU/mL	26.6	28.4	27.6
	0.015625 IU/mL	33.6	33.8	34.4
	0.0078125 IU/mL	44.6	45.2	44.8
Sample	1/40,000(0.000025 mL)	15.0	14.4	15.0
	1/80,000(0.0000125 mL)	17.6	17.4	16.8
	1/160,000(0.00000625 mL)	21.0	20.6	21.4
	1/320,000(0.000003125 mL)	26.0	25.2	26.6
	1/640,000(0.0000015625 mL)	33.0	30.0	33.0
	1/1,280,000(0.00000078125 mL)	43.0	40.0	42.0
Water LAL	1	>60	>60	>60

Data 1, 2 and 3 are measurements on independent series of dilutions.

Transform the dose into log and responses into double log to carry out analysis of variance.

Estimate dose-response regression lines by the parallel line assay method. (See Chapter V Statistical Analysis of Results for the calculation)

Figure 5:



There is a linear relationship between logarithmic dose and double logarithmic reaction time.

Calculate relative potencies and their 95% confidence intervals

Table 12:

	Relative activity	95% Confidence limits	
Sample	12099.9	11391.1	12856

Chromogenic techniques:

A set of example data of a chromogenic kinetic assay that measures the rate of colour development is given below.

Table 13:

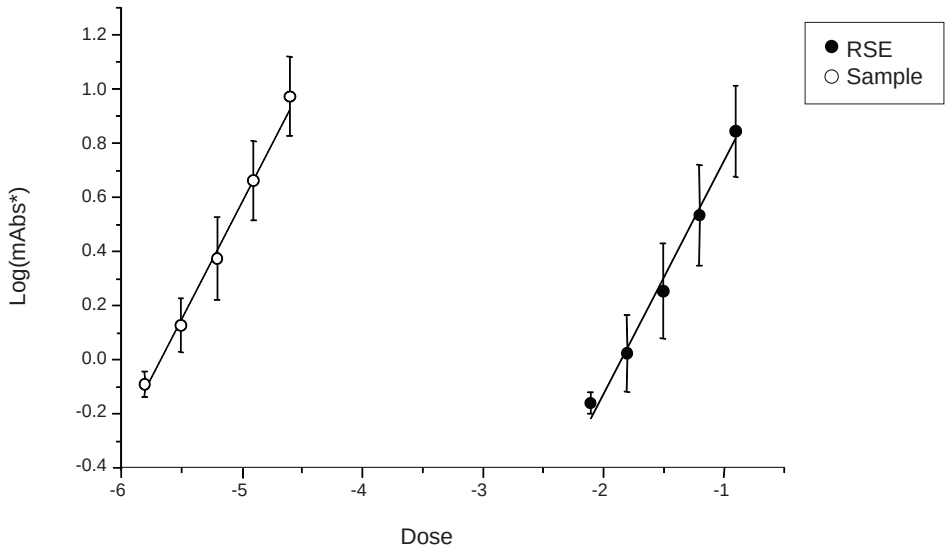
	Concentration	Reaction rate(mAbs/min)		
		1	2	3
RSE	0.125 IU/mL	15.4	15.6	15.2
	0.0625 IU/mL	18.2	18.8	18.0
	0.03125 IU/mL	22.0	22.4	22.0
	0.015625 IU/mL	26.6	28.4	27.6
	0.0078125 IU/mL	33.6	33.8	34.4
Sample	1/40,000(0.000025 mL)	15.0	14.4	15.0
	1/80,000(0.0000125 mL)	17.6	17.4	16.8
	1/160,000(0.00000625 mL)	21.0	20.6	21.4
	1/320,000(0.000003125 mL)	26.0	25.2	26.6
	1/640,000(0.0000015625 mL)	33.0	30.0	33.0
Water LAL	1	0.3	0.2	0.4

Data 1, 2 and 3 are measurements on independent series of dilutions.

Transform the dose into log and responses into double log to carry out analysis of variance.

Estimate dose-response regression lines by the parallel line assay method. (See Chapter V Statistical Analysis of Results for the calculation)

Figure 6:



* Change in absorbance x 1,000/min

There is a linear relationship between logarithmic dose and log-transformed reaction rate.

Calculate relative potencies and their 95% confidence intervals

Table 14:

	Relative activity	95% Confidence limits	
Sample	6715.5	5988.1	7546.4

Assay validity criteria:

Table 15: Gel-clot techniques:

	Solution	Addition of endotoxin	Diluent	Serial dilution
1)	Water LAL	-	-	-
2)	Water LAL	+	Water LAL	+
3)	Sample dilution	+	Sample	+
4)	Sample dilution	-	-	-

a) should be negative.

b) should be within ±2 times of the labelled sensitivity of the reagent.

c) should not differ more than ±2 times from "b".

d) If all "a" to "c" were met, the result d is valid.

e) If "b" was not met, should be retested.

f) If "c" was not met, the sample dilution should be changed to lower concentrations for eliminating interfering effect.

Table 16: Turbidimetric and chromogenic techniques:

	Solution	Addition of endotoxin	Diluent	Serial dilution
1)	Water LAL	-	-	-
2)	Water LAL	+	Water LAL	+
3)	Sample dilution	+	Sample	+
4)	Sample dilution	-	-	+

a) should be undetected.

b) should be straight linear.

c) should be parallel to "b" throughout the dose range.

d) If all "a" to "c" were met, the result "d" is valid.

Pass criteria:

Endotoxin content of the sample must not exceed the specified limit value (Please see Validation/establishment of the assay), for the sample to pass the test.

Fail to pass:

If endotoxin content of the sample is not within the specified limit value, the sample should be retested.

Gel-clot techniques:

Combine all individual dilution series of log-transformed endotoxin content to calculate mean and variance. 95 % confidence interval of the mean is calculated by $t_{(0.05,n-1)}\sqrt{s^2/n}$, where is variance, is number of individual result. (See Chapter V Statistical Analysis of Results for the details). If the specified limit value was outside the confidence interval, the sample fails to pass.

Turbidimetric and chromogenic techniques:

Calculate weighted mean of log endotoxin content (relative potency to RSE) by using inverse of estimated variance of relative potency and 95% confidence interval. Weighted mean endotoxin content of the sample is IU/mL. If the specified limit value was below the lower limit of the confidence interval, the sample fails to pass.

Data monitoring:

Gel-clot techniques:

Test results on batches to see consistency of products.
Test results on RSE to see accuracy and reliability of the test.

Turbidimetric and chromogenic techniques:

Test results on batches to see consistency of products.

Validation/establishment of the assay:

The bacterial endotoxin test is affected by temperature, pH, cationic and anionic ions, adjuvant gel and other factors, and influence of these factors may differ depending on the reagent to be used. Test procedures should be validated by spiking a known amount of endotoxin to a sample or sample dilutions to ensure that the spiked endotoxin is correctly detectable.

a) For turbidimetric or chromogenic techniques, measure serial dilutions as wide as possible of the same preparation repeatedly and try log and other transformations of the response values to calculate and compare variances of the doses to choose the most appropriate transformation for achieving straight linearity of dose-response and homogeneity of variance.

b) It is recommended to accumulate sum of squares for doses (*Sxx*) and covariances for dose and response (*Sxy*) of each sample in assays for estimating population regression coefficient which is useful for ensuring validity of dose-response in each assay. (See Statistics section, **Assay validation)**

c) No internationally accepted limit for endotoxin exists for whole cell pertussis vaccine. For national/or in-house endotoxin limit value: Measure endotoxin contents of as many lots as possible to specify endotoxin content of normal lots as $10^{mean\ log\ IU+2SD}$. If endotoxin-related adverse events were found not tolerable, the initially specified limit should be revised according to the clinical outcomes.

References

1) Levin, J. and Bang, F.B. 1964. The role of endotoxin in the extracellular coagulation of limulus blood. Bull. Johns. Hoplins. Hosp. 115: 265-274.

2) Levin, J. and Bang, F.B. 1968. Clottable protein in Limulus; its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. 19: 186-197.

3) Tsuchiya M, Oishi H, Takaoka A, Fusamoto M, Matsuura S. Discrimination between endotoxin and (1-3)-beta-D-glucan using turbidimetric kinetic assay with Limulus amebocyte lysate. Chem Pharm Bull (Tokyo). 1990 Sep; 38(9):2523-6.

4) Tsuchiya M, Takaoka A, Tokioka N, Matsuura S. Development of an endotoxin-specific Limulus amebocyte lysate test blocking beta-glucan-mediated pathway by carboxymethylated curdlan and its application. Nippon Saikingaku Zasshi. 1990 Nov; 45(6): 903-11.

5) Obayashi, T., Tamura, H., Tanaka, S., Ohki, M., Takahashi, S., Arai, M., Masuda, M. and Kawai, T. 1985. A new chromogenic endotoxin-specific assay using recombined Limulus coagulation enzymes and its clinical applications. Clinica. Chimica. Acta. 149: 55-65.

IV.3 Methods used for strain characterization and identity tests

IV.3.1 Slide agglutination test for detection of fimbriae 2 and 3 (also refer as AGG 2 and 3)

Principle:

Examination of the expression of serotype specific fimbriae by *Bordetella pertussis* bacteria can be carried out by traditional slide agglutination assay. This assay can be used as an identity test for whole cell pertussis vaccine and monitoring the consistency of production. This tests may be performed either on the cultures of the seed strains to confirm their identity or on unabsorbed vaccine bulk material.

The general guidance on how to perform these tests is given below. Individual laboratories may adopt these procedures according to their own conditions.

Materials:

microscope slides
monospecific antisera (polyclonal or monoclonal)
negative rabbit serum
matt black plate
pipette

Experimental procedure:

1) Take four microscope slides.
2) On each slide, place one drop of the *B. pertussis* cell suspension.
3) Add the three monospecific antisera to one slide each and mix.
4) Add to the fourth slide normal rabbit serum and mix.
5) Rock the slides gently and let it rest.
6) After 3 minutes observe the slides on a black background.

Criteria for acceptance:

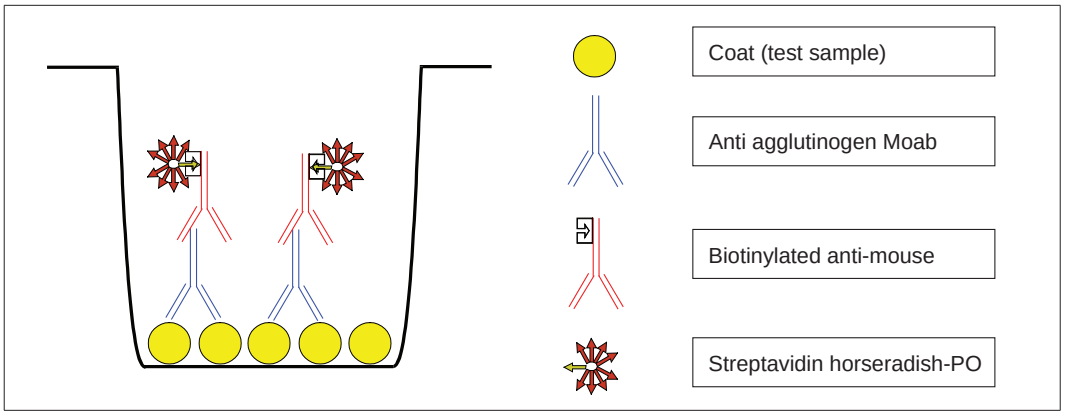
Rapid, clear agglutination should be observed with all three monospecific antisera within 3 minutes.

IV.3.2 ELISA test for detection of agglutinogens (also refer as fimbriaes)

The bacterial suspension under test is adsorbed onto a microplate after which the presence of all three agglutinogens is detected by subsequent incubation with the respective highly specific mouse monoclonal antibody, biotinylated anti-mouse antibodies, streptavidin horseradish peroxidase and substrate. If the mean optical density (OD) for each agglutinogen is equal to or higher than three times the background OD, the relevant AGG is considered being present.

Since there is only limited experience using this test for DTPw product, individual laboratories that wish to use this assay are recommended to carry out their own validation for setting up this test.

Figure 7:
Schematic presentation of ELISA for the determination of agglutinogens



Materials:

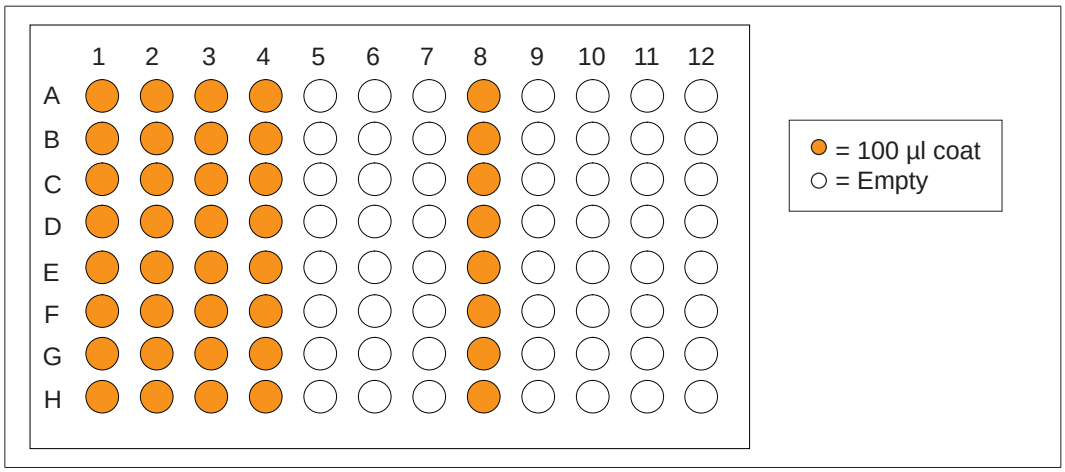
Polysorb ELISA plates
Phosphate Buffered Saline (PBS) pH 7.2
Bovine Serum Albumin (BSA)
Block buffer: PBS + 0.5% BSA
Monoclonal antibodies*:
  anti-AGG-1
  anti-AGG-2
  anti-AGG-3
Skimmed milk (commercial available)
Biotinylated sheep-anti-mouse Ig (commercial available)
Streptavidin horseradish peroxidase (commercial available)
Distilled water
Sodium acetate buffer, pH 5.5, 1.1 mol/l
3,3`,5,5´Tetra-Methyl-Benzidin (TMB, commercial available) 6 mg/ml in ethanol.
Peroxidase 30% (commercial available)
2M sulphuric acid
ELISA reader with 450 nm filter
Tween 80
Diluent: PBS + 0.1% Tween 80
Positive Control Vaccine containing AGG-1, AGG-2 and AGG-3
Data recording form

* obtained from CBER, USA or NIBSC, UK

Experimental procedure:

1) Dilute the test sample to a concentration of 20 OU/ml in PBS.
2) Put 100 µl volumes of the test sample in the wells of columns 1 to 4 and column 8 of an ELISA plate.
3) Reconstitute one vial Kh 85/1 (positive control) in 10 ml PBS to a concentration of 20 OU/ml
4) Put 100 µl volumes of Kh 85/1 in the wells of columns 1 to 4 and column 8 of another ELISA plate.
5) Incubate the plates overnight at 37°C without plate cover so that the well content can evaporate.

Figure 8:

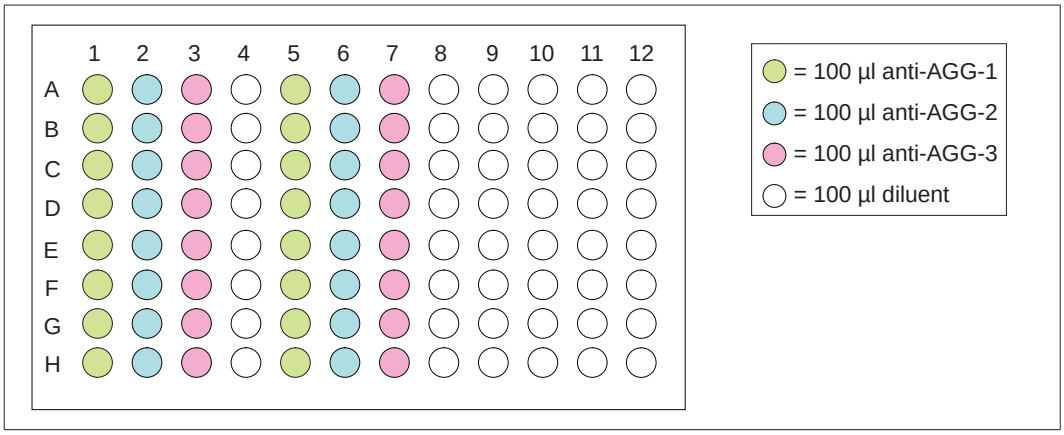


Next day

6) Wash the plates.
7) Block the plates by addition of 125 µl block buffer in each well.
8) Incubate for 1 hour at 37°C.
9) Wash the plates.
10) Dilute all three monoclonal antibodies 1/5000 in diluent.
11) Introduce 100 µl of the monoclonals:

anti-AGG-1 (BP G 10) in wells of columns 1 and 5.
anti-AGG-2 (118 E 10) in wells of columns 2 and 6.
anti-AGG-3 (48 B 5) in wells of columns 3 and 7.

Figure 9:



12) Add 100 µl diluent in the wells of column 4 and 8.

13) Incubate for 1 hour at 37°C.

14) Wash the plates.

15) Dilute the biotinylated sheep-anti-mouse 1/1000 in diluent.

16) Introduce 100 µl of the diluted sheep-anti-mouse in the wells of columns 1 to 8.

17) Incubate for 1 hour at 37°C.

18) Wash the plates.

19) Dilute Streptavidin horseradish peroxidase 1/5000 in diluent + 0.5% skimmed milk.

20) Incubate for 1 hour at 37°C.

21) Wash the plates.

22) Prepare the substrate consisting of:

    45 ml distilled water
    5 ml sodium acetate buffer pH 5.5
    0.83 ml TMB/ethanol
    10 µl peroxidase

23) Introduce 100 µl of the substrate to all wells.

24) Incubate for 10 minutes at room temperature.

25) Stop the reaction by addition of 100 µl sulphuric acid per well.

26) Measure the OD´s at 450 nm.

27) Calculate for each column the mean OD.

Result:

The sample is considered to contain:

AGG-1 if mean OD column 1 is greater than or equal to 3 x mean OD column 5
AGG-2 if mean OD column 2 is greater than or equal to 3 x mean OD column 6
AGG-3 if mean OD column 3 is greater than or equal to 3 x mean OD column 7

Requirements for test validity:

The positive control vaccine should be positive for all three AGG´s.

References

1) NVI Manual Quality Control of DTP Vaccines, NVI RIVM/BIS/VTTC 1998

IV.3.3 Immunodiffusion assay

Principles:

The double diffusion (Ouchterlony) assay may be used to demonstrate the presence of whole-cell pertussis antigens in both adsorbed and non-adsorbed pertussis and combined vaccines. This method is suitable for testing both final filling lots and bulk preparations of DTP vaccines.

The general guidance on how to perform the test is given below. Individual laboratories may adopt these procedures according to their own laboratory conditions.

Materials:

Negative control

Both diphtheria and tetanus toxoids can be used as negative control. Here use diphtheria as an example.

Purified diphtheria toxoid unabsorbed is used as a control preparation. Concentrated toxoid is stored infinitely at +4°C. The control dose of diphtheria toxoid is prepared at a concentration of 100 Lf/ml in sterile PBS and stored for up to 1 year at +4°C.

Whole-cell Pertussis antiserum

This is prepared in-house by injecting a rabbit intramuscularly into the rear leg with one single human dose (0.5 ml) of pertussis whole cell vaccine (NIBSC 88/522). Boost after 4 weeks with the same dose by sub-cutaneous route. Bleed after a further 2 weeks and store sera infinitely as 200 µl aliquots at -20°C. When thawed, store at +4°C for up to 1 year with the expiry date written on the container. There is no requirement for a set titre.

Whole-cell Pertussis antigen solution (+ve control)

This is prepared in-house by growing one vial of *B. pertussis* (Wellcome 28, Phase I, stored indefinitely at -70ºC) in 100ml of Stainer and Scholte liquid media for 48 hours at +37ºC. Centrifuge for 10 minutes at 10,000 g. Discard the supernatant and re-suspend the pellet in 10 ml Empigen BB. Re-centrifuge the solution for 10 min at 10,000 g, discard pellet and store supernatant (antigen solution) indefinitely at +4°C with the preparation date written on the container. There is no requirement for a set dose.

Phosphate buffered saline (PBS) pH 7.4

NaCl	10 g
KCl	0.75 g
$Na_2HPO_4.12H_2O$	1.44 g
KH_2PO_4 (anhydrous)	0.125 g

Dissolve to a final volume of 1 litre in distilled water. Decant 100 ml into 150 ml glass bottles and sterilize by autoclaving at a minimum of 15 lbs (121ºC) for at least 15 minutes. Store at 2-8ºC for up to 2 years.

Stainer and Scholte Liquid medium for B. pertussis

Basal Medium

L-Glutamic acid (mono Na salt)	10.72 g
L-Proline	0.24 g
NaCl	2.5 g
KH_2PO_4(anhydrous)	0.5 g
KCl	0.2 g
$MgCl_2.6H_2O$	0.1 g
$CaCl_2.2H_2O$	0.02 g
Tris	1.525 g

Make up to 1 litre with distilled water and pH to 7.3 - 7.4. Autoclave at 15 lbs for 20 mins.

Shelf life = 2 months

Supplement

L-Cystine	0.4 g *
$FeSO_4.7H_20$	0.1 g
Ascorbic acid	0.2 g
Nicotinic acid	0.04 g
Glutathione (red.)	1.0 g

Dissolve all without heating and make up to 100 ml. Filter sterilise.

Shelf life = 5 weeks

Immediately before use add 1 ml of supplement to 100 ml basal medium.

Empigen BB

Commercially available detergent

Experimental procedures:

Preparation of test vaccines

a) Desorption of adsorbed DTP vaccines:

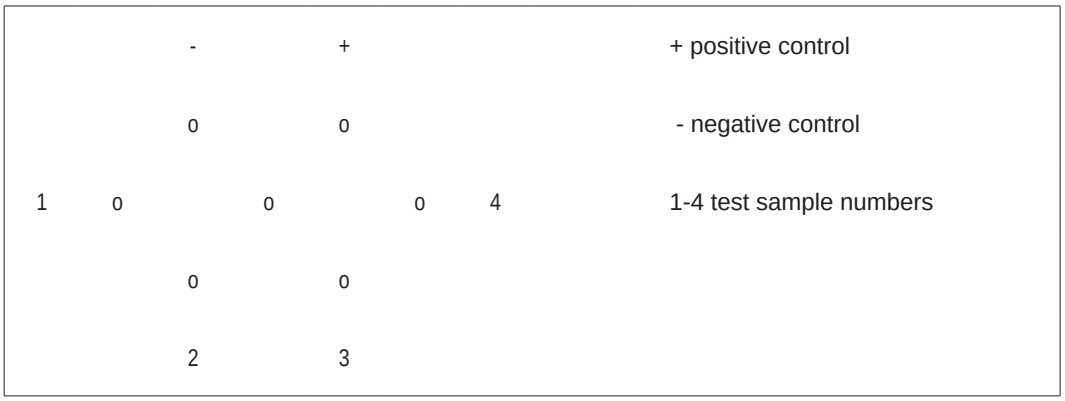
1) Add 1ml of vaccine to 0.1g tri-sodium citrate in a microfuge tube.
2) Mix until tri-sodium citrate has dissolved using a vortex mixer and incubate for 16-20 hours at +37°C.
3) Centrifuge in microcentrifuge at 13000 rpm for 10 minutes to give a clear supernatant, free from adjuvant.
4) Remove supernatant and resuspend pellet in 10 µl of Empigen BB and incubate for 90 minutes at +37°C.
5) Centrifuge in microcentrifuge at 13000 rpm for 10 minutes, remove supernatant and store for up to 1 week at +4°C until required for test.

