2.2.1.1.1 Specificity

The experimental design is:

- One test is performed to verify the presence of reaction between the anti-PTxd antibody and a sample of Hexaxim supernatant (production batch);
- One other test is performed to verify the absence of reaction between the anti-PTxd antibody and a sample of Hexaxim matrix supernatant without PTxd (batch BBO-09-083[a]).

2.2.1.1.1.1 Analytical Results

Analytical results are provided in Table 24.

Table 24: Absorbance of the Hexaxim Matrix Supernatants without PTxd Antigen versus Absorbance of the Hexaxim Matrix Supernatant

Absorbance established as the detection threshold (previously determined with 30 blanks independent values)	Absorbance measured for the Hexaxim matrix supernatant without PTxd	Absorbance measured for the Hexaxim supernatant (production batch)
0.109	0.087 0.098	1.440 1.493

2.2.1.1.1.2 Analysis

The PTxd antigen is detected in presence of PTxd antigen in Hexaxim sample.

The PTxd antigen is not detected in absence of PTxd antigen in Hexaxim sample.

2.2.1.1.2 Limit of Detection

The experimental design is: 3 tests are performed to verify that the absorbance measured for a sample containing a concentration of 0.023 µg/mL PTxd in Hexaxim supernatant is detectable and inferior to the positive control of the test (2.5 µg/mL). The limit of detection has been established based on previous experiments and requirements for this test.

2.2.1.1.2.1 Analytical Results

Analytical results are provided in Table 25.

[a] Hexaxim matrix without PTxd valence

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Table 25: Absorbance of the Hexaim Matrix Supernatant without PTxd Antigen Spiked with 0.023 µg/mL of PTxd versus Absorbance at the Detection Threshold and Absorbance of the PTxd Positive Control

Absorbance established as the detection threshold (previously determined with 30 blanks independent values)	Absorbance measured for the Hexaxim matrix supernatant spiked with 0.023 µg/mL of PTxd	Absorbance measured for the PTxd positive control at 2.5 µg/mL
0.109	0.414 0.418	2.430
	0.522 0.562	2.450
	0.409 0.455	2.512

2.2.1.1.2.2 Analysis

Detection limit for the PTxd limit assay is verified to be 0.023 µg/mL.

2.2.1.1.3 Conclusion

The method is specific.

The limit of detection is verified to be 0.023 µg/mL and meets the needs of the assay.

The method is valid to detect PTxd in the supernatant of Hexaxim vaccine.

2.2.1.2 Validation of the Limit Assay for Measuring Non-Adsorbed FHA in the Hexaxim Supernatant

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

The results of the validation are summarized in the Table 26:

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Table 26: Titration of FHA by ELISA in the Hexaxim Supernatant - Validation Summary

Characteristics	Acceptance criteria	Results
Specificity	FHA antigen is detected in the presence of FHA in a sample of Hexaxim supernatant	FHA antigen is detected when FHA is present in the sample of Hexaxim supernatant
	FHA antigen is not detected in absence of FHA in a sample of Hexaxim matrix supernatant (containing the other antigens)	FHA antigen is not detected when FHA is not present in the sample of Hexaxim matrix supernatant
Limit of Detection	Detection limit will be established at 0.043 µg/mL if the absorbance of the assay (Hexaxim matrix supernatant spiked with 0.043 µg/mL of FHA antigen) is above the absorbance determined as the detection threshold	The absorbance of the assay is above the absorbance determined the detection threshold Detection limit = 0.043 µg/mL
	Detection limit will be satisfactory if the absorbance of the assay (Hexaxim matrix supernatant spiked with 0.043 µg/mL of FHA antigen) is below the absorbance measured for the positive control (2.5 µg/mL)	The absorbance of the assay is below the absorbance measured for the positive control Detection limit of the method is satisfactory

The method is relevant for measuring non-absorbed FHA in Hexaxim supernatant.

2.2.1.3 Specificity

The experimental design is:

- One test is performed to verify the presence of reaction between the anti-FHA antibody and a sample of Hexaxim supernatant (production batch)
- One other test is performed to verify the absence of reaction between the anti-FHA antibody and a sample of Hexaxim matrix supernatant without FHA (matrix containing all other antigens without FHA).

