2.1.1.3.2 *In-Vitro* Phase: Titration of Serum Sample to Detect the Presence of Antibody

1) Antigen coating: microplates are coated with a 5 µg/mL *haemophilus influenzae* b polysaccharide. 100 µL of this solution is filled in each well of microplates and then incubated for 16-20 hours at +37°C ± 2°C;
2) Saturation: wells of microplates are saturated with goat serum 10% in Phosphate Buffer Saline (PBS) 1X and then incubated for approximately 1 hour at +37°C ± 2°C;
3) Titration of antibodies:

- Samples, controls and standards are diluted at 1/10 with in PBS-octoxynol 0.1%, goat serum 5%;
- 100 µL of goat serum 5% in PBS-Triton 0.1% is dispensed in each well of microplates;
- Then 100 µL of diluted samples diluted negative controls, diluted standards, diluted internal controls and blanks are dispensed as appropriate in wells of microplates;
- Microplates are incubated under agitation for approximately 1.5 hours at +37°C ± 2°C;

4) Conjugate addition: 100 µL is dispensed in each well of microplates and then incubated under agitation for approximately 1.5 hours at +37°C ± 2°C;
5) Colorimetric reaction: the complex is revealed using 100 µl of a peroxidase substrate: OPD. The reaction is stopped by adding 50 µL of sulfuric acid 2 N in each well of microplates.

2.1.1.4 Reading – Calculations - Results

2.1.1.4.1 Reading

The optical density (OD) is read at 492 nm and at 620 nm.

2.1.1.4.2 Calculations

The absorbance of the mouse antiserum reference standard curve is plotted as a function of the anti-PRP concentration using a linear/logarithmic regression program. The different concentrations are calculated from the OD of each sera. The anti-PRP concentration corresponds to the mean of the concentrations of each sera taking into account the dilution factors.

Hib immunogenicity is expressed in EU/mL with the following formula:

$OD = d + (a - d) / (1 + (X/c)^b)$

Where:

- d: Asymptote as X approaches 0;
- a: Asymptote as X approaches infinity;
- X: Concentration (UE/mL);
- c and b: parameters that define the curve shape.

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2.1.1.4.3 Results

Mice are seroconverted if their *haemophilus* immunogenicity is not less than 4 times that of the pooled control serum.

Mice are not seroconverted if their *haemophilus* immunogenicity is less than 4 times that of the pooled control serum.

2.1.1.5 Validity Criteria

2.1.1.5.1 Validity Criteria for Antibody Titration

- Optical density of the blank must be less than 0.200. When the average of the blank is more than 0.200, it is possible to eliminate 1 aberrant result;
- The average of optical density of negative control first dilution must be less than 0.200.

2.1.1.5.2 Validity Criteria for Immunogenicity Test

- The pool of non-immunized control mice sera must be negative. The optical density of the lowest dilution must be less than 0.200;
- Mean titer of a group must be calculated with at least 6 mice among 8.

2.1.2 Non-Adsorbed PT and Non-Adsorbed FHA

The method is based on Ph. Eur. 2.7.1 (Immunochemical methods).

2.1.2.1 Principle

- The method is carried out as a limit test.
  - Quantification of pertussis toxoid:

    The method is based on the reaction of pertussis toxoid with sheep anti-pertussis antibody bound to microtiter wells. Then a secondary antibody, mouse anti-pertussis toxin antibody, is added to the wells. Goat anti-mouse antibody conjugated to alkaline phosphatase is added next, followed by substrate.
  - Quantification of FHA:

    The method is based on the reaction of FHA with goat anti-FHA antibody bound to microtiter wells. Then a secondary antibody, mouse anti-FHA antibody, is added to the wells. Goat anti-mouse antibody conjugated to alkaline phosphatase is added next, followed by substrate.
- For both ELISA, the intensity of color is proportional to the concentration of antigen (respectively pertussis toxoid and FHA).