* Dissolve first in 1 ml 6 N HCl & 4-5 mls H_2O

b) Gel Preparation

For whole-cell pertussis, melt Seakem agarose and allow to cool to pouring temperature, then dispense 3ml immediately onto a clean plastic immunodiffusion plate. Allow to set for 15-30 minutes. A maximum of two sets of wells are cut in a six-shooter design not less than 10 mm apart (pattern).

Figure 10: Pattern



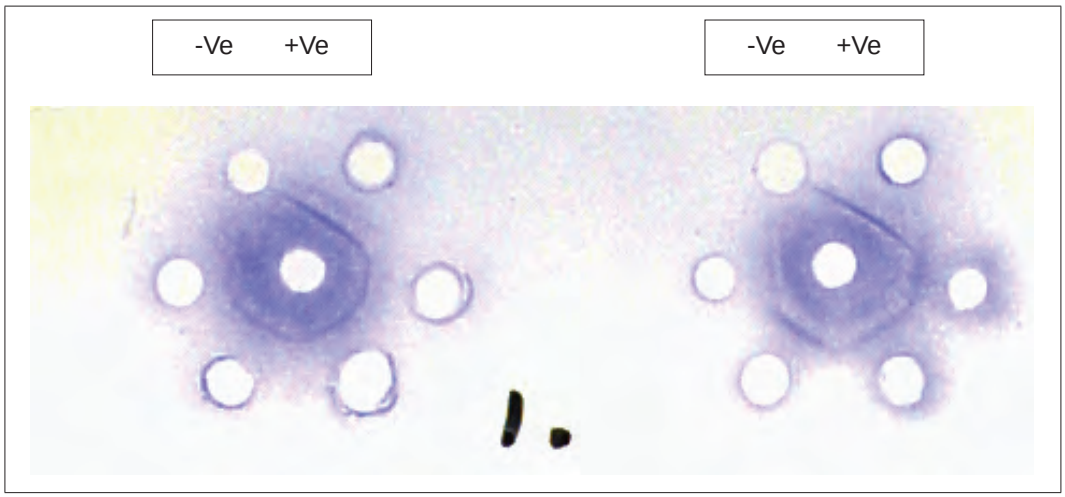
c) Addition of Samples and Controls

For identity of whole-cell pertussis component, place 10 µl (2 lots of 5 µl, allowing the first 5 µl addition to absorb into the gel before adding the next 5 µl) of whole-cell pertussis antiserum in the central well. Add 10 µl (2 lots of 5 µl, allowing the first 5 µl addition to absorb into the gel before adding the next 5 µl) of controls and test samples to the surrounding wells according to the layout (pattern).

d) Identity test

The plates are covered with lids and incubated for 16 to 48 hours in a humidified box at room temperature. Remove lids and secure gels to plates with elastic bands. The gels are soaked in sterile PBS for 4 to 24 hours. Cut right-hand top corner of the gel to aid in orientation. Remove the gels from the plates and place onto an alcohol cleaned glass sheet. Fill wells with sterile PBS and cover with a sheet of filter paper and a thick layer of paper towels. Apply a heavy weight to flatten the gels onto the glass sheet for 20 minutes. Change the paper towels and reapply the weight for a further 20 minutes. Remove weight and paper towels and dry filter paper and glass at +37°C for up to 10 minutes, then removefilter paper and allow glass sheet and gels to air dry for up to 60 minutes (advisory note: during these drying stages, check that the gel is not drying out too much). Stain gels by immersion in Coomassie blue stain for 3 minutes. Immerse gels in destain solution until the background is clear and the blue precipitate ring for diphtheria and tetanus antigens, and blue precipitation line for pertussis component is clearly visible to the naked eye.

Figure 11:



Assay validity criteria:

For the identity test to be valid the positive control should demonstrate a blue precipitin ring for diphtheria and tetanus antigens, and blue line of precipitin for pertussis component. The negative control should show no blue precipitin line or rings. If positive control does not demonstrate a blue precipitin ring/line, repeat test.

It is impossible to set detection limits for whole-cell pertussis vaccines, due to the large number of different antigens present. Because of this, per well, there must no less than 10 µl of desorbed vaccine, which is equivalent to 0.08 SHD.

Pass criteria:

The vaccine passes the test if a blue precipitin line is observed for whole-cell pertussis components.

Fail to pass:

If test vaccine does not demonstrate a blue precipitin line, repeat test at least twice. If the test vaccine gives positive response in both repeated tests, the vaccine passes. If the test vaccine does not show a blue precipitin line, the lot fails to pass.

Data monitoring:

The positive control must show a line of precipitate and the negative control must not show a line of precipitate.

Contents

Chapter V:
Statistical analysis of results

V.1 The use of biostatistics in vaccine testing

V.1.1 Introduction

This Section of the manual provides guidance on the statistical design and analysis of bioassays used for vaccine testing and potency determination. Methods of calculation are limited to the assays described in the manual, and are intended for use by those who do not have extensive training or experience in statistics. Professional advice is recommended for situations where laboratory constraints require customized assay designs or where analysis outside the scope of this manual is required.

The potency assays described in this manual are assumed to be 'dilution assays'. That is, the test and reference vaccines are assumed to contain the same active principle and behave as unknown dilutions of each other. It is also assumed that neither test nor reference vaccine contains additional components which affect the response of the bioassay. That is, the two preparations are assumed to be 'similar'. If there are 'interfering' components which differ between the test and reference vaccines, then the estimates of potency may be unpredictably affected depending on the way in which the interference affects the biological response. In some cases, the interfering components may affect the response and cause non-parallelism, so that the assay fails to meet statistical validity criteria. However, these criteria, while necessary, are not sufficient, to show that the test and reference vaccines are similar. There may be interfering substances with 'parallel' activity in the assay system. This situation is illustrated for some hypothetical cases in Table V.1.1. The critical factors are the components which differ between test and reference vaccines and how these interact with the assay system. These can only be considered on a case by case basis. The introduction of new combined vaccines and active principles has increased the complexity of factors which must be considered.

For a dilution assay, the ratio of the doses of the test and reference vaccines giving the same response in the bioassay is the relative potency, and this ratio is assumed to be the same for all doses. Consequently the mathematical functions which describe the dose – response curves are identical for reference and test vaccines except for a possible shift of 'dose' by the relative potency (Figure V.1.1). When considered over the entire dose range, the relationship of the biological responses to dose typically follows a sigmoid shaped curve which extends from the 'natural' biological status of the system at zero dose of vaccine to the maximum response which can be achieved by the system. For the assays described in this manual, experience has shown that response (or transformed response) is linearly related to the logarithm of dose over a sufficiently wide range of doses to permit comparison of test vaccines with reference vaccines using this linear part of the relationship. This gives the 'parallel-line' model.

Figure V.1.1.

Parallel line assays. Figure a) shows the complete response range for two parallel curves A and B with constant displacement. Figure b) shows the log-dose response lines for linear regions of dose response curves. This provides the basis for calculation of log potency, *Mp*, determined as the shift in position of log-dose response lines for the test product relative to the reference standard. Y_R denotes the response for the reference and Y_P denotes that for the test preparation. X_R and X_P denote the log doses for the reference and test preparations respectively.

Figure A:

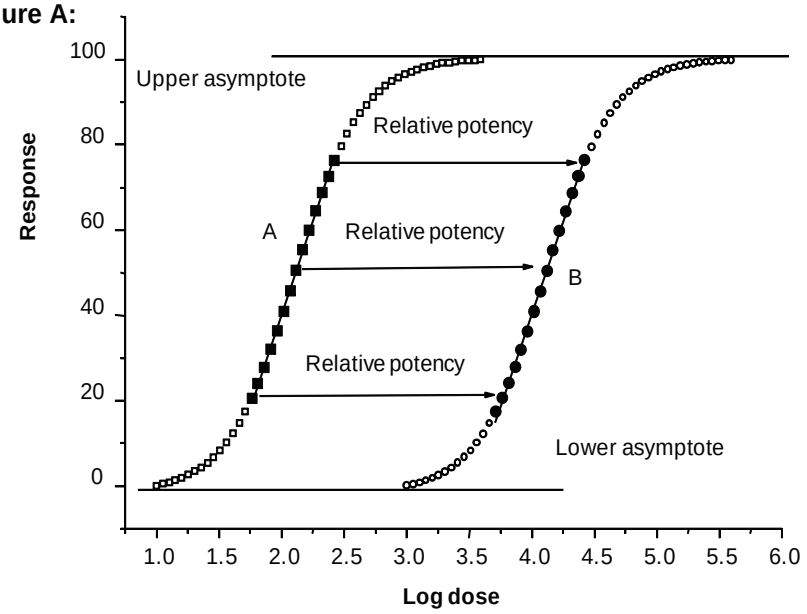
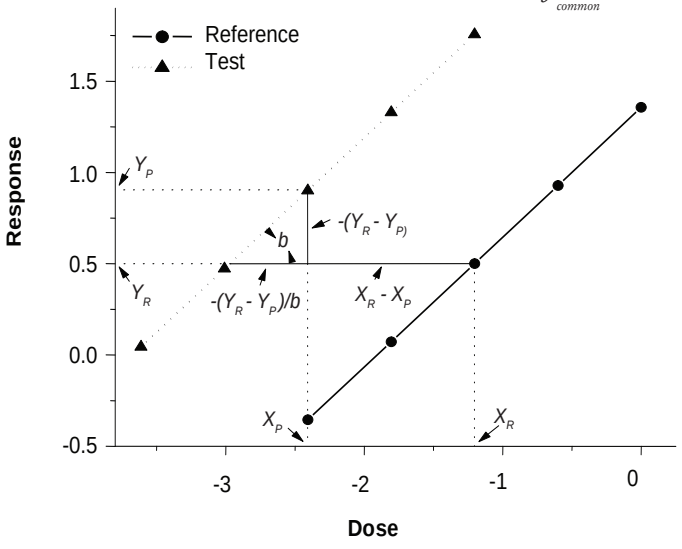


Figure B:

$$M_p = \bar{x}R - \bar{x}p - \frac{\bar{y}_R - \bar{y}_p}{b_{common}}$$



The analysis for the potency assays given in this manual is based on assessing the data for conformity with the parallel-line model. If the analysis shows that there is a significant linear relationship between the response and log dose and if there are no statistically significant deviations from linearity and parallelism, then the assay is considered to be statistically valid for the purposes of potency estimation. Statistical validity, that is, the absence of significant deviations from linearity or parallelism, does not 'prove' that the lines are linear and parallel. It only establishes that any deviations are smaller than the capacity of the assay system to detect them. The probability with which the statistical tests detect any deviations from the model will depend on the precision of the assay and the existence and magnitude of any differences from the model. However, for the assays discussed here, statistical validity is considered sufficient to allow potency estimation.

Although the primary aim of these assays is to estimate potency of the test product, the statistical analysis described here also provides some information about both the assay system and the vaccine quality. For example, occurrence of significant deviations from parallelism may indicate a change in the quality or composition of the product, occurrence of significant deviations from linearity may indicate a change in the way the assay system is performing. If significant deviations from linearity or parallelism occur in an individual assay, the data and the specific test sample must be further examined to determine possible reasons and the appropriate action. If significant deviations from linearity or parallelism occur consistently and more frequently than the expected 5% of assays, then the correct action may be to reconsider the assay validation (Section V.6).

Table V.1.1. Hypothetical examples of comparisons of test and reference preparations which contain different components, demonstrating that the relative potency depends on the interaction between any dis-similarity of the reference and test preparations and any non-specificity of the assay system. ***A*** denotes the active principle of the vaccine. ***X***, ***Y*** and ***Z*** denote different additional components. If the assay system is highly specific and responds only to the active ingredient, then the relative potency will be correctly determined even if the reference and test vaccines contain different 'inert' components. For each of the hypothetical cases shown below, the 'correct' potency of the test vaccine in terms of the reference for the active principle, A is 0.5.

Table V.1.1:

Composition of		Assay	Relative Potency given by assay
Reference vaccine	Test vaccine		
'Ideal' assay specific for A only			
AAXXY	AYYZZ	'Ideal' assay responds only to A	0.5 Correct
'Ideal' composition *			
A(XYZ)A(XYZ)	A(XYZ)	Any assay which detects A	0.5 Correct
Non-ideal compositions and assays **			
AAXYY	AXXZZ	Assay detects A and X equally; assay does not detect Y or Z	1.0 Not correct
		Assay detects A and X, but X has half the effect of A; assay does not detect Y or Z	0.8 Not correct
		Assay detects A and Z equally; assay does not detect X or Y	1.5 Not correct
		Assay detects A and Y equally; assay does not detect X or Z	0.25 Not correct

* Components other than A, which may themselves be active in the assay system, are identical and occur in the same relative proportions with A in both reference and test vaccines.

** The statistical tests for validity may or may not distinguish between different compositions, depending on the way in which the different components interact.

V.1.2 Experimental design and randomization

In the ideal situation, an assay would give the 'true' potency of the test vaccine without error. However, the biological systems which form the basis of bioassays are inherently variable, reflecting the responsiveness of biological functions to a wide variety of environmental and other factors. This responsiveness gives bioassays their high specificity and sensitivity for measuring the biological activity of vaccines. This responsiveness also means that good experimental design is essential to ensure that all factors apart from the applied treatment are carefully controlled in order to reduce variability and the possibility of bias. Control of many factors is achieved to some extent by inclusion of a reference standard in each assay. Inclusion of both reference standard and test product in the same correctly designed assay means that they are equally influenced by any extraneous factors.

The aim of experimental design is to select and to allocate the 'experimental units' to the defined treatments, i.e. doses of vaccine or controls, in such a way that, as far as possible, the only factor which affects the response is the assigned treatment. Positional effects, time and / or order effects, effects due to different operators, influence of food and other known factors may be controlled by appropriate experimental design. Animals from different batches or shipments may differ in their responsiveness and may be a biased sample from the population. Many differences between animals can be controlled by selecting healthy animals with restricted sex, age, weight range and other characteristics. Genetically homogeneous inbred strains might reduce animal variation, but in practice healthy out-bred strains are widely used without apparent problems. Failure to control for such factors may lead to bias when treatments are compared. That is, the both the treatment and the uncontrolled factors will influence the response, and their separate effects can not be distinguished. If, for example, an assay used animals restricted in weight to 18g to 20g, but animals with weights from 18g to 19g were assigned to one treatment and animals with weights from 19g to 20g were assigned to another treatment, comparison of the two groups would be biased. Any difference in mean responses could be due to differences in weight and not due to differences in treatment.

Some differences will inevitably remain. No two animals are identical; the same operator can not administer a treatment simultaneously to different animals; all animals can not be in the same cage simultaneously. The role of randomization of the animals is to ensure that any remaining differences and any uncontrolled factors are spread with equal probability among all treatments. This avoids bias and the effects of any unknown or unspecified factors to link to particular treatments. Thus only the specified treatments differentially influence the responses.

Complete randomization of all animals or experimental units may not be practical or feasible. Nevertheless, highly structured and systematic designs should be avoided.

For example, complete randomization of all wells on a microtitre plate is not feasible. However, microtitre plates are frequently used with highly systematic designs. The standard preparation may be always positioned in row A or in columns 1 and 2. Such designs should be avoided. Alternative approaches to complete randomization include randomizing the order in which treatments are assigned to rows or columns, and ensuring that there are replicate independent rows or columns (possibly separated on the plate) with the same treatment so that the variation due to serial dilution is to some extent measured.

Similarly, it may not be feasible to use a completely randomized design for some in vivo assays. Nevertheless, systematic designs must be avoided, for example assay designs in which low, medium and high doses of reference standard are administered in order followed by those for the test product. Animals within a cage may, for example, all be given the same treatment. From a statistical point of view, this confounds any effects due to the particular cage position or other cage effect with the effects due to the assigned treatment. As far as possible, animals should be randomly assigned to cages, treatments should be blinded and assigned to cages in randomized order, and replicate cages should be used to provide some indication of the magnitude of any cage effects. It may be possible to design experiments in which cages are 'blocks' with the number of animals in a cage being the same as the total number of treatments, and with each animal in the cage receiving a different treatment. A simple example of a 'block' design is given. Professional advice should be taken to ensure the best analysis if more complex designs are used.

An objective procedure should be specified and followed for randomization. Simple physical procedures can be used, and there are also computer based techniques which provide random sequences of numbers. A description of the assay design and the randomization procedure used is an essential part of the report of an assay.

V.1.3 Variation

The variation among the responses of the treated animals forms the basis for the probable range of values for the estimated potency or other assay parameters. Many of the statistical methods used for the analysis of assays involve calculating the total variance among the responses and then partitioning it, or assigning it to the different specified sources. The residual variance is the variation among animals which have been commonly treated. Variances due to other factors are compared to this residual variance. Thus, for most assays, the first step in the analysis is to calculate the 'analysis of variance'. The way in which this is done is dependent on the assay design, and on the specified sources of variation. Examples are given in the following sections.

Valid interpretation of the analysis of variance and assignment of probabilities requires that the responses are independent, are normally distributed about their expected values, and are homogeneous.

Statistical tests for normality may be considered but are not powerful for small group sizes, and are thus not recommended for individual bioassays.

There are a variety of statistical tests for homogeneity of variances and for outliers. However, because of the small group sizes typical of bioassays, these tests are not powerful, and can give misleading results. The issue of homogeneity should be addressed during assay validation. Responses should not be excluded from analysis except where there are clear technical reasons for omission of an observation as an outlier.

Independence is essential for valid estimation of variance, and no statistical method can identify or 'adjust for' non-independence of responses. Failure to randomize frequently leads to non-independent responses and as a result, potency estimates may be biased by confounding factors.

V.1.4 Principles of bioassay validation

Introduction of any assay method into a laboratory requires some validation, or assurance that the method is suitable for its intended purpose.

In taking decisions about recommended assays which are considered appropriate for calibration of International Standards, the Expert Committee on Biological Standardization of WHO selects as far as possible assays which are 'ideal', that is, which are specific for the active principle of the vaccine and are not affected by the customary non-specific components of the reference and test materials. It may be impossible to achieve the ideal assay, but the recommended assay will be that which is deemed by experts in the field to most nearly achieve the ideal for the majority of test products. In the case of these Standard or official methods, such as many of those described in this manual, validation in the individual laboratory may be limited to ensuring that the staff in the laboratory have adequate training, selecting an assay design suitable for the available facilities, determining a suitable dose range for the available animals and conditions and assessing the precision of the method using the laboratory facilities and staff.

Assay methods and products are subject to on-going development. Thus, within laboratories assays have been developed for a number of vaccines and validated for use under specifically defined conditions. Introduction of these methods into another laboratory requires validation for use within these conditions. *In vitro* methods have been developed for their convenience in some instances. For some assays mice are more readily available and preferred to guinea pigs. Frequently these alternate methods are sensitive to the matrix or other non-specific components of either the product or the International Standard (IS) for the vaccine. Such assays raise the question of how the International Unit (IU) is determined for the in-house reference and subsequent calibration of the test product in IU in terms of the in-house reference. This topic is discussed in ***Section V.6.3****. Validation of in house specific procedures and 'transferability' of IU.*

If a newly developed or modified method is under consideration, stringent and thorough validation is required. Many issues must be addressed, including the suitability and specificity of the method for the materials to be tested, the theoretical and statistical nature of the dose–response relation, appropriate assay designs, and accuracy, precision, robustness and sensitivity of the method. If the method is to be considered as an alternative to or replacement for an existing method, a multi-laboratory comparative study of the methods is likely to be required. The statistical methods required for such validation are outside the scope of this manual, and professional statistical advice may be required.

V.1.5 Consistency of assay parameters

A number of statistical parameters, such as the slope of the dose – response line for the standard, responses of control groups to defined doses, dose giving 50% response (ED_{50}), residual variance of responses, and so on, are calculated for each bioassay. These calculated values may not directly determine the validity of the assay. Nevertheless, for an assay which is carried out frequently or routinely, the calculated parameters should be broadly consistent across assays. It is thus good practice to maintain charts of these values. Deviations in these values should not be the basis for rejection of assays. However, any marked change in the value or a consistent trend indicating a 'shift' in the values should be explored, as this may indicate that factor(s) affecting the assay have unexpectedly changed.

V.1.6 Retesting and combination of estimates

Assay repeats or retesting of products may be necessary for a variety of reasons. In the procedures specified for carrying out an assay, the different types of 'failure' should be defined and there should be a specified plan of action to take if a 'failure' occurs.

Technical accidents or errors may occur, such as incorrect storage of a sample, accidental use of an incorrect reagent, use of an incorrect injection technique by an inexperienced member of staff. A valid potency estimate cannot be obtained from such an assay and the assay must be repeated. Action to guard against the identified accident in future may be needed, but no usable estimate of potency can be obtained from these data.

Assays may fail to meet defined criteria for validity. For example, one pharmacopoeial requirement for the Kendrick test is that the ED_{50} for each vaccine should lie between the largest and smallest immunizing doses. For 'three dilution' assays, the statistical tests should not show significant deviations from the theoretical 'parallel-line' model. That is, the data should not show significant deviations of the log dose–(transformed) response lines for the test and reference vaccines from linearity and parallelism to one another. Actions in response to these 'statistical' failures must be considered in terms of the cause of the failure and the available historical information about both the assay and the test product. Although the defined validity criteria are not met, it may nevertheless be possible to obtain a valid potency estimate.

Some assays may conform statistically to the theoretical model, but the potency estimate may fail to achieve the required precision. The criteria for the Kendrick test require that the estimate of potency for the test product should be not less than 4.0 IU per single human dose and that the lower 95% limit for the estimate should be not less than 2.0 IU. An assay may give an estimate greater than 4.0 IU, but the lower limit may not meet this requirement since assays vary in their precision for a number of reasons. In such cases, it may be necessary to repeat the assay so that the combined estimate from two or more assays achieves the required precision.

Whenever two or more valid assays have been carried out, the estimates from all assays must be combined. Methods for combination are given in Section V.5.

V.1.7 3Rs in bioassays

Any scientist carrying out bioassays using animals should be aware of the 3Rs, as described by Russell and Burch (1959). Thus, *in vivo* bioassays should only be used if scientifically valid *in vitro* or other techniques are not available. Refinement should be introduced as far as possible in *in vivo* bioassays. For example, several of the assays described here employ 'humane endpoints'. Techniques are being developed which allow use of the same animals for serologically based testing of more than one component in some combined vaccines. Replacement of *in vivo* bioassays is encouraged, but it must be based on sound scientific principles and requires stringent validation.

