Tests	Acceptance criteria	T0*	1 month	3 months	6 months	9 months	12 months	18 months	24 months	30 months	36 months
Non-adsorbed D-antigen content	For information										
Type 1		27.9	NP	27.0	26.3	NP	25.6	25.3	On-going	On-going	On-going
Type 2		6.1	NP	5.9	5.8	NP	5.2	4.6	On-going	On-going	On-going
Type 3		27.5	NP	25.9	25.3	NP	26.3	25.3	On-going	On-going	On-going
Percent adsorption – hepatitis B (ELISA)	For information (%)	88	86	80	84	86	83	78	On-going	On-going	On-going
Hepatitis B *in-vitro* relative potency (IVRP)	For information (relative potency)	1.11	NP	1.26	1.30	1.36	1.38	1.17	On-going	On-going	On-going
Hepatitis B immunogenicity	Upper confidence limit (P = 0.95) of the estimated relative potency is not less than 1.0	1.16	NP	NP	1.59	NP	1.45	1.19	On-going	On-going	On-going
Lower limit		0.568	NP	NP	0.965	NP	0.821	0.771	On-going	On-going	On-going
Upper limit		2.041	NP	NP	2.855	NP	2.607	1.83	On-going	On-going	On-going
Bacterial and fungal sterility test	No microbial growth	Conforms	NP	NP	NP	NP	NP	NP	NP	NP	On-going
Pyrogen test	Conforms to Ph. Eur. criterion	0.63 (0.14 - 0.40 - 0.09)	NP	NP	NP	NP	NP	NP	NP	NP	On-going
Specific toxicity for diphtheria and tetanus components	There must be no toxic reactions or deaths. All animals must maintain a healthy appearance during the period of observation and weigh no less at the end of the test than at the time of injection	Conforms	NP	NP	NP	NP	NP	NP	NP	NP	On-going

Tests	Acceptance criteria	T0*	1 month	3 months	6 months	9 months	12 months	18 months	24 months	30 months	36 months
Integrity test	The CCIT‡‡ is acceptable if no presence of dye is detected in the contents of any of the tested single-dose vials	Conforms	NP	NP	NP	NP	Conforms	NP	On-going	NP	On-going

* All the results are obtained from the T0 of the Filled Product excepted for the following tests: free formaldehyde content, osmolality measurement, diphtheria and tetanus potency, histamine-sensitizing activity, pertussis immunogenicity anti-FHA and anti-PT, haemophilus immunogenicity, non-adsorbed PT and FHA, rat immunogenicity assay for IPV, hepatitis B immunogenicity and specific toxicity for diphtheria and tetanus components. For these tests, the results are release results of the Final Bulk Product.

† Not Planned as per protocol

‡ Test invalid and not retested because of the proximity of the next time-point (T24 months)

§ The reference vaccine for pertussis immunogenicity test was changed at T18 months. The qualification of this reference standard was performed by a statistical comparison to the previous one

** Expected results: Not less than 50% of the vaccinated mice are seroconverted. Their titer is not less than 4 times that of the pooled control serum

†† Expected value: ≤ 2.5 µg/mL

‡‡ CCIT: Container Closure Integrity Test

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Table 18: Stability Study Results for Val de Reuil Filled Product Batch S4314 at +5°C ± 3°C

