# Work Protocol ver. 6

ATTENTION: the work protocol is a guidance. Product specific adaptation is highly recommended.

Procedure for the immunisation of mice and preparation of serum samples for the Pertussis Serological Potency Test

(MS-PSPT work protocol)

*Note: any deviation from the proposed work protocol should be reported.*

*In case of any doubt kindly refer to 'Laura Viviani'* *l.viviani@dcvmn.net*

**1. Introduction**

This Protocol describes the procedure for the immunisation of mice and preparation of serum samples for wP-ELISA test to determine the titers of whole-cell Pertussis (wP)-vaccine-induced mouse antibodies that specifically recognize the whole-cell *Bordetella pertussis* coating antigen of strain 18323. The wP-ELISA is described in the document PSPT-ELISA work protocol.

Furthermore, this procedure also describes the production of a mouse negative serum (coded wP-ms-Neg) and a mouse positive serum (wP-ms-Pos) that are necessary to perform the ELISA test as a part of the PSPT study (see paragraph 12).

The wP-ms-Neg needs to be prepared prior to the initiation of PSPT.

2. PSPT procedures

Mice to be used for each vaccine lot are randomly subdivided into four (4) dilution groups. Not fewer than 12 mice are allocated to each dilution group. Each group of animals is immunised at day 0 with one of the 4 dilutions of a Reference vaccine or test vaccine, respectively.

Animals are immunised intraperitoneally (i.p.) with a dose of 0.5 ml dilution/mouse.

Twenty-eight days after immunisation, all animals are bled under anaesthesia (see blood collection) and individual blood samples are collected, processed, divided in aliquots (200 mcl) and stored at -20°C (Fig. 1). A volume of 500-1500 mcl blood can be obtained per mouse.



**Fig. 1:** Schematic overview of the mouse immunisation procedure over time.

3. Animals

For each vaccine lot (and reference preparation) allocate not fewer than 48 healthy mice of either one sex, or an equal distribution of both genders over the four groups, within the weight range of 20-24 g. Divide the animals randomly for each dilution group, but in case of both genders on the condition of equal distribution of genders.

NOTE:

All animals are of SPF quality and certified by the respective commercial breeder or by the manufacturer’s breeding facility. In particular, it is important that it is certified that the animals are free of *B. bronchiseptica and B parapertussis*.

The health status of the animals should be recorded at arrival and monitored during the experiment by inclusion of sentinel animals.

**3.1 Sentinel mice**

Manufacturers have usually a sentinel programme in place in their animal facility where experiments for QC purposes are carried out. When this is not the case, kindly follow the procedure given below.

Allocate nine animals of the same cohort of animals as those used for KT or PSPT, to be used as sentinel animals.

Sentinel mice will not be immunised and will be housed in one or two cage(s) in the animal room.

If more than one animal room is used, each additional room shall include 6 sentinel mice in one or two cage(s).

Few days before to start the potency study, bleed 3 of the 9 sentinel animals and screen them individually for antibodies for *B. pertussis* by the PSPT-ELISA (see work protocol for ELISA).

The sentinel mouse serum, pre-diluted 1/400 – 1/800 will be considered negative in ELISA if the OD is below 0.35-0.45.

The absence of infection by *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* in the mice colony can also be confirmed by other appropriate methods (e.g. cultural or serological assay). In case commercial kits are available these can also be used.

Even if one mouse in sentinel group is found positive for pre-existing antibodies against at least one of three species *Bordetella* bacteria (*B. pertussis*, *B. parapertussis* or *B. bronchiseptica*), the batch of animals is not suitable for the experiment and must not be immunized. Important is to find out what the reason of the positive pre-test has been.

At the end of the experiment (28 days), on the bleeding day (post-experiment), serum samples obtained from the 6 sentinel mice/room should be tested in PSPT-ELISA or any other serological assay (e.g. agglutination assay) to detect whether the sentinel animals are positive for *B. pertussis* antibodies. If the sentinel mice are positive the entire experiment is invalid. If negative, proceed with the testing of all mice sera.