Reduction, a key element of the 3Rs has been described as the mode of progress 'most obviously, immediately, and universally advantageous in terms of efficiency' without loss of scientific information. Good experimental design is a key element in reduction. Thus, in vivo assays should be designed to use the minimum number of animals consistent with giving an estimate with the required precision. Assays described in WHO Guidelines and Recommendations frequently indicate the numbers of animals which have been found to be suitable for recommended assays, typically for three dilution assays, in which three dilutions for each of the test and reference vaccines are used. However, following assay validation and experience of consistency with a given product, further reduction may be possible, and in some cases 'single dilution' assays have been suggested (SectionV.6.4.).

One technique frequently employed to reduce the total number of animals is to design assays to include several test preparations (different batches of the same product, or in control laboratories batches of similar products from different sources) to be compared with a single reference preparation. From a solely theoretical statistical perspective, it is sometimes suggested that data from such an assay should be treated as a 'multiple'

assay and all data simultaneously analyzed. However, for control purposes, estimates of potency for the test batches should be calculated by pair wise comparison of each test batch in turn with the reference preparation. That is, the analysis should be restricted only to the comparison of direct interest, and should not be affected by other factors (in particular, test preparations from different sources) which are not relevant to this comparison. It must be noted that estimates determined in this way are not independent of one another and cannot be combined using the methods of Section V.5. This is not relevant if there are two different test products, and no reason for combination of their estimates. However, it is not correct to test two vials of the same batch of test product in an assay with a single standard and then attempt to use this assay to provide two independent estimates for the batch. These estimates are not independent and the methods of Section V.5 cannot be used to combine them.

V.1.8 Statistical software

A wide variety of computing resources are now available and it is expected that most laboratories will have access to some form of statistical software. The available software ranges from large complex packages requiring a good statistical background and computing skills to packages designed for easy use by a less experienced user. There are also numerous web based resources.

Before using any type of software, it is essential to demonstrate that the software is performing correctly. The examples given in this manual can be used as test data but these examples are not exhaustive and cover a limited range of conditions.

Many individual laboratories have developed in house programs and calculation procedures based on the use of spreadsheets such as Excel. These are likely to continue to be used where the laboratories have validated them and found them to be suitable. Particular caution should be exercised in the interpretation of results from such programs if the data do not conform exactly to the specifications for which the procedures were developed.

There are many commercial packages of which two examples are given. CombiStats (*www.combistats.edqm.eu*) was initially developed for use by the Official Medicines Control Laboratory network in Europe for analysis of data from biological dilution assays, but is now available more widely. SAS (www.sas.com) is a powerful and versatile commercial software package providing many options and extensive programming capacity and is widely used by the pharmaceutical industry.

Free 'shareware' is available on the internet, but should be accessed with caution and specific applications may be limited. R is a programming language and software widely used for statistical computing and graphics (*www.r-project.org*). It is available free, but its use requires computing and programming skills.

Individual users and laboratories have developed software and some may make these available on an ad hoc basis. One example is a software package, 'Bioassay Assist', developed for use in routine bioassays by National Institute of Infectious Diseases (NIID), Japan. NIID has donated this software to WHO now. Any request from the member states of WHO shall be addressed to Dr Dianliang Lei, QSS/EMP/HIS/WHO at leid@who.int.

V.1.9 Further reading

A wide variety of statistical text books and other resources are available. Most national pharmacopoeias contain a statistical chapter, frequently with descriptions of the methods of calculation and with examples.

A limited selection of texts is indicated. Many references and discussions of specific methods and designs are found in the biological literature.

The classical source for statistical analysis of bioassays is:

1) Finney DJ. (1978, 3rd Edition) Statistical Method in Biological Assay, Charles Griffin and Company Ltd., High Wycombe, UK.

A text for more general statistical analysis is:

1) Sokal RR and Rohlf FJ (1994, 3rd Edition) Biometry, W.H. Freeman

A classical text describing a wide variety of designs is:

1) Cochran WG and Cox GM (1957) Experimental Designs, John Wiley and Sons Ltd.

Brief discussion of the general issues of design for biologists is given in:

1) Festing MFW, Overend P, Gaines Das R, Cortina Borja M, Berdoy M. (2002) The Design of Animal Experiments. Laboratory Animal Handbooks No. 14. The Royal Society of Medicine Press Ltd. London.

The three Rs are described in:

1) Russell WMS, Burch RL (1959) The Principles of Humane Experimental Technique. Reprinted 1992. Wheathampstead, Universities Federation for Animal Welfare.

2) The principles of quality control / assurance are widely discussed in the statistical literature. The guidelines for the system(s) under which the laboratory operates should be consulted.

V.2 Parallel line analysis, quantitative responses

V.2.1 Introduction

Parallel line analysis is used for assays with quantitative responses. The log dose-response lines are assessed for linearity and parallelism and used for analysis. For some assay responses, the raw responses must be transformed to give a more nearly linear relation with log dose. Suggested response transformations are indicated with the details of the assay.

For some types of assay, for example ELISAs, where a dose–response curve for a wide range of doses is produced, the extended curve may be non-linear. Typically such curves have a sigmoid shape with a lower and upper asymptote. There may not be a convenient transformation which gives linearity over the complete response range. However, for many assays, a suitable transformation of the responses will give linearity over much of the curve (typically for responses falling between about 20 per cent and 80 per cent of the asymptotic limits). Parallel line analysis using only responses in this nearly linear region is satisfactory in many cases. Statistical techniques are available for analyses incorporating all responses. However, the complexity of the statistical issues raised by the various approaches is outside the scope of this manual. If analysis of all non-linear responses is desired, professional advice is recommended.

V.2.2 Assay design

Many assay designs are possible, depending on the facilities within a laboratory. For all assays, it is essential that the design is clearly specified, that the procedure for random assignment of treatments to animals is specified, and that the analysis used is appropriate to the design. These issues should be addressed when the assay is validated for use in the particular laboratory.

Other factors which contribute to optimizing the assay precision should also be considered. The ratio between the residual error and the slope of the log dose–(transformed) response line (sometimes referred to as the index of precision) should be as small as possible. The range of doses should be as large as possible, provided that these remain in the linear part of the dose–response range. The response range for the test and reference preparations should be similar.

The analyses and examples given here are for the more straight forward designs, and typically for the completely randomized design. If the total group of animals is reasonably homogeneous, then allocation of treatments to animals is made randomly, subject to the restriction that each treatment is as far as possible allocated to the same number of animals. Where the animals can be divided into recognizable sub-groups, then these sub-groups should be considered separately, and treatments allocated randomly within the sub-group. For example, for the challenge assay for tetanus vaccine potency, if both male and female animals are used, then there must be equal numbers of males and females in each treatment group. The experimental records should include details of the sex with the response, so that data for male and female animals can be separately analyzed, or so that the difference in response between them can be included in the analysis of variance. Animals might similarly be put into sub-groups on the basis of weight or age, if these factors have not been strictly limited when the total group of animals is selected. These identified sub-groups must also be equally distributed among the treatment groups.

V.2.3 Analysis of variance

As a preliminary step before any analysis, it is recommended that the assay data should be plotted and visually examined for consistency with the expected model.

Calculation of the analysis of variance is simplified if there are equal numbers of animals in each treatment group, the same number of treatment groups for each preparation, and an equal interval (on a logarithmic scale) between doses. These simplifications are made for the formulae given here. These restrictions are not necessary for a valid and correct assay, but the more general computational formulae are outside the scope of this text. Where suitable software is available, the calculations present no difficulty.

V.2.3.1 Calculation of analysis of variance

The formulae given here are for data having k doses and n repeats of measurement for each of the ***Reference*** preparation and q test samples as shown in the table below. It is assumed that the response values have been assessed during assay validation for normality and homogeneity and appropriately transformed if required (Section V.6.). The calculations given below are for a control or release assay which would customarily use data only for the Reference and a single Test lot. Where suitable software is available, it may not be necessary to refer to these detailed calculations.

Notation is shown in Table V.2.1, with calculated means and variances.

The formulae for the general analysis of variance over all preparations in an assay are given (Table V.2.2.). The combined slope over all preparations and the pooled residual variance over all preparations may sometimes be considered as giving more stable and reliable estimates for these terms, since they are based on larger amounts of data. However, the reliability of these estimates depends on the assumption that each of the preparations included in the assay has the same slope and variance. This assumption may not be true and can never be guaranteed when different test preparations are compared. If for example, one preparation had a different slope than the others, the common slope would be affected by this different slope. Thus, for product release only the direct comparison of the test sample and the reference preparation should be considered (Table V.2.3.), so that the effect of any other possibly different preparations does not influence the result.

The analysis described here is sometimes carried out although the assay design is not completely randomized. For example, animals may be randomly assigned to cages, and the cages may be positioned using a random design. Nevertheless, if all animals in the same cage receive the same treatment, there are likely to be additional cage effects which can influence the response of all animals in the same cage. The estimation of variance among the animals within a cage does not include the additional variation in response due to the cage effect. A consequence of this is that the residual variance may be underestimated. This variance is the denominator of the F test used to assess linearity and parallelism, and if it is incorrectly too small, then the value of F may be incorrectly too large, and thus the deviations from linearity and parallelism may appear to be statistically significant. If this occurs, then the assay design should be carefully considered to determine whether there may be non-random factors influencing the responses. Non-random factors may also lead to bias in the estimate of potency, and to statistically significant deviations from homogeneity when estimates are combined.

Table V.2.1: Notation for assays with measurement of a continuous variable. Y denotes the (transformed) continuous response variable, Z denotes dose, X denotes log-dose, n denotes the number of responses at the specified dose. The subscripts identify the preparation, dose, and replicate. For example, Y_{R11} denotes the first response to dose Z_{R1}. of preparation R, Y_{Rkn} denotes the n_kth response to dose Z_{Rkn} of preparation R

Sample		Dose	Individual Responses		Log Dose	Mean	Variance
Source Details	ID						
Reference	R	Z_{R1}	$Y_{R11}, Y_{R12}, Y_{R13}, \ldots, Y_{R1j}, \ldots, Y_{R1n}$	n_{R1}	X_{R1}	$\Sigma Y_{R1j}/n_{R1}$	$\Sigma(Y_{R1j}-\bar{Y}_{R1})^2/(n_{R1}-1)$
		Z_{R2}	$Y_{R21}, Y_{R22}, Y_{R23}, \ldots, Y_{R2j}, \ldots, Y_{R2n}$	n_{R2}	X_{R2}	$\Sigma Y_{R2j}/n_{R2}$	$\Sigma(Y_{R2j}-\bar{Y}_{R2})^2/(n_{R2}-1)$
		...	...	...	...	...	...
		Z_{Ri}	$Y_{Ri1}, Y_{Ri2}, Y_{Ri3}, \ldots, Y_{Rij}, \ldots, Y_{Rin}$	n_{Ri}	X_{Ri}	$\Sigma Y_{Rij}/n_{Ri}$	$\Sigma(Y_{Rij}-\bar{Y}_{Ri})^2/(n_{Ri}-1)$
		...	...	...	...	...	...
		Z_{Rk}	$Y_{Rk1}, Y_{Rk2}, Y_{Rk3}, \ldots, Y_{Rkj}, \ldots, Y_{Rkn}$	n_{Rk}	X_{Rk}	$\Sigma Y_{Rkj}/n_{Rk}$	$\Sigma(Y_{Rkj}-\bar{Y}_{Rk})^2/(n_{Rk}-1)$
Manufactured Lot	T	Z_{T1}	$Y_{T11}, Y_{T12}, Y_{T13}, \ldots, Y_{T1j}, \ldots, Y_{T1n}$	n_{T1}	X_{T1}	$\Sigma Y_{T1j}/n_{T1}$	Similarly as for R (with subscript R replaced by T)
		Z_{T2}	...	n_{T2}	X_{T2}	$\Sigma Y_{T2j}/n_{T2}$	
		...	...		...	...	
		Z_{Tk}	$Y_{Tk1}, Y_{Tk2}, Y_{Tk3}, \ldots, Y_{Tkj}, \ldots, Y_{Tkn}$	n_{Tk}	X_{Tk}	$\Sigma Y_{Tkj}/n_{Tk}$	
Manufactured Lot	U	Z_{U1}	Similarly as for T for U and any other manufactured product				

For each preparation, the sums of squares (SS) and slope are calculated as follows.

(Reminder of notation: The ***i***th log dose of the ***p***th sample including the Reference (***p=1~q=R***, $T_1,\ldots,T_U$) is denoted X_{pi} , the number of repeats of measurement at log dose X_{pi} as n_{pi}, and the value of the ***j***th response measurement as Y_{pij}.)

SS of deviations of log doses *x* for preparation ***p***

$$Sxx_p = \sum (x_{pi} - \bar{x}_p)^2 = \sum n_{pi} \cdot x_{pi}^{\ 2} - (\sum x_{pi} \cdot n_{pi})^2 / \sum n_{pi}$$

SS of deviations of responses *y* for preparation ***p***

$$Syy_p = \sum (y_{pij} - \bar{y}_{pi})^2 = \sum y_{pij}^{\ 2} - (\sum y_{pij})^2 / \sum n_{pi}$$

SS of deviations of *x and y* for preparation ***p***

$$Sxy_p = \sum (x_{pi} - \bar{x}_p)(y_{pij} - \bar{y}_p) = \sum x_{pi} y_{pij} - (\sum x_{pi})(\sum y_{pij}) / \sum n_{pi}$$

Regression coefficient (slope) for preparation ***p***

$$b_p = Sxy_p \Big/ Sxx_p$$

The overall sums of squares for all samples to give the analysis of variance are calculated as:

SS total: $_T Syy = \sum (y_{pij} - \bar{y})^2 = \sum y_{pij}^{\ 2} - (\sum y_{pij})^2 / N$

Where $\bar{y} = \Sigma y_{pij} \Big/ N$, $N = \sum n_{pi}$

SS between all preparations: $_P Syy = {}_T Syy - \sum Syy_p$

SS within doses (Error)
(pooled over all doses and preparations): $_E Syy = \sum \sum (y_{pij} - \bar{y}_{pi})^2$

SS between all doses: $_D Syy = {}_T Syy - {}_E Syy$

SS regression: $S_{reg} = \sum Sxy_p^{\ 2} \Big/ \sum Sxx_p$

SS parallelism: $S_{para} = \sum Sxy_p^{\ 2} \Big/ Sxx_p - Sreg$

SS linearity: $S_{lin} = {}_D Syy - {}_P Syy - S_{reg} - S_{para}$

The analysis of variance given by these calculations is summarized in Tables V.2.2. (multiple test preparations) and V.2.3. (pair wise comparison of reference preparation and one test preparation).

Table V.2.2. Analysis of variance for assays with multiple preparations

Source of variation	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio
Preparation	${}_pSyy$	q - 1	${}_pSyy/(q - 1)$	$({}_pSyy/(q - 1))/({}_ESyy/(N - \Sigma\Sigma k))$
Regression	S_{reg}	1	$S_{reg}/1$	$(S_{reg}/1)/({}_ESyy/(N - \Sigma\Sigma k))$
Parallelism	S_{para}	q - 1	$S_{para}/(q - 1)$	$(S_{para}/(q - 1))/({}_ESyy/(N - \Sigma\Sigma k))$
Linearity	S_{lin}	$\Sigma\Sigma k - 2q$	$S_{lin}/(\Sigma\Sigma k - 2q)$	$(S_{lin}/(\Sigma\Sigma k - 2q))/({}_ESyy/(N - \Sigma\Sigma k))$
Between Doses	${}_DSyy$	$\Sigma\Sigma k - 1$	${}_DSyy/(\Sigma\Sigma k - 1)$	$({}_DSyy/(\Sigma\Sigma k - 1))/({}_ESyy/(N - \Sigma\Sigma k))$
Error(Residual)	${}_ESyy$	$N - \Sigma\Sigma k$	${}_ESyy/(N - \Sigma\Sigma k)$	
Total	${}_TSyy$	N - 1		

q: total number of samples tested
N: over all number of measurements
k: number of treatments (doses)

Table V.2.3. Analysis of variance for pairwise comparison of reference and a single test preparation.

Source of variation	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio
Preparations	${}_pS_{yy}$	1	${}_pS_{yy}/1$	$({}_pS_{yy}/1)/({}_ES_{yy}/(N - \Sigma\Sigma k))$
Regression	S_{reg}	1	$S_{reg}/1$	$(S_{reg}/1)/({}_ES_{yy}/(N - \Sigma\Sigma k))$
Parallelism	S_{para}	1	$S_{para}/1$	$(S_{para}/1)/({}_ES_{yy}/(N - \Sigma\Sigma k))$
Linearity	S_{lin}	$\Sigma\Sigma k - 4$	$S_{lin}/(\Sigma\Sigma k - 4)$	$(S_{lin}/(\Sigma\Sigma k - 4))/({}_ES_{yy}/(N - \Sigma\Sigma k))$
Linearity (R)	S_{linR}	$k_R - 2$	$S_{linR}/(k_R - 2)$	$(S_{linR}/(k_R - 2))/({}_ES_{yy}/(N - \Sigma\Sigma k))$
Linearity(T)	S_{linT}	$k_T - 2$	$S_{linT}/(k_T - 2)$	$(S_{linTp}/(k_T - 2))/({}_ES_{yy}/(N - \Sigma\Sigma k))$
Between treatments	${}_DS_{yy}$	$\Sigma\Sigma k - 1$	${}_DSyy/(\Sigma\Sigma k - 1)$	$({}_DS_{yy}/(\Sigma\Sigma k - 1))/({}_ES_{yy}/(N - \Sigma\Sigma k))$
Residual	${}_ES_{yy}$	$N - \Sigma\Sigma k$	${}_ESyy/(N - \Sigma\Sigma k)$	
Total	${}_TS_{yy}$	N - 1		

V.2.3.2 Interpretation of the analysis of variance

The probability of the variance ratios, or the F values, in the analysis of variance table are determined using suitable software, or the values are compared with tabulated values using the degrees of freedom as shown in the analysis. The order in which the variance ratios are generally considered is that given below.

There are three requirements for statistical validity of the assay.

1) **The regression of the response on log dose must be statistically significant**, or else the assay is not valid. For a valid assay, the responses to a large dose of vaccine should differ significantly from the responses to a small dose of vaccine. If the regression is not significant the assay response does not change significantly as the dose of vaccine changes, and no potency estimation is possible. The regression should be highly significant, typically with probability less than 0.01.

2) **The deviations from parallelism must not be statistically significant**. Significant deviations from parallelism indicate fundamental invalidity of the assay. That is, the assumption that the test preparation and the reference preparation act as dilutions of one another is false. Potency calculation is not valid, and the vaccine must be retested.

3) **The deviations from linearity must not be statistically significant**. That is, the log dose–response lines should be linear. Deviations from linearity may be statistically significant if doses used do not give responses in the linear range. Changes in the assay conditions may lead to a shift in the dose–response curves so that the doses used are not in the linear range. For example, a different shipment of animals may have a different sensitivity to the vaccine. If historical information indicates that the shift is not too great, and there is good information about the consistency of the product, then the doses outside the linear range may be omitted and analysis of the remaining doses carried out. A vaccine may be more potent than expected, and although three dilutions have been tested, the maximum possible response may be given by the largest dilution and a near maximum response by the second largest dilution. Depending on the historical information about the product, it may in this case be possible to consider analysis based on only two lower doses of the test vaccine after omitting the largest dose.

The difference between preparations should ideally be small. That is, the response range for the test and reference preparations should be similar, so that the overall mean responses are similar. A significant difference between preparations does not invalidate the assay, but may reduce the precision, and in some cases the accuracy, of the estimate of potency. In some cases a large difference may be the consequence of an assay design using a multiple dilution dose–response line for the reference preparation and a limited number of dilutions of a test vaccine in an assay where several different test preparations are being assessed. If a large difference is observed, the position of the response of the test preparation on the dose–response line for the reference preparation should be examined, and the response of the test preparation should lie within the response range of the reference preparation.

V.2.4 Potency estimation

Mean dose for the reference preparation $\bar{x}_R = \sum(x_{Ri} \cdot n_{Ri}) \Big/ \sum n_{Ri}$

Mean response $\bar{y}_R = \sum y_{Rij} \Big/ \sum n_{Ri}$

Mean dose for the pth sample $x_p = \sum(x_{pi} \cdot n_{pi}) \Big/ \sum n_{pi}$

Mean response $\bar{y}_p = \sum y_{pij} \Big/ \sum n_{pi}$

Regression coefficient is calculated by the following equation for samples R and T.(note that p denotes samples 1, 2$\cdots$,p,$\cdots q$ including R and T)

$$b_{common} = \sum Sxy_p \Big/ \sum Sxx_p$$

Relative potency of each sample referring to the reference preparation can be calculated using those values by the following equation.

Relative potency of the pth sample

$$M = \bar{x}_R - \bar{x}_p - \frac{\bar{y}_R - \bar{y}_p}{b_{common}}$$

Confidence interval of the relative potency can be calculated by following equations.

Median value of the confidence interval

$$M_c = \bar{x}_R - \bar{x}_p + \frac{M - \bar{x}_R + \bar{x}_p}{1 - g_p}$$

Width of the confidence interval

$$t\sqrt{V_M} = \frac{t \cdot s}{b_{common}(1-g_p)} \sqrt{(1-g_p)\left(\frac{1}{\sum n_R} + \frac{1}{\sum n_p}\right) + \frac{(M - \bar{x}_R + \bar{x}_p)^2}{Sxx_R + Sxx_p}}$$

Where degrees of freedom based on numbers for R and T is $df=\Sigma n_{pi}-\Sigma k_{pi}$

$$g_p = \frac{t^2 \cdot s^2}{b_{common}{}^2 \cdot (Sxx_R + Sxx_p)}$$

Upper and lower limits of confidence interval are given by

$$M_U, M_L = M_c \pm t\sqrt{V_M}$$

Where

$$V_M = \frac{s^2}{b_{common}{}^2(1-g_p)^2}\left((1-g_p)\left(\frac{1}{\sum n_R} + \frac{1}{\sum n_p}\right) + \frac{(M - \bar{x}_R + \bar{x}_p)^2}{Sxx_R + Sxx_p}\right)$$

is the estimate of variance of relative potency M.

For large values of g, due to large variance or small degrees of freedom, these calculations may not be possible. In such cases, the protocol of the test should be revised to employ a wider range of doses or increased repeats of measurement to improve the accuracy.

When doses were transformed into logarithm, 10^M, 10^{ML}, 10^{MU} are relative potency and lower and upper limits of confidence interval, respectively. If unit value of the reference or standard preparation was given as U, the values of relative potency and its confidence limits need to be multiplied by U to obtain unit value and its confidence interval of a sample. When no transformation was made for doses, M, ML, MU multiplied by the unit value of reference preparation are relative potency and confidence limits of samples.

V.2.5 Examples

V.2.5.1 Example 1. Histamine sensitization test by Temperature method

A reference standard, R, and a test product, sample T, were tested using 3 doses of reference standard and two doses of test product. There was sufficient historical information about the test vaccine to assure its linearity so that a two dilution test can be used. A completely randomized design was followed. Temperatures were recorded following histamine challenge. For 10 mice in each of three groups for the reference preparation and the two groups for T. Data are shown in Table V.2.5, and plot of data is given in Figure V.2.1.

Calculations are shown in detail. However, if suitable software is used, the results as shown in the analysis of variance, Table V.2.6, may be confirmed.

Table V.2.4. Data for Example1.
Histamine sensitization assay by temperature method.
Responses are temperatures recorded following histamine challenge for 10 mice in each group.

