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Single Vaccine Dilution Assay for testing the potency of D & T components of combination Vaccine

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V.6.4.1 Conditions for use of single dilution assays

Before initiating use of a single dilution assay system, the control laboratory must have recorded adequate experience with multiple-dilution assays on the specific product vaccine to be tested in this way. This experience must provide:

- evidence of consistency in production and testing;
- evidence of a highly significant regression of the dose – response line for the vaccine and justification of the assumptions of linearity and of parallelism with the dose – response line for the reference preparation;
- guidance for the selection of the single-dilution system parameters, namely number of animals and dilution level to be used for the reference vaccine;
- prediction of the behavior of the single dilution system.

In practice, it is recommended that data from a series of 10 to 20 recent and consecutive multiple-dilution assays should be available for study and confirmation of the above conditions.

Different products will require separate evidence that these conditions are met. Following the introduction of changes in the vaccine production process (e.g. purification, adjuvant, formulation) or in the testing method, evidence that the conditions are met must be provided.

Principle of Single dilution assay : WHO TRS 980 Pg No 295

A one-dilution assay is based on the same principles for evaluating the response as three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 30 IU (or the minimum requirement for the product expressed as an SHD), that elicits a minimum protective effect (or antibody response) in immunized animals; the effect of the reference vaccine is compared with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P \leq 0.05$), the potency of the test vaccine is satisfactory.

One-dilution assays provide assurances that the potency significantly exceeds the minimum requirement. A disadvantage of this approach is that it is not possible to obtain strictly quantitative estimates of vaccine potency. Therefore, in order

FOR MULTIPLE DILUTIONS VALIDITY Criteria : the statistical analysis should show a significant regression ($P < 0.05$) of the log dose–response lines without significant deviation from linearity and parallelism ($P > 0.05$); WHO TRS 980 Annexure 04 pp No 231

1.Consistency of Production and Testing Diphtheria component

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LPV				
S No	Batch No	Diphtheria Potency (IU/SHD)		
		RP	LCL	UCL
1	220112518	61.55	44.44	86.79
2	220112618	77.91	56.26	110.68
3	220112718	57.92	39.40	87.45
4	220112818	64.76	44.05	98.80
5	220112918	59.36	40.83	88.85
6	220113018	58.58	40.37	87.41
7	220113118	65.69	45.25	99.01
8	220113218	67.35	47.22	99.51
9	220113318	59.73	40.05	92.53
10	220113418	63.89	43.04	99.60
11	220113518	65.11	43.56	102.24
12	220113618	75.44	50.33	120.79
13	220100119	71.71	48.09	113.36
14	220100219	61.98	42.02	94.86
15	220100319	63.45	43.23	97.14
16	220100419	81.34	52.09	139.96
17	220100519	70.46	48.92	105.08
18	220100619	64.31	44.74	95.03
19	220100719	66.76	44.43	105.74
20	220100819	75.39	50.22	121.18
21	220100919	73.29	50.17	112.43
22	220101019	60.40	42.32	88.21
23	220101119	70.41	49.49	103.37
24	220101219	72.62	50.81	107.43
25	220101319	67.54	47.06	100.36
26	220101419	60.13	42.04	87.92
27	220101519	59.41	40.63	89.41
28	220101619	74.81	48.81	123.95
29	220101719	68.04	44.24	111.55
MEAN		66.58	45.47	101.69
SD		6.42	4.22	12.98
RSD(%) or CV		9.64	9.29	12.77

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- Obtain an estimate of the mean potency and its variance for each type of vaccine under consideration.
- Obtain an estimate of the observed mean regression slope, its level of significance and the mean range of fiducial limits obtained with the product and by the laboratory concerned.
- Derive from the mean potency estimate and its level of significance above an estimate of the minimum number of animals needed in the assay in order to release a product having the "historically" observed mean potency.

The SD of RP is 66.58 and LCL is 45.47 from their mean values and the CV is 9.64 and 9.29 respectively. In both the cases the CV is less than 20% which is acceptable in case of in vivo testing. This clearly indicates the consistency in production and testing of Diphtheria potency .