NOTE

If the PSPT *in vivo* part of the study (immunization) is not performed by testing of all vaccine lots in one analytical session, then in each further session the sentinels have to be included as well as the Reference vaccine and the sample FL1.

**Table 1: Overview of time schedule for the MS-PSPT study**

|  |  |  |
| --- | --- | --- |
| **Day**  | **Activity** | **NOTE** |
| **2-3 days prior to immunization** | Test 3 sentinel mice for absence of a positive signal in ELISA with *B. pertussis* coating antigen | All 3 animals should be negative to *Bordetella* antibodies*.* If animals show a response in ELISA or other appropriate test, animals should not be used for the main study. |
| **0** | Start of PSPT study - *Immunisation*  | Reference vaccine  |
| Test vaccine lots  |
| **1-27** | Check health status of animals  | Report it in the lab-journal  |
| **28** | End of study  | Euthanize and bleed all mice. Blood of each individual animal is collected in sterile tubes  |
| Bleed the remaining sentinel mice and verify that these animals have not been infected by *B. pertussis*, *B. parapertussis or B. bronchiseptica* during the 28 days  |

4. Reference vaccine

Recommended Reference vaccine in the PSPT assay is either the Regional Working Reference Standard (RWRS) of Pertussis Vaccine (IPRS/20/PERT) or WHO Pertussis Reference vaccine IS4, 94/532 (potency of 40 IU/ampoule) or an in-house Reference vaccine calibrated against the RWRS or WHO Reference vaccine.

1. **Vaccine lot alteration process**

Based on **Table 6 of the WHO report *Temperature Sensitivity of Vaccines*** (WHO/IVB/06.10, August 2006), please find below a table showing the percent of original (unaltered) potency remaining after 1, 2, 3 and 4-weeks incubation at 46°C. The value of 6.7% loss per day at 46°C was used from Table 6.

|  |  |
| --- | --- |
| **Days at 46°C** | **Potency (%)** |
| 0 | 100% |
| 7 | 64% |
| 14 | 40% |
| 21 | 26% |
| 28 | 16% |

**NOTE:** 43-45°C is recommended to reduce the chance of protein aggregation, which occurs at 46°C and above.

Recommended procedure for lot alteration, i.e., preparation of a subpotent lot:

* Place the vaccine final lot in an incubator or water bath at 43-45°C under agitation.
* The agitation must be **slow but sufficient to** move the fluid in the vials.

If an agitator/shaker is not available, the vials should be manually gently inverted 3 times per day.

* Alteration duration: 21 days.
1. Preparation of test vaccines and Reference vaccines

Prepare, in a safety cabinet, 4 two-fold dilutions series of the test lots and the Reference vaccine.

Prepare vaccine dilutions not more than one hour before immunisation.

6.1 Scheme of testing vaccines all in one experiment

Overview vaccine samples to be used:

|  |  |  |
| --- | --- | --- |
| Vaccine samples | No of animals (as a minimum) | Explanation  |
| NA | 6/room | Sentinel mice (no treatment) |
| RWRS or WHO wP IS4 or in-house Reference | 48 | 4 dilution series. 12 animals per dilution  |
| Vaccine Final lot (FL) 1 | 48 | This lot will be used as in-house Reference vaccine in consistency testing  |
| Vaccine FL2A | 48 | 4 dilution series. 12 animals per dilution  |
| Vaccine FL2B | 48 | 4 dilution series. 12 animals per dilution |
| Vaccine FL3 | 48 | 4 dilution series. 12 animals per dilution |
| Vaccine FL3-altered | 48 | 4 dilution series. 12 animals per dilution |

* 1. Scheme of testing vaccines in two experiments

Overview vaccine samples to be used:



When testing the vaccines in two experiments, the Reference vaccine, the vaccine lot FL1 and the sentinel mice must be used again.