Tests	Acceptance criteria	T0*	1 month	3 months	6 months	9 months	12 months	18 months	24 months	30 months	36 months
Appearence	Whitish and cloudy suspension	Conforms	NP†	Conforms	Conforms	Conforms	Conforms	Conforms	On-going	On-going	On-going
pH measurement	6.5 – 7.5	7.20	NP	7.22	7.22	7.27	7.25	7.27	On-going	On-going	On-going
Free formaldehyde content	≤ 30 µg/mL	1.36	NP	NP	NP	NP	< 0.36	NP	On-going	NP	On-going
Extractable volume	At least the nominal volume	Conforms	NP	NP	NP	NP	NP	NP	NP	NP	On-going
Aluminium content	0.40 – 0.80 mg/dose	0.62	NP	NP	NP	NP	NP	NP	NP	NP	On-going
Osmolality measurement	300 - 400 mosmol/kg	336	NP	NP	NP	NP	NP	NP	NP	NP	On-going
Non-adsorbed PRP	≥ 16 µg/mL	19.8	18.6	19.5	21.8	20.8	20.8	19.9	On-going	On-going	On-going
Depolymerized PRP	For information (%)	12.3	16.7	20.3	23.7	26.1	29.9	36.1	On-going	On-going	On-going
Diphtheria potency	Activity ≥ 30 IU/mL										
Activity	Lower confidence limit (*P = 0.95*) of the estimated potency ≥ 20 IU/mL	76	NP	NP	74	NP	49	54	On-going	On-going	On-going
Lower limit		57	NP	NP	55	NP	27	40	On-going	On-going	On-going
Upper limit		113	NP	NP	102	NP	86	73	On-going	On-going	On-going
Tetanus potency	Lower confidence limit (*P = 0.95*) of the estimated potency ≥ 40 IU/mL										
Activity		705	NP	NP	819	NP	685	394	On-going	On-going	On-going
Lower limit		485	NP	NP	584	NP	499	197	On-going	On-going	On-going
Upper limit		1017	NP	NP	1166	NP	933	620	On-going	On-going	On-going
Histamine-sensitizing activity	≥ 95% survival (%)	100	NP	NP	NP	NP	100	NP	On-going	On-going	On-going

Tests	Acceptance criteria	T0*	1 month	3 months	6 months	9 months	12 months	18 months	24 months	30 months	36 months
Pertussis immunogenicity anti-FHA	Anti-Filamentous Hemagglutinin (FHA) antibody titer obtained for the vaccine is not significantly *(P = 0.95)* less than that of the reference vaccine	Conforms	NP	NP	Conforms	NP	Conforms	Conforms‡	On-going	On-going	On-going
Pertussis immunogenicity anti-PT	Anti-Pertussis Toxoid (PTxd) and antibody titer obtained for the vaccine is not significantly *(P = 0.95)* less than that of the reference vaccine	Conforms	NP	NP	Conforms	NP	Conforms	Conforms‡	On-going	On-going	On-going
Haemophilus immunogenicity	For information§	Conforms	NP	NP	Conforms	NP	Conforms	Conforms	On-going	On-going	On-going
Non-adsorbed PT	For information (µg/mL)**	< 2.5	NP	NP	< 2.5	NP	< 2.5	NP	On-going	NP	On-going
Non-adsorbed FHA	For information (µg/mL)**	< 2.5	NP	NP	< 2.5 at 10 months	NP	< 2.5	NP	On-going	NP	On-going
Percent adsorption – tetanus toxoid	For information (%)	18	NP	39	25	NP	34	35††	On-going	On-going	On-going
Percent adsorption – diphtheria toxoid	For information (%)	52	NP	59	56	NP	59	63	On-going	On-going	On-going
Rat Immunogenicity Assay for IPV	For information (relative potency)										
Type 1		0.6	NP	NP	NP	NP	0.9	NP	On-going	NP	On-going
Type 2		1.2	NP	NP	NP	NP	1.0	NP	On-going	NP	On-going
Type 3		3.1	NP	NP	NP	NP	0.6	NP	On-going	NP	On-going