2.2.1.3.1 Analytical Results

Analytical results are provided in Table 27.

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Table 27: Absorbance of the Hexaxim Matrix Supernatants without FHA Antigen versus Absorbance of the Hexaxim Matrix Supernatant

Absorbance established as the detection threshold (previously determined with 30 blanks independent values)	Absorbance measured for the Hexaxim matrix supernatant without FHA	Absorbance measured for the Hexaxim supernatant (production batch)
0.116	0.092	0.331

2.2.1.3.2 Analysis

The FHA antigen is detected in presence FHA in Hexaxim sample.

The FHA antigen is not detected in absence of FHA in Hexaxim sample.

2.2.1.4 Limit of Detection

The experimental design is: 3 tests are performed to verify that the absorbance measured for a sample containing a concentration of 0.043 µg/mL FHA in Hexaxim supernatant is detectable and inferior to the positive control of the test (2.5 µg/mL). The limit of detection has been established based on previous experiments and requirements for this test.

2.2.1.4.1 Analytical Results

Analytical results are provided in Table 28.

Table 28: Absorbance of the Hexaxim Matrix Supernatant without FHA Antigen Spiked with 0.043 µg/mL of FHA versus Absorbance at the Detection Threshold and Absorbance of the FHA Positive Control

Absorbance established as the detection threshold (previously determined with 30 blanks independent values)	Absorbance measured for the Hexaxim matrix supernatant spiked with 0.043 µg/mL FHA	Absorbance measured for the FHA positive control at 2.5 µg/mL
0.116	0.136	2.233
	0.132	2.267
	0.134	2.367

2.2.1.4.2 Analysis

Detection limit for the FHA limit assay is verified to be 0.043 µg/mL.

2.2.1.5 Conclusion

The method is specific.

The limit of detection is verified to be 0.043 µg/mL and meets the needs of the assay.

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The method is valid to detect FHA in the supernatant of Hexaxim vaccine.

2.2.2 Percent Adsorption – Tetanus Toxoid (Rocket)

This section describes the validation of non-adsorbed purified tetanus toxoid quantification by immunoelectrophoresis method in the Hexaxim vaccine, according to the ICH guideline.

Since the method is a quantitative assay, the studied characteristics are specificity, linearity, accuracy and precision.

The results of the validation are summarized in the following Table 29:

Table 29: Percent Adsorption – Tetanus Toxoid (Rocket) – Validation - Summary

Characteristics	Acceptance criteria	Results
Specificity	Formulation without purified tetanus toxoid: Absence of significant response. Hexaxim vaccine adjusted with 5% of supernatant: The average recovery between the measured quantity and the mean of precision results should be included between 80 and 120%.	Formulation without purified Tetanus Toxoid: Absence of significant response. Hexaxim vaccine adjusted with 5% of supernatants matrix: The average recovery between the measured quantity and the mean of precision results is equal to 93%.
Linearity	$P_{linearity}$ ≤ 0.01 $P_{Lack\ of\ Fit}$ > 0.05	$P_{linearity} < 0.0001$ $P_{LOF} = 0.08$ $Y = 0.061 + 0.951.X$ Where X = theoretical concentration of purified tetanus toxoid (log Lf/mL) and Y = measured concentration of purified tetanus toxoid (log Lf/mL). $R^2 = 0.9941$ Linearity range: **[2.3 - 23.8] Lf/mL** equivalent to **[89 - 0]%** of adsorption
Accuracy	The average percent recovery calculated for the 5 theoretical concentration levels should be included between 80 and 120%	According to the concentration level, the mean percent recovery is from 99 to 115%
Precision (Lf/mL)	95% confidence interval of repeatability should be lower or equal than x/÷ 1.4 95% confidence interval of intermediate precision should be lower or equal than x/÷ 1.4	Overall mean: $\bar{m} = 1.11$ that is **12.9 Lf/mL** in arithmetic form. Relative standard deviation of repeatability and intermediate precision are respectively: 3.6% **and** 4.5% 95% confidence interval of repeatability for **1 measurement**: ± 0.033 that is **x/÷ 1.08** in arithmetic form 95% confidence interval of intermediate precision for **1 run with 1 measurement**: ± 0.041 that is **x/÷ 1.10** in arithmetic form

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Characteristics	Acceptance criteria	Results
Precision (% of adsorption)	For information	Overall mean: $\overline{m}$ = 35 % Relative standard deviation of repeatability and intermediate precision are respectively: 6.7% and 8.3% 95% confidence interval of repeatability for **1 measurement:** ± 5% 95% confidence interval of intermediate precision for **1 run with 1 measurement:** ± 6 %

The method is specific, linear and accurate on the range [2.3 - 23.8] Lf/mL, and precise.

2.2.2.1 Results

2.2.2.1.1 Specificity

The specificity consists in following steps:

- For the formulation without purified tetanus toxoid: the absence of significant response should be demonstrated;
- For the vaccine adjusted with 5% of supernatants matrix without purified tetanus toxoid: the average recovery between the measured quantity and the mean of precision results should be included between 80% and 120%.

The experimental design was: 1 assay was performed (batch No. IND09014 of Hexaxim Final Bulk Product, batch No. FA269112 of Hexaxim supernatant and batch No. BBO09-078 of matrix without purified tetanus toxoid).

Batches used for the study is representative of the production.

Table 30: Specificity: Results

Matrix without purified tetanus toxoid (Height Rocket)	Batch of Hexaxim + 5% supernatant (Lf/mL)
0	10.68

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2.2.2.1.1.1 Analysis

- For the formulation without purified tetanus toxoid: there is an absence of significant response;
- For the Hexaxim vaccine adjusted with 5% of matrix supernatant: the average recovery between the measured quantity (10.68 Lf/mL) and the mean of precision results is equal to 93%.

There is no interference of the matrix. So, the method is specific.

2.2.2.1.2 Linearity

The experimental design was: 3 separated series were performed by 2 operators, on different days. Each run included the assay of a range of 5 concentrations of purified tetanus toxoid in Hexaxim vaccine (5 spikes of purified tetanus toxoid – Batch No. FA269112 in supernatants of Hexaxim matrix without purified tetanus toxoid – Batch No. BBO09-078).

Batches used for the study are representatives of the production.

2.2.2.1.2.1 Analytical Results

The data subjected to analysis is the concentration in purified tetanus toxoid in Hexaxim vaccine, expressed in Lf/mL.

Table 31 summarizes the results of the study.

Table 31: Linearity: Measured Concentrations versus Theoretical Concentrations (Lf/mL)

Level	Theoretical concentration (Lf/mL)	Measured concentration (Lf/mL)		
		Serie 1	Serie 2	Serie 3
+5	20.00	21.20	19.21	20.78
+4	15.00	15.60	15.41	15.37
+3	10.00	9.78	10.15	9.71
+2	5.00	5.07	4.93	5.18
+1	2.50	3.18	2.67	2.80

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2.2.2.1.2.2 Analysis

The linearity over the chosen range is tested through the following steps, applied to the data in Table 31:

- Homogeneity of bound variances is verified by Cochran's test;
- The dependance between the theoretical concentration of purified tetanus toxoid and measured concentration of purified tetanus toxoid and the linearity of this relation, are tested by an unweighted linear regression using least squares method. A significant slope and a non-significant deviation from linearity must be shown.

→ Cochran's test shows that the variances of the 3 series are homogeneous.

→ There is a linear dependence between the theoretical concentration and the measured concentration.

Table 32: Linearity - Equation of the Straight Regression Line

Equation of the straight regression line	Coefficient of determination	Linearity range in Lf/mL
$Y = (0.061 \pm 0.043) + (0.951 \pm 0.044).X$	$r^2 = 0.9941$	2.30 - 23.76]
with: X = Theoretical concentration of non adsorbed purified tetanus toxoid (log(Lf/mL)) Y = Measured concentration of non adsorbed purified tetanus toxoid (log(Lf/mL))		
$P_{linearity}$ < 0.0001 P_{LOF} = 0.08		

The method is linear over the range [2.30 - 23.76] Lf/mL. All acceptance criteria are satisfied, so the method is linear.

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