Experiment n. 1

|  |  |  |
| --- | --- | --- |
| Vaccine samples | No of animals (as a minimum) | Explanation  |
| N/A | 6/room | Sentinel mice (no treatment) |
| RWRS or WHO wP IS4 or in-house Reference | 48 | 4 dilution series. 12 animals per dilution  |
| Vaccine Final lot FL1 | 48 | This lot will be used as in-house Reference vaccine in consistency testing  |
| Vaccine FL2A | 48 | 4 dilution series. 12 animals per dilution  |
| Vaccine FL2B | 48 | 4 dilution series. 12 animals per dilution |

Experiment n. 2

|  |  |  |
| --- | --- | --- |
| Vaccine samples | No of animals (as a minimum) | Explanation  |
| N/A | 6/room | Sentinel mice (no treatment) |
| RWRS or WHO wP IS4 or in-house Reference | 48 | 4 dilution series. 12 animals per dilution  |
| Vaccine Final lot (FL)1 | 48 | This lot will be used as in-house Reference vaccine in consistency testing  |
| Vaccine FL3 | 48 | 4 dilution series. 12 animals per dilution |
| Vaccine FL3-altered | 48 | 4 dilution series. 12 animals per dilution |

* 1. Reference vaccine preparation

Reference vaccine should be reconstituted or diluted to obtain 10 IU per mL. This represents the first dilution.

NOTE: Prepare at least a volume of 20 mL (10 IU/mL) of the first dilution, if 12 mice per dilution are used.

An example of how to proceed is given below for the RWRS and the WHO IS4.

* Regional Working Reference Standard (RWRS) of Pertussis Vaccine (IPRS/20/PERT): reconstitute x vials of the Reference vaccine with sterile PBS/saline to obtain 10.0 IU/ mL. You need at least 20 ml (10 IU/ml) to prepare the RWSR dilutions.
* RWRS1 = 10.0 IU/ml
* RWRS2 = 5.0 IU/ml: 10 ml dilution RWRS1 + 10 ml PBS/saline
* RWRS3 = 2.5 IU/ml: 10 ml dilution RWRS2 + 10 ml PBS/saline
* RWRS4 = 1.25 IU/ml: 10 ml dilution RWRS3 + 10 ml PBS/saline
* In case the WHO pertussis Reference vaccine IS4, 94/532 (40 IU/ampoule)is used,reconstitute x ampoules to obtain 10.0 IU/ ml. You need at least 20 mL (10 IU/ml) to prepare the dilutions.
* R1 = 10.0 IU/ml PBS/saline
* R2 = 5.0 IU/ml: 10 ml dilution R1 + 10 ml PBS/saline
* R3 = 2.5 IU/ml: 10 ml dilution R2 + 10 ml PBS/saline
* R4 = 1.25 IU/ml: 10 ml dilution R3 + 10 ml PBS/saline
	1. Preparation of wP combined test vaccine (16 – 40 Opacity Units (OU)/ml)
* D1; dose/mouse = 50 µl of vaccine + 450 µl diluent for final volume of 0.5 ml:

Prepare at least 20 ml (for group of 12 mice): 2 ml undiluted vaccine in 18 ml of PBS/saline

* D2; dose/mouse = 25 µl of vaccine + 475 µl diluent for final volume of 0.5 ml:

Preparation (for group of 12 mice): 10 ml vaccine dilution D1 + 10 ml PBS/saline

* D3; dose/mouse = 12.5 µl of vaccine + 487.5 µl diluent for final volume of 0.5 ml:

Preparation (for group of 12 mice):10 ml vaccine dilution D2 + 10 ml PBS/saline

* D4; dose/mouse = 6.25 µl of vaccine + 493.75 µl diluent for final volume of 0.5 ml:

 Preparation (for group of 12 mice):10 ml vaccine dilution D3 + 10 ml

 PBS/saline

7. Immunisation of mice

Immunization is performed using 4 dilutions of each of the vaccine lots and Reference vaccine. Use a work sheet/ lab journal for reporting of the test details.