Experiment 1					
Sample information	NCL Reference L2			Manufacturer A DTP	
Sample ID	R			T	
Dose	0.0625	0.25	1	0.333333333	1
Responses	37.0	36.1	29.7	33.4	35.1
	34.2	35.3	31.1	35.2	30.6
	31.6	33.0	36.0	32.6	30.7
	36.4	32.0	31.8	33.0	31.4
	34.9	31.8	31.1	31.4	32.9
	32.9	37.7	30.7	33.9	31.6
	36.4	35.2	31.0	33.8	30.5
	36.6	33.8	36.4	33.1	31.5
	35.7	34.2	37.5	32.8	32.4
	33.8	35.7	31.3	34.0	32.9
Log dose	-1.2041	-0.6021	0.0000	-0.4771	0.0000
n	10	10	10	10	10
Mean	34.95	34.48	32.66	33.32	31.96
Variance	3.223	3.526	7.932	1.022	2.005

Table V.2.5. Analysis of variance for Example 1.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	Probability F Ratio
Between Treatments	4	61.691	15.42280	4.35	0.005
Preparations	1	23.18520	23.18520	6.55	0.014
Regression	1	34.58357	34.58357	9.77	0.003
Parallelism	1	0.88493	0.88493	0.25	0.62
Linearity	1	3.03750	3.03750	0.86	0.36
Residual	45	159.36500	3.54144		
Total	49	221.05620			

Details of calculations are given below:

The sums of square for the individual preparations are calculated as follows:

SS of deviations of *x*

For R: Sxx_R = - 1.204^2x10 - 0.602^2x10+0^2x10 - (- 1.204x10 - 0.602x10+0x10)^2/30=7.250

For T: Sxx_T = - 0.4771^2x10+0^2x10 - (- 0.4771x10+0x10)^2/20=1.138

SS of deviations of *y*

For R: Syy_R=37.0^2+34.2^2+···+37.5^2+31.3^2 - (37.0+34.2+···+31.3)^2/30

=34,902.6 - 1,020.9^2/30=161.383

For T: Syy_T=33.4^2+35.2^2+···+32.9^2 - (33.4+35.2+···+32.9)^2/20=32,343.88 - 652.8^2/20=36.488

SS of deviations of *x* and *y*

For R: Sxy_R = - 1.204x37.0 - 1.204x34.2+···+0x37.5+0x31.3

- 10x(- 1.204 - 0.602+0)x(37.0+34.2+···+31.3)/30= - 628.43 - 18439.3/30= - 13.787

For T: Sxy_T = - 0.4771x33.4 - 0.4771x35.2+···+0x32.4+0x32.9

- 10x(- 0.4771+0)x(33.4+35.2+···+32.9)/20= - 158.98 - 3114.65/20= - 3.2444

Regression coefficients for the reference preparation R and test sample T.

For R: $b_R = Sxy_R / Sxx_R$ = - 13.787 / 7.2495= - 1.902

For T: $b_T = Sxy_T / Sxx_T$ = - 3.2442 / 1.1382= - 2.850

The calculations of the Sums of square for the analysis of variance are shown below to give the results shown in Table V.2.5.:

SS total: ${}_TSyy$=37.0^2+34.2^2+···+32.4^2+32.9^2 - (37.0+34.2+···+32.4+32.9)^2/50

=56,246.5 - 1,673.7^2/50=221.056 (*df*=50 - 1=49)

SS preparation: ${}_PSyy = {}_TSyy - \Sigma Syy_p$=221.056 - (161.38+36.488)

=23.185 (*df*=2 - 1=1)

SS within doses (Residual) : ${}_ESyy$=3.223x9+3.526x9+····+1.022x9+2.005x9

=159.365 (*df*=(10 - 1)x5=45)

SS between doses (Treatments): ${}_{D}Syy={}_{T}Syy - {}_{E}Syy$=221.056 - 159.365=61.691 (*df*=5 - 1=4)

SS regression: $S_{reg}=(Sxy_p)^2/\Sigma Sxx_p$=(- 13.787 - 3.2442)²/(7.2495+1.1382)= 34.583 (*df*=1)

SS parallelism: $S_{para}=\Sigma(Sxy_p{}^2/Sxx_p) - S_{reg}$
=(- 13.787)²/7.2495+(- 3.2442)²/1.1382 - 34.583
=0.885 (*df*=2 - 1=1)

SS linearity: $S_{lin}={}_{D}Syy-{}_{P}Syy-S_{reg}-S_{para}$=61.691 - 23.185 - 34.583 - 0.885
=3.038 (*df*=3 - 2=1)

Summary of assay parameters:

Sample R: Slope = -1.9018 Response intercept = 32.8850

Sample T: Slope = -2.8502 Response intercept = 31.9600

Common Slope = b = -2.0305 Variance of common slope = 0.42221

Standard deviation = s = 1.88187 = $\sqrt{3.54144}$

Index of precision = s/b = -0.92679 Finney's g = 0.41579

Interpretation of the analysis of variance and assessment of the assay validity

1) Regression of response on log dose is significant; $F_{1,45} = 9.77$, $p < 0.05$.
2) Deviations from parallelism are not significant; $F_{1,45} = 0.25$, $p > 0.05$.
3) Deviations from linearity are not significant; $F_{1,45} = 0.86$, $p > 0.05$.

Although the responses for the test and reference vaccine differ significantly ($F_{1,45} = 6.55$, $p < 0.05$) this does not mean that the assay is not valid. Examination of the mean responses for the individual dilutions of the two preparations, and of the plot shown in Figure V.2.1 indicate that the response range for the reference vaccine broadly encompasses that for the test vaccine, but that the overall mean for the reference vaccine is larger than that for the test vaccine since an additional dilution has been used.

The assay satisfies statistical validity criteria and an estimate of relative potency with its limits can be calculated.

Relative activity

Mean dose

Reference preparation: $\bar{x}_R = (-1.2041 - 0.6021 - 0)\times 10/30 = -0.6021$

Sample 1: $\bar{x}_{S1} = (-0.477 + 0)\times 10/20 = -0.2386$

Mean response

Reference preparation: $\bar{y}_R = (37.0 + 34.2 + \cdots + 37.5 + 31.3)/30 = 34.0$

Test sample T: $\bar{y}_{S1} = (33.4 + 35.2 + \cdots + 32.4 + 32.9)/20 = 32.6$

Common slope: $b_{common} = \sum Sxy_p / \sum Sxx_p = (-13.787 - 3.244)/(7.250 + 1.138) = -2.031$

Relative activity

Sample1: $M_T = \bar{x}_R - \bar{x}_p - \frac{\bar{y}_R - \bar{y}_p}{b_{common}} = -0.6021 + 0.239 - \frac{34.0 - 32.6}{-2.031} = 0.321$

Confidence interval

Median value of confidence interval

Sample 1: $M_c = \bar{x}_R - \bar{x}_p + \frac{M - \bar{x}_R + \bar{x}_p}{1 - g_p}$

$$= -0.6021 + 0.239 + \frac{0.321 + 0.602 - 0.239}{1 - 0.415} = 0.806$$

Where

Degrees of freedom for Sample 1: $df = \Sigma n_i - \Sigma k_i = 50 - 5 = 45$

g for Sample1: $g_{S1} = \frac{t^2 \cdot s^2}{b_{common}{}^2 \cdot (Sxx_R + Sxx_T)} = \frac{2.014^2 \cdot 3.541}{(-2.031)^2 \cdot (7.250 + 1.138)} = 0.415$

Variance of M

Sample 1

$$V_M = \frac{s^2}{b_{common}{}^2 (1-g)^2}\left((1-g)\left(\frac{1}{N_R} + \frac{1}{N_T}\right) + \frac{(M - \bar{x}_R + \bar{x}_T)^2}{Sxx_R + Sxx_T}\right)$$

$$= \frac{3.541}{(-2.031)^2 \cdot (1-0.415)^2}\left((1-0.415) \cdot \left(\frac{1}{30} + \frac{1}{20}\right) + \frac{(0.321 + 0.6021 - 0.239)^2}{7.250 + 1.138}\right)$$

$$= 0.263$$

Upper and lower limits of confidence interval

$$M_L, M_U = M_c \pm t\sqrt{V_M} = 0.806 \pm 2.014\sqrt{0.263} = -0.225 \sim 1.840$$

Log relative activity of Sample T and 95% confidence interval

0.321(- 0.225 ~ 1.840)

Weight = 3.804

Relative activity (antilog) : 2.094 (0.595 ~ 69.2)

Multiply unit value of the reference preparation for obtaining unit value of test preparation T.

V.2.5.2 Example 2. Histamine sensitization assay by Temperature method

The same assay method as described in Example1 was used. For this assay, 4 doses of reference standard and 3 doses of test product were tested using a completely randomized design. The sample S1 of Example 1 was measured in a second assay to obtain following results. Data are give in Table V.2.6 and a plot of these data is shown in figure V.2.2. Detailed calculations follow the formulae given above and are not given in detail.

SS deviations for X for R: $Sxx_R = 18.137$ and for T: $Sxx_T = 9.7712$

SS deviations for Y for R: $Syy_R = 151.691$ and for T: $Syy_T = 63.519$

SS of deviations of x and y for R: $Sxy_R = 39.7494$ and for T: $Sxy_T = 17.3345$

Regression coefficients for R:

$b_R = Sxy_R / Sxx_R = 2.1917$ and $b_T = Sxy_T / Sxx_T = 1.7740$

Assay parameters are calculated as:

Sample ID R: Slope = -2.1917 Response intercept = 32.3353

Sample ID S1: Slope = -1.7740 Response intercept = 32.4248

Common Slope = b = -2.0455 Variance of common slope = 0.05522

Standard deviation = s = 1.24140 = $\sqrt{1.54108}$

Index of precision = s/b = -0.60691 Finney's g = 0.05274

Interpretation of the analysis of variance and assessment of the assay validity:

1) Regression of response on log dose is significant; $F_{1,63} = 75.8$, $p < 0.05$.
2) Deviations from parallelism are not significant; $F_{1,63} = 0.72$, $p > 0.05$.
3) Deviations from linearity are not significant; for reference preparation R the ratio $F_{2,63} = 0.08$, $p > 0.05$ and for test sample T the ratio $F_{1,63} = 0.00$, $p > 0.05$.

The mean responses for the test and reference vaccine do not differ significantly ($F_{1,63} = 1.10$, $p > 0.05$) as confirmed by the plotted data, although this is not required for assay validity.

The assay satisfies statistical validity criteria and an estimate of relative potency with its limits can be calculated.

Log potency with 95% limits: 0.13667 (-0.15781 to 0.44975) ; weight = 43.344
Potency with 95% limits: 1.36983 (0.69533 to 2.81674.

Comparison of the results from Examples 1 and 2 indicate the way in which alterations in the assay design can affect the information obtained. Thus, for Example 1, there was no test for linearity of the log dose – response line for the test vaccine, and it was necessary to assume that this was known. The estimate of potency obtained for Example 2 is more precise than the estimate obtained in Example 1. This reflects both the greater precision of Example 2 compared with Example 1 and the greater number both of dilutions and of mice used for Example 2.

Table V.2.6. Example 2. Data from histamine sensitization assay by temperature method. Responses are temperatures recorded following histamine challenge for 10 mice in each group.

Experiment 2							
Sample information	NCL Reference L2				Manufacturer A DTP		
Sample ID	R				T		
Dose	0.015625	0.0625	0.25	1	0.0268	0.134	0.67
Responses	35.1	33.8	35.2	31.9	34.8	34.7	31.7
	35.5	32.7	34.7	33.2	34.5	33.8	33.5
	37.5	33.9	32.5	33.1	35.9	34.2	36.7
	37.1	35.7	32.5	30.8	35.0	33.9	31.8
	36.7	36.2	35.3	30.5	34.2	34.3	33.5
	36.0	34.9	33.7	31.6	35.2	33.7	33.7
	37.1	36.0	34.9	32.2	35.9	34	33
	35.6	34.5	34.4	31.5	35.9	33.5	31
	36.4	36.5	30.9	36.4	35.2	33.4	30.7
	35.5	35.6	33.7	31.3	35.5	34.3	31.7
Log dose	-1.8062	-1.2041	-0.6021	0.0000	-1.5719	-0.8729	-0.1739
n	10	10	10	10	10	10	10
Mean	36.25	34.98	33.78	32.25	35.21	33.98	32.73
Variance	0.685	1.526	2.044	2.892	0.361	0.162	3.118

Table V.2.7. Analysis of variance for Example 2.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	Probability F Ratio
Between Treatments	6	120.12286	20.02048	12.00	<0.000
Preparations	1	2.00119	2.00119	1.10	0.26
Regression	1	116.76294	116.76294	75.77	<0.000
Parallelism	1	1.10768	1.10768	0.72	0.40
Linearity (R)	2	0.25038	0.12519	0.08	0.92
Linearity (T)	1	0.00067	0.00067	0.00	0.98
Residual	63	97.0880	1.54108		
Total	69	217.21086			

Figure V.2.1. Data for Example 1.

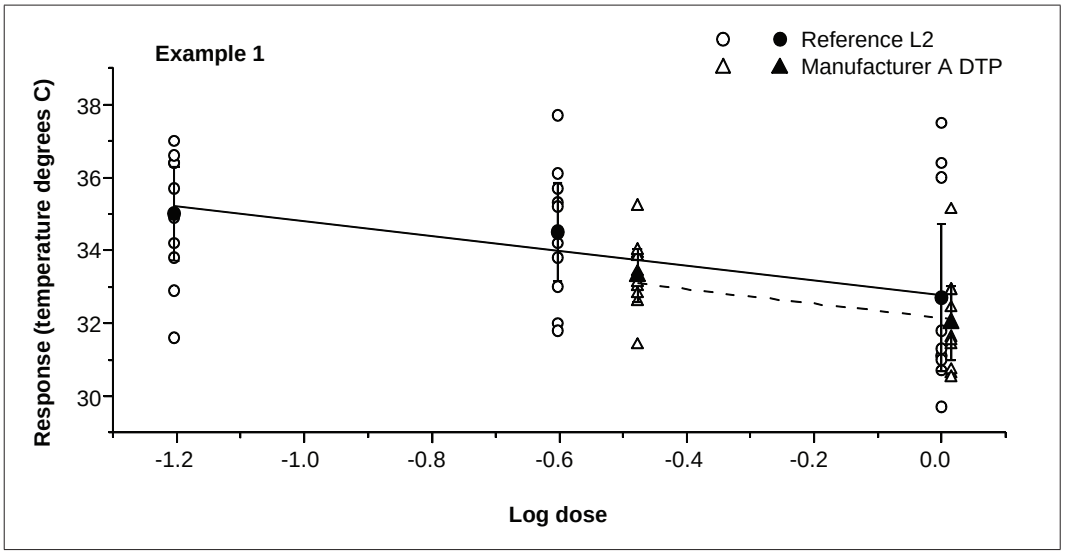
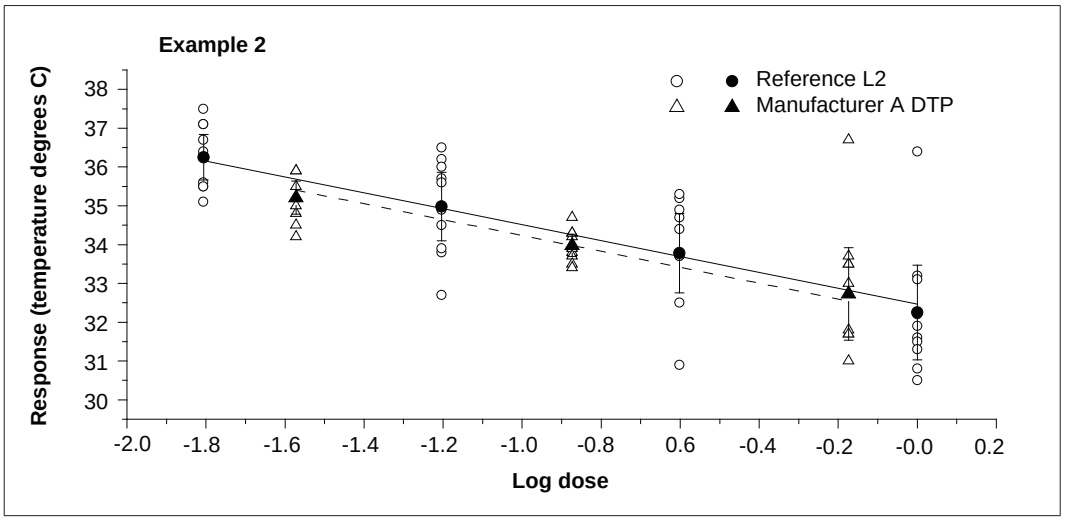


Figure V.2.2. Data for Example 2.



V.2.5.3 Example 3. Toxin binding inhibition assay of tetanus antitoxin in mice

A further example of the calculation method is given for data obtained in a toxin binding inhibition assay of tetanus antitoxin from mice immunized with diphtheria-tetanus-pertussis vaccine.

Data are shown in Table V.2.8. and Figure V.2.3. Detailed calculations are not given.

SS deviations for X for R: $Sxx_R = 3.6248$ and for T: $Sxx_T = 3.6248$

SS deviations for Y for R: $Syy_R = 142.875$ and for T: $Syy_T = 145.875$

SS of deviations of x and y for R: $Sxy_R = 18.3628$ and for T: $Sxy_T = 18.0618$

Regression coefficients for R:

$b_R = Sxy_R / Sxx_R = 5.0659$ and $b_T = Sxy_T / Sxx_T = 4.9829$

Assay parameters are calculated as:

Sample ID R: Slope = 5.0659 Response intercept = -0.9819

Sample ID T: Slope = 4.9414 Response intercept = -2.7783

Common Slope = b = 5.0244 Variance of common slope = 0.24786

Standard deviation = s = 1.34463 = $\sqrt{1.80804}$

Index of precision = s/b = 0.26790 Finney's g = 0.03976

Interpretation of the analysis of variance and assessment of the assay validity:

1) Regression of response on log dose is significant; $F_{1,56} = 101$, $p < 0.05$.
2) Deviations from parallelism are not significant; $F_{1,56} = 0.007$, $p > 0.05$.
3) Deviations from linearity are not significant; for reference preparation R the ratio $F_{2,56} = 1.338$, $p > 0.05$ and for test sample T the ratio $F_{2,56} = 0.0314$, $p > 0.05$.

The mean responses for the test and reference vaccine do not differ significantly ($F_{1,56} = 1.244$, $p > 0.05$) as confirmed by the plotted data, although this is not required for assay validity.

The assay satisfies statistical validity criteria and an estimate of relative potency with its limits can be calculated.

Log potency with 95% limits and weight:

-0.376 (0.516 to 0.241) weight = 220.64

Potency with 95% limits: 0.421 (0.3047 to 0.5737).

Figure V.2.3. Plotted data for Example 3.

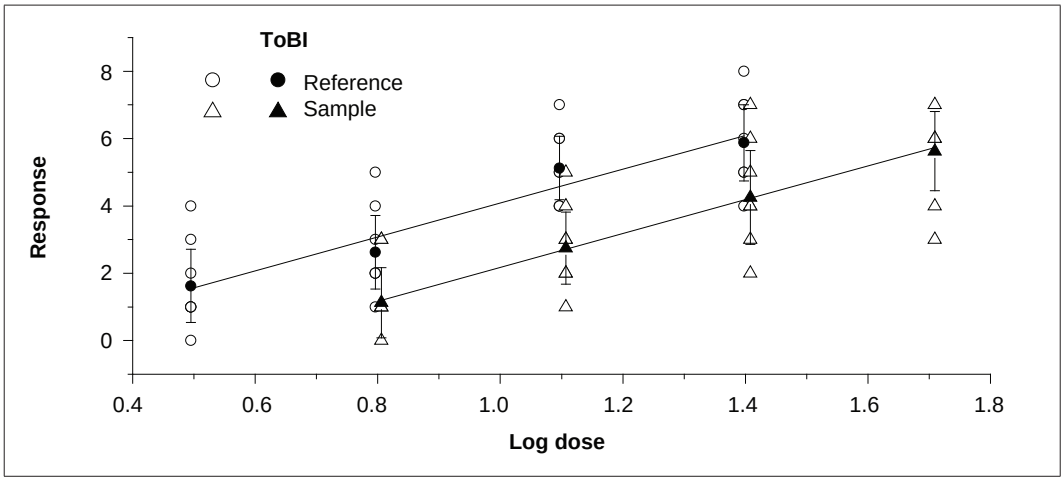


Table V.2.8. Data for example 3, with calculated means and variances.

Tetanus toxin binding inhibition assay								
Sample	Reference vaccine				Test vaccine			
Dose, µl	25	12.5	6.25	3.125	50	25	12.5	6.25
Responses	8	4	2	1	6	2	3	1
	7	5	2	4	6	3	2	3
	5	4	1	1	6	3	2	1
	4	6	3	3	3	4	4	3
	6	4	2	1	4	6	1	0
	7	5	5	0	7	7	5	0
	5	6	2	2	7	4	2	0
	5	7	4	1	6	5	3	1
Log Dose	1.3979	1.0969	0.7959	0.4949	1.699	1.3979	1.0969	0.7959
n	8	8	8	8	8	8	8	8
Mean	5.875	5.125	2.625	1.625	5.625	4.25	2.875	1.125
Variance	1.8393	1.2679	1.6964	1.6964	1.9821	2.7857	1.5536	1.5536

Table V.2.9. Analysis of variance for Example 3.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	Probability F Ratio
Between Treatments	7	189.7500	27.1071	14.99	<0.000
Preparations	1	2.25	2.25	1.244	0.31
Regression	1	183.01	183.01	101.222	<0.000
Parallelism	1	0.0125	0.0125	0.00691	0.89
Linearity (Ref)	2	4.35000	2.17500	1.338	0.31
Linearity (Test)	2	0.125	0.0625	0.0314	0.91
Residual	56	101.25	1.8080		
Total	63	291			

V.2.5.4 Example 4. Endotoxin test (LAL): Turbidimetric technique

A plot of the raw data of gelation time, Figure V.2.4a, shows that the log dose–untransformed response lines are not linear. However, experience with this assay during development and validation have indicated that a log log transformation of the responses may give linearity over much of the response range. Thus the responses are transformed to log log values, as shown in Figure V.2.4b, and these responses are analyzed. For data of this type, rounding errors may be a problem.

Table V.2.10a. Raw data for Example 4.

	Concentration	Gelation time(min)			X	y=log(log(min))			Syyi
		1	2	3		1	2	3	**Σyi2-(Σyi)2/n**
RSE	0.25 IU/mL	15.4	15.6	15.2	-0.602	0.075	0.077	0.073	0.000009
	0.125 IU/mL	18.2	18.8	18.0	-0.903	0.100	0.105	0.099	0.000023
	0.0625 IU/mL	22.0	22.4	22.0	-1.204	0.128	0.130	0.128	0.000004
	0.03125 IU/mL	26.6	28.4	27.6	-1.505	0.154	0.162	0.159	0.000037
	0.015625 IU/mL	33.6	33.8	34.4	-1.806	0.184	0.184	0.187	0.000005
	0.0078125 IU/mL	44.6	45.2	44.8	-2.107	0.217	0.219	0.218	0.000001
Sample	1/40,000(0.000025 mL)	15.0	14.4	15.0	-4.602	0.070	0.064	0.070	0.000029
	1/80,000(0.0000125 mL)	17.6	17.4	16.8	-4.903	0.095	0.094	0.088	0.000027
	1/160,000(0.00000625 mL)	21.0	20.6	21.4	-5.204	0.121	0.119	0.124	0.000015
	1/320,000(0.000003125 mL)	26.0	25.2	26.6	-5.505	0.151	0.147	0.154	0.000026
	1/640,000(0.0000015625 mL)	33.0	30.0	33.0	-5.806	0.181	0.169	0.181	0.000096
	1/1,280,000(0.00000078125 mL)	43.0	40.0	42.0	-6.107	0.213	0.205	0.210	0.000037
Water LAL	1	>60	>60	>60	>60	>60	>60	>60	-

Gelation time: time for exceeding predetermined threshold turbidity due to gelation

Table V.2.10.b. Transformed data for Example 4, used for calculation of analysis of variance and potency estimation. Transformed responses are obtained as log(log(time)).

