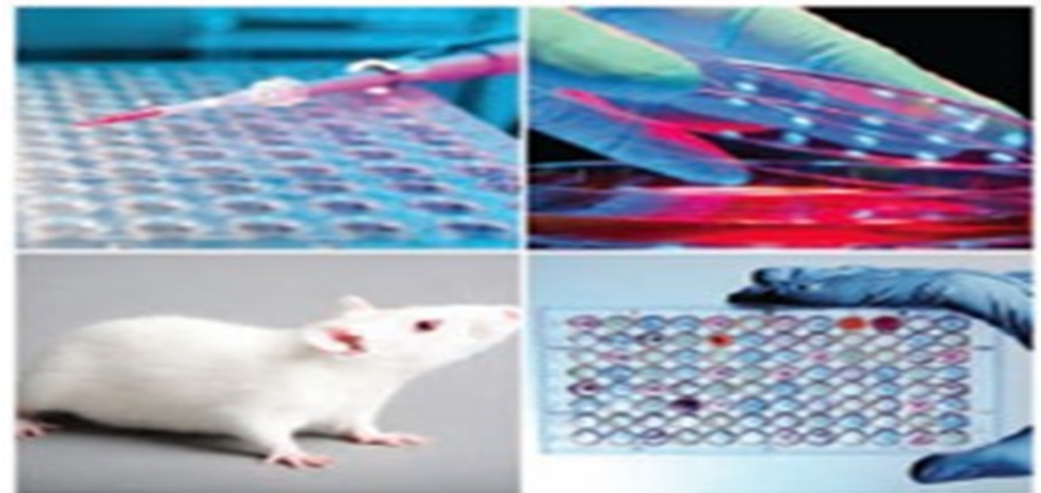


Diphtheria Absence of Toxin (Specific Toxicity) and Irreversibility of Toxoid: Vero-cells assay

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Specific toxicity test: Diphtheria: Ref WHO TRS 980 (Annex 04)

Stage	Test
A.3.3.4 Detoxification and purification	At harvest stage, Detoxification has been shown to be complete by performance of a specific toxicity test either by suitably validated in vivo or in vitro method.
A.3.4.4: Specific toxicity	Each bulk purified toxoid should be tested for the presence of diphtheria toxin. The test may be performed in vivo using guinea-pigs or in vitro using a suitable cell culture assay
A.3.4.5 Reversion to toxicity	Each bulk purified toxoid should be tested to ensure that reversion to toxicity does not take place during storage. The test may be performed in vivo using guinea-pigs or in vitro using a suitable cell culture assay, such as the Vero cell assay.

*A detailed procedure Vero cell method is described in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines, WHO IVB 11.11/ EP 11.2/IP 2022.



		1	2	3	4	5	6	7	8	9	10	11	12	
		(Fresh) Lf/mL			(2-8°C) Lf/mL			(34-37°C) Lf/mL			Empty	Control		
		100 Lf	50 Lf	25 Lf	100 Lf	50 Lf	25 Lf	100 Lf	50 Lf	25 Lf				
Sample Diluted with PBS + Media + Cell	A	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	White	Blue	Blue	Cell control
	B	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	White	Blue	Blue	
	C	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	White	Blue	
Toxin + Media + Cell	D	White	White	White	Blue	Blue	Blue	Blue	Blue	Blue	White	White	White	Blank
		1x10 ⁻⁴	5x10 ⁻⁵	3x10 ⁻⁵	1x10 ⁻⁵	6x10 ⁻⁶	3x10 ⁻⁶	2x10 ⁻⁶	8x10 ⁻⁷	4*10 ⁻⁷				
Sample Diluted with PBS + DAT (50 Lf) + Cell	E	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	White	Blue	Blue	DAT Control
	F	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	White	Blue	Blue	
	G	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	White	Blue	Blue	
Toxin + Media + Cell	H	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	White	Blue	Blue	Control Toxoid
		1x10 ⁻⁴	5x10 ⁻⁵	3x10 ⁻⁵	1x10 ⁻⁵	6x10 ⁻⁶	3x10 ⁻⁶	2x10 ⁻⁶	8x10 ⁻⁷	4*10 ⁻⁷				

Acceptance Criteria :

The geometric mean optical density value (OD) of the wells containing Vero cells together with the control toxoid should be calculated and divided by 2 to obtain the 50% OD value.