Inject each immunisation group (12 animals as a minimum/dilution) of mice with one dilution of each prepared wP test vaccine lot (D1, D2, D3, D4) or Reference vaccine (1, 2, 3, 4 of RWRS or WHO or in-house Reference). The cages are numbered consecutively.

Inject 0.5 ml intraperitoneally (i.p.) in each mouse, using a 2.5 ml syringe fitted with a 23 G × 1*"* needle. Tilt the syringe gently between the injections in order to maintain a homogeneous suspension.

Observe the animals daily for 4 weeks and report any peculiarity on the work sheet/ lab journal.

8. Blood collection

All mice are bled on day 28 after immunization. Blood collection is performed by heart puncture (retro-ocular puncture) under isoflurane (or other suitable procedure) anaesthesia (mixture of 50% nitrous oxide, 50% O2 and 1-2 % isoflurane), and euthanize the animals humanely. Blood of each individual animal is collected in sterile tubes/vials (e.g. Eppendorf-tubes, NO coating with EDTA or Heparin) identified by the animal number as given in the worksheet.

**NOTE**: bleeding by tail-vein puncture will not provide the same volume of 500-1500 mcl blood that can be obtained by heart puncture or retro-ocular puncture.

9. Preparation of serum specimens

When filled with blood, the vial/tube of each individual mouse is inverted six times.

The tube is left at 37°C for 2 h followed by 2 h at + 4°C.

Centrifuge for 20 min at 800 x *g* at room temperature.

Transfer the serum into sterile tubes, properly identified with animal number, group number vaccine lot and reference code.

Serum should be stored below -20°C in aliquots of 200 mcl.

10. Antibody titration by ELISA

See PSPT-ELISA Work Protocol.

11. Potency calculation

Based on the individual antibody titres, the potency of the test vaccine can be calculated by parallel line analysis.

See Potency Calculation Work Protocol.

12. Production of mouse negative and positive serum

Participants are also required to prepare a mouse negative serum and a wP positive mouse serum to be used respectively as negative and positive controls in ELISA.

|  |  |
| --- | --- |
| NOTE  | Number of animals  |
| Negative serum – negative control in ELISA (wP-ms-Neg) | 10 |
| wP positive serum – positive control in ELISA(wP-ms-Pos) | 15 |

**12.1 Negative mouse control serum** (coded wP-ms-Neg) **for the PSPT-ELISA**

This serum should be prepared as soon as possible.

A wP negative control serum (coded wP-ms-Neg) is obtained by pooling the sera from 10 non-immunized mice of the same strain as used for the PSPT studies, after having checked the absence of antibodies against the *B. pertussis* coating antigen in the sentinel mice.

The pooled serum is stored in aliquots of 200 mcl at -20°C.

The negative control serum will be used as negative control in all wP-ELISA plates.

**12.2 Positive mouse control serum** (coded wP-ms-Pos) **for the PSPT-ELISA**

The positive mouse control serum can be prepared in parallel with the main experiment.

A dedicated group of 15 mice is immunized with the highest dose (first dilution) of the RWRS, WHO wP IS4 Reference or the in-house Reference.

At day 28 after immunization, mice are bled.

The serum of each animal is assayed in PSPT-ELISA.

**NOTE:** as the volume of serum obtained from each animal can vary as well as the titre, by pooling the serum of all 15 animals, independently from the titre, a *wP-ms-Pos* with a low titer (< 1.0 as OD in ELISA) can be obtained.

Therefore, to have a *wP-ms-Pos* with a high titre, it is advisable to pool only individual sera with similar titre.

The *wP-ms-Pos* serum needs to be included in all ELISA plates to allow establishment of the antibody titres of the individual test or reference sera. This is done by assigning an arbitrary unitage of 100 EU/mL to the *wP-ms-Pos* serum.