Preparation	Transformed Dose, X	Transformed Responses, Y			Mean	Variance
Reference	-2.10721	0.21731	0.21883	0.21782	0.21799	0.000001
	-1.80618	0.18365	0.18438	0.18655	0.18486	0.000002
	-1.50515	0.15378	0.16236	0.15864	0.15826	0.000019
	-1.20412	0.12789	0.13041	0.12789	0.12873	0.000002
	-0.90309	0.10039	0.10522	0.09874	0.10145	0.000011
	-0.60206	0.07464	0.07669	0.07256	0.07463	0.000004
Test Sample	-6.10721	0.21311	0.20468	0.21038	0.20939	0.000019
	-5.80618	0.18142	0.16942	0.18142	0.17742	0.000048
	-5.50515	0.15075	0.14656	0.15378	0.15036	0.000013
	-5.20412	0.12130	0.11855	0.12399	0.12128	0.000007
	-4.90309	0.09535	0.09361	0.08825	0.09240	0.000014
	-4.60206	0.07044	0.06384	0.07044	0.06824	0.000015

Detailed calculations of the sums of squares for the Reference and Test preparations:

SS of deviations of *x*

For Reference preparation R:

Sxx_R=(- 0.602)²x3+(- 0.903)²x3+···+(- 1.806)²x3+(- 2.107)x3
- (- 0.602x3 - 0.903x3+··· - 2.107x3)²/18=4.758

For Test sample T:

Sxx_T=(- 4.602)²x3+(- 4.903)²x3+···+(- 6.107)²x3
- (- 4.602x3 - 4.903x3+··· - 6.107x3)²/18=4.758

SS of deviations of *y*

Syy_R=0.075²+0.077²+···+0.219²+0.218² - (0.075+0.077+···+0.218)²/18
=0.4176 - 2.598²/18=0.0427

Syy_T=0.070²+0.064²+···+0.210² - (0.070+0.064+···+0.210)²/18
=0.3777 - 2.457²/18=0.0423

SS of deviations of *x* and *y*

Sxy_R= - 0.602x0.075 - 0.602x0.077+···- 2.107x0.219 - 2.107x0.218
- 3x(- 0.602 - 0.903+··· - 2.107)x(0.075+0.077+···+0.218)/18
= - 3.969 - (- 24.383)x2.598/18= - 0.450

Sxy_T= - 4.602x0.070 - 4.602x0.064+···- 6.107x0.205 - 6.107x0.210
- 3x(- 4.602 - 4.903+···- 6.107)x(0.070+0.064+···+0.210)/18
= - 13.605 - (- 96.383) x 2.457/18= - 0.447

Regression coefficients for the reference preparation and sample 1.

For Reference preparation R: $b_R = Sxy_R / Sxx_R = -0.450 / 4.758 = -0.0946$
For Test sample T: $b_T = Sxy_S / Sxx_S = -0.447 / 4.758 = -0.0940$

For the analysis of variance, the total sums of squares are calculated as:

SS total: ${}_TSyy = 0.075^2+0.077^2+\cdots+0.205^2+0.210^2 - (0.075+0.077+\cdots+0.205+0.210)^2/36 = 0.795 - 5.055^2/36 = 0.0855$ (df=36 - 1=35)

SS preparation: ${}_PSyy = {}_TSyy - \Sigma Syy_p = 0.0855 - (0.0427+0.0423) = 0.00055$ (df=2 - 1=1)

SS within doses: ${}_ESyy = 0.00009+0.000023+\cdots+0.000096+0.000037 = 0.00031$ (df=(3 - 1)x12=24)

SS between doses: ${}_DSyy = {}_TSyy - {}_ESyy = 0.0855 - 0.00031 = 0.0852$ (df=12 - 1=11)

SS regression: $S_{reg} = (Sxy_p)^2/\Sigma Sxx_p = (-0.450 - 0.447)^2/(4.758+4.758) = 0.0846$ (df=1)

SS parallelism: $S_{para} = \Sigma(Sxy_p{}^2/Sxx_p) - S_{reg}$
$= (-0.450)^2/4.758+(-0.447)^2/4.758 - 0.0846 = 0.000001$ (df=2 - 1=1)

SS linearity: $S_{lin} = {}_DSyy - {}_PSyy - S_{reg} - S_{para} = 0.0852 - 0.00055 - 0.0846 - 0.000001 = 0.00010$ (df=2x(6 - 2)=8)

Table V.2.11. Analysis of variance for Example 4.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio	Probability F Ratio
Between Treatments	11	0.08520	0.00775	601.97	<0.000
Preparations	1	0.00055	0.00055	42.60	<0.000
Regression	1	0.08456	0.08456	6571.56	<0.000
Parallelism	1	0.00000	0.00000	0.07	0.79
Linearity (Ref)	4	0.00005	0.00001	0.88	0.36
Linearity (T)	4	0.00005	0.00001	0.99	0.33
Residual	24	0.00031	0.00001		
Total	35	0.08551			

Assay parameters are calculated using transformed data shown in Table below as:

Sample ID R: Slope = -0.09459 Response intercept = 0.01619

Sample ID S1: Slope = -0.09395 Response intercept = -0.8470

Common Slope = b = -0.09427 Variance of common slope = $(0.00116)^2$

Standard deviation = s = 0.00359 = $\sqrt{0.00001}$

Index of precision = s/b = -0.03805 Finney's g = 0.00065

Interpretation of the analysis of variance and assessment of the assay validity:

1) Regression of response on log dose is significant; $F_{1,24} = 6571$, $p < 0.05$.
2) Deviations from parallelism are not significant; $F_{1,24} = 0.07$, $p > 0.05$.
3) Deviations from linearity are not significant; for reference preparation R the ratio $F_{4.24} = 0.88$, $p > 0.05$ and for test sample T the ratio $F_{4,24} = 0.99$, $p > 0.05$.

There is a significant difference between preparations ($F_{1,24} = 43$, $p < 0.05$) but this does not mean that the assay is invalid. The plotted data confirm that this reflects the overall larger responses of the Reference than the Test preparation, and the relatively large number of doses for each preparation which makes this comparison precise. Nevertheless, the responses for two preparations cover broadly the same range, as shown by plotted data.

The transformed doses and responses (Table V.2.10.b) give log potency

1.08279 with 95% limits 1.05655 to 1.10913 and weight 6176

and relative potency

12.10004 with 95 % limits 11.39070 to 12.85673

which must be multiplied by 1000 to adjust for the transformation of doses in this table.

Relative activity

Mean dose

Reference preparation: $\bar{x}_R = (-0.602 - 0.903 - \cdots - 1.806 - 2.107) \times 3/18 = -1.355$

Sample: $\bar{x}_S = (-4.602 - 4.903 - \cdots - 5.806 - 6.107) \times 3/18 = -5.355$

Mean response

Reference preparation: $\bar{y}_R = (0.075 + 0.077 + \cdots + 0.219 + 0.218)/18 = 0.144$

Sample: $\bar{y}_S = (0.070 + 0.064 + \cdots + 0.205 + 0.210)/18 = 0.137$

Common slope:

$$b_{common} = \sum Sxy_p / \sum Sxx_p = (-0.450 - 0.447)/(4.758 + 4.758) = -0.0943$$

Relative potency

Sample: $$M_S = \bar{x}_R - \bar{x}_s - \frac{\bar{y}_R - \bar{y}_s}{b_{common}} = -1.355 + 5.355 - \frac{0.144 - 0.137}{-0.0943} = 4.083$$

Confidence interval

Median value of confidence interval

Sample 1: $$M_c = \bar{x}_R - \bar{x}_p + \frac{M - \bar{x}_R + \bar{x}_p}{1 - g_p}$$

$$= -1.355 + 5.355 + \frac{4.083 + 1.355 - 5.355}{1 - 0.00065} = 4.083$$

Where

Degrees of freedom for Sample 1: $df = \Sigma n_i - \Sigma k_i = 36\text{-}12 = 24$

Variance of M

Sample 1

$$V_M = \frac{s^2}{b_{common}{}^2(1-g)^2}\left((1-g)\left(\frac{1}{N_R}+\frac{1}{N_T}\right)+\frac{(M-\bar{x}_R+\bar{x}_T)^2}{Sxx_R+Sxx_T}\right)$$

$$= \frac{0.000013}{(-0.0943)^2\cdot(1-0.00065)^2}\left((1-0.00065)\cdot\left(\frac{1}{18}+\frac{1}{18}\right)+\frac{(4.083+1.355+5.355)^2}{4.758+4.758}\right)$$

=0.000162

Upper and lower limits of confidence interval

$$M_L, M_U = M_c \pm t\sqrt{V_M} = 4.083 \pm 2.064\sqrt{0.00016} = 4.057$$

Log relative activity of test preparation T and 95% confidence interval

4.083(4.057 ~ 4.109)

Relative activity (antilog) is: 12,099.9(11,391.1 ~ 12,856.0)

Adjust appropriately, if necessary, unit value of the reference preparation for calculating unit value of sample

Figure V.2.4. Endotoxin test, LAL Turbidimetric assay

Figure V.2.4a. Plot of untransformed data showing non-linear log dose – response lines

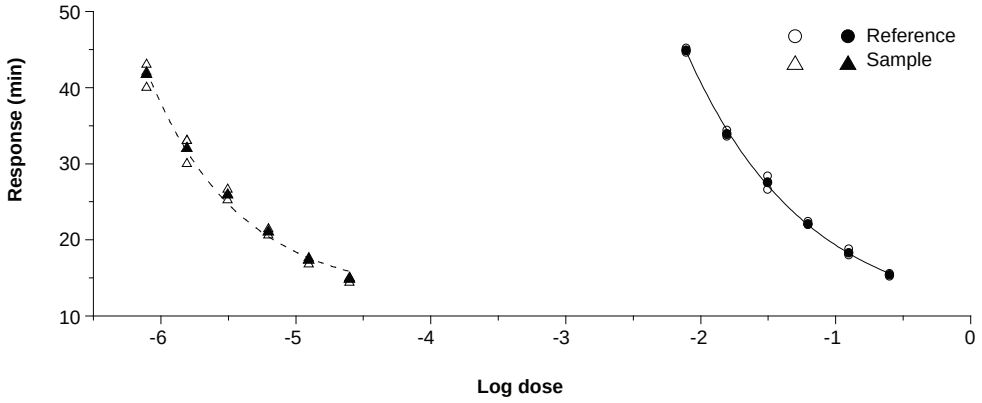
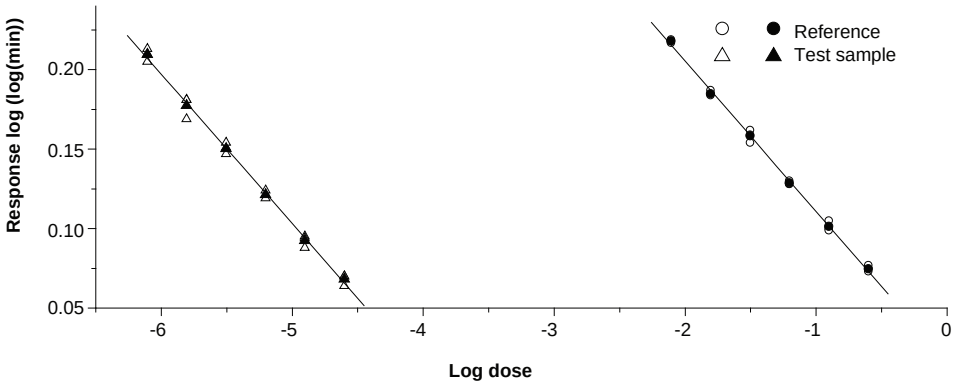


Figure V.2.4b. Plot of log10 log10 responses, showing graphically approximately linear log dose–log log transformed responses.



V.2.6 References

1) Finney DJ. (1978, 3rd Edition) Statistical Method in Biological Assay, Charles Griffin and Company Ltd., High Wycombe, UK.

2) The Statistical Chapters of National Pharmacopoeia may also be consulted for formulae and examples.

V.3 Parallel line assay analysis, quantal responses (Probit analysis)

V.3.1 Introduction

In some bioassay systems, the determination of a quantitative response to the treatment is not possible, or not feasible, and only a recognized binary, or quantal, response can be attributed to the animal. Binary responses are obtained when groups of animals have received different doses of vaccine and are then classified as 'protected' or 'not protected' following challenge with the pathogen from which the vaccine should provide protection. Examples include the Kendrick assay for whole cell pertussis vaccine and the lethal challenge assay for diphtheria vaccine in guinea pigs.

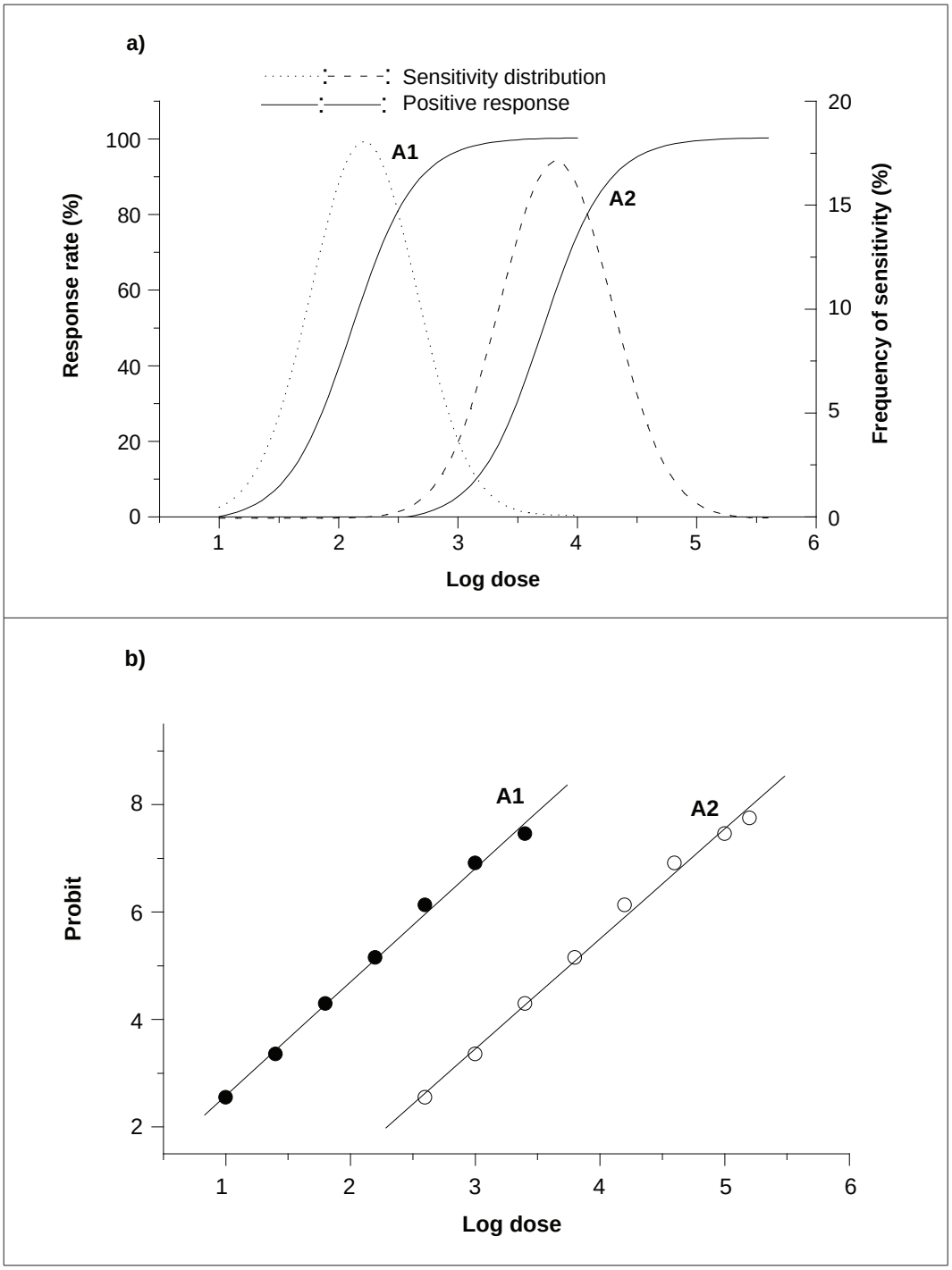
Binary responses should generally not be used if quantitative measures are available. Analysis based on quantal responses may be less efficient or precise than analysis based on quantitative responses. One example of a change from binary to quantitative responses is the replacement of the lethal response to challenge with diphtheria by a serological response. In changing to use of quantitative responses, doses of vaccine and amounts of challenge giving optimized assays with binary responses may not give optimized assays for quantitative responses.

Although a binary response is recorded for the individual animals, these are not generally used for analysis. Instead the 'tolerance' of probability of the 0 or 1 binary response is considered. An overall response to the dose of vaccine is recorded as the proportion of the group 'protected' from the defined amount of challenge. Potency is then estimated by using the relationship between the transformed proportions responding and the dose of vaccine, and it is normally assumed that these proportions have minimum 0% response and maximum 100% response. This procedure is directly analogous to the use of the relationship between the mean (transformed) response and the log dose for assays based on quantitative responses.

The transformation of the proportion responding is based on theoretical assumptions about the distribution of the tolerances of the animals to the treatments. The assumption that the log tolerances follow a normal distribution and, therefore, the cumulative tolerance rate to serial log doses (a) leads to the probit transformation to give a straight linear dose-response (b) which is widely used for potency assays (Fig. V.3.1). Other distributions may be used, but are not considered here. Differences in results based on the different distributions are generally not large provided the proportions responding lie between about 10-15% and 85-90%.

Particular attention must be given to the ways in which any analysis handles extreme responses of 0% or 100%. Different approaches may be taken by different analysts and procedures. The occurrence of extreme responses may lead to different results if different methods of analysis are used. Moreover, the distribution of the sums of squares calculated for the analysis of variance is based on assumptions that the expected numbers responding are not extreme, so the occurrence of extreme responses may mean that the interpretation of the results is not reliable.

Figure V.3.1: Probit transformation



V.3.2 Assay design

Good assay design for bioassays based on quantal responses follows the same principles as good design for parallel line assays based on quantitative responses, Sections V.1.2 and V.2.2. A critical assumption for the analysis is that the animals tested respond independently. Thus, the proportion of a group responding should reflect only the different treatments (or vaccine doses) and not bias which can result from non-independent groups. Randomization is essential if this assumption is to be met.

As for parallel line assays based on quantitative responses, the range of doses should be as large as possible and the response range for the test and reference preparations should be similar. The probit transformed responses are not equally variable, as the variances depend on the theoretical proportion responding. Thus a weighting coefficient must be determined, and this coefficient gives greater weight to theoretical proportions near 50% and smaller weight to more extreme proportions. The assignment of animals to doses must therefore be a compromise between using doses for which the maximal weight can be achieved, and using more widely spaced doses which give a better estimate of the slope of the log dose – transformed response line. For proportions less than 10% or greater than 90% the weighting coefficient is small, and doses which give responses in these regions should be avoided. Efficient designs generally have doses which give responses evenly spaced around 50%, but which avoid extreme responses. Consideration of the 3Rs would also support the use of more efficient designs for assays using animals.

V.3.3 Analysis of variance

The theoretical base of the analysis is explained here using the notation given in Table V.3.1.

Table V.3.1. Notation used for probit analysis. It is assumed that all subjects (animals) were appropriately randomized and respond independently. For each of the Reference preparation and q test samples there are k doses and test subjects with r of the n subjects giving a positive response.

Sample					Response	
Manufacturer	Lot	Preparation name	ID	Log Dose	Total number (n)	Responded (r)
NCL	*1*	*Reference*	*R*	X_{R1}	n_{R1}	r_{R1}
				X_{R2}	n_{R2}	r_{R2}
				-	-	-
				X_{ri}	n_{Ri}	r_{Ri}
				-	-	-
				X_{Rk}	n_{Rk}	r_{Rk}
A	*L1*	*T*	*T1*	X_{T11}	n_{T11}	r_{T11}
				X_{T12}	n_{T12}	r_{T12}
				-	-	-
				X_{T1l}	n_{T1l}	r_{T1l}
				-	-	-
				X_{T1k}	n_{T1k}	r_{T1k}
B	L_2	*T*	T_2	-	-	-
-	-	-	-	-	-	-
P	L_p	*T*	T_p	-	-	-
-	-	-	-	-	-	-
Q	L_q	*T*	T_q	-	-	-

It is assumed that the tolerances for subjects treated with log dose x follow a normal distribution with mean μ and standard deviation,σ. From this, the probability of subjects to show positive response (population positive rate) *P* can be given by

$$P = \frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^{x} e^{\frac{(x-\mu)^2}{2\sigma^2}} dx$$

The probit, Y, is defined by increasing the value of the normal equivalent deviate $z = \frac{x-\mu}{\sigma}$ by 5.0, giving $Y = z + 5$, to obtain

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{Y-5} e^{\frac{z^2}{2}} dx$$

Thus defined *Y* is Probit of p.

Under the assumption of normality, a linear dose-response equation $Y = \beta x + \alpha$ can be fitted to the regression of Probit *Y* on treatment (dose) *x*. Note however, that for the case where *P = 0, 1* the probit is not defined. Differences in the way in which calculation procedures handle observed responses of 0 and 1 can lead to different results, and caution must be used in interpreting results where such responses occur.

The regression coefficient β corresponds to width of the distribution. β is smaller for wider distributions. The regression coefficient and intercept can be expressed in terms of the parameters of the normally distributed tolerances as

$$\beta = \frac{1}{\sigma}$$

$$\alpha = 5 - \frac{\mu}{\sigma}$$

Calculations require an iterative process. Initial estimates for β and α are obtained using empirical probits calculated for the observed response rates $p = r/n$. Fit a regression equation $y' = b_1 . x + a_1$ to *Y* on *x* using the method of least-squares. Using the equation, estimated probit *y'* can be obtained as the first approximation of the population value of *Y*. The first approximation of the population value of *p* is estimated using $y' = z + 5$. Working probit *Y* and weighting factor *W* can be obtained as

$$y = y' + \left(\frac{p - P}{Z}\right)$$

$$w = \frac{Z^2}{P(1-P)}$$

Where *Z* is calculated from *P* by the substitution $z = y' - 5$

$$Z = \frac{\delta P}{\delta Y} = \frac{1}{\sqrt{2\pi}} e^{-\frac{(y'-5)^2}{2}}$$

Using those values, an improved regression equation $y' = b_2 . x + a_2$ is estimated. Population values are estimated by iterating the calculation until reaching sufficient agreement of estimated probit y_i',y_{i+1}' and working probit *y*. The calculations are explained using the example data shown above.