Tests	Acceptance criteria	T0*	1 month	3 months	6 months	9 months	12 months	18 months	24 months	30 months	36 months
D-antigen content	Type 1: 20 – 43 DU/dose Type 2: 5 – 9 DU/dose Type 3: 17 – 36 DU/dose	26.5 6.8 24.0	NP NP NP	24.9 6.5 22.3	28.0 7.3 26.1	NP NP NP	26.4 7.2 24.6	26.4 6.6 21.9	On-going	On-going	On-going
Non-adsorbed D-antigen content	For information										
Type 1		27.8	NP	26.6	28.7	NP	26.1	24.4	On-going	On-going	On-going
Type 2		5.9	NP	5.7	5.6	NP	5.2	4.2	On-going	On-going	On-going
Type 3		24.8	NP	23.9	26.4	NP	24.2	21.3	On-going	On-going	On-going
Percent adsorption – hepatitis B (ELISA)	For information (%)	90	88	86	87	84	85	87	On-going	On-going	On-going
Hepatitis B *in-vitro* relative potency (IVRP)	For information (relative potency)	1.45	NP	1.46	1.27	1.44	1.43	1.25	On-going	On-going	On-going
Hepatitis B immunogenicity	Upper confidence limit (P = 0.95) of the estimated relative potency is not less than 1.0	1.17	NP	NP	1.14	NP	1.28	1.02	On-going	On-going	On-going
Lower limit		0.644	NP	NP	0.631	NP	0.712	0.577	On-going	On-going	On-going
Upper limit		2.268	NP	NP	2.176	NP	2.356	1.954	On-going	On-going	On-going
Bacterial and fungal sterility test	No microbial growth	Conforms	NP	NP	NP	NP	NP	NP	NP	NP	On-going
Pyrogen test	Conforms to Ph. Eur. criterion	Conforms	NP	NP	NP	NP	NP	NP	NP	NP	On-going
Specific toxicity for diphtheria and tetanus components	There must be no toxic reactions or deaths. All animals must maintain a healthy appearance during the period of observation and weigh no less at the end of the test than at the time of injection	Conforms	NP	NP	NP	NP	NP	NP	NP	NP	On-going

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Tests	Acceptance criteria	T0*	1 month	3 months	6 months	9 months	12 months	18 months	24 months	30 months	36 months
Integrity test	The CCIT‡‡ is acceptable if no presence of dye is detected in the contents of any of the tested single-dose vials	Conforms	NP	NP	NP	NP	Conforms	NP	On-going	NP	On-going

* All the results are obtained from the T0 of the Filled Product excepted for the following tests: free formaldehyde content, osmolality measurement, diphtheria and tetanus potency, histamine sensitizing activity, pertussis immunogenicity anti-FHA and anti-PT, haemophilus immunogenicity, non-adsorbed PT and FHA, rat immunogenicity assay for IPV, hepatitis B immunogenicity and specific toxicity for diphtheria and tetanus components. For these tests, the results are release results of the Final Bulk Product.

† Not Planned as per protocol

‡ The reference vaccine for pertussis immunogenicity test was changed at T18 months. The qualification of this reference standard was performed by a statistical comparison to the previous one

§ Expected results: Not less than 50% of the vaccinated mice are seroconverted. Their titer is not less than 4 times that of the pooled control serum

** Expected value: ≤ 2.5 µg/mL

†† Retest (average value of three complementary tests: 38% - 36% - 31%) as the first result was atypical (5%)

‡‡ CCIT: Container Closure Integrity Test

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1.7 Study 5: Val de Reuil Filled Product – Optimized Formulation – Stability Data on Accelerated Storage Conditions at +25°C ± 2°C

6 months stability data with the optimized formulation on 3 batches of VDR FP are presented in Table 19 to Table 21.

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Table 19: Stability Study Results for Val de Reuil Filled Product Batch S4312 at +25°C ± 2°C

Tests	Acceptance criteria	T0*	1 month	3 months	6 months
Appearence	Whitish and cloudy suspension	Conforms	Whitish cloudy suspension with white particles	Conforms	Conforms
pH measurement	6.5 – 7.5	7.29	7.33	7.34	7.36
Non-adsorbed PRP	≥ 16 µg/mL	21.1	25.2	28.1	30.5
Depolymerized PRP	For information (%)	10.9	33.9	64.8	77.3
Diphtheria potency	Activity ≥ 30 IU/mL				
Activity	Lower confidence limit (*P = 0.95*) of the estimated potency ≥ 20 IU/mL	42	NP†	46	46
Lower limit		34	NP	36	28
Upper limit		52	NP	59	75
Tetanus potency	Lower confidence limit (*P = 0.95*) of the estimated potency ≥ 40 IU/mL				
Activity		556	NP	283	245
Lower limit		280	NP	196	186
Upper limit		853	NP	393	335
Pertussis immunogenicity anti-FHA	Anti-Filamentous Hemagglutinin (FHA) antibody titer obtained for the vaccine is not significantly *(P = 0.95)* less than that of the reference vaccine	Conforms	NP	Conforms	Conforms
Pertussis immunogenicity anti-PT	Anti-Pertussis Toxoid (PTxd) and antibody titer obtained for the vaccine is not significantly *(P = 0.95)* less than that of the reference vaccine	Conforms	NP	Conforms	Conforms
Haemophilus immunogenicity	For information‡	Conforms	NP	Conforms	Conforms
Non-adsorbed PT	For information (µg/mL)§	< 2.5	NP	< 2.5	< 2.5