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6524

2.1.2.2 Reagents

- Coating antibody: sheep anti-*Bordetella* pertussis toxin: NIBSC code 97/572 and goat anti-FHA sera in-house production;
- Standard reference antigen: pertussis toxoid stabilised in 50% v/v glycerol and FHA in solution;
- Secondary antibody: in-house mouse anti-*Bordetella* pertussis toxin serum and in-house mouse anti-FHA serum;
- Conjugate: alkaline-phosphatase labelled goat (Fab')2 anti-mouse Fc antibody;
- PBST: Phosphate Buffer Saline 0.05% Tween 80;
- Blocking solution: Phosphate Buffer Saline - Bovine Serum Albumin (BSA) 2% (the percentage of BSA can be adapted depending on the BSA batch);
- Sample diluent (blank): PBST 1% BSA solution (the percentage of BSA can be adapted depending on the BSA batch);
- Chromogenic substrate: pNPP (p-nitrophenyl phosphate, disodium).

2.1.2.3 Operating Procedure

- Antigen coating: microplates are coated with coating antibody and then incubated overnight at +37°C ± 2°C for FHA and at +5°C ± 3°C for PT;
- Saturation: wells of microplates are saturated with blocking solution and then incubated 1 hour at +37°C ± 2°C;
- Using the sample diluent dilute the standard reference antigen to the concentration of 2.5 µg/mL. Product is centrifuged and the supernatant is deposited pure on the ELISA plate and then incubated 1.5 hours at +37°C ± 2°C;
- Secondary antibody: secondary antibody is added to each well and then incubated 1.5 hours at +37°C ± 2°C;
- Conjugate addition: conjugate is dispensed in each well of microplates and then incubated 1.5 hours at +37°C ± 2°C;
- Colorimetric reaction: pNPP previously diluted at 1 mg/mL with diethanolamine buffer is filled into each well and then incubated 30 minutes at room temperature. The reaction is stopped by adding sodium hydroxide 2.5 N in each well of microplate.

2.1.2.4 Reading – Calculations - Results

After the chromogenic reaction with alkaline phosphatase substrate, the absorbance of each well is recorded at 405 nm and 620 nm.

- Compare the OD of the product to the average OD of the standard reference at 2.5 µg/mL;

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- If both the OD of the product is less than to the OD of the standard reference, the concentration is less than to 2.5 µg/mL;
- If both the OD of the product is superior or equal to the OD of the standard reference, the concentration is superior or equal to 2.5 µg/mL.

2.1.2.5 Validity Criteria

- Blank control OD must be not more than 0.2
- OD coefficient of variation of the standard reference at 2.5 µg/mL must be not more than 30%;
- OD of the standard reference must be greater than OD of the blank +3 relative standard deviation determined historically (eg: 0.109 for Pertussis Toxoid in solution and 0.116 for FHA in solution);
- Both the 2 individual OD of sample must be less than or greater than the average OD of the standard reference at 2.5 µg/mL.

2.1.3 Percent Adsorption - Tetanus Toxoid (Rocket)

The adsorption percentage of tetanus toxoid is determined by the double gel Rocket Immunoelectrophoresis (RIE) method.

2.1.3.1 Principle

The purpose of the test is to determine the concentration of non-adsorbed purified tetanus toxoid on the hydroxide aluminum gel in the Hexaxim combination vaccine containing both Polyribosyl Ribitol Phosphate conjugated to tetanus protein (PRP) and purified tetanus toxoid. This method is based on the migration under an electric field of the supernatant of the vaccine in a 1% agarose gel containing two distinct areas:

- In order to avoid PRP-T interference, the first part contains *Haemophilus influenzae* type b specific antibodies able to capture total PRP antigens (gel 1);
- The second part contains tetanus-specific antibodies, which react with the free tetanus toxoid (gel 2), present in the sample vaccine.

Rocket-shaped precipitin lines are formed at equivalence. The heights of the Rocket-shaped curves in the second part of the gel are proportional to the amount of tetanus toxoid present in the sample.

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2.1.3.2 Operating Procedure

- Preparation of the gel

Gel 1 and gel 2 are prepared separately from a 1% agarose solution in which the correct amount of each antiserum (rabbit anti-PRP serum in gel 1 and horse anti-tetanus serum in gel 2) is added. The dilution of antiserum is determined during the qualification step.

Gel 1 is poured. After solidification, gel 2 is poured next to gel 1.

- Preparation of the samples

The reference standard is the mix of 2 references:

- In-house purified tetanus toxoid lot;
- In-house PRP lot.

The reference range includes 4 dilutions: pure - 0.5 - 0.25 - 0.125. The pure reference sample is prepared to obtain concentrations of tetanus toxoid and PRP equivalent to their respective concentration in the vaccine. Dilutions use a 1xC barbital buffer solution. Each dilution is loaded in duplicate on both sides of the agarose gel 1 wells.

Tested products: The products are centrifuged at around 5200 g during 5 min at +5°C. The supernatants are collected and loaded pure in duplicate on the agarose gel 1 wells.

Migration control used is bromophenol blue loaded in one or more free wells.

- Rocket immunoelectrophoresis

Samples (reference standard, tested products and migration control) are loaded as described above and the immunoelectrophoresis is performed.

The gel is washed (using NaCl 0.9% and purified water), dried with a ventilated air system, stained using a Coomassie staining solution and faded using a methanol-acetic acid solution.

2.1.3.3 Reading – Calculations - Results

The rockets heights are measured in centimeters from the baseline of the tetanus toxoid rockets to the culminant point of each rocket.

The parameters a (slope), b (intercept) of the standard curve are calculated:

Log_{10} of the tetanus toxoid concentration (Lf/dose) = a.(log_{10} of the rocket heights (cm) + b

The tetanus toxoid adsorption on aluminum gel is expressed in percentage of adsorption.

The percent adsorption of tetanus toxoid in the sample is calculated as follows:

$$\text{Adsorption of Tetanus Toxoid (\%)} = 100 - \frac{\text{Concentration of Tetanus Toxoid measurment in the supernatant}}{\text{Theorical concentration of Tetanus Toxoid in initial vaccine}} \times 100$$

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2.1.3.4 Validity Criteria

- The rocket height measured in gel 2 of the most concentrated reference antigen is comprised between 10 and 40 mm;
- Rockets corresponding to PRP/anti-PRP antibody reactions visible on gel 1 have to not pass the baseline of tetanus rockets (you could say should not be visible in gel 2);
- The rockets have to be correctly colored;
- The rocket borders have to be neat and contrasted;
- The correlation coefficient of reference curve has to not be less than 0.98;
- The percentage CV calculated on rocket height of each reference and sample dilutions must not be more than 20%.

2.1.4 Rat Immunogenicity Assay for IPV

The method is based on Ph. Eur. 2.7.20 (*In-vivo* assay of poliomyelitis vaccine (inactivated)).

2.1.4.1 Principle

The rat immunogenicity assay is to confirm that the vaccine induces a consistent antibody response in rats, when compared to animals injected with a qualified reference vaccine. The test is based on the Ph. Eur. compendial 2.7.20 "*In-vivo* assay for poliomyelitis vaccine". This is a micro metabolic inhibition test based on the premise that a sufficiently potent poliovirus vaccine Inactivated would elicit polio neutralizing antibodies in rats. The results are compared with an in-house reference vaccine.

2.1.4.2 Reagents

2.1.4.2.1 *In-Vivo* Phase: Immunization of the Rats

- Vaccine samples;
- In-house Pediacel (DTaP-IPV-PRP-T) reference standard;

2.1.4.2.2 *In-Vitro* Phase: Virus Neutralization Test

- Challenge virus: poliovirus monovalent sabin strains passaged in Hep-2 cells;
- Naphthol blue black.

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2.1.4.3 Operating Procedure

2.1.4.3.1 *In-Vivo* Phase: Immunization of the Rats

1) Test vaccine samples and reference standard vaccine are appropriately diluted and injected intramuscularly into groups of 10 female rats per vaccine dilution. The weight of the rats is from 175 to 250 g;
2) A group of 5 uninjected rats is used as a control;
3) Rats are bled on day 21 and the sera from individual rats are collected.

2.1.4.3.2 *In-Vitro* Phase: Virus Neutralization Test

1) Serum samples are heat activated at approximately +56°C for around 30 minutes;
2) Immune sera (0.025 mL) are two-fold serially diluted as appropriate and dispensed in wells of microtiter plates containing 0.15 mL Hep-2 cell as the indicator cell. Immune sera are added to microplate for poliovirus type 1, type 2 and type 3;
3) Each virus (type 1: lot R174, type 2: lot R227 and type 3: lot R371) is prepared to contain approximately 50 $TCID_{50}$[a] per 0.025 mL and added to each well. Microplates are incubated at approximately +37°C for around 3 hours and overnight at +2°C to +8°C;
4) Hep-2 cells diluted to contain 1000 cells/well in 0.15 mL are added to each well. Microplates are then incubated at +37°C ± 1.0°C for 5 to 7 days;
5) Each plate also includes type-specific standard sera as positive controls, cel-toxicity controls (no virus), virus controls and cell controls to assure test validity;
6) Staining of plates by a 0.1% naphthol blue black staining solution;
7) Each microplate also includes type-specific standard sera as positive controls, cell-toxicity controls (no virus), virus controls and cell controls to assure test validity.

2.1.4.4 Reading – Calculations - Results

Intact cell monolayers stained dark blue indicate specific neutralization of the virus or lack of viral activity. Absence of stained cell monolayers or empty wells indicates viral activity.

The serum titer is expressed as the $\log_2$ 50% end points which is the highest dilution of the serum that causes neutralizatin of the virus as indicated by the stained cell monolayer.

The 50% end-point for each serum is calculated and expressed as mean titers (logarithm to base 2). Vaccine potency values are determined by comparison to the reference vaccine.

[a] $TCID_{50}$: Tissue Culture Infectious Dose 50

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2.1.4.5 Validity Criteria

The following criteria must be satisfied for a test to be considered valid:

- The in-house standard serum titers are within the range of:
  - Type 1: 16-64;
  - Type 2 : 4-16;
  - Type 3: 16-64.
- The virus dose must be within the range of 10 to 1000 $TCID_{50}$ per 0.025 mL;
- The medium control must be free of contamination by microscopic examination prior to staining;
- For virus typing, the 3 types of poliovirus must be type correctly. There should be no stained cells in the 4 wells containing the other 2 types of antisera;
- The toxicity control should not be toxic. If toxicity is observed in the toxicity control, the serum is toxic and result is invalid at that level. This is to ensure that the neutralization is due to the antibody response and is not caused by the serum being toxic to the cells alone;
- The average titer of the sera from negative rats is less than 1/4. A minimum of 3 negative controls is required for a valid assay;
- A minimum of 8 rats from each dilution group of rats is required to be tested for a valid dilution group;
- Probit result is invalid if a calculated relative potency cannot be obtained, e.g., Probit result of non-parallel, non-linear, no significant dose-response relationship and/or estimates diverging relative to the vaccine reference preparation;
- The ED_{50} values for both the test and reference vaccine must lie between the smallest and the largest doses given to the animals;
- The 95% confidence limit of the estimated relative potency of sample must be between 25% and 400% of the estimated potency.

2.1.5 Specific Toxicity for Diphtheria and Tetanus Components

The specific toxicity test is used to confirm the absence of any residual tetanus and diphtheria toxicity in the combined diphtheria, tetanus, acellular pertussis, hepatitis B, inactivated poliomyelitis trivalent concentrate and *haemophilus influenzae* type b vaccine. The test is performed according to the method described in WHO Technical Report Series No. 800, 1990, Annex 2 "Requirements for Diphtheria, Tetanus, Pertussis and Combined Vaccines" and Ph. Eur. Monograph 2067 " Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated) and *haemophilus* type b conjugate vaccine (adsorbed)".

2.1.5.1 Principle

This test involves injecting guinea pigs with the test sample and observing the animals over a period of 42 days for weight loss and any symptoms specific to diphtheria toxicity and tetanus toxicity.

2.1.5.2 Operating Procedure

Each of seven guinea pigs of the same sex, weighing between 250 g to 350 g are injected subcutaneously in the abdominal area. 5 of them receive 2.5 mL of the test vaccine (equal to 5 human doses injected/animal) and 2 of them receive a negative control (0.9% sodium chloride).

2.1.5.3 Reading – Calculations - Results

The animals are observed during 42 days and weighed at regular intervals. The weight gain of animals is calculated for the period.

Furthermore, the animals are examined daily for 42 days for symptoms of diphtheria toxicity or tetanus. If an animal dies on test, it is subjected to necropsy to determine the cause of death.

2.1.5.4 Validity Criteria

- The test is valid if at least 80% of treated animals survived the observation period;
- The test is invalid if more of 20% of treated animals die or show symptoms of disease due to non specific or intercurrent causes. The test must be repeat on the same type of animal;
- The eventual presence of a palpable subcutaneous node in the injection site is considered normal.

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2.1.6 Integrity Test

2.1.6.1 Principle

The container closure integrity test is carried out to demonstrate that the container integrity of pre-filled syringes and single-dose vials are maintained after filling and packaging processes and during the product shelf-life program. The testing procedure is outlined below.

2.1.6.2 Reagents

- Riboflavin solution at 0.1% w/v.

2.1.6.3 Operating Procedure

A 0.1% riboflavin dye tracer solution is used to submerge twenty product syringes or vials in a horizontal position together with 5 (single-dose vials) or 3 (syringes) system suitable control containers.

A system suitable control container is a system used to verify that the test is satisfactory. Concerning the integrity test, the system suitable control container used for example syringes or vials with an intended integrity defect specifically built for this test. These containers are tested similarly to the samples.

The chamber is closed and the air is evacuated to obtain a pressure ≤ 0.7 bar and hold for 30 ± 1 minute at room temperature.

30 minutes are necessary to let the atmospheric pressure returns. The containers are then removed from the bath, rinsed under water and dried.

2.1.6.4 Reading – Calculations - Results

- The content of the containers are examined under UV at a wavelength of 365 nm;
- The integrity of the container is observed if a yellow fluorescence is not observed.

2.1.6.5 Validity Criteria

The results are considered valid when the following conditions are met:

- All containers with the system suitability control are completely submerged into a dye tracer solution;
- The content of all system suitability control and the unexposed positive product control must show the presence of fluorescence when exposed to the source of UV at a wavelength of 365 nm;
- The content of the unexposed negative control must show no presence of fluorescence when exposed to the source of UV at a wavelength of 365 nm.

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2.2 Summary of Validation of Analytical Procedures

2.2.1 Non-Adsorbed PT and Non-Adsorbed FHA

This section describes the validation of non-adsorbed PT and non-adsorbed FHA: determination as limit test of PTxd and FHA by ELISA in the supernatant of Hexaxim vaccine, according to the ICH guideline.

The validation is divided into 2 parts: one for each pertussis components (pertussis toxoid and FHA).

2.2.1.1 Validation of the Limit Assay for Measuring Non-Adsorbed Pertussis Toxoid in the Hexaxim Supernatant

Since the method is a limit assay, the studied characteristics are specificity and limit of detection.

The results of the validation are summarized in the following Table 23:

Table 23: Titration of Pertussis Toxoid by ELISA in the Hexaxim Supernatant - Validation Summary

Characteristics	Acceptance criteria	Results
Specificity	PTxd antigen is detected in the presence of PTxd antigen in a sample of Hexaxim supernatant	PTxd antigen is detected when PTxd is present in the sample of Hexaxim supernatant
	PTxd antigen is not detected in absence of PTxd in a sample of Hexaxim matrix supernatant (containing the other antigens)	PTxd antigen is not detected when PTxd is not present in the sample of Hexaxim matrix supernatant
Limit of detection	Detection limit will be established at 0.023 µg/mL if the absorbance of the assay (Hexaxim matrix supernatant spiked with 0.023 µg/mL of PTxd antigen) is above the the adsorbance determined as the detection threshold	The absorbance of the assay is above the absorbance determined the detection threshold Detection limit = 0.023 µg/mL
	Detection limit will be satisfactory if the absorbance of the assay (Hexaxim matrix supernatant spiked with 0.023 µg/mL of PTxd antigen) is below the absorbance measured for the positive control (2.5 µg/mL)	The adsorbance of the assay is below the absorbance measured for the positive control Detection limit of the method is satisfactory

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