Assume the *i*th dose of the *p*th sample including the reference preparation as X_{pi}, and response rate at the dose as r_{pi}/n_{pi}. As explained above, calculate empirical probit y and subsequently the estimated probit y' using the regression equation of y on X_{pi}. The first estimate of population value of P, namely working probit y, and weighing factor w are estimated using those values. Using y and w, following estimation is made by the method of the weighted least squares.

$$\bar{x}_p = \sum nw_{pi}x_{pi} \Big/ \sum nw_{pi} \qquad \bar{y}_p = \sum nw_{pi}y_{pi} \Big/ \sum nw_{pi}$$

$$nwSxx_p = \sum nwx_{pi}^{\,2} - \left(\sum nwx_{pi}\right)^2 \Big/ \sum nw_{pi}$$

$$nwSyy_p = \sum nwy_{pi}^{\,2} - \left(\sum nwy_{pi}\right)^2 \Big/ \sum nw_{pi}$$

$$nwSxy_p = \sum nwx_{pi}y_{pi} - \left(\sum nwx_{pi}y_{pi}\right)^2 \Big/ \sum nw_{pi}$$

$$b_p = nwSxy_p \Big/ nwSxx_p$$

$$a_p = \bar{y}_p - b_p \cdot \bar{x}_p$$

Using the regression equation obtained here, estimate *y's* to make iterated calculations to improve the estimation of population values of dose-response regression.

Using the result of the calculation, linearity of dose-response, therefore normality of sensitivity distribution, is tested as shown below.

$$\chi_0^{\,2} = nwSyy_p - \frac{nwSxy^2}{nwSxx} \quad (df = \text{dose number - 2})$$

If $X_0^{\,2}$ was found significant $(X_0^2 > \chi^2_{(k-2)(\alpha)})$, the cause might be inadequate transformation of dose x or biased groups due to insufficient randomization.

Calculation of the dose for causing positive response in 50 % of subjects (median effective dose; $\boldsymbol{D}_{50}$) is given in *V.3.5.1*

On the samples *p (p=1- q)* subjected for the analysis,

Linearity can be tested by

$$\chi^2_{lin} = \sum nwSyy_p - \sum \frac{nwSxy_p^{\,2}}{nwSxx_p} \quad \text{(df=dose number-2)}$$

Parallelism can be tested by

$$\chi^2_{para} = \sum \frac{nwSxy_p^{\,2}}{nwSxx_p} - \frac{\left(\sum nwSxy_p\right)^2}{\sum nwSxx_p} \quad \text{(df=Sample number-1)}$$

Common regression coefficient (slope) is

$$b_{common} = \sum nwSxy_p \Big/ \sum nwSxx_p$$

V.3.4 Potency estimation

Potency of the *p*th sample relative to the reference preparation is

$$M_p = \bar{x}_R - \bar{x}_p - \frac{\bar{y}_R - \bar{y}_p}{b_{common}}$$

Its confidence interval is calculated by

$$ML, MU = M_c \pm t\sqrt{V_M}$$

using

median value of confidence interval

$$M_c = \overline{x_R} - \overline{x_p} + \frac{M - \bar{x}_R + \bar{x}_p}{1 - g_p}$$

and the interval

$$t\sqrt{V_M} = \frac{t_{\infty(\alpha)}}{b_{common}(1-g_p)}\sqrt{(1-g_p)\left(\frac{1}{\sum nw_R} + \frac{1}{\sum nw_p}\right) + \frac{(M - \overline{x_R} + \overline{x_p})^2}{Sxx_R + Sxx_p}}$$

Where

$$g_p = \frac{t^2{}_{\infty(\alpha)}}{b_{common}{}^2 \cdot (nwSxx_R + nwSxx_p)}$$

When doses were transformed into logarithm, 10^M, 10^{ML}, 10^{MU} are relative potency and lower and upper limits of confidence interval, respectively. If unit value of the reference or standard preparation was given as *U*, the values of relative potency and its confidence limits need to be multiplied by *U* to obtain unit value and its confidence interval of a sample. When no transformation was made for doses, *M, ML, MU* multiplied by the unit value of reference preparation are relative potency and confidence limits of samples.

V.3.5 Estimation of LD_{50}

V.3.5.1 Estimation using probit transformation

The dose for causing positive response in 50 % of subjects (median effective dose; D_{50}) is calculated by

$$D_{50} = \bar{x}_p + \frac{5 - \bar{y}_p}{b_p}$$

and its confidence interval by

$$D_{50} + \frac{g(D_{50} - \bar{x})}{1-g} \pm \frac{t_{\infty(\alpha)}}{b(1-g)}\sqrt{\frac{1-g}{\sum nw} + \frac{\left(D_{50} - \bar{x}\right)^2}{nwSxx}}$$

Where, $g = \dfrac{t^2{}_{\infty(\alpha)}}{b^2 nwSxx}$

V.3.5.2 Estimation using the Spearman-Karber method

The Spearman-Karber method was developed at a time when computing facilities were limited, and provides a relatively simple way of estimating the LD_{50}. For application of this method, it is assumed that responses span the range 0% to 100%.

This method is not recommended if the iterative probit method is available, but may be relevant in some instances.

Calculations are as follow:
Assume that equally spaced log doses $x_1, x_2, \ldots x_k$ are tested and that at dose x_i there are r_i of n_i responses so that an estimate of the response rate at x_i is given by $p_i = r_i / n_i$. The difference between successive doses is denoted $d = x_{i+1} - x_i$.

Then the mean of the log tolerance distribution may be estimated by
$m = \Sigma \{(p_{i+1} - p_i)(x_i + x_{i+1}) / 2\}$
and its variance may be estimated by
$V(m) = d^2 \Sigma (p_i q_i / (n_i - 1))$.

V.3.6 Examples

The user should be aware that extreme responses, that is, 0% or 100%, may cause problems in analysis, depending on the methods of analysis used. The statistical tests for linearity and parallelism are based on approximations to the chi-square distribution. This is not valid for expected numbers of responses less than 5 for any group, and use of groups with very small expected values should be avoided as far as possible.

It is likely that statistical software will be used to carry out the calculations shown above. The process followed for groups with extreme responses may not be consistent between different programs, and differences in results may occur. In particular, the question may be raised whether any attempt should be made to fit a slope to the data if there is only one non-extreme response.

V.3.6.1 Example 1. Tetanus vaccine potency assay

The reported data for this assay of tetanus vaccine Test product in terms of Reference standard are given in Table V.3.2. Different methods of calculation are shown for comparison. Three out of the six doses tested give extreme responses, two extreme responses for three doses of reference preparation and one of three doses for the test sample. For data such as this, with many extreme responses, the results from any method of calculation should be interpreted with caution.

Table V.3.2. Data for tetanus vaccine assay

Vaccine Preparation	**Dose, µl**	Log dose, x	Total Number Tested	Number Responding	Proportion Responding, p	Percent Response
Reference R	2.00	0.30103	14	14	1.000	100%
	1.00	0.0	14	10	0.714	71.4%
	0.50	-0.30103	14	0	0.000	0%
Test Sample T	5.00	0.69897	13	13	1.000	100%
	2.50	0.39794	14	6	0.429	42.9%
	1.25	0.09691	14	1	0.071	7.1%

Table V.3.3. Analysis of variance: summation after the fourth and fifth iteration. (1) is Preparation, with R denoting reference and T denoting test sample; (2) is iteration number; (3) is the empirical probit; (4) is the expected probit; (5) is the calculated probit, Y, calculated from the regression of the empirical probit on log dose; (6) is the working probit, y. Tables of empirical probits and weighting factors can be found in the references, which the reader is advised to consult for further details of methods and formulae if software for these calculations is not available.

(1)	(2)	x	p	(3)	(4)	Y(5)	nw	y (6)	nwx	nwy	nwxx	nwyy	nwxy	X2
R		0.30	1.00	1.803	1.000	3.793	0.016	4.060	0.005	0.005	0.001	0.259	0.019	0.001
		0.00	0.71	0.566	0.713	0.563	7.935	0.566	0.000	4.491	0.000	2.542	0.000	0.000
	4	-0.30	0.00	-1.803	0.004	-2.667	0.476	-3.003	-0.143	-1.430	0.043	4.294	0.430	0.054
	sum													0.055
		0.30	1.00	1.803	1.000	4.132	0.004	4.400	0.001	0.018	0.000	0.079	0.005	0.000
		0.00	0.71	0.566	0.714	0.566	7.927	0.566	0.000	4.486	0.000	2.539	0.000	0.000
	5	-0.30	0.00	-1.803	0.001	-3.001	0.203	-3.306	-0.061	-0.670	0.018	21217	0.202	0.019
	sum						8.134		-0.060	-3.834	0.018	4.835	0.207	0.019
T		0.70	1.00	1.769	0..967	1.838	2.210	2.286	1.545	5.502	1.080	11.551	3.531	0.444
		0.40	0.43	-0.180	0..517	0.043	8.907	-0.179	3.544	-1.597	1.410	0.286	-0.635	0.440
	4	0.10	0.07	-1.466	0..040	-1.752	2.703	-1.385	0.262	-3.745	0.025	5.188	-0.363	0.363
	sum													1.247
		0.70	1.00	1.769	0..967	1.838	2.209	2.286	1.544	5.051	1.079	11.549	3.531	0.444
		0.40	0.43	-0.180	0..517	0.043	8.907	-0.179	3.544	-1.597	1.410	0.286	-0.635	0.440
	5	0.10	0.07	-1.466	0..040	-1.752	2.703	-1.385	0.262	-3.744	0.025	5.187	-0.363	0.363
	sum						13.819		5.350	-0.290	2.514	17.022	2.533	1.248

For the reference R:

$$\bar{x}_R = \sum nwx_R \Big/ \sum nw_R = -0.060/8.134 = -0.007$$

$$\bar{y}_R = \sum nwy_R \Big/ \sum nw_R = 3.834/8.134 = 0.471$$

$$nwSxx_R = \sum nwx_R{}^2 - (\sum nwx_R)^2 \Big/ \sum nw_R = 0.018 - (-0.060)^2/8.134 = 0.018$$

$$nwSyy_R = \sum nwy_R{}^2 - (\sum nwy_R)^2 \Big/ \sum nw_R = 4.835 - 3.834^2/8.134 = 3.028$$

$$nwSxy_R = \sum nwxy_R - (\sum nwx_R)\cdot(\sum nwy_R) \Big/ \sum nw_R = 0.207 - (-0.06 \cdot 3.834)/8.134 = 0.235$$

Slope (b_R) = $nwSxy_R / nwSxx_R = 0.235/0.018 = 13.06$

Standard error of b_R = $\sqrt{1/nwSxx_R} = \sqrt{1/0.018} = 7.45$

Note: *This estimate of slope is based on only one non-extreme response, and for this calculation the assumptions have been made that responses were 0.5/14 at the smaller dose and 13.5/14 at the larger dose. This estimate is not reliable, and to some extent this is reflected in the relatively large standard error.*

Log ED_{50}(LED) = $[\ \bar{y}_R - b_R \cdot x_R\ ]\ /\ b_R$ = [0.471 – (-0.007 · 13.06)] / 13.06
= -0.043
ED_{50} = 0.91

Standard error of ED_{50} (σED)
= $\sqrt{(1/nw_R + (LED_R - \bar{x}_R)^2/nwSxx_R)/b_R{}^2}$
= $\sqrt{(1/8.134 + (-0.043 - 0.007)^2/0.018)/13.06^2} = 0.034$

Limits for ED_{50}:
Lower limit = antilog(LED – 1.96 · σED) = antilog (-0.110) = 0.78
Upper limit = antilog(LED + 1.96 · σED) = antilog (0.024) = 1.06

For the test sample T:

$$\bar{x}_T = \sum nwx_T \Big/ \sum nw_T = 5.350/13.819 = 0.387$$

$$\bar{y}_T = \sum nwy_T \Big/ \sum nw_T = -0.290/13.819 = -0.021$$

$$nwSxx_T = \sum nwx_T{}^2 - (\sum nwx_T)^2 \Big/ \sum nw_T = 2.514 - 5.350^2/13.819 = 0.444$$

$$nwSyy_T = \sum nwy_T{}^2 - (\sum nwy_T)^2 \Big/ \sum nw_T = 17.022 - (-0.290)^2/13.819 = 17.016$$

$$nwSxy_T = \sum nwxy_T - (\sum nwx_T)\cdot(\sum nwy_T) \Big/ \sum nw_T = 2.533 - (-0.290 \cdot 5.350)/13.819 = 2.645$$

Slope (b_T) = $nwSxy_T/nwSxx_T$ = 2.645/0.444 = 5.96
Standard error of b_T = $\sqrt{1/nwSxx_T}$ = $\sqrt{1/0.444}$ = 1.50

Note: *Although this estimate of slope is based on two non-extreme responses, one extreme response (13/13) has been replaced by 12.5/13), and this will affect the estimate of the slope*.

Log ED_{50} (LED) = [$\bar{y}_T - b_T \cdot \bar{x}_T$] / b_T = [-0.021 – (0.471 – (-0.007 · 13.06)]/ 5.96 = 0.391
ED_{50} = 2.46

Standard error of ED_{50} (σED)
= $\sqrt{(1/nw_T + (LED_T - \bar{x}_T)^2 / nwSxx_T) / b_T^2}$
= $\sqrt{(1/13.819 + (0.391 - 0.387)^2 / 0.444) / 5.96^2}$ = 0.045

Limits for ED_{50}:
Lower limit = antilog(LED – 1.96 · σED) = antilog (0.303) = 2.01
Upper limit = antilog(LED + 1.96 · σED) = antilog (0.479) = 3.02

Calculation of common slope, analysis of variance and potency:

Deviations from linearity are tested by
$\chi^2 = \sum nwSyy - \sum (nwSxy^2 / nwSxx)$
= (3.028 + 17.016) – (0.235^2 / 0.018 + 2.645^2/ 0.444)
= 20.044 – 18.825 = 1.22
degrees of freedom = total number of doses – 2×number of preparations
= 3 +3 – 2 x 2 = 2
For this value of χ_2^2 probability is 0.54 > 0.05
Deviations from linearity are not significant.

Deviations from parallelism are tested by
$\chi^2 = \sum (nwSxy^2 / nwSxx) - (\sum nwSxy)^2 / \sum nwSxx$
= (0.235^2/0.018 + 2.645^2/0.444) – $(0.235 + 2.645)^2$/(0.018 + 0.444)
=18.825 – 17.953 = 0.87
with 1 degree of freedom (number of preparations less 1)
For this value of χ_1^2 probability is 0.36 > 0.05
Deviations from parallelism are not significant

Common slope (*cb*) = $\sum nwSxy / \sum nwSxx$ = 2.880/0.462 = 6.23
Log relative potency (*M*) = $\bar{x}_R - \bar{x}_T - (\bar{y}_R - \bar{y}_T) / cb$
=-0.007 -0.387 – (0.471 – 0.021) / 6.23 = -0.466
Relative potency, *R* = antilog (*M*) = 0.342

Fiducial limits for M are given by:
$((M - \bar{x}_R + \bar{x}_T) \pm t\sqrt{K} / cb) / (1 - g)$
where *t* = 1.96
$g = (t^2 \cdot s^2) / (cb^2 \cdot \sum nwSxx)$
= (– 1.96^2 · 1) /(– 6.23^2 · 0.4628) = 0.214
$K = [(1-g) \cdot \sum (1/\sum nw)] + [(M - \bar{x}_R + \bar{x}_T)^2 / \sum nwSxx]$
=[(1/8.314 + 1/13.819)] + [$(-0.072)^2$/0.462]
giving limits
(-0.072 ± 0.127)/0.786 = -0.253 , 0.070

Limits for the relative potency *R* are obtained as the antilog of limits of M giving 0.225, 0.474

If the potency of the reference vaccine is 735.0 IU / ml, then the potency of the test vaccine is 735.0×0.342 = 251.35 IU / ml, with limits 165.62, 348.55.These limits are 66% to 139% of the calculated potency.

In the procedure shown above, the iterations are carried out after the extreme proportions are replaced with the proportion which would have been observed if 0.5 animals had responded or failed to respond. For example the extreme proportion of 0 for the Reference is replaced by 0.5/14 = 0.036 to give an empirical probit of approximately -1.803. The Test extreme proportion of 1.0 is replaced by 12.5/13 = 0.962 to give an empirical probit of approximately 1.769.

Results are shown for iterations and analysis of variance after the fourth and fifth iterations.

Results from maximum likelihood procedure:

Calculation using a maximum likelihood based procedure with the extreme proportions not replaced for the first iteration, but using working probits for these doses in the subsequent iterations gives results as shown below:

Statistical tests:

Significance of the common slope (regression): Normal Equivalent Deviate = 5.092 with probability <0.000.

Total deviations from the parallel line model: Chi-square with 3 degrees of freedom = 3.815 with probability p ~ 0.28 > 0.05 and therefore no significant deviations from the theoretical model. That is, the theoretical model is not contradicted.

No valid slope can be fitted independently for the Reference, since there is only one non-extreme response. Thus the validity of any test for parallelism is questionable. In this case the acceptance of the model is based on the probability of total deviations from the model.

Calculated parameters:

Common slope = b = 7.2264 with variance of b = 2.01404 and Finney's g = 0.148

Log potency = -0.447 with weight = 302

Relative potency = 0.357 with 95% limits 0.269 to 0.473 or limits as % of potency 75% to 132%

Comments on different methods of calculation:

Based on 5 iterations with simplified substitution for the extreme proportions, the estimate of relative potency is 0.342 with limits 0.225 to 0.474 and based on an iterative maximum likelihood procedure the estimate of relative potency is 0.357 with limits 0.269 to 0.473.

These differences reflect the different ways in which the two procedures deal with the extreme responses.

V.3.6.2 Example 2: Potency assay for tetanus vaccine

Table V.3.3. Data for Example 2.

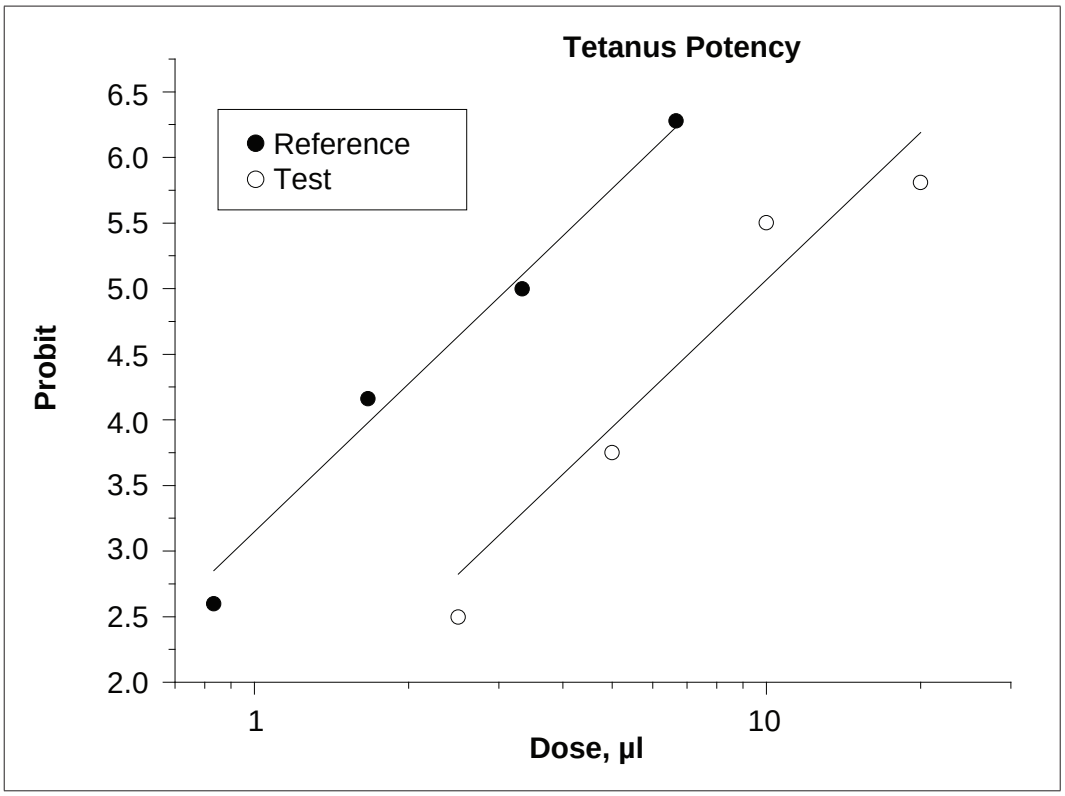
Vaccine Preparation	**Dose, µl**	Log dose, x	Total Number Tested	Number Responding	Proportion Responding, p	Percent Response
Reference R	6.667	0.8239	10	9	0.90	90%
	3.333	0.5229	10	5	0.50	50%
	1.667	0.2218	10	2	0.20	20%
	0.833	-0.0794	10	0	0.00	0%
Test Sample T	20	1.3010	10	8	0.80	80%
	10	1.0000	10	7	0.70	70%
	5	0.6990	10	1	0.10	10%
	2.5	0.3979	10	0	0.00	0%

Table V.3.4. Data for Example 2 with details required for manual iteration.

Sample	**Dose, µl**	Log dose	Total number tested	Number survived	Empirical Probit	Estimated Probit(Y')	nw	Working Probit(Y)	nwY	nwY^2	nwXY	nwX	nwX^2	Estimated Probit(Y'')
Reference	6.667	0.8239	10	9	6.282	6.209	3.674	6.278	23.063	144.799	19.002	3.027	2.494	6.233
	3.333	0.5229	10	5	5.000	5.147	6.317	4.999	31.576	157.846	16.510	3.303	1.727	5.106
	1.667	0.2218	10	2	4.158	4.085	4.666	4.161	19.415	80.786	4.307	1.035	0.230	3.978
	0.833	-0.0792	10	0		3.023	1.361	2.598	3.536	9.186	-0.280	-0.108	0.009	2.850
T1	20	1.3010	10	8	5.842	6.090	4.083	5.808	23.711	137.707	30.848	5.312	6.910	6.190
	10	1.0000	10	7	5.524	5.028	6.364	5.502	35.014	192.629	35.014	6.364	6.364	5.068
	5	0.6990	10	1	3.718	3.966	4.273	3.750	16.021	60.076	11.198	2.986	2.087	3.946
	2.5	0.3979	10	0		2.904	1.110	2.497	2.771	6.919	1.103	0.442	0.176	2.823

Sample	mean X	mean Y	nwSxx	nwSxy	nwSyy	b	a
Reference	0.453	4.844	1.171	4.387	16.756	3.746	3.147
T1	0.954	4.897	1.126	4.198	17.728	3.728	1.340

Figure V.3.2. Plotted data for Example 2.



Using the values from Table V.3.4. gives the following results (details are shown in a few cases only):

$$nwSxx_R = \sum nwX_R^2 - (\sum nwX_R)^2 / \sum nw_R = 1.171$$
$$2.494+1.727+0.230+0.009–(3.027+3.303+1.035-0.108)^2/(3.674+6.317+4.666+1.361)$$

$$nwSxx_{T1} = \sum nwX_{T1}^2 - (\sum nwX_{T1})^2 / \sum nw_{T1} = 1.126$$

$$nwSyy_R = \sum nwY_R^2 - (\sum nwY_R)^2 / \sum nw_R = 16.756$$

$$nwSyy_{T1} = \sum nwY_{T1}^2 - (\sum nwY_{T1})^2 / \sum nw_{T1} = 17.728$$
$$= 137.707+192.629+60.076+6.919–$$
$$(23.711+35.014+16.021+2.771)^2/(4.083+6.364+4.273+1.110)$$

$$nwSxy_R = \sum nwXY_R - (\sum X_R)(\sum Y_R) / \sum nw_R = 4.387$$
$$=19.002+16.510+4.307–0.280–(3.027+3.303+1.035–$$
$$0.108)(23.063+31.576+19.415+3.536)/ (3.674+6.317+4.666+1.361)$$

$$nwSxy_{T1} = \sum nwXY_{T1} - (\sum X_{T1})(\sum Y_{T1}) / \sum nw_{T1} = 4.199$$

Linearity:

$\chi_2^2 = Syy_R - \frac{Sxy_R^2}{Sxx_R} = 0.322$ for the Reference preparation.

$\chi_2^2 = Syy_{T1} - \frac{Sxy_{T1}^2}{Sxx_{T1}} = 2.076$ for the test sample.

Combined linearity: $\chi_4^2 = \sum Syy - \sum \frac{Sxy^2}{Sxx} = Syy_R + Syy_{T1} - \frac{Sxy_R^2}{Sxx_R} - \frac{Sxy_{T1}^2}{Sxx_{T1}} = 2.398$

Parallelism: $\chi_1^2 = \sum \frac{Sxy^2}{Sxx} - \frac{(\sum Sxy)^2}{\sum Sxx} = \frac{Sxy_R^2}{Sxx_R} + \frac{Sxy_{T1}^2}{Sxx_{T1}} - (\frac{Sxy_R^2}{Sxx_R} + \frac{Sxy_{T1}^2}{Sxx}) = 0.00018$

Individually fitted slopes:

$b_R = nwSxy_R / nwSxx_R = 3.746$

$b_{T1} = nwSxy_{T1} / nwSxx_{T1} = 3.728$

Relative potency:

The common slope $b_{common} = (nwSxy_R + nwSxy_{T1}) / (nwSxx_R + nwSxx_{T1}) = 3.737$

Relative potency and 95 % confidence interval

M= -0.487(-0.683 ~ -0.287)

Antilog= 0.326 (0.207 – 0.517)

V.3.6.3 Example 3: Kendrick test for pertussis vaccine

This example indicates the type of statistical issues which need to be addressed. In some cases professional advice may be helpful.

Summary of data for vaccinated mice: IU denotes international units and SHD denotes single human dose. * denotes a cage with only 8 mice at the time of challenge; ** denotes a cage with one death in the first 48 hours after challenge.

Table V.3.5. Data for Example 2, vaccinated mice

Preparation	Dilution	Female		Male		Total	Percent
Reference	0.05 IU	6/9	1/9	5/9	0/9	12/36	33.3
	0.25	7/8*	8/9	7/9	9/9	31/35	88.6
	1.25	9/9	8/8**	9/9	8/9	34/35	97.1
Test 1	0.01 SHD	1/9	2/9	1/9	4/9	8/36	22.2
	0.05	9/9	5/9	8/9	4/9	26/36	72.2
	0.25	7/9	9/9	8/9	8/9	32/36	88.9
Test 2	0.01 SHD	3/9	4/9	1/9	3/9	11/36	30.6
	0.05	8/9	7/9	8/9	3/9	26/36	72.2
	0.25	8/9	9/9	8/9	9/9	34/36	94.4

Table V.3.6. Summary of data for control mice used to determine LD50 of challenge suspension

Dilution of challenge	Female	Male
1	0/10	1/10
0.1	5/10	3/10
0.01	9/10	10/10
0.001	9/10	10/10

A summary of the results of the pairwise statistical analysis for each of the two test sample is shown below.

Statistical tests for sample T1:

Significance of the common slope (regression): Normal Equivalent Deviate = 7.685 with probability <0.000

Total deviations from the parallel line model: Chi-square with 3 degrees of freedom = 4.202 with probability p ~ 0.24 > 0.05 and therefore no significant deviations from the theoretical model. That is, the theoretical model is not contradicted. Total deviations from the model may be subdivided as shown below, although the separate terms are only approximately distributed as chi-square variables.

Deviations from linearity (total): Chi-square with 2 degrees of freedom = 3.406 with probability p ~ 0.182 > 0.05. That is, the theoretical assumption of linearity is not contradicted.

Deviations from parallelism: Chi-square with 1 degree of freedom = 0.796 with p ~ 0.372 > 0.05. That is, the theoretical assumption of parallelism is not contradicted.

***Calculated parameters for sample T1*:**

Individually fitted slopes with deviations from linearity are:

Ref : slope = 1.8930 and chi-square with 1 degree of freedom = 1.809 with p ~ 0.179

Test T1: slope = 1.4651 and chi=square with 1 degree of freedom = 1.597 with p ~ 0.206

Common slope = b = 1.6293 with variance of b = 0.04495 and Finney's g = 0.065

Log potency = 0.396 with weight = 60

Relative potency = 2.491 with 95% limits 1.333 to 4.444 or limits as % of potency 54% to 178%

Statistical tests for sample T2:

Significance of the common slope (regression): Normal Equivalent Deviate = 7.571 with probability <0.000

Total deviations from the parallel line model: Chi-square with 3 degrees of freedom = 2.206 with probability p ~ 0.53 > 0.05 and therefore no significant deviations from the theoretical model. That is, the theoretical model is not contradicted. Total deviations from the model may be subdivided as shown below, although the separate terms are only approximately distributed as chi-square variables.

Deviations from linearity (total): Chi-square with 2 degrees of freedom = 1.834 with probability p ~ 0.400 > 0.05. That is, the theoretical assumption of linearity is not contradicted.

Deviations from parallelism: Chi-square with 1 degree of freedom = 0.372 with p ~ 0.542 > 0.05. That is, the theoretical assumption of parallelism is not contradicted.

Calculated parameters for sample T2:

Individually fitted slopes with deviations from linearity are:

Ref : slope = 1.8930 and chi-square with 1 degree of freedom = 1.809 with p ~ 0.179

Test T2: slope = 1.5164 and chi=square with 1 degree of freedom = 0.025 with p ~ 0.876

Common slope = b = 1.6715 with variance of b = 0.04874 and Finney's g = 0.067

Log potency = 0.515 with weight = 62

Relative potency = 3.275 with 95% limits 1.787 to 5.835 or limits as % of potency 55% to 178%

Calculation of LD_{50} for challenge:

The determination of the LD_{50} of the challenge suspension for the combined data is 0.68 with 95% limits 0.034 to 0.143. These data show that the challenge given to the vaccinated mice is about 20 LD_{50}. (See below.)

Interpretation of results:

Examination of the statistical tests for validity indicates that each of these assays is statistically valid, in that there are no significant (p > 0.05) deviations from linearity or parallelism. In addition to the estimate of relative potency, the log10 of the potency and the weight (the reciprocal of the variance of the log potency) are also given. The log potency is approximately normally distributed with mean given by the estimated log potency and variance given by the reciprocal of the weight. This information can be used to determine the probability of particular values for subsequent estimates.

The requirements for potency of pertussis vaccine are not satisfied by either of these two samples although both were expected to be satisfactory on the basis of information about their formulation. The question then arises whether these estimates are consistent with the requirements, and whether further assays should be carried out which would give combined estimates which are consistent with the requirements.

For Test 1, the estimated potency is 2.49 IU per SHD with 95% limits 1.33 to 4.44. A combined mean estimate of 4 IU would require an estimate of 6.4 IU from a subsequent assay. Assuming that all assays have equal precision, and that the 'true' potency of the vaccine is 4 IU per SHD, the probability of this is less than 10%. Moreover, the two estimates, 2.49 IU and 6.4 IU, would be heterogeneous and thus could not be validly combined. The statistical decision should therefore be not to repeat the assay, and to report that the vaccine is shown to be sub-potent.

For Test 2, the estimated potency is 3.27 IU per SHD with 95% limits 1.79 to 5.84 IU. A combined mean estimate of 4 IU would require an estimate of 4.9 IU from the next assay. Assuming, as for Test 1, equal precision for a subsequent assay and a 'true' potency of 4 IU per SHD, the probability of this is about 25%. If, on the other hand, the 'true' potency is 3.27 (or less), then the probability of an estimate as large as 4.9 is about 10% (or less). A subsequent assay should clarify the situation, and because the vaccine was expected to be potent, the decision should be to repeat the assay.

Although mice were randomly assigned to treatment groups, they were subsequently kept together in the same cage. The possibility of 'cage effects' should therefore be considered. Male and female mice were separately allocated in equal numbers to each treatment group, and sex might be considered as a blocking factor, or, if there were reason to believe that the separate sexes responded differently, data for each sex might be separately analysed as an assay. Analysis carried out jointly for the three preparations considering all cages separately gave total deviations from the linear parallel line model of $x^2_{32} = 51.27$ compared with $x^2_5 = 4.23$ when the data for each of the four 'identically treated cages' were combined. Thus $x^2_{27} = 47.04$ (significant, $p \sim 0.01$) reflects differences between cages and sexes. When this is subdivided, $x^2_{18} = 40.67$ between cages is significant ($p \sim .002$) and $x^2_9 = 6.37$ between male and female mice is not significant ($p \sim 0.3$). The reasons for significant between cage differences are not known. However, the occurrence of such differences emphasizes the need for randomisation of treatments to cages and the need for continued vigilance in examining the assumptions which underlie the statistical analysis. Alternative methods of analysis based on 'cage responses', for example, a cage score of some description, might offer a more valid statistical approach to these data if the occurrence of such cage effects is a regular feature of these assays, and cannot be accounted for or reduced.

V.3.6.4 Example 4: Estimation of LD_{50}

The data obtained for the challenge controls in the Kendrick test for pertussis vaccine shown in ***V.2.6.2*** above have been used for estimation of the LD_{50}.

Table V.3.7. Data for challenge controls for Example 2, and calculations for Spearman-Karber method

Dilution of challenge,	log dilution	Female	Male	Response rate, p	*diff(p)= pi+1-pi*	*d= (xi+xi+1)/2*	*diff(p) x d*
1	0	0/10	1/10	0.05(p1)			
0.1	-1	5/10	3/10	0.4(p2)	0.35	-0.5	-0.175
0.01	-2	9/10	10/10	0.95(p3)	0.55	-1.5	-0.825
0.001	-3	9/10	10/10	0.95(p4)	0.00	-2.5	0.00

Use of maximum likelihood iteration of probit transformed responses for the response rate *p* gives a significant regression with Normal Equivalent Deviate = 5.450 with probability < 0.000, deviations from linearity chi-square with 2 degrees of freedom = 4.826 with probability ~ 0.09, slope = -1.269, log LD_{50} = -1.1660 with weight 45, LD_{50} = 0.068 with 95% limits 0.034 to 0.143

Calculations using the Spearman-Karber method, the mean of the log tolerance distribution may be estimated by

$m = \Sigma \{(p_{i+1} - p_i)\,(x_i + x_{i+1}) / 2\}$
so for these data
$m = (-0.165) + (-0.825) + (0.0) = -1.0$
and hence
LD_{50} = anti-log (-1.0) = 0.1
and its variance may be estimated by
$V(m) = d^2 \Sigma\,(p_i q_i / (n_i - 1))$.

V.3.7 References

1) D. J. Finney. Probit Analysis, 3rd Edition, Cambridge University Press, 1971.

V.4 Comparison of two independent groups

V.4.1 Introduction

Requirements for some product characteristics may be satisfied if it can be shown that the characteristic significantly exceeds some minimum reference standard. For such characteristics, if a group of animals treated with the test product is shown to give a significantly greater response than a group of animals treated with the minimum reference standard then the requirement is met. Similarly, the requirement may be that the characteristic is less than some maximum reference standard and this is met if the response to the test product is significantly less than the response to the maximum reference standard.

The tests given here cannot be used to show that two preparations are 'consistent' or 'equivalent' or to determine 'non-inferiority' of one preparation with respect to another. Thus, these tests should not be used if it is required to show, for example, that a new batch of product does not differ from a previous (clinical) batch. In assessing equivalence, clear definitions must be given for what is meant by 'equivalent' and how large a difference will be allowed before two preparations are said to be not equivalent.

The capacity of the tests described below to detect any difference between two independent groups (the power of the test) depends on the variation among animals, the number of animals used, and the size of the difference between the reference standard and the test product. A large difference between reference standard and test product may be detectable using a relatively small number of animals. In contrast, if the test product and reference standard are very similar for the measured characteristic, it may not be possible to detect a difference unless a very large number of animals is used. Software for calculations of the power and sample size is available.

V.4.2 Student's t test

This test is used to determine whether the mean responses for two independent groups of quantitative responses differ significantly from one another. The probability levels determined for the test depend on the assumption of normality of responses. The widely used formula for Student's t-test also depends on the assumption of equality of variances of the responses for the two groups. This assumption is tested using Fisher's F-test, and the probabilities determined for this test also depend on the assumption of normality.

V.4.2.1 Calculation of Student's t

Table V.4.1. Terms and notation used for calculations. Greek letters denote the theoretical values of parameters.

μ_T	mean response for animals treated with test vaccine
μ_R	mean response for animals treated with reference vaccine
δ_T	standard deviation of responses for animals treated with test vaccine
δ_R	standard deviation of responses for animals treated with reference vaccine
n_T	number of animals treated with test vaccine
n_R	number of animals treated with reference vaccine
$\bar{x}_t$	observed mean response for animals treated with test vaccine (sum of n_T responses divided by n_T)
$\bar{x}_r$	observed mean response for animals treated with reference vaccine
S_T	observed standard deviation of responses for animals treated with test vaccine (sum of squares of deviations of n_T responses from the observed mean response, $\bar{x}_t$, divided by n_T - 1
S_R	observed standard deviation of responses for animals treated with reference vaccine

The null hypothesis is that the mean response for the test dilution is less than or equal to the mean response for the reference dilution. The alternative hypothesis is that the mean response to the test dilution is greater than the mean response to the reference dilution.

$H_0 : \mu_T \leq \mu_R$
$H_A : \mu_T > \mu_R$
Level of significance $\alpha = 0.025$ (one-tailed test)

Equality of the variances of the two groups is assessed by comparing the ratio of the larger of the two variances, S_T^2 and S_R^2, with that for Fisher's F distribution or by determining whether the probability of the observed ratio is smaller than the significance level (customarily 0.05).

If the two variances do not differ significantly from one another, then the pooled variance, S^2 with $(n_T + n_R - 2)$ degrees of freedom may be calculated as

$$s^2 = ((n_T - 1)s_T{}^2 + (n_R - 1)S_R{}^2)/(n_T + n_R - 2)$$

and Student's t statistic is calculated as

$$t = \frac{((\bar{x}_T - \bar{x}_R)/s)}{\sqrt{(n_T \cdot n_R)/(n_T + n_R)}}$$

The probability of this value is then determined by reference to the Student t distribution with $(n_T + n_R - 2)$ degrees of freedom if the null hypothesis is true.

If the variances differ significantly, then an approximate t statistic is calculated as

$$t = \frac{(\bar{x}_T - \bar{x}_R)}{\sqrt{(s_T^2 / n_T) + (s_R^2 / n_R)}}$$

The probability for this value is determined by reference to the Student t distribution with degrees of freedom calculated as

$$df = \frac{((s_T^2 / n_T) + (s_R^2 / n_R))^2}{((s_T^2 / n_T)^2 / (n_T - 1) + (s_R^2 / n_R)^2 / (n_R - 1))}$$

If the probability of the calculated t statistic is less than α then the two groups differ significantly from one another at significance level α. If it is more convenient, the calculated t statistic can be compared with tabulated values.

If the probability of the calculated t statistic is greater than α then the two groups do not differ significantly. This does not mean that the two groups are the same, but that the difference between them is smaller than the detection capability of this test.

V.4.2.2 Power and sample size determinations for Student's t test

The null hypothesis, H_0, for Student's t test in its general form is that two independent groups of observations with equal variances come from distributions with the same mean responses, with the alternative hypothesis, H_A, being that the two means are not equal.

$H_0 : \mu_T = \mu_R$
$H_A : \mu_T \neq \mu_R$

The significance level of the test, α, is the probability that H_0 will be rejected when it is true and is set to be small. A common value for the significance level is $\alpha = 0.05$. The power of the test, β, is the probability that H_A will be accepted, and H_0 rejected, when H_0 is false and H_A is true. However, H_A is not a single value, but covers a range of values. Moreover, the power, β, depends on the 'true' value of the mean under H_A, the magnitude of the difference between the means of the two groups, the size of the two groups and the variance. The power can only be determined when these values are known or assigned in some way. Some values for the power are shown in Table V.4.2, and Figure V.4.1 shows power functions for this test. Thus for two groups each of size 10, a difference between the two means equivalent to the standard deviation will be detected with probability 0.56. That is, for approximately 40% of such tests, the null hypothesis would not be rejected, even though the true difference between means was as large as 1 standard deviation.

Table V.4.2. Power for Student's t test for the null hypothesis that the means of two samples from populations having the same variance are the same against the alternative hypothesis that the two means are different. The effect size is the difference in the means of the two samples divided by the standard deviation of the population. The significance level for the test is 0.05 and the two groups are assumed to have the same sample size.

Power **α = 0.05** Two-sample t-test		Effect Size Index = Difference / Std. Dev. (Difference in means of two samples)					
		0.2	0.5	0.8	1.0	1.5	2.0
n = number of animals in each sample	5	0.06	0.11	0.20	0.29	0.55	0.79
	10	0.07	0.19	0.40	0.56	0.89	0.99
	20	0.09	0.34	0.69	0.87	0.996	
	50	0.17	0.70	0.98			

V.4.3 Mann-Whitney U test

The Mann-Whitney U test is sometimes viewed as the non-parametric equivalent of Student's t-test. The aim of the test is to determine whether the responses from one of two groups of independent sample are larger than those from the other. It is required that the responses can be ordered, but normality of distributions is not required. If quantitative responses have been observed, then each response from the total set of responses from both groups is assigned its order in the set. The sums of the ranks for the separate groups are calculated and the larger of the two values is compared with a table of probabilities for this test (or directly calculated if suitable software is available).

If the data are quantitative and the conditions for Student's t-test are satisfied, then the t test is more powerful that the Mann-Whitney U test. That is, in comparison with the Mann-Whitney U test, Student's t-test is capable of detecting a smaller difference with the same number of animals

V.4.4 Fisher's exact test

Fisher's exact probability test can be used with data from single dilution assays based on quantal responses to show that the test product has better performance than the reference standard at the minimum acceptable level.

This test summarizes the exact binomial probabilities of the observed occurrence of proportions, and of all possible more extreme proportions. The data take the form of a 2x2 table, as shown below. There are two preparations, reference vaccine denoted R and test vaccine denoted T. The quantal response either occurs or does not, that is, each animal tested either responds or does not respond.

Table V.4.3. Notation for Fisher's exact test.

	Reference, R	Test, T	Total
Responder	a	b	(a+b)
Non-responder	c	d	(c+d)
Total	(a+c)	(b+d)	N=a+b+c+d

The exact probability of the occurrence of this observed table, with ! denoting factorial, is

(a+b)!(c+d)!(a+c)!(b+d)! / a!b!c!d!N!

The marginal totals remain fixed, and the more extreme occurrences are determined by selecting the smallest of a, b, c, d. This smallest value is then successively reduced by 1 in such a way as to keep the marginal totals fixed to give all more extreme tables. The exact probabilities of the observed and the more extreme tables are determined, and the probability of the occurrence of the observed or any more extreme table is obtained as the sum of these probabilities.

V.4.5 Examples

V.4.5.1 Student's t test

This example is based on a diphtheria potency test by toxin neutralization in vitro in the Vero cell assay. The null hypothesis is that the mean response for the test dilution is less than or equal to the mean response for the reference dilution. The alternative hypothesis is that the mean response to the test dilution is greater than the mean response to the reference dilution.

$H_0 : \mu_T \leq \mu_R$
$H_A : \mu_T > \mu_R$
Level of significance $\alpha = 0.025$ (one-tailed test)

Data consisting of two independent groups of (unpaired) observations

Group	Responses	n	Mean	Variance
Reference	2, 2, 2, 3, 6, 8, 2, 1, 4, 2	10	3.2	4.844
Test	6, 4, 8, 3, 7, 4, 3, 8, 3, 8	10	5.4	4.933

For the test dilution

$$n_T = 10 \quad \bar{x}_T = 5.40 \quad s_T = 2.221 = \sqrt{4.933}$$

For the reference dilution

$$n_R = 10 \quad \bar{x}_R = 3.20 \quad s_R = 2.201 = \sqrt{4.844}$$

Fisher F-test: Ratio of larger to smaller variance = 4.933 / 4.844 = 1.018

From the F table with 9,9 degrees of freedom, the critical value is 3.18. (Alternatively, the probability of an F(9,9) value of 1.02 or larger is 0.98, which is greater than 0.025)

Conclusion: The variances do not differ significantly; accept the assumption of equal variances for Student's t-test.

Calculate the pooled variance as

$s^2 = [(9 \times 4.933) + (9 \times 4.844)] / (10 + 10 - 2) = 2.211^2$

and calculate t as

$$t = \frac{((5.4 - 3.2)/2.211)}{\sqrt{10 \cdot 10/(10+10)}} = (2.2/2.211)\sqrt{5} = 2.225$$

From tables of probability for Student's t values, the critical value with probability 0.025 is 2.101. The observed t value is larger than this, so the null hypothesis of equal means is rejected and the alternative hypothesis that the test dilution has a larger mean than that for the reference standard is accepted.

V.4.5.2 Mann-Whitney U test

The same responses as used above for Student's t test (V.4.5.1) are used. The total of 20 responses are ordered and ranked with ranks taking the values 1 to 20. Tied values are assigned the mean rank. If there are an excessive number of tied values, the probability may be compromised.

Table V.4.4. Response data with ranks used for Mann-Whitney U test.

Response	1	2	2	2	2	2	3	3	3	3	4	4	4	6	6	7	8	8	8	8
Rank values	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Ranks assigned	1	4	4	4	4	4	8.5	8,5	8,5	8,5	12	12	12	14.5	14.5	16	18.5	18.5	18.5	18.5
Group	R	R	R	R	R	R	T	T	T	R	T	T	R	T	R	T	T	T	T	R

Total of assigned ranks for the reference dilution = 74.5
Total of assigned ranks for test product dilution = 135.5

The smaller of the two totals is referred to the table of probabilities or its probability is calculated as 0.023. This probability is less than the significance level of 0.025 and thus the hypothesis of equal distributions is rejected and the alternative hypothesis that the test dilution gives larger responses than those for the reference standard is accepted.

V.4.5.3 Fisher's exact test

For a reference vaccine a response rate of 2/16 was observed compared with a response rate of 9/16 for the test vaccine.

The observed 2x2 table, and more extreme tables are shown below.

Table V.4.5. Data for calculation of probability for Fisher's exact test.