Tests	Acceptance criteria	T0*	1 month	3 months	6 months
Non-adsorbed FHA	For information (µg/mL)§	< 2.5	NP	< 2.5 at 4 months	< 2.5
Percent adsorption – tetanus toxoid	For information (%)	25	53	44	44
Percent adsorption – diphtheria toxoid	For information (%)	54	61	73	75
D-antigen content	Type 1: 20 – 43 DU/dose Type 2: 5 – 9 DU/dose Type 3: 17 – 36 DU/dose	26.9 5.5 26.3	26.4 6.2 25.1	24.8 6.3 22.7 at 4 months	23.7 5.8 24.6
Percent adsorption – hepatitis B (ELISA)	For information (%)	88	67	61	60
Hepatitis B *in-vitro* relative potency (IVRP)	For information (relative potency)	1.32	1.35	1.20	0.83
Hepatitis B immunogenicity	Upper confidence limit (P = 0.95) of the estimated relative potency is not less than 1.0	1.21	NP	1.00	1.21
Lower limit		0.628	NP	0.580	0.722
Upper limit		2.561	NP	1.738	1.991
Bacterial and fungal sterility test	No microbial growth	Conforms	NP	NP	Conforms
Integrity test	The CCIT is acceptable if no presence of dye is detected in the contents of any of the tested single-dose vials	Conforms	NP	NP	Conforms

* All the results are obtained from the T0 of the Filled Product excepted for the following tests: diphtheria and tetanus potency, pertussis immunogenicity anti-FHA and anti-PT, haemophilus immunogenicity, non-adsorbed PT and FHA and hepatitis B immunogenicity. For these tests, the results are release results of the Final Bulk Product.

Not Planned as per protocol

Expected results: Not less than 50% of the vaccinated mice are seroconverted. Their titer is not less than 4 times that of the pooled control serum

Expected value: ≤ 2.5 µg/mL

Table 20: Stability Study Results for Val de Reuil Filled Product Batch S4313 at +25°C ± 2°C

Tests	Acceptance criteria	T0*	1 month	3 months	6 months
Appearence	Whitish and cloudy suspension	Conforms	Whitish cloudy suspension with white particles	Conforms	Conforms
pH measurement	6.5 – 7.5	7.18	7.22	7.23	7.21
Non-adsorbed PRP	≥ 16 µg/mL	21.1	23.1	27.0	30.2
Depolymerized PRP	For information (%)	13.9	22.0	61.4	76.7
Diphtheria potency	Activity ≥ 30 IU/mL				
Activity	Lower confidence limit (*P = 0.95*) of the estimated potency ≥ 20 IU/mL	57	NP†	84	73
Lower limit		43	NP	59	52
Upper limit		82	NP	128 at 3.5 months	109
Tetanus potency	Lower confidence limit (*P = 0.95*) of the estimated potency ≥ 40 IU/mL				
Activity		584	NP	439	197
Lower limit		413	NP	314	155
Upper limit		795	NP	631	252
Pertussis immunogenicity anti-FHA	Anti-Filamentous Hemagglutinin (FHA) antibody titer obtained for the vaccine is not significantly *(P = 0.95)* less than that of the reference vaccine	Conforms	NP	Conforms	Conforms
Pertussis immunogenicity anti-PT	Anti-Pertussis Toxoid (PTxd) and antibody titer obtained for the vaccine is not significantly *(P = 0.95)* less than that of the reference vaccine	Conforms	NP	Conforms	Conforms
Haemophilus immunogenicity	For information‡	Conforms	NP	Conforms	Fails
Non-adsorbed PT	For information (µg/mL)§	< 2.5	NP	< 2.5	< 2.5

