Single Dilution Assay for Diphtheria and Tetanus potency test: Reduction

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Methods available for Diphtheria and Tetanus Potency test

Following are the methods for Diphtheria Potency test

- Lethal Challenge test in G.Pigs (Multi-dilution / Single dilution Assay)
- Intradermal Challenge Method in G.Pigs
- Antibody Induction Method
- Any other Serological Method approved by NRA

Following are the methods for Tetanus Potency test

- Lethal Challenge Method in G.Pigs / Mice (Multi-dilution / Single dilution Assay)
- Determination of antibodies in G.Pig
- Any other validated Serological assay in G.Pigs / Mice approved by NRA

Implementation of 3 R's approaches at Biological E. Limited and Animal Reduction Per batch

Before 3 R's Implementation				
Potency	**Method**	**Animal model**	**Duration of Test (days)**	**Number of animals per lot**
Tetanus Potency (Multi dilution Assay)	Lethal challenge assay	Mice	33	184
Diphtheria Potency (Multi dilution Assay)	Lethal challenge assay	Guinea Pigs	33	144

After 3 R's Implementation					
Potency	**Method**	**Animal model**	**Duration of Test (days)**	**Number of animals per lot**	**% Reduction in animal consumption**
Tetanus Potency (Single dilution Assay)	Lethal challenge assay	Mice	33	64	~ 65%
Diphtheria Potency (Single dilution Assay)	Lethal challenge assay	Guinea Pigs	33	64	~ 55%

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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.

Principle of Single dilution assay : WHO TRS 980 Page No 233

A one-dilution assay is based on the same principles for evaluating the response as three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 30 IU (or the minimum requirement for the product expressed as an SHD), that elicits a minimum protective effect (or antibody response) in immunized animals; the effect of the reference vaccine is compared with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P \leq 0.05$), the potency of the test vaccine is satisfactory.

One-dilution assays provide assurances that the lower limit of the estimated potency exceeds the minimum requirement. A disadvantage of such an approach is that a strictly quantitative estimate of vaccine potency will not be obtained.

Lot release requirements WHO TRS 980 Page No 233

- Lot release based upon the use of a simplified approach requires periodic review to ensure that the validity of all procedures (including assumptions of linearity and parallelism) is maintained.
- The timing of the review should be decided on a case by case basis, depending on the number of lots of vaccine produced annually, or by time schedule (at least every two years), and should be approved by the NRA.
- It should be noted that if there is a significant change in the production process, testing should revert to the full multiple-dilution assay, and production consistency should be reconfirmed before the reduction scheme is reintroduced

1.Consistency of Production and Testing Diphtheria component in LPV

WHO VSQ 97.04 Chapter 27

- Obtain an estimate of the mean potency and its variance for each type of vaccine under consideration.
- Obtain an estimate of the observed mean regression slope, its level of significance and the mean range of fiducial limits obtained with the product and by the laboratory concerned.
- Derive from the mean potency estimate and its level of significance above an estimate of the minimum number of animals needed in the assay in order to release a product having the "historically" observed mean potency.

2. Evidence of highly significant regression and justification of the assumptions of linearity and parallelism of the dose response curve:

The probability values of regression, linearity and parallelism derived from the statistical calculation using software tools like Combistats software (EDQM) from minimum recent 20 batches :

NOTE: Probability value less than 0.05 is “Significant”
Probability value less than 0.01 is “Highly Significant”
Probability value less than 0.001 is “Even More significant”

Remarks:

The following criteria should be met in order for the potency estimate to be statistically valid: (WHO TRS 980 Page No 231)

- the statistical analysis should show a significant regression ($P < 0.05$) of the log dose–response curves without significant deviation from linearity and parallelism ($P > 0.05$);
- The 50% protective dose should lie between the smallest and largest.

3. Guidance for the selection of the single-dilution system parameters, namely number of animals and dilution level to be used for the reference vaccine

Selection of dilution:

- For the reference vaccine, the recent 20 batches data results shall be collected to contain number of international units known to elicit an immune response in the lower part of the dose response curve.
- As per WHO/IVB/11.11 page No 296, about 10-20% protection is considered acceptable. The mean protection for reference vaccine shall be calculated.
- The % survival of the regular dilutions of the reference standard should be identified to know the protection %.

3.1 Selection of Dilution of Reference Standard

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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.

- To get the dose producing protection level of about 10%, ED_{10} of the reference standard was calculated using combistats software or any validated software (Quantal response) for minimum of 10-15 sets (One reference + One Sample).
- Once data is generated take Geomean of all the test. Now to calculate dose required to Elicit 10% Protection = Potency of Reference Std (IU/mL)/ Average ED_{10} value obtained in reference std.
- Further to get dilution fold required for reference vaccine, calculate Potency of ref std IU/mL / Dose required to elicit 10% protection.
- The dilution fold of the reference standard is rounded off that will be considered for the single dilution assay.

Selection of dilution for test vaccine

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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.

WHO VSQ 97.04 Chapter 27 Page No 172

- To verify that the single vaccine dilution method is likely to work, compare responses to the lower dilution levels of the reference (which should be chosen so as to elicit a minimal response) with the responses to the higher dilution levels of the test vaccines.