	Observed response (1)			More extreme response (2)			Most extreme response(3)		
	R	T	Total	R	T	Total	R	T	Total
Responder	2	9	11	1	10	11	0	11	11
Non-resp	14	7	21	15	6	11	16	5	21
Total	16	16	32	16	16	32	16	16	32

Calculated exact probabilities are:
(1) 0.011 = [(2+9)!(14+7)!(2+14)!(9+7)!]/[2!9!14!7!32!]
(2) 0.00099
(3) 0.000034

The probability of the observed or a more extreme response is thus the sum of the three probabilities or 0.012 which is less than 0.05. Thus at the 5% significance level, the test vaccine has shown a higher response than the reference vaccine.

V.4.5 References

1) Amitage P, Berry G, Matthews, JNS (2002). Statistical Methods in Medical Research. Blackwell.

V.5 Combination of estimates

V.5.1 Introduction

In order to meet the requirements for estimation of potency for some products, it may be necessary to carry out two or more assays. The conditions for repeating an assay or retesting a batch of product should be specified with the procedure used to assess the assay results, that is should be part of any Standard Operating Procedure.

Where two or more statistically valid assays have been carried out and thus two or more estimates of potency are available, a composite estimate is needed. The question then is how the estimates should be combined. The methods given here are approximate, but should be generally reliable for the assays described in this manual. It is assumed that the estimates have been obtained in independent valid dilution assays.

Estimates may be considered to come from a common distribution for various reasons, for example, because they have been determined by the same assay method, within the same laboratory, for comparison of the same test product and reference standard. If the estimates have not come from a common distribution, then their combination may not be meaningful.

V.5.2 Notation

Assume that relative potency of a sample T was measured repeatedly in $\boldsymbol{n}$ separate independent experiments, each using the same reference preparation $\boldsymbol{R}$ to obtain relative log potencies M_i with their estimates of variances V_i. and weights $w_i = 1 / V_i$ for i = 1 to n. The weights are obtained using the within assay variance.

V.5.3 Homogeneity of estimates

If the potency of a test preparation is estimated in two or more assays, and the assays are valid dilution assays, then the two estimates of potency should not differ by a greater amount than that due to the sampling error of the assays. Thus, before potency estimates are combined, they should be tested for consistency.

The homogeneity of the $\boldsymbol{n}$ estimates can be tested using

$$\chi_0^2 = \sum w_i (M_i - \overline{M})^2 = \sum w_i M_i^2 - \frac{(\sum w_i M_i)^2}{\sum w_i}$$

The probability of $\boldsymbol{X}_0^2$ can be determined by reference to appropriate tables, or calculation from validated software. If this probability is greater than the selected significance level, usually 0.05, then the hypothesis of consistency or homogeneity is not contradicted and the estimates of relative potency can be considered homogeneous.

Estimates of potency show significant deviations from homogeneity because there is some underlying inconsistency between them. For example, different assay methods may have been used. Estimates of potency may also show significant deviations from homogeneity because the within assay variances which form the basis of this test have been underestimated and there are sources of variability between assays which have not been included. Before combining estimates, the possible causes of heterogeneity should be considered.

V.5.4 Weighted mean of homogeneous estimates

In determining the combined estimate, greatest importance should be attached to the estimate which is most precise or reliable, and thus a weighted mean is needed. For the assays described in this manual, an estimate of the variance of the log potency, M, is determined. Provided that the degrees of freedom for this estimate are not small, preferably about 20 or more, a weighted mean of the individual estimates may be determined using weights inversely proportional to the variances.

If the estimates of potency do not differ significantly from homogeneity, the weighted mean of the potency estimates is used,

$$\overline{M} = \sum M_i \cdot w_i \Big/ \sum w_i$$

Variance of $\overline{M}$ can be estimated by

$$\overline{V}_M = 1 \Big/ \sum w_i$$

Confidence limits (M_L, M_U) of the weighted mean are given by

$$M_L, M_U = \overline{M} \pm t\sqrt{\overline{V}_M} \quad (df=n-1)$$

V.5.5 Combination of heterogeneous estimates

If the individual potency estimates differ significantly from homogeneity, then the estimates differ by more than would have been predicted from the estimates of the confidence limits of the individual estimates. If it is nevertheless considered that the estimates come from a common distribution, for example, all estimates have been obtained in the same laboratory using the same assay method, an unweighted combination of the estimates may be used. In this case the variance among the potency estimates is directly determined from the estimates themselves, and is not based on the within assay variances.

The k separate estimates of M from the k independent assays are combined to give the mean potency $\overline{M} = \sum M \Big/ k$ and the standard deviation of $\overline{M}$ can be estimated as

$$s_{\overline{M}} = \sqrt{s_{\overline{M}}^2} = \sqrt{\sum (M - \overline{M})^2 \Big/ (k-1)}$$

Confidence limits are calculated as

$$\overline{M} \pm t\sqrt{s_{\overline{M}}^2} = \overline{M} \pm t \cdot s_{\overline{M}}$$

where t is Student's t with (k-1) degrees of freedom.

When k is small, the value of t is large (for 95% limits, the value of t is 12.7 when k=2).

V.5.6 Examples

V.5.6.1 Weighted mean of homogeneous estimates.

Data for the two examples of parallel line assay used to illustrate the calculations for the parallel line assay method gave the results shown below.

Exp.	Sample	M_i	V_{M_i}	$w = 1/V_{M_i}$	wM	$\overline{M} = \sum wM / \sum w$	$w(M_i - \overline{M})^2$	X^2	$V_{\overline{M}} = 1/\sum W_i$	$t\sqrt{V_{\overline{M}}}$
Exp 1	sample 1	0.321	0.263	3.804	1.221	0.152	0.109	0.119	0.021	0.285
Exp 2	sample 1	0.137	0.023	43.344	5.930		0.010			

There is no significant difference between the two experiments (X^2=0.119, probability 0.73 > 0.05).

	Relative potency	Lower limit	Upper limit
Log	0.152	-0.134	0.437
Antilog	1.418	0.735	2.736

V.5.6.2. Combination of heterogeneous estimates

Potency of an endotoxin preparation was measured relative to a standard endotoxin using a chromogenic kinetic technique. The same assay design and methods and calculations using parallel line analysis were repeated nine times, and gave the following results.

Relative Potency, R	95% Confidence Limits		M =Log R	Variance of M V(M)	Weight = 1/V(M) = W	WM	$W(M_i-M)^2$
	Lower	Upper					
1.414	1.382	1.446	0.1504	0.000024	41263	6205.85	101.9046
1.349	1.313	1.385	0.1299	0.000033	30088	3907.63	25.6000
1.257	1.235	1.280	0.0995	0.000015	65037	6469.81	0.0973
1.242	1.224	1.261	0.0943	0.000011	92666	8736.72	3.8199
1.257	1.215	1.300	0.0992	0.000055	18276	1813.76	0.0390
1.126	1.105	1.147	0.0514	0.000017	59305	3048.96	144.0898
1.265	1.170	1.368	0.1020	0.000290	3453	352.18	0.0058
1.325	1.200	1.464	0.1221	0.000469	2132	260.26	0.9754
1.393	1.342	1.446	0.1439	0.000067	14962	2152.91	27.9046
			Mean= 0.1103 s^2= 0.00091		Sum = 327182	Sum= 32948	Sum= χ^2= 304.436

The value of x^2 (8 = 9 - 1 degrees of freedom) of 304.4 has probability < 0.05, and the estimates of relative activity therefore differ significantly from homogeneity. Examination of the assay design showed that the dilution series for both reference and test samples were obtained using serial dilutions, and that each assay was carried out on one microtitre plate. One possible cause of the apparent heterogeneity in this

example is that the variance of the log potency is artificially small because the responses are not independent, having come from serial dilutions and neighboring wells on a micro-titre plate.

All estimates were obtained following the same procedures, and are thus expected to have a common distribution. We can therefore use the log potency estimates and directly determine the mean and variance. The 95% confidence limits are determined using values from the t distribution with 8 degrees of freedom.

Mean log potency with 95% confidence interval: 0.1103, limits 0.0407 to 0.1779
Relative potency with 95% limits: 1.289, limits 1.098 to 1.513

V.6 Assay validation

V.6.1 Introduction

Assay validation is required to show that the assay is suitable for its intended purpose. In the most general sense assay validation is outside the scope of this manual. The assays described here are assays which have been extensively validated in individual laboratories, and in most cases, in international collaborative studies. However, these assays have been validated for particular types of products and materials, and under defined conditions. Extension to other types of products may raise questions about the need for further validation, with some combination vaccines being of particular concern.

Introduction of any assay method into a laboratory or any changes in staff or facilities requires validation to ensure that the assay continues to perform suitably. For many of the methods described in this manual, this may be limited to ensuring that the staff in the laboratory have adequate training and are appropriately certified, selecting an assay design suitable for the available facilities, determining a suitable dose range for the available animals and conditions and assessing the precision of the method in the laboratory using its facilities and staff.

Assays from some assay systems may fail the statistical tests for validity with a greater frequency than expected. The nature of the statistical tests means that if a 5% significance level is used then 5% of the tests are expected to show statistical invalidity. If a greater number of tests show statistical invalidity, it may be necessary to reconsider the assay validation. A larger than expected proportion of assays showing statistical invalidity may reflect features of the assay design or conditions which have changed over time. It may also indicate that there have been changes in the test product. On-going analysis of assay parameters for consistency (Section V.7.) may be helpful in assessing whether possible changes have occurred.

In some cases, laboratories may develop specific in vitro or other assays which they wish to use in conjunction with or in partial replacement for the in vivo assays. In this case, the general considerations of validation apply within the laboratory, and the extent to which these are carried out will determine the usefulness of the in vitro assay.

V.6.2 In house validation of official methods

The first consideration when a new method is introduced to a laboratory is to ensure that there are appropriate facilities and suitably trained staff to carry out the required procedures. Having ensured this, the next step is to set up pilot assays which demonstrate that there is a significant dose – response relation for a known preparation, for example, for an international reference preparation, or for a product of known potency and efficacy. Pilot assays will also provide information about the suitability of the response transformation recommended with the method, and about the variability of the responses in this laboratory, and whether they are likely to be homogeneous over the response range of the assay.

At this stage, the assay design to be used should be carefully considered and the procedures for random assignment of treatments to animals should be put in place.

Validation is an on-going process. Records of statistical parameters and assay characteristics for the standard preparation should be kept and accessed (see for example, Section V.7). Calibration and validation of equipment and instruments used in the assay must be maintained.

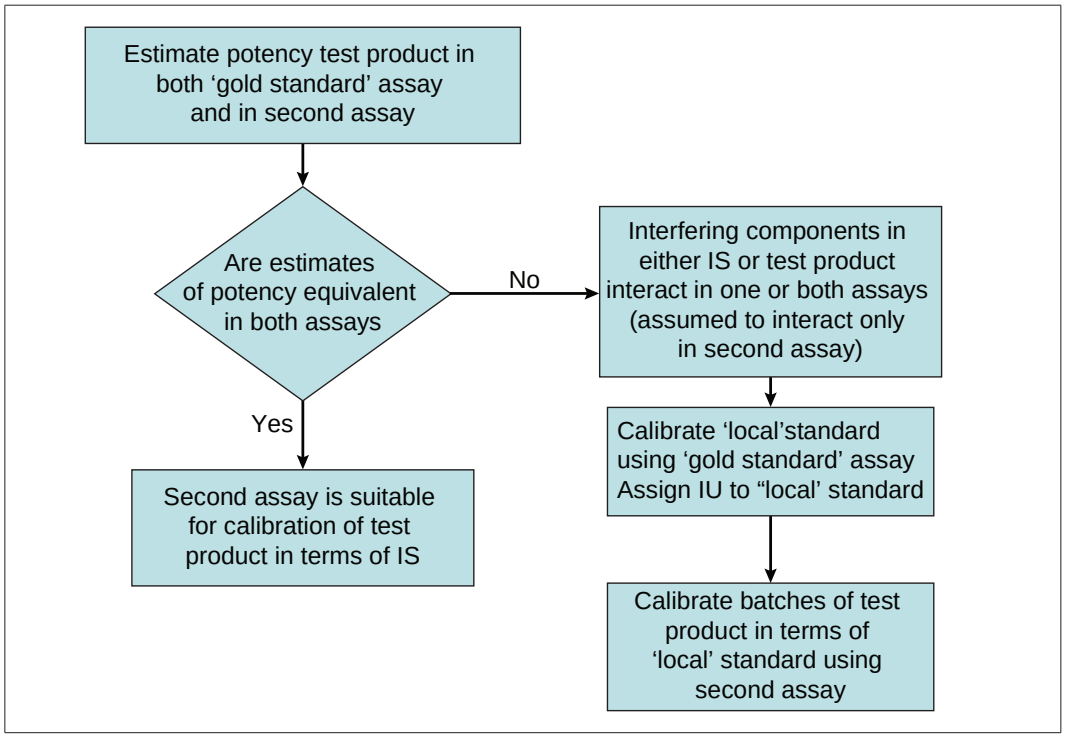
V.6.3 Validation of in house specific procedures and 'transferability' of IU

Alternative assays may be preferred to the assay used for calibration of the International Standard. In some cases the alternative assays may show different specificities for components of particular products. Moreover, the supply of International Standards is limited and in house reference standards may be more appropriate.

The validation process for comparisons among IS, 'local' (i.e. in-house, regional, or other localized) reference and test batches of product in an alternative assay is shown in Figure V.6.1. An example is the potency assay for tetanus toxoid. The IS is calibrated using assays in guinea pigs. In contrast to guinea pigs, different mouse strains appear to respond differently to tetanus toxoids of different origins. Nevertheless a number of laboratories prefer potency assays using mice in conjunction with a local standard from having the same origin as the test products. In this case, the assay in mice is considered as a 'second' assay and must be assessed as shown in Figure V.6.1.

If the purpose of the assay is the demonstration of consistency, for example in immunogenicity in comparison with a clinical lot of known safety and efficacy, different considerations may apply to alternative assays (See Introduction TRS 927 page 139 on the purpose of potency tests). In such cases, the transferability of the IU may not be an issue.

Figure V.6.1. Assessment of suitability of a second assay for calibration of test products, and transferability of the International Unit when the second assay reacts to 'interfering' components in the International Standard or the test product. The 'gold standard' assay is used for calibration of the International Standard. Potency determined by the 'gold standard' assay would be decisive if there is dispute about the potency of a product.



If there is doubt about the similarity of components of the local reference and the test product, then estimate the potency of the test product in terms of the local reference using both gold standard assay and second assay. If these estimates of potency are not the same in both assays, then the local reference can not be used in the second assay. Consequently the potency of the product under test cannot be expressed in IU by using the second assay. A more appropriate 'local' standard may also be considered.

V.6.4 Validation for dilution assays with reduced numbers of dilutions

Concern with reduction in the use of laboratory animal has resulted in proposals for 'single dilution' assays for products which have been shown to be produced consistently and for which adequate assay validation has been carried out. In this procedure, one group of animals is treated with a single dilution of test vaccine and a comparable group is treated with a single dilution of the reference vaccine.

This procedure does not permit a check of assay validity by testing linearity and parallelism of dose-response lines and does not provide an estimate of potency. This procedure cannot be applied if an actual estimate of potency is required or if both upper and lower limits are required for the potency of the vaccine. It can only be applied to show that a vaccine meets a defined minimal (or maximal) specification.

It may be possible to achieve reductions less extreme than the single dilution assay where the experience with multiple-dilution assays provides:

- evidence of consistency in production and testing
- evidence of a highly significant regression of the dose-response line for the vaccine and justification of the assumption of linearity and of parallelism with the dose-response line for the reference preparation.

For example, if the information for a particular product is considered sufficient to establish linearity and the appropriate dilution levels for this, reduction from three to two doses might be considered. An assay with two dilutions each for reference standard and test product would reduce by one-third the number of animals, would provide a test for deviations from parallelism (although not for linearity) and would provide an estimate of potency with limits.

This Following Section is intended to provide guidance on the use of the single dilution assay.

V.6.4.1 Conditions for use of single dilution assays

Before initiating use of a single dilution assay system, the control laboratory must have recorded adequate experience with multiple-dilution assays on the specific product vaccine to be tested in this way. This experience must provide:

- evidence of consistency in production and testing;
- evidence of a highly significant regression of the dose – response line for the vaccine and justification of the assumptions of linearity and of parallelism with the dose – response line for the reference preparation;
- guidance for the selection of the single-dilution system parameters, namely number of animals and dilution level to be used for the reference vaccine;
- prediction of the behavior of the single dilution system.

In practice, it is recommended that data from a series of 10 to 20 recent and consecutive multiple-dilution assays should be available for study and confirmation of the above conditions.

Different products will require separate evidence that these conditions are met. Following the introduction of changes in the vaccine production process (e.g. purification, adjuvant, formulation) or in the testing method, evidence that the conditions are met must be provided.

V.6.4.2 Selection of appropriate dilutions for single dilution assays

For the reference vaccine, historical data are used to select a dilution containing a number of International Units known to elicit an immune response in the lower part of the dose – response curve. For a quantal response, about 10-20% protection is considered acceptable.

For the vaccine under test, all test products are assumed to contain the minimum required potency (e.g. 30 IU per single human dose of diphtheria vaccine). Based on this assumption, a dilution of the test vaccine is made which hypothetically contains the same number of International Units as the reference vaccine.

V.6.4.3 Statistical design and evaluation of single dilution assays

When planning single dilution assays it is essential that the two treatments are randomly assigned to the total number of animals to be used, and that the two groups resulting after assignment of treatment are not handled in such a way that the groups can be otherwise dissimilarly treated. For example, if all animals treated with the test vaccine are kept in one cage and all animals treated with the reference vaccine are kept in another cage, then the test is not valid, since any treatment difference is confounded with possible cage differences. If animals receiving the same vaccine are kept in the same cage, then the cage is the 'experimental unit'. In the above example of a single cage for each treatment, there are thus only two independent responses, one for each cage, without regard for the number of animals in each cage.

Selection of the number of animals for single dilution assays requires information about the type of response, and also requires historical information about the dose – response curves for both reference and test vaccine, the expected variability of the responses and the expected potency level of the test vaccine. If the responses are variable and the potency level of the test vaccine is close to the minimum requirement, then a large number of animals may be required to show that the minimum requirement is met. If response variability can be decreased, or if previous history indicates that batches of the test vaccine typically substantially exceed the minimum requirement, then fewer animals may be required to show that the minimum requirement is met. Statistical software for calculation of required numbers is available when the historical information is supplied, and there are also published tables of required numbers.

The aim of statistical evaluation of a single dilution comparison of reference and test vaccines is to determine whether the responses of the group treated with the test vaccine differ significantly from the responses of the group treated with the reference vaccine. If the difference is significant, then it can be concluded that the test vaccine achieves at least the minimum potency. The methods of Section V.4 can be used with the statistical test depending on the type of response data obtained and the assumptions which can be made about the distribution of the responses.

V.6.5 References

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
ICH Harmonized Tripartite Guidelines
Validation of Analytical Procedures: Text and Methodology
Q2(R1)
Current Step 4 version
Parent Guideline dated 27 October 1994

(Complementary Guideline on Methodology dated 6 November 1996 incorporated in November 2005)

V.7 Consistency of calculated parameters

V.7.1 Introduction

In laboratories where tests are carried out frequently and / or on a routine basis, the methods of quality control may be helpful in assessing the consistency of the assay performance, and may provide useful information if there are questions about individual assays or estimates of potency. Control charts for the parameters associated with the reference standard may be of value in assessing whether or how animal populations or other assay factors change over time. The way in which quality control techniques are applied will depend on the type of assay, the number and types of preparations included in the assay, the relevant assay parameters and the facilities in the individual laboratory. Control charts for slopes of log dose-response lines, for ED_{50} values and for residual variance are among the more widely recorded. Unexpected occurrence of significant deviations from linearity or parallelism may sometimes be attributed to an unexpectedly small value of the residual variance.

Section V.7.2. illustrates how data from a sequence of assays might be considered.

V.7.2 Trend analysis and validity evaluation of an assay

b) Estimation of population values of slope and variance

Assume that results of repeated runs of the assay on ,in total, n samples including reference preparation were accumulated to calculate variations of doses, $Sxx_1 \cdots Sxx_i \cdots Sxx_n$, covariations of dose and response, $Sxy_1 \cdots Sxy_i \cdots Sxy_n$ and error variations, ${}_E Syy_1 \cdots {}_E Syy_i \cdots {}_E Syy_n$, with $N_1 \cdots N_i \cdots N_n$ of data and $k_1 \cdots k_i \cdots k_n$ doses.

Where $${}_E Syy_1 = \sum \left(y_{1k_1} - \overline{y_1}\right)^2 \cdots {}_E Syy_i = \sum \left(y_{ik_i} - \overline{y_i}\right)^2 \cdots {}_E Syy_n = \sum \left(y_{nk_n} - \overline{y_n}\right)^2$$

Regression coefficient $\boldsymbol{b}_i$ of ***i th*** sample is

$$b_i = Sxy_i \Big/ Sxx_i$$

Its confidence interval is obtained by

$$bi \pm t\sqrt{s^2{}_i \Big/ Sxx_i}$$ (Degree of freedom of t **:** N - k)

Where $$s^2{}_i = {}_E Syy_i \Big/ (N-k)$$

Common slope $\overline{b}$ is obtained by

$$\overline{b} = \frac{\sum Sxy_i}{\sum Sxx_i}$$

If variances for the whole results do not show significant deviations from homogeneity as tested by Bartlett's method, homogeneity of regression coefficients, therefore parallelism of regression lines, can be tested for ***n*** regression lines by

$$F = \frac{\left[\sum\left(Sxy_i^2 / Sxx_i\right) - \left(\sum Sxy_i\right)^2 / \sum Sxx_i\right] / (n-1)}{\sum Syy_i / \sum (N_i - k_i)}$$

(Degrees of freedom:f_1=number of regression lines-*1*, f_2=*N-k*)

If *F* was not significant, regressions can be considered not to deviate significantly from parallelism.

Utility of common slope for evaluating validity of a test

When the range of lower and upper confidence interval of a

$bi \pm t\sqrt{s^2_i / Sxx_i}$ slope includes common slope,

the dose response regression in the assay could be considered within the normal range.

After accumulating sufficient data, the distribution pattern of individual slopes can be analysed.

> Fistly, calculate cumulative rate of occurrence of slope values to transform into Normal Equivalent Deviate (NED) which can be calculated by transforming into probit and then substracting 5 from the probit values.
> If the plot of NED on slope value was found to be straight linear, the linear range of slope can be regarded as normally distributing.
> Therefore, if b_i observed in an assay was within the range of the normal distribution, the assay results could be considered normal in terms of dose-response regression.

Variance

When deviation of variances from homogeneity was not significant for results of whole experiments as tested by Bartlett's method,

> Common variance which is an estimate of population variance is obtained by
> $\overline{s^2} = \sum Syy_i / \sum (N_i - k_i)$

Utility of common variance for evaluating validity of a test

Compare error variance of each test, $s^2_i = {}^E Syy_i / (N-k)$ with the common variance $\overline{s^2} = \sum Syy_i / \sum (N_i - k_i)$, by calculating *F* value by $F = \frac{s_i^2}{\overline{s^2}}$.

If this *F* value was not significant, the test results could be considered not affected by abnormal variation factors.

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