Tests	Acceptance criteria	T0*	1 month	3 months	6 months
Non-adsorbed FHA	For information (µg/mL)§	< 2.5	NP	< 2.5 at 4 months	< 2.5
Percent adsorption – tetanus toxoid	For information (%)	27	50	47	33
Percent adsorption – diphtheria toxoid	For information (%)	55	65	68	77
D-antigen content	Type 1: 20 – 43 DU/dose Type 2: 5 – 9 DU/dose Type 3: 17 – 36 DU/dose	28.8 6.0 25.7	27.6 6.7 26.5	25.1 6.9 26.5 at 4 months	21.4 6.1 25.0
Percent adsorption – hepatitis B (ELISA)	For information (%)	88	74	76	65
Hepatitis B *in-vitro* relative potency (IVRP)	For information (relative potency)	1.11	1.17	1.09	0.77
Hepatitis B immunogenicity	Upper confidence limit (P = 0.95) of the estimated relative potency is not less than 1.0	1.16	NP	0.93	1.25
Lower limit		0.568	NP	0.539	0.765
Upper limit		2.041	NP	1.705	2.034
Bacterial and fungal sterility test	No microbial growth	Conforms	NP	NP	Conforms
Integrity test	The CCIT is acceptable if no presence of dye is detected in the contents of any of the tested single-dose vials	Conforms	NP	NP	Conforms

* All the results are obtained from the T0 of the Filled Product excepted for the following tests: diphtheria and tetanus potency, pertussis immunogenicity anti-FHA and anti-PT, haemophilus immunogenicity, non-adsorbed PT and FHA and hepatitis B immunogenicity. For these tests, the results are release results of the Final Bulk Product.

† Not Planned as per protocol

‡ Expected results: Not less than 50% of the vaccinated mice are seroconverted. Their titer is not less than 4 times that of the pooled control serum

§ Expected value: ≤ 2.5 µg/mL

Table 21: Stability Study Results for Val de Reuil Filled Product Batch S4314 at +25°C ± 2°C

Tests	Acceptance criteria	T0*	1 month	3 months	6 months
Appearence	Whitish and cloudy suspension	Conforms	Whitish cloudy suspension with white particles	Conforms	Conforms
pH measurement	6.5 – 7.5	7.20	7.25	7.28	7.28
Non-adsorbed PRP	≥ 16 µg/mL	19.8	21.9	26.6	29.4
Depolymerized PRP	For information (%)	12.3	27.7	61.3	78.7
Diphtheria potency **Activity** **Lower limit** **Upper limit**	Activity ≥ 30 IU/mL Lower confidence limit (*P = 0.95*) of the estimated potency is ≥ 20 IU/mL	 76 57 113	 NP† NP NP	 76 53 115 at 4 months	 45 30 67
Tetanus potency **Activity** **Lower limit** **Upper limit**	Lower confidence limit (*P = 0.95*) of the estimated potency ≥ 40 IU/mL	 705 485 1017	 NP NP NP	 314 226 432	 273 204 359
Pertussis immunogenicity anti-FHA	Anti-Filamentous Hemagglutinin (FHA) antibody titer obtained for the vaccine is not significantly *(P = 0.95)* less than that of the reference vaccine	Conforms	NP	Invalid test‡	Conforms
Pertussis immunogenicity anti-PT	Anti-Pertussis Toxoid (PTxd) and antibody titer obtained for the vaccine is not significantly *(P = 0.95)* less than that of the reference vaccine	Conforms	NP	Invalid test‡	Conforms
Haemophilus immunogenicity	For information§	Conforms	NP	Conforms	Conforms
Non-adsorbed PT	For information (µg/mL)**	< 2.5	NP	< 2.5	< 2.5

Tests	Acceptance criteria	T0*	1 month	3 months	6 months
Non-adsorbed FHA	For information (µg/mL)**	< 2.5	NP	< 2.5 at 4 months	< 2.5 at 10 months
Percent adsorption – tetanus toxoid	For information (%)	18	35	37	38
Percent adsorption – diphtheria toxoid	For information (%)	52	61	71	73
D-antigen content	Type 1: 20 – 43 DU/dose Type 2: 5 – 9 DU/dose Type 3: 17 – 36 DU/dose	28.1 5.8 24.6	27.0 6.9 21.9 at 2 months	22.6 6.5 22.4 at 4 months	19.4 6.0 19.0
Percent adsorption – hepatitis B (ELISA)	For information (%)	90	75	71	71
Hepatitis B *in-vitro* relative potency (IVRP)	For information (relative potency)	1.45	1.50	1.16	0.96
Hepatitis B immunogenicity	Upper confidence limit (P = 0.95) of the estimated relative potency is not less than 1.0	1.17 0.644 2.268	NP NP NP	1.37 0.820 2.450	0.96 0.554 1.689
Bacterial and fungal sterility test	No microbial growth	Conforms	NP	NP	Conforms
Integrity test	The CCIT is acceptable if no presence of dye is detected in the contents of any of the tested single-dose vials	Conforms	NP	NP	Conforms