2. Evidence of highly significant regression and justification of the assumptions of linearity and parallelism of the dose response curve:

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S No	Batch No	Probability values of		
		Regression	Non-Parallelism	Non-Linearity
1	220112518	0.000	0.640	0.598
2	220112618			
3	220112718	0.000	0.864	0.771
4	220112818			
5	220112918			
6	220113018	0.000	0.387	0.108
7	220113118			
8	220113218	0.000	0.767	0.532
9	220113318	0.000	0.964	0.387
10	220113418			
11	220113518	0.000	0.622	0.759
12	220113618			
13	220100119			
14	220100219	0.000	0.774	0.956
15	220100319			
16	220100419	0.000	0.625	0.537
17	220100519	0.000	0.627	0.642
18	220100619			
19	220100719	0.000	0.961	0.581
20	220100819			
21	220100919	0.000	0.807	0.574
22	220101019	0.000	0.463	0.813
23	220101119			
24	220101219			
25	220101319	0.000	0.818	0.903
26	220101419			
27	220101519	0.000	0.964	0.83
28	220101619	0.000	0.692	0.824
29	220101719			

The probability values of regression, linearity and parallelism derived from the statistical calculation using Combistat software (EDQM) for the recent 29 batches (15 sets) are tabulated :

NOTE: Probability value less than 0.05 is “Significant”
Probability value less than 0.01 is “Highly Significant”
Probability value less than 0.001 is “Even More significant”

Remarks:

- The probability values of non-parallelism and non-linearity are greater than 0.05 and are non-Significant.
- This means there is a significant parallelism and linearity between the dose response curve of reference and test vaccine
- Based on the values from the above table, it is concluded that there is highly significant regression of the dose response for the vaccine and no significant deviation of linearity and parallelism of the dose response test vaccine and the reference standard.

3. Guidance for the selection of the single-dilution system parameters, namely number of animals and dilution level to be used for the reference vaccine

S No	Dilution of Reference from 40 IU/mL		
	1/10 (4 IU/mL)	1/20 (2 IU/mL)	1/40 (1 IU/mL)
1	13/17	8/16	0/17
2	13/17	8/16	2/17
3	12/17	7/18	0/18
4	12/17	7/17	1/18
5	10/16	8/17	1/17
6	11/17	7/19	2/18
7	13/16	7/16	1/17
8	13/17	6/16	2/17
9	12/18	6/17	0/16
10	12/18	10/19	1/18
11	13/18	7/17	0/16
12	12/19	7/16	1/18
13	13/18	8/19	2/17
14	13/18	5/17	2/17
15	12/17	8/19	2/17
Average	12.27/17.33	7.27/17.27	1.13/17.20
% Survival	70.74	42.10	6.57

3. Selection of dilution:

- For the reference vaccine, the recent 29 batches (15 sets) results are used to select a dilution containing number of international units known to elicit an immune response in the lower part of the dose response curve.
- As per WHO/IVB/11.11 page #296, about 10-20% protection is considered acceptable. The mean protection level are calculated in the below mentioned table
- The % survival of the regular dilutions of the reference standard showed that the 1/20 dilution dose the protection level is about 42.10% and the 1/40 dilution dose protection level is 6.57%.

3.1 Selection of Dilution of ref std

S No	ED10/mL
1	32.7846
2	43.2032
3	33.6526
4	34.3453
5	37.7317
6	38.2629
7	35.6199
8	41.0892
9	46.0668
10	30.7431
11	42.4045
12	33.0131
13	38.2624
14	36.301
15	45.7374
Average	37.95
Dose required to Elicit 10% protection or ED10	1.05

V.6.4.2 Selection of appropriate dilutions for single dilution assays

For the reference vaccine, historical data are used to select a dilution containing a number of International Units known to elicit an immune response in the lower part of the dose – response curve. For a quantal response, about 10-20% protection is considered acceptable.