This 50% OD value (plate specific) is used as a threshold value for the test toxoid samples. If the test sample OD is greater than the 50% threshold value the toxoid passes the test.

Validity Criteria:

As per Indian Pharmacopeia and European Pharmacopeia

- ❖ The test is invalid if, 5×10^{-5} Lf/mL of reference diphtheria toxin in 100 Lf/mL toxoid has no cytotoxic effect on Vero cells or the cytotoxic effect of this amount of toxin is not neutralised in the wells containing diphtheria antitoxin.

As per WHO IVB 11.11

- ❖ The assay is valid if, the sensitivity and specificity of the assay are confirmed: i.e. the control toxin is toxic to Vero cells and this toxic effect is neutralised in duplicate wells containing toxin and antitoxin.
- ❖ The toxoid control OD value is not less than 50% of the geometric mean optical density of cell control.
- ❖ No toxicity is seen following the addition of diphtheria antitoxin or control toxoid.

Retest: If non-specific toxicity is observed (i.e. toxin effects that are not neutralised in the presence of antitoxin) the test sample should be dialysed against the toxoid diluent prior to used in a repeat assay.

Layout for Pass batch

		1	2	3	4	5	6	7	8	9	10	11	12	
		(Fresh) Lf/mL			(2-8°C) Lf/mL			(34-37°C) Lf/mL						
		100 Lf	50 Lf	25 Lf	100 Lf	50 Lf	25 Lf	100 Lf	50 Lf	25 Lf	Empty	Control		
Sample Diluted with PBS + media + Cell	A	●	●	●	●	●	●	●	●	●	○	●	●	Cell control
	B	●	●	●	●	●	●	●	●	●	○	●	●	
	C	●	●	●	●	●	●	●	●	●	○	○	○	
Toxin + Media +Cell	D	●	●	●	●	●	●	●	●	●	○	○	○	
Sample Diluted with PBS + DAT (50 Lf) + Cell	E	●	●	●	●	●	●	●	●	●	○	●	●	DAT Control
	F	●	●	●	●	●	●	●	●	●	○	●	●	
	G	●	●	●	●	●	●	●	●	●	○	●	●	
Toxin + DAT(50Lf)+Cell	H	●	●	●	●	●	●	●	●	●	○	●	●	

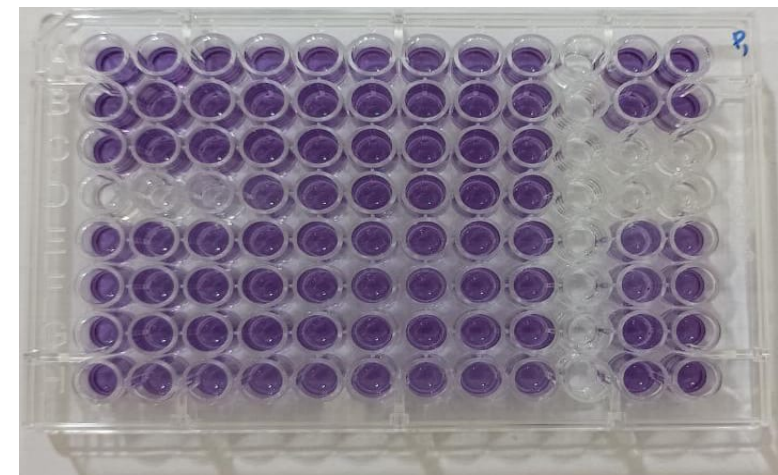
If toxin present in sample layout look

		1	2	3	4	5	6	7	8	9	10	11	12	
		(Fresh) Lf/mL			(2-8°C) Lf/mL			(34-37°C) Lf/mL						
		100 Lf	50 Lf	25 Lf	100 Lf	50 Lf	25 Lf	100 Lf	50 Lf	25 Lf	Empty	Control		
Sample Diluted with PBS + media + Cell	A	○	○	○	○	○	○	○	○	○	○	●	●	Cell control
	B	○	○	○	○	○	○	○	○	○	○	●	●	
	C	○	○	○	○	○	○	○	○	○	○	○	○	
Toxin + Media +Cell	D	○	○	○	●	●	●	●	●	●	○	○	○	
Sample Diluted with PBS + DAT (50 Lf) + Cell	E	●	●	●	●	●	●	●	●	●	○	●	●	DAT Control
	F	●	●	●	●	●	●	●	●	●	○	●	●	
	G	●	●	●	●	●	●	●	●	●	○	●	●	
Toxin + DAT(50Lf)+Cell	H	●	●	●	●	●	●	●	●	●	○	●	●	