* All the results are obtained from the T0 of the Filled Product excepted for the following tests: diphtheria and tetanus potency, pertussis immunogenicity anti-FHA and anti-PT, haemophilus immunogenicity, non-adsorbed PT and FHA and hepatitis B immunogenicity. For these tests, the results are release results of the Final Bulk Product.

† Not Planned as per protocol

‡ No retest proximity of next time point

§ Expected results: Not less than 50% of the vaccinated mice are seroconverted. Their titer is not less than 4 times that of the pooled control serum

** Expected value: ≤ 2.5 µg/mL

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2 Analytical Procedures

The list of all analytical procedures used for the different stability studies performed with the combined diphtheria, tetanus, acellular pertussis, hepatitis B, inactivated poliomyelitis trivalent concentrate and *haemophilus influenzae* type b vaccine Drug Product are provided in Table 22.

Table 22: Test Methods Used for Stability Studies

Test	Reference	Test method		Study number
		Method	Validation	
Appearance	Ph. Eur. 2.9.20, current edition Visual inspection	/	/	1, 2, 3, 4 and 5
pH measurement	Ph. Eur. 2.2.3, current edition Potentiometric method	/	/	1, 2, 3, 4 and 5
Free formaldehyde content	Based on Ph. Eur. 2.4.18, current edition Colorimetric assay	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Exctractable volume	Ph. Eur. 2.9.17, current edition Volume = mass/density	/	/	2, 3, 4 and 5
Aluminium content	Based on Ph. Eur. 2.5.13, current edition Complexometry assay (EDTA)	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 3 and 5
Osmolality measurement	Ph. Eur. 2.2.35, current edition Physico-chemical method	/	/	1, 2, 3, 4 and 5
Non-adsorbed PRP	Ph. Eur. 2.2.29, current edition High Performance Anion Exchange Chromatography - Pulse Amperometric Detection (HPAEC-PAD)	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Depolymerized PRP		Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Diphtheria potency	Ph. Eur. 2.7.6, current edition Injection of the vaccine into animals by intradermal route	/	/	1, 2, 3, 4 and 5

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Test	Reference	Test method		Study number
		Method	Validation	
Tetanus potency	Ph. Eur. 2.7.8, current edition Injection of the vaccine into animals by subcutaneous route	/	/	1, 2, 3, 4 and 5
Histamine-sensitizing activity	Ph. Eur. 1356, current edition Injection of the vaccine into mice by intraperitoneal route followed by the injection of an histamine base solution	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Pertussis immunogenicity	Ph. Eur. 2.7.16, current edition Immunogenicity test in mice (serological assay: ELISA method)	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Haemophilus immunogenicity	Immunogenicity test in mice (serological assay: ELISA method)	Section 3.2.P.8.3	Section 3.2.P.8.3	1, 2, 3, 4 and 5
Non-adsorbed PT	Based on Ph. Eur. 2.7.1, current edition ELISA method	Section 3.2.P.8.3	Section 3.2.P.8.3	1, 2, 3, 4 and 5
Non-adsorbed FHA	Based on Ph. Eur. 2.7.1, current edition ELISA method	Section 3.2.P.8.3	Section 3.2.P.8.3	1, 2, 3, 4 and 5
Percent adsorption – tetanus toxoid	Rocket Immunoelectrophoresis method	Section 3.2.P.8.3	Section 3.2.P.8.3	1, 2, 3, 4 and 5
Percent adsorption – diphtheria toxoid	Rocket Immunoelectrophoresis method	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Rat immunogenicity assay for IPV	Based on Ph. Eur. 2.7.20, current edition In vivo assay on rats	Section 3.2.P.8.3	Section 3.2.P.8.3	1, 3 and 5
Poliomyelitis potency in chicken	Ph. Eur. 2.7.20, current edition In vivo assay on chicken	/	/	2 and 4
D-antigen content	Ph. Eur. 2.7.1, current edition ELISA method	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Non-adsorbed D-antigen content*	Ph. Eur. 2.7.1, current edition ELISA method	Section 3.2.P.5.2	Section 3.2.P.5.3	3 and 5