The dilution fold of the reference standard should be rounded off to xx fold to identify the lower dilutions level of the reference std which should be chosen, so as to elicit a minimal response with the responses higher dilution level of test vaccine.

For the vaccine under test, all the products are assumed to contain the minimum required potency i.e. 30 IU/SHD (For ex Diphtheria component. Based on this, dilution of the test vaccine which hypothetically contains the same number of international unit as reference standard is calculated below:

Minimum expected Potency of regular batches	60 IU/mL (30 IU/0.5mL/SHD)
Dilution required for test vaccine	Minimum expected potency 60 IU/mL / Dose obtained in ref vaccine to Elicit 10% Protection .

3.2 Minimum number of animal for single dilution assay

WHO VSQ 97.04 Chapter 27 Page No 166

Obtain an estimate of the observed mean regression slope, its level of significance and the mean range of fiducial limits obtained with the product and by the laboratory concerned.

Table 15: Minimum potency excess factor[a]: Effect of slope and number of animals

No. of animals per vaccine	Minimum true response[b] (%)	Slope = 2	Slope = 3	Slope = 4	Slope = 5	Slope = 6
10	81.7	12.39	5.35	3.52	2.74	2.31
15	70.0	8.00	4.00	2.82	2.30	2.00
20	59.8	5.82	3.24	2.41	2.02	1.80
25	54.0	4.91	2.89	2.22	1.89	1.70
30	49.5	4.31	2.65	2.08	1.79	1.63
35	46.1	3.91	2.48	1.98	1.72	1.57
40	42.9	3.56	2.33	1.89	1.66	1.53

[a] The "minimum potency excess factor" relates to the potency associated with the "minimum true response" and is calculated from the probit of the "minimum true response" and the slope in probits per $\log_{10}$.

The "minimum potency excess factor" is multiplied by the minimum required potency to yield the minimum true potency which is expected to be released in 97.5% of the assays, e.g. when entering the table at a slope value of 4 and using 20 animals per vaccine, a tetanus or diphtheria-tetanus vaccine will have to contain at least 2.41 x 40 = 96.4 IU/single human dose. If the true potency of a given producer's vaccine is known approximately, this value is divided by the minimum required potency to obtain a "potency excess factor" which can be used to enter Table 15 at a given slope value, and estimate the number of animals needed for 97.5% release.

[b] The "minimum true response" is calculated using the exact binomial distribution corresponding to the number of animals considered. To yield the 97.5% of observed responses necessary for acceptance, a test vaccine must have a true potency associated with this "minimum true response", given a true response of 10% for the reference vaccine.

- The minimum number of animals likely to be assuring the potency and for the releases of a product can be derived from the estimate of the observed mean potency and the mean common slope. The rounded values of the mean slope to absolute numbers with no decimal point shall be considered for the same.
- Calculate common slope value obtained for each individual assay and take geomean of all the assay.

❖ Round off the value to single digit.

❖ Calculate common slope in Probits per $\log_{10}$ from each of the common slope value and then take geomean for all the assay.

❖ The data obtained in the common slope in Probit per $\log_{10}$.

❖ The value obtained in common slope probits per $\log_{10}$ should me checked with the table 15 as mentioned in the WHO VSQ 97.04.

❖ This design will be give minimum number of animals required for the single dilution assay.

3.3 Prediction of the behavior of the single dilution assay:

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prediction of the behavior of the single dilution system.

V.4.4 Fisher's exact test Page No 285

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

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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.

- Base on the dilution finalized for the reference standard (xx fold) and test vaccine (xx fold) for the single vaccine dilution assay, data collected from the multi dilution assay which is close to the proposed single vaccine dilution shall be taken for the prediction of the behaviour of the single vaccine dilution assay.
- The survival data of xx fold of the reference standard and xx fold for the test vaccine are calculated with the Fisher exact probability test (one side) or by combistat

NOTE: Probability value less than 0.05 is "Significant"

Probability value less than 0.01 is "Highly Significant"

Probability value less than 0.001 is "Even More Significant"

The calculation says that the test vaccine has shown a significantly higher response than the reference vaccine

Validation of single dilution assay:

- Based on the overall assessment, for the validation of single dilution assay following conclusion are arrived.
- The dilution finalized is for reference standard xx fold from unitage assigned from reference vaccine 40IU/mL
- The dilution finalized for test vaccine is xx fold.
- Number of animals to be used for the single dilution is 20 or as mentioned in Table 15 WHO VSQ 97.04.
- Applying above criteria 3 single dilution tests shall be performed simultaneously along with the multiple dilution assay for the validation.

1. **Retest:**
   - During regular testing of batches for release, if a batch fails in single dilution assay, the sample shall be retested by following multi dilution assay to decide the fate of the batch.

Retest criteria is not applicable for the validation study and only applicable for release testing of batches.

1. **ACCEPTANCE CRITERIA**

The sample passes if, the probability value falls in any of the following categories:

- Probability value less than 0.05 is “Significant”
- Probability value less than 0.01 is “Highly Significant”
- Probability value less than 0.001 is “Even More Significant”

Thank You

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