- To get the dose producing protection level of about 10%, ED_{10} of the reference standard was calculated using the combistat software and the result is calculated below:
- Based on the above table, the dilution required to obtain a protection of 10% is calculated as follows.

Potency of Reference Standard	40 IU/mL
Dose required to Elicit 10% protection or ED10	1.05 IU/mL
Dilution fold to obtain 1.05 IU/mL	38.09 fold approx.. 38.0 fold.

- The dilution fold of the reference standard is rounded off on the lower side, to increase the dose of the reference that will be considered for the single dilution assay.

Selection of dilution for test vaccine

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For the vaccine under test, all test products are assumed to contain the minimum required potency (e.g. 30 IU per single human dose of diphtheria vaccine). Based on this assumption, a dilution of the test vaccine is made which hypothetically contains the same number of International Units as the reference vaccine.

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- To verify that the single vaccine dilution method is likely to work, compare responses to the lower dilution levels of the reference (which should be chosen so as to elicit a minimal response) with the responses to the higher dilution levels of the test vaccines.

The dilution fold of the reference standard is rounded off to 38 fold to identify the lower dilutions level of the reference std which should be chosen so as to elicit a minimal response with the responses higher dilution level of test vaccine.

For the vaccine under test all the products are assumed to contain the minimum required potency i.e 30 IU/SHD. Based on this dilution of the LPV which hypothetically contains the same number of international unit as reference standard is calculated below:

Minimum expected Potency of regular batches	60 IU/mL (30 IU/0.5mL/SHD)
Dilution top obtain 1.05 IU/mL	57.14 fold approx.. 57.0 fold.

3.2 Minimum number of animal for single dilution assay

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Obtain an estimate of the observed mean regression slope, its level of significance and the mean range of fiducial limits obtained with the product and by the laboratory concerned.

Table 15: Minimum potency excess factor[a]: Effect of slope and number of animals

No. of animals per vaccine	Minimum true response[b] (%)	Slope = 2	Slope = 3	Slope = 4	Slope = 5	Slope = 6
10	81.7	12.39	5.35	3.52	2.74	2.31
15	70.0	8.00	4.00	2.82	2.30	2.00
20	59.8	5.82	3.24	2.41	2.02	1.80
25	54.0	4.91	2.89	2.22	1.89	1.70
30	49.5	4.31	2.65	2.08	1.79	1.63
35	46.1	3.91	2.48	1.98	1.72	1.57
40	42.9	3.56	2.33	1.89	1.66	1.53

[a] The "minimum potency excess factor" relates to the potency associated with the "minimum true response" and is calculated from the probit of the "minimum true response" and the slope in probits per $\log_{10}$.

The "minimum potency excess factor" is multiplied by the minimum required potency to yield the minimum true potency which is expected to be released in 97.5% of the assays, e.g. when entering the table at a slope value of 4 and using 20 animals per vaccine, a tetanus or diphtheria-tetanus vaccine will have to contain at least 2.41 x 40 = 96.4 IU/single human dose. If the true potency of a given producer's vaccine is known approximately, this value is divided by the minimum required potency to obtain a "potency excess factor" which can be used to enter Table 15 at a given slope value, and estimate the number of animals needed for 97.5% release.

[b] The "minimum true response" is calculated using the exact binomial distribution corresponding to the number of animals considered. To yield the 97.5% of observed responses necessary for acceptance, a test vaccine must have a true potency associated with this "minimum true response", given a true response of 10% for the reference vaccine.

- The minimum number of animals likely to be assuring the potency and for the releases of a product can be derived from the estimate of the observed mean potency and the mean slope. The rounded values of the mean slop to absolute numbers with no decimal point shall be considered for the same.
- The following are the calculated slop values derived from the recent 29 batches (15 sets):

Test No	Common Slope value	Common Slope in probit per log 10
1	1.86572	4.29488744
2	1.47554	3.39669308
3	1.50529	3.46517758
4	1.61766	3.72385332
5	1.39968	3.22206336
6	1.3508	3.1095416
7	1.45866	3.35783532
8	1.22971	2.83079242
9	1.58116	3.63983032
10	1.33004	3.06175208
11	1.46145	3.3642579
12	1.59233	3.66554366
13	1.54286	3.55166372
14	1.45955	3.3598841
15	1.22445	2.8186839
Mean Slopoe		3.372047558
Approx.		3

The calculation shows mean slope is 3, and if we use 20 animals for single dilution vaccine have to contain 30 X 3.24= 97.20 IU/SHD for Diphtheria potency

3.3 Prediction of the behavior of the single dilution assay:

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prediction of the behavior of the single dilution system.

S No	Batch No	Survival with reference vaccine 1/40 Dilution	Survival with test vaccine 1/40 Dilution	P-value
1	220112518	0/17	11/17	0.000
2	220112618	0/17	14/16	0.000
3	220112718	2/17	12/16	0.000
4	220112818	2/17	11/16	0.001
5	220112918	2/17	13/18	0.000
6	220113018	0/18	11/17	0.000
7	220113118	0/18	12/16	0.000
8	220113218	1/18	10/17	0.001
9	220113318	1/17	10/17	0.001
10	220113418	1/17	10/17	0.001
11	220113518	2/18	11/16	0.001
12	220113618	2/18	13/17	0.000
13	220100119	2/18	11/16	0.001
14	220100219	1/17	11/16	0.000
15	220100319	1/17	13/19	0.000
16	220100419	2/17	13/19	0.001
17	220100519	0/16	11/16	0.000
18	220100619	0/16	9/16	0.000
19	220100719	1/18	10/16	0.001
20	220100819	1/18	12/17	0.000
21	220100919	0/16	13/17	0.000
22	220101019	1/18	10/17	0.001
23	220101119	1/18	12/18	0.000
24	220101219	1/18	11/17	0.000
25	220101319	2/17	13/17	0.000
26	220101419	2/17	11/17	0.002
27	220101519	2/17	10/17	0.005
28	220101619	2/17	15/19	0.000
29	220101719	2/17	11/17	0.002

V.4.4 Fisher's exact test

Fisher's exact probability test can be used with data from single dilution assays based on quantal responses to show that the test product has better performance than the reference standard at the minimum acceptable level.

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The aim of statistical evaluation of a single dilution comparison of reference and test vaccines is to determine whether the responses of the group treated with the test vaccine differ significantly from the responses of the group treated with the reference vaccine. If the difference is significant, then it can be concluded that the test vaccine achieves at least the minimum potency. The methods of Section V.4 can be used with the statistical

- Base on the dilution finalized for the reference standard (38fold) and test vaccine (57 fold) for the single vaccine dilution assay, data collected from the multi dilution assay which is close to the proposed single vaccine dilution are taken for the prediction of the behaviour of the single vaccine dilution assay.
- The survival data of 40 fold of the reference standard and 40 fold for the test vaccine are calculated with the Fisher exact probability test (one side)..

NOTE: Probability value less than 0.05 is "Significant"

Probability value less than 0.01 is "Highly Significant"

Probability value less than 0.001 is "Even More Significant"

The calculation says that the test vaccine has shown a significantly higher response than the reference vaccine

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Validation of single dilution assay:

- Based on the overall assessment, for the validation of single dilution assay following conclusion are arrived.
- The dilution finalized is for reference standard 39 fold from 40IU/mL
- The dilution finalized for test vaccine is 57 fold.
- Number of animals to be used for the single dilution is 20.

- Applying above criteria 3 single dilution tests shall be performed simultaneously along with the multiple dilution assay for the validation.

1. **Retest:**
   - During regular testing of batches for release, if a batch fails in single dilution assay, the sample shall be retested by following multi dilution assay to decide the fate of the batch.

Retest criteria is not applicable for the validation study and only applicable for release testing of batches.

1. **ACCEPTANCE CRITERIA**

The sample passes if, the probability value falls in any of the following categories:

- Probability value less than 0.05 is “Significant”
- Probability value less than 0.01 is “Highly Significant”
- Probability value less than 0.001 is “Even More Significant”

Validation of single dilution assay

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Dilution	Volume of diluted Reference Standard	Volume of Physiological Saline (0.85% w/v)
1/10	2 mL of 40 IU/mL	18 mL
1/38	10 mL from 1/10 dilution	28 mL

- 1/38 dilution used for immunization.
- The test sample was diluted as per bellow table:

Dilution	Volume of Sample	Volume of Physiological Saline (0.85% w/v)
1/20	1 mL of sample	19 mL
1/57	15 mL from 1/20 dilution	27.75 mL

- 1/57 dilution used for immunization.

Test No	Batch No	Probability values (P<0.05)	Significant category	Compliance
1	220102516A (36M RTS)	0.000	Even more Significant	Complies
	220102516B (36M RTS)	0.022	Significant	Complies
	220102516C (36M RTS)	0.010	Highly Significant	Complies
2	220105419	0.001	Even more Significant	Complies
	220105519	0.000	Even more Significant	Complies
	220105619	0.000	Even more Significant	Complies
3	220105719	0.001	Even more Significant	Complies
	220105819	0.000	Even more Significant	Complies
	220105919	0.000	Even more Significant	Complies

- The calculation clearly proved that there is significance difference in response between the reference and test vaccine.
- **Acceptance criteria:**
- The sample passes if; the probability value falls in any of the following categories:

  Probability value less than 0.05 is “Significant”

  Probability value less than 0.01 is “Highly Significant”

  o Probability value less than 0.001 is “Even More Significant

- The test vaccine dilutions includes significantly higher immune response than the reference vaccine in the single dilution assay, it may be concluded that the test vaccine contains greater than the least minimum required potency i.e., ≥30 IU/SHD.

Hence the single dilution assay may be implemented after approval from NRA for testing diphtheria potency in LPV vaccine

Conditions applicable for single dilution assay

- If a significant change in the production process, testing should revert to the multiple-dilution assay.
- To monitor production consistency, full multiple dilution assay shall be performed as approved by National Regulatory authority.
- If there is a change in reference standard, minimum three independent multi-dilution assays shall be performed to identify the suitable single dilution by statistical analysis.
- If a batch fails to meet the single dilution assay requirement i.e., $P<0.05$, the sample shall be retested by following multi dilution assay to decide the fate of the batch.

1.Consistency of Production and Testing of tetanus component in LPV vaccine

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LPV				
S No	Batch No	Tetanus Potency (IU/SHD)		
		RP	LCL	UCL
1	220112418	1071.35	667.76	1800.28
2	220112518	984.59	616.25	1636.29
3	220112618	742.00	451.70	1245.25
4	220112718	835.62	507.77	1421.98
5	220112818	939.86	595.85	1531.52
6	220112918	891.63	566.31	1444.62
7	220113018	1113.35	670.02	2008.21
8	220113118	1032.52	623.15	1838.73
9	220113218	860.19	573.76	1326.95
10	220113318	960.02	637.64	1501.21
11	220113418	672.84	423.81	1078.55
12	220113518	488.57	307.91	762.28
13	220113618	753.57	439.03	1326.57
14	220100119	717.25	417.98	1255.38
15	220100219	861.75	500.82	1543.65
16	220100319	1233.58	760.66	2126.765
17	220100419	895.26	561.935	1474.775
18	220100519	801.45	504.525	1304.12
19	220100619	756.925	456.6855	1284.82
20	220100719	997.865	609.925	1721.425
21	220100819	1069.385	651.665	1863.995
22	220100919	1375.745	855.125	2363.04
23	220101019	992.685	650.585	1567.41
24	220101119	929.22	610.95	1455.61
25	220101219	978.97	642.06	1543.08
26	220101319	1082.24	689.63	1768.57
27	220101419	870.46	559.59	1388.61
28	220101519	1002.62	647.18	1612.68
29	220101619	1249.45	765.08	2191.73
30	220101719	809.62	504.37	1337.59
MEAN		914.11	570.23	1521.22
SD		183.64	117.06	338.16
RSD(%) or CV		20.09	20.53	22.23

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- Obtain an estimate of the mean potency and its variance for each type of vaccine under consideration.
- Obtain an estimate of the observed mean regression slope, its level of significance and the mean range of fiducial limits obtained with the product and by the laboratory concerned.
- Derive from the mean potency estimate and its level of significance above an estimate of the minimum number of animals needed in the assay in order to release a product having the "historically" observed mean potency.

The SD of RP is 183.64 and LCL is 117.06 from their mean values and the CV is 20%. In both the cases the CV is about 20% which is acceptable in case of in vivo testing. This clearly indicates the consistency in production and testing of tetanus potency in LPV vaccine

2. Evidence of highly significant regression and justification of the assumptions of linearity and parallelism of the dose response curve:

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LPV				
S No	Batch No	Probability values of		
		Regression	Non-Parallelism	Non-Linearity
1	220112418	0.000	0.141	0.931
2	220112518			
3	220112618	0.000	0.474	0.942
4	220112718			
5	220112818	0.000	0.142	0.927
6	220112918			
7	220113018	0.000	0.597	0.971
8	220113118			
9	220113218	0.000	0.115	0.488
10	220113318			
11	220113418	0.000	0.546	0.959
12	220113518			
13	220113618	0.000	0.534	0.964
14	220100119			
15	220100219			
16	220100319	0.000	0.100	0.438
17	220100419			
18	220100519			
19	220100619	0.000	0.736	0.935
20	220100719	0.000	0.258	0.857
21	220100819			
22	220100919	0.000	0.891	0.466
23	220101019	0.000	0.950	0.478
24	220101119			
25	220101219			
26	220101319	0.000	0.684	0.802
27	220101419			
28	220101519	0.000	0.601	0.872
29	220101619	0.000	0.055	0.8
30	220101719			

The probability values of regression, linearity and parallelism derived from the statistical calculation using Combistat software (EDQM) for the recent 29 batches (15 sets) are tabulated :

NOTE: Probability value less than 0.05 is “Significant”
Probability value less than 0.01 is “Highly Significant”
Probability value less than 0.001 is “Even More significant”

Remarks: The probability values of non-parallelism and non-linearity are greater than 0.05 and are non-Significant. This means there is a significant parallelism and linearity between the dose response curve of reference and test vaccine.

Based on the values from the above table, it is concluded that there is highly significant regression of the dose response for the vaccine and no significant deviation of linearity and parallelism of the dose response test vaccine and the reference standard.

3. Guidance for the selection of the single-dilution system parameters, namely number of animals and dilution level to be used for the reference vaccine

S No	Dilution of Reference from 100 IU/mL			
	1/10 (10 IU/mL)	1/20 (5 IU/mL)	1/40 (2.5 IU/mL)	1/80 (1.25 IU/mL)
1	19/20	17/20	11/20	3/20
2	19/20	16/20	11/20	6/20
3	18/20	17/20	12/20	3/20
4	17/20	13/19	11/20	5/20
5	20/20	17/20	13/20	2/20
6	20/20	18/20	15/20	6/20
7	19/20	17/20	11/20	6/20
8	19/20	16/20	13/20	3/20
9	18/20	16/20	11/20	5/20
10	20/20	14/20	11/20	4/20
11	18/20	15/20	10/20	4/20
12	19/20	17/20	12/20	4/20
13	19/20	16/20	11/20	3/20
14	18/20	14/20	10/20	4/20
15	17/20	16/20	12/20	4/20
Average	18.67/20	15.93/19.93	11.60/20	4.13/20
% Survival	93.35	79.65	58.00	20.65

Potency of Reference Standard	100 IU/mL
Dose required to Elicit 20% protection	1.25 IU/mL
Dilution fold to obtain 1.25 IU/mL	80.0 fold

3. Selection of dilution:

- For the reference vaccine, the recent 30 batches (15 sets) results are used to select a dilution containing number of international units known to elicit an immune response in the lower part of the dose response curve. As per WHO/IVB/11.11 page #296, about 10-20% protection is considered acceptable. The mean protection level are calculated in the below mentioned table:
- As per WHO/IVB/11.11 page #296, about 10-20% protection is considered acceptable. The mean protection level are calculated in the below mentioned table.
- The % survival of the regular dilutions of the reference standard showed that the 1/40 dilution dose the protection level is about 58% and the 1/80 dilution dose protection level is about 20%.

V.6.4.2 Selection of appropriate dilutions for single dilution assays

For the reference vaccine, historical data are used to select a dilution containing a number of International Units known to elicit an immune response in the lower part of the dose – response curve. For a quantal response, about 10-20% protection is considered acceptable.

Selection of dilution for test vaccine

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For the vaccine under test, all test products are assumed to contain the minimum required potency (e.g. 30 IU per single human dose of diphtheria vaccine). Based on this assumption, a dilution of the test vaccine is made which hypothetically contains the same number of International Units as the reference vaccine.

WHO VSQ 97.04 Chapter 27

- To verify that the single vaccine dilution method is likely to work, compare responses to the lower dilution levels of the reference (which should be chosen so as to elicit a minimal response) with the responses to the higher dilution levels of the test vaccines.

Based on this dilution of the LPV which hypothetically contains the same number of international unit as reference standard is calculated below:

Minimum expected Potency of regular batches	120 IU/mL
Dilution top obtain 1.25 IU/mL	96.0 fold

3.2 Minimum number of animal for single dilution assay

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Obtain an estimate of the observed mean regression slope, its level of significance and the mean range of fiducial limits obtained with the product and by the laboratory concerned.

Table 15: Minimum potency excess factor[a]: Effect of slope and number of animals

No. of animals per vaccine	Minimum true response[b] (%)	Slope = 2	Slope = 3	Slope = 4	Slope = 5	Slope = 6
10	81.7	12.39	5.35	3.52	2.74	2.31
15	70.0	8.00	4.00	2.82	2.30	2.00
20	59.8	5.82	3.24	2.41	2.02	1.80
25	54.0	4.91	2.89	2.22	1.89	1.70
30	49.5	4.31	2.65	2.08	1.79	1.63
35	46.1	3.91	2.48	1.98	1.72	1.57
40	42.9	3.56	2.33	1.89	1.66	1.53

[a] The "minimum potency excess factor" relates to the potency associated with the "minimum true response" and is calculated from the probit of the "minimum true response" and the slope in probits per $\log_{10}$.

The "minimum potency excess factor" is multiplied by the minimum required potency to yield the minimum true potency which is expected to be released in 97.5% of the assays, e.g. when entering the table at a slope value of 4 and using 20 animals per vaccine, a tetanus or diphtheria-tetanus vaccine will have to contain at least 2.41 x 40 = 96.4 IU/single human dose. If the true potency of a given producer's vaccine is known approximately, this value is divided by the minimum required potency to obtain a "potency excess factor" which can be used to enter Table 15 at a given slope value, and estimate the number of animals needed for 97.5% release.

[b] The "minimum true response" is calculated using the exact binomial distribution corresponding to the number of animals considered. To yield the 97.5% of observed responses necessary for acceptance, a test vaccine must have a true potency associated with this "minimum true response", given a true response of 10% for the reference vaccine.

- The minimum number of animals likely to be assuring the potency and for the releases of a product can be derived from the estimate of the observed mean potency and the mean slope. The rounded values of the mean slop to absolute numbers with no decimal point shall be considered for the same.
- The following are the calculated slop values derived from the recent 30 batches (15 sets):

Test No	Common Slope value	Common Slope in probit per log 10
1	0.977913	2.251155726
2	0.910061	2.094960422
3	1.00516	2.31387832
4	0.873557	2.010928214
5	1.19434	2.74937068
6	1.0683	2.4592266
7	0.830331	1.911421962
8	0.983987	2.265138074
9	0.876832	2.018467264
10	0.913711	2.103362722
11	0.983674	2.264417548
12	1.14454	2.63473108
13	1.03181	2.37522662
14	1.03431	2.38098162
15	0.942585	2.16983067
Mean Slopoe		2.256079909
Approx.		2

The calculation shows mean slope is 2, and if we use 20 animals foe single dilution vaccine have to contain 60 X 5.82= 349.20 IU/SHD for tetanus potency

The mean LCL of 30 LPV batches minimum required is 349.20 IU/SHD (Mean LCL is 570.23 IU/SHD). So 20 number of animals will be sufficient for the single dilution assay.

3.3 Prediction of the behavior of the single dilution assay:

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prediction of the behavior of the single dilution system.

LPV

S No	Batch No	Survival with reference vaccine	Survival with test vaccine	P-value
		1/80 dilution	1/120 dilution	
1	220112418	3/20	20/20	0.000
2	220112518	3/20	19/20	0.000
3	220112618	6/20	18/20	0.000
4	220112718	6/20	19/20	0.000
5	220112818	3/20	19/20	0.000
6	220112918	3/20	19/20	0.000
7	220113018	5/20	20/20	0.000
8	220113118	5/20	19/20	0.000
9	220113218	2/20	20/20	0.000
10	220113318	2/20	20/20	0.000
11	220113418	6/20	19/20	0.000
12	220113518	6/20	19/20	0.000
13	220113618	6/20	18/20	0.000
14	220100119	6/20	18/20	0.000
15	220100219	6/20	19/20	0.000
16	220100319	3/20	20/20	0.000
17	220100419	3/20	19/20	0.000
18	220100519	3/20	18/20	0.000
19	220100619	5/20	19/20	0.000
20	220100719	4/20	19/20	0.000
21	220100819	4/20	20/20	0.000
22	220100919	4/20	20/20	0.000
23	220101019	4/20	20/20	0.000
24	220101119	4/20	20/20	0.000
25	220101219	4/20	19/20	0.000
26	220101319	4/20	19/20	0.000
27	220101419	4/20	19/20	0.000
28	220101519	4/20	19/20	0.000
29	220101619	4/20	20/20	0.000
30	220101719	4/20	17/20	0.000

- Base on the dilution finalized for the reference standard and test vaccine for the single vaccine dilution assay, data collected from the multi dilution assay which is close to the proposed single vaccine dilution are taken for the prediction of the behaviour of the single vaccine dilution assay. The survival data of 80 fold of the reference standard and 120 fold for the test vaccine are calculated with the Fisher exact probability test (one side) to the significance of the difference between the response of reference standard and test vaccine

NOTE: Probability value less than 0.05 is “Significant”

Probability value less than 0.01 is “Highly Significant”

Probability value less than 0.001 is “Even More Significant”

The calculation says that there is significant difference in deference between reference and test vaccine.

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Validation of single dilution assay:

- Based on the overall assessment, for the validation of single dilution assay following conclusion are arrived.
- The dilution finalized is for reference standard 80 fold from 100IU/mL
- The dilution finalized for test vaccine is 96 fold.
- Number of animals to be used for the single dilution is 20.
-

- Applying above criteria 3 single dilution tests shall be performed simultaneously along with the multiple dilution assay for the validation

1. **Retest:**
   - During regular testing of batches for release, if a batch fails in single dilution assay, the sample shall be retested by following multi dilution assay to decide the fate of the batch.

Retest criteria is not applicable for the validation study and only applicable for release testing of batches.

1. **ACCEPTANCE CRITERIA**

The sample passes if, the probability value falls in any of the following categories:

- Probability value less than 0.05 is “Significant”
- Probability value less than 0.01 is “Highly Significant”
- Probability value less than 0.001 is “Even More Significant”

Conditions applicable for single dilution assay

- If a significant change in the production process, testing should revert to the multiple-dilution assay.
- To monitor production consistency, full multiple dilution assay shall be performed as approved by National Regulatory authority.
- If there is a change in reference standard, minimum three independent multi-dilution assays shall be performed to identify the suitable single dilution by statistical analysis.
- If a batch fails to meet the single dilution assay requirement i.e., $P<0.05$, the sample shall be retested by following multi dilution assay to decide the fate of the batch.