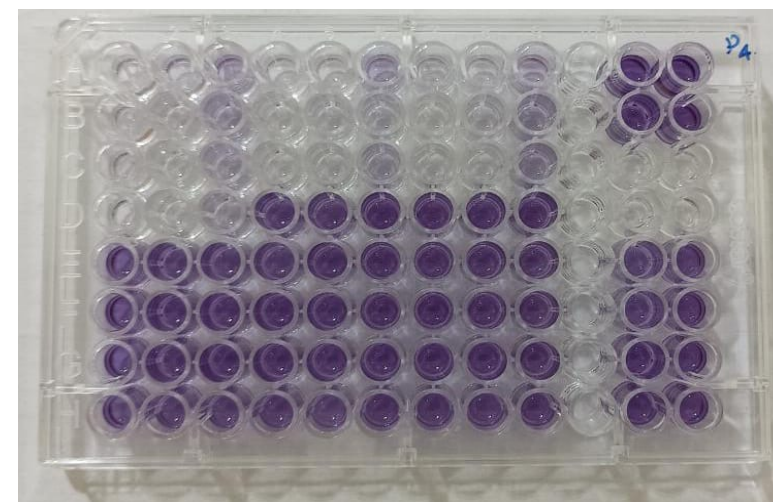
Pass Result

	1	2	3	4	5	6	7	8	9	10	11	12	Cell Control
A	0.743	0.723	0.73	0.632	0.685	0.707	0.612	0.648	0.686	0.047	0.729	0.702	0.697130
B	0.695	0.68	0.679	0.588	0.62	0.661	0.608	0.601	0.675	0.046	0.641	0.72	0.348565
C	0.696	0.646	0.643	0.596	0.628	0.633	0.573	0.671	0.655	0.046	0.041	0.041	
D	0.05	0.055	0.267	0.398	0.525	0.612	0.58	0.587	0.643	0.046	0.043	0.041	
E	0.664	0.612	0.602	0.643	0.59	0.583	0.595	0.558	0.584	0.05	0.56	0.581	
F	0.682	0.607	0.591	0.641	0.577	0.576	0.601	0.551	0.58	0.047	0.581	0.587	Control Toxoid
G	0.707	0.635	0.597	0.629	0.61	0.59	0.626	0.576	0.587	0.048	0.577	0.648	0.614548
H	0.677	0.589	0.551	0.513	0.55	0.519	0.594	0.55	0.515	0.046	0.578	0.66	0.307274



Fail Result

	1	2	3	4	5	6	7	8	9	10	11	12	Cell Control
A	0.047	0.058	0.052	0.053	0.048	0.053	0.059	0.047	0.046	0.047	0.673	0.767	0.681211
B	0.05	0.048	0.049	0.055	0.049	0.045	0.058	0.048	0.047	0.046	0.658	0.634	0.340605
C	0.052	0.05	0.047	0.06	0.051	0.048	0.062	0.045	0.047	0.045	0.042	0.043	
D	0.044	0.047	0.176	0.338	0.469	0.518	0.53	0.474	0.495	0.046	0.041	0.056	
E	0.636	0.553	0.556	0.564	0.582	0.54	0.531	0.523	0.562	0.047	0.545	0.534	
F	0.659	0.566	0.556	0.535	0.554	0.541	0.544	0.552	0.566	0.047	0.569	0.57	Control Toxoid
G	0.682	0.581	0.573	0.562	0.583	0.585	0.567	0.558	0.579	0.047	0.63	0.653	0.663536
H	0.696	0.57	0.599	0.598	0.613	0.579	0.572	0.628	0.541	0.046	0.62	0.76	0.331768



Conclusion

In Vivo Test

- For specific Toxicity, 5 animals required and observed for 42 days.
- For Irreversibility, 42 days incubation and 2 days animal test.
- Sensitivity by Subcutaneous method is 0.1Lf/mL, Intradermal method 3×10^{-4} Lf/mL.
- Result observation by manual.

In Vitro (Vero cell assay)

- For Specific Toxicity only cells required and observation is 7 days.
- For Irreversibility 42 days incