

# **FREEZE-DRIED HAEMOPHILUS TYPE b VACCINE (TETANUS TOXOID CONJUGATE)**

## **1. Descriptive definition**

This product is a freeze-dried preparation containing "*Haemophilus influenzae* type b polysaccharides conjugated to tetanus toxoid". The polyribosylribitol phosphate, purified capsular polysaccharides extracted from a *Haemophilus influenzae* type b strain (hereinafter referred to as "*Influenzae* type b strain"), is covalently linked to tetanus toxoid. When reconstituted, it becomes a colorless transparent liquid preparation.

## **2. Production control**

### 2.1 Source materials

#### 2.1.1 Strains for production

*Influenzae* type b strain and *Clostridium tetani* Harvard strain or any other strains with equivalent or higher toxinogenicity shall be used.

#### 2.1.2 Culture medium

No high-molecular-weight polysaccharide shall be added to the medium used for culture of *Influenzae* type b strain. If the medium added with any blood-derived ingredient has been used, such blood-derived ingredient must be removed by a suitable method.

The medium used for production of tetanus toxoid shall be free from substances of horse or human origin, potential human blood-typing antigens, or any other ingredients possibly inducing pronounced allergic reactions in humans.

## 2.2 Bulk material

### 2.2.1 *Influenzae* type b polysaccharides

#### 2.2.1.1 Fermentation

*Influenzae* type b strain is cultured at  $37\pm 2^{\circ}\text{C}$ . At the end of the culture, contamination with any other bacteria must not be detected by microscopy and an appropriate culture method.

#### 2.2.1.2 Bacteriostasis, extraction and purification

Growth of *Influenzae* type b strain shall be stopped by adding formalin to the culture medium. Capsular polysaccharides shall be precipitated by adding cetrимide to the culture medium supernatant obtained by centrifugation and *Influenzae* type b polysaccharides (hereinafter referred to as "PRP") are obtained by treating with phenol and ethanol for purification followed by freeze-drying.

#### 2.2.1.3 *Influenzae* type b polysaccharide AH (adipic hydrazide) derivative

*Influenzae* type b polysaccharide AH derivative (hereinafter referred to as "PRP-AH") shall be obtained by adding cyanogen bromide to PRP for activation followed by reaction with adipic hydrazide and concentration by ultrafiltration, etc. The PRP-AH shall be subjected to the test specified in 3.1.

### 2.2.2 Tetanus toxoid

#### 2.2.2.1 Fermentation

*Clostridium tetani* Harvard strain is cultured at  $35\pm 1^{\circ}\text{C}$ . At the end of the culture, contamination with any other bacteria must not be detected by microscopy and an appropriate culture method.

#### 2.2.2.2 Toxoidation and purification

Formalin shall be used for toxoidation. Purification shall be performed either before or after

toxoidation. Tetanus toxoid after purification shall be concentrated by ultrafiltration, etc.. This solution containing purified toxoid shall be used as the concentrated tetanus toxoid solution. The concentrated tetanus toxoid solution shall be subjected to the test specified in 3.2.

#### 2.2.3 *Influenzae* type b polysaccharides conjugated with tetanus toxoid

PRP-AH shall be conjugated with concentrated tetanus toxoid in the presence of ethyl dimethylaminopropyl carbodiimide (hereinafter referred to as “EDAC”), followed by sucrose density-gradient centrifugation for purification. The purified fraction diluted with a buffer shall be used as the stock solution of *Influenzae* type b conjugated with tetanus toxoid (hereinafter referred to as “PRP-T”).

The stock solution shall be subjected to the test specified in 3.3.

#### 2.3 Final bulk and freeze-drying

The final bulk shall be obtained by diluting, if necessary, the stock solution and can be added some suitable excipient(s). The final bulk shall be diluted to conform to the test specified in 3.4.6.

The final bulk shall be dispensed and freeze-dried.

### **3. Control tests**

#### 3.1 Tests on PRP-AH solution

##### 3.1.1 Test for Cyanide content

Determine the cyanide content in the PRP-AH solution as directed under the Gas Chromatography in the General Tests, Processes and Apparatuses of Japanese Pharmacopoeia: it must be not more than 5 µg/ml.