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Test	Reference	Test method		Study number
		Method	Validation	
Percent adsorption – hepatitis B (ELISA)	Ph. Eur. 2.7.1, current edition ELISA method	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Hepatitis B *in-vitro* relative potency (IVRP)	Ph. Eur. 2.7.15, current edition ELISA method	Section 3.2.P.5.2	Section 3.2.P.5.3	1, 2, 3, 4 and 5
Hepatitis B immunogenicity	Ph. Eur. 2.7.15, current edition ELISA method	/	/	1, 2, 3, 4 and 5
Bacterial and fungal sterility test	Ph. Eur. 2.6.1, current edition Membrane filtration	/	/	1, 2, 3, 4 and 5
Pyrogen test	Ph. Eur. 2.6.8, current edition Measuring rise of body temperature in animals	/	/	2, 3, 4 and 5
Specific toxicity for diphtheria and tetanus components	Based on Ph. Eur. 2067, current edition Specific toxicity	Section 3.2.P.8.3	Section 3.2.P.8.3	3 and 5
Integrity test	Container closure integrity test with riboflavin 0,1% w/v	Section 3.2.P.8.3	Section 3.2.P.8.3	3 and 5

* The only difference between the D-antigen content test and the non-absorbed D-antigen content test is the preparation of the samples. For the non-absorbed D-antigen content test, the samples are not desorbed but centrifuged. The assay is performed on the supernatant.

Analytical methods are identical to those applied to release the Drug Product, except for the 7 additional tests listed below, only carried out during stability studies. Methods used for Drug Product release are described and validated in 3.2.P.5.2 Analytical Procedures and 3.2.P.5.3 Validation of Analytical Procedures whereas methods specifically used during stability studies are described hereafter:

- Haemophilus immunogenicity;
- Non-adsorbed PT;
- Non-adsorbed FHA;
- Percent adsorption – tetanus toxoid;
- Rat immunogenicity assay for IPV;
- Specific toxicity for diphtheria and tetanus components;
- Integrity test.

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2.1 Summary of Analytical Procedures

2.1.1 *Haemophilus* Immunogenicty

2.1.1.1 Principle

Mice female aged of 22-24 g are injected subcutaneously with ¼ of the human dose at day 0 (primovaccination) and day 14 (booster). Mice are bled at day 21 under anaesthesia.

Anti-*haemophilus* type b polysaccharide antibody titration is performed on individual sera by an ELISA method.

2.1.1.2 Reagents

- Coating antigen prepared by dilution of *haemophilus* type b polysaccharide in ultrafiltered demineralised wate;
- Reference: pool of immunised mice (against a PRP polysaccharide batch or a combined vaccine containing the PRP) serum. This solution is tittered in EU[a]/mL in comparison to a previous arbitrarily defined pool;
- Internal control: pool of immunised mice (against a PRP polysaccharide batch or a combined vaccine containing the PRP) serum. *haemophilus* immunogenicity (titer) is calculated in comparison to the reference;
- Negative control: pool of non-immunised mice serum;
- Conjugate: Mouse anti-IgG goat antibody conjugated to peroxydase.

2.1.1.3 Operating Procedure

2.1.1.3.1 *In-Vivo* Phase: Inoculation of Animals

1) Mice are injected in groups of 8 with 0.5 mL at day 0 and day 14, subcutaneously with a dilution of *haemophilus* type b polysaccharide conjugate vaccine or a control vaccine. The dose is 2.5 µg polysaccharide/0.5 mL per mouse for both materials;
2) Mice are bled at day 21;
3) An additional group of 8 non-injected mice is bled at day 21, as negative controls (sentinel mice);
4) Blood samples are collected individually before centrifugation;
5) After centrifugation, serum samples are pooled by group product and stored at ≤ -20°C.

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[a] EU : ELISA Units

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