#### 3.2 Tests of concentrated tetanus toxoid solution

##### 3.2.1 Detoxication test

Dilute the specimen to 2 aliquots with a concentration of 15 Lf/mL which is estimated to be equivalent to the final bulk concentration. Store one aliquot at  $37\pm 1^{\circ}\text{C}$  and the other at  $5\pm 3^{\circ}\text{C}$  for 42 days. Perform the following test using these aliquots as the samples. Use healthy five (5) guinea pigs weighing from 250 to 350g. Inject 5mL each of the sample per guinea pig subcutaneously, and observe the animals for 21 days. In a case where at least 80 % of the animals survive and none of the animals show any toxic symptoms attributable to the tetanus toxin, “Conformity” shall be judged. In a case where at least one animal shows any toxic symptoms attributable to the tetanus toxin or dies from toxicity, “Non-conformity” shall be judged. In a case where at least one animal dies from an unidentified cause, retest shall be performed and “Non-conformity” shall be judged when at least one animal dies.

#### 3.2.2 Specific toxicity test

Dilute the specimen to 800 Lf/mL. Use healthy five (5) guinea pigs weighing from 250 to 350g. Inject 2.5 mL each of the sample per guinea pig subcutaneously, and observe the animals for 21 days. In a case where at least 80 % of the animals survive and none of the animals show any toxic symptoms attributable to the tetanus toxin, “Conformity” shall be judged. In a case where at least one animal shows any toxic symptoms attributable to the tetanus toxin or dies from toxicity, “Non-conformity” shall be judged. In a case where at least one animal dies from an unidentified cause, retest shall be performed and “Non-conformity” shall be judged when at least one animal dies.

#### 3.2.3 Purity test

After precipitation with 40 w/v% trichloroacetic acid, determine the protein nitrogen as directed under the Nitrogen Determination (semi-micro Kjeldahl method) in the General Tests, Processes and Apparatuses of Japanese Pharmacopoeia or by an equivalent methodology and determine the

Lf by test tube precipitation based on the antibody variation method using the tetanus antitoxin titrated with the WHO International Tetanus Standard (for flocculation): not less than 1500Lf/mg of toxoid must be contained in each mg of protein nitrogen.

### 3.3 Tests on bulk material

#### 3.3.1 Polysaccharide/protein ratio test

Calculate the polysaccharide content from the phosphorus content determined by measuring the absorbances of the solutions obtained after mineralization of the stock solution and the standard phosphate solution and coloration by a suitable method, at a wavelength of 825 nm in comparison with the control solution. Determine the protein content as directed under the Protein Determination in the General Tests or by an equivalent method. Calculate the ratio of the polysaccharide content to the protein content: it must be between 0.30 and 0.55.

#### 3.3.2 Test for EDAC (ethyl dimethylaminopropyl carbodiimide) content

Calculate the EDAC content from the absorbance at 599 nm measured after adding the dimethylbarbiturate reagent and acetate-pyridine reagent to the stock solution: it must be less than 10  $\mu\text{mol/L}$ .

#### 3.3.3 Test for phenol content

Mix exactly equal amounts of the specimen, an alkaline buffer (pH 9.0), 4-aminoantipyrine and potassium ferricyanate, and allow the mixture to stand for 10 minutes under protection from light and use this solution as the sample solution. Separately, proceed similarly with the 1, 2, 3, 4 and 6  $\mu\text{g/mL}$  standard phenol solutions and use the resulting solutions as the standard solutions. Separately, proceed similarly with water and use the resulting solution as the control solution. Determine the absorbances of the sample solution and the standard solutions in comparison with the control solution at a wavelength of 546 nm and calculate the phenol content in the sample

solution: it must be less than 1 µg/mL.

#### 3.3.4 Test for molecular-size distribution

Fractionate the stock solution by size exclusion chromatography using a sepharose CL-4B column and a 0.2 mol/L sodium chloride solution, take a fraction eluted before a  $K_D$  value of 0.20 and obtain the polysaccharide content by determining the phosphorus content according to the test in 3.4.6. Calculate the rate of the polysaccharide content in the fraction eluted before a  $K_D$  value of 0.20 to the polysaccharide content in the entire fraction: it must be not less than 60%.

#### 3.3.5 Test for free tetanus toxoid content

Determine the free tetanus toxoid content according to the SDS polyacrylamide gel electrophoresis method: it must be less than 1%.

#### 3.3.6 Test for free polysaccharide content

Subject the supernatant obtained by ultracentrifugation to appropriate hydrolysis and determine the free polysaccharide content as directed under the Liquid Chromatography in the General Tests, Processes and Apparatuses of Japanese Pharmacopoeia: the rate of free polysaccharide content to the total polysaccharide content determined after similar appropriate hydrolysis must be less than 20%.

#### 3.3.7 Sterility test

Perform the test as directed under the Sterility Test in the General Tests, the requirements must be met.

### 3.4 Tests on final product

#### 3.4.1 Test for moisture content

Perform the test as directed under the Water Determination in the General Tests, the water content must be not more than 3.0 %.

#### 3.4.2 Test for pH

Perform the test as directed under the pH Determination in the General Tests, the pH must be between 6.5 and 7.5.

#### 3.4.3 Sterility test

Perform the test as directed under the Sterility Test in the General Tests, the requirements must be met.

#### 3.4.4 Test for freedom from abnormal toxicity

Perform the test as directed under the test for freedom from abnormal toxicity in the General Tests, the requirements must be met.

#### 3.4.5 Bacterial endotoxins test

Perform the test as directed under the Bacterial Endotoxins Test in the General Tests, the endotoxin level must be less than 100EU/container.

#### 3.4.6 Polysaccharide content test

Calculate the polysaccharide content from the phosphorus content determined by measuring the absorbances of the solutions obtained after mineralization of the specimen and the standard phosphate solution and coloration by a suitable method, at a wavelength of 825 nm in comparison with the control solution: it must be between 80 and 120 % of the labeled content.

#### 3.4.7 Identity test

*Influenzae* type b polysaccharides and tetanus toxoid shall be identified using the double diffusion method (Ouchterlony technique) or any other suitable method.

### **4. Storage and expiry date**

Storage: The temperature for storage shall be 2-8°C.

The expiry date shall be 3 years after the manufacturing date.

## 5. Other requirements

### 5.1 Reconstituent solvent

The reconstituent solvent shall be a 0.4 % sodium chloride solution.

The following one Item shall be added next to the Item of “Acetone (special grade)” in the Paragraph of “Reagent and Test Solution” in Part C of the General Tests.

#### 4-Aminoantipyrine solution

Dissolve 1 g of 4-aminoantipyrine in a pH 9.0 alkaline buffer to make 1000 mL. Store this solution at  $5 \pm 3^{\circ}\text{C}$ .

The following one Item shall be added next to the Item of “1-Amino-2-naphtol-4-sulfonic acid” in the Paragraph of “Reagent and Test Solution” in Part C of the General Tests.

#### Alkaline buffer, pH 9.0

Dissolve 6.18 g of boric acid and 7.46 g of potassium chloride in water to make 1000 mL. Mix 1000 mL of this solution with 420 mL of a solution of sodium hydroxide (1 in 250). Store this solution at  $5 \pm 3^{\circ}\text{C}$ .

The following two Items shall be added next to the Item of “Phenol (special grade)” in the Paragraph of “Reagent and Test Solution” in Part C of the General Tests.

1, 2, 3, 4 and 6  $\mu\text{g}/\text{mL}$  standard phenol solutions

Dilute the 1g/L standard phenol stock solution hundredfold with water. Pipette 0.5, 1, 1.5, 2, and 3

mL of this solution, and add exactly 4.5, 4, 3.5, 3, and 2 mL of water, respectively, to prepare the 1, 2, 3, 4 and 6 µg/mL standard phenol solutions, respectively. Prepare just before use.

1 g/L standard phenol stock solution

It contains 1 g of phenol per 1000 mL.

Preparation: Weigh 1.11 g of 90% phenol solution, add 0.1 mol/L hydrochloric acid to make 1000 mL, and perform the following standardization.

Standardization: Determination by iodometry

Note: After standardization, subdivide this solution, and store at  $5\pm 3^{\circ}\text{C}$ .

The following one Item shall be added next to the Item of “Sucrose test solution for 50 % fractionation” in Paragraph “Reagent and Test Solution” in Part C of the General Tests.

Potassium hexacyanoferrate (III) solution

Dissolve 50 g of potassium hexacyanoferrate (III) in water to make 1000 mL. Store this solution at  $5\pm 3^{\circ}\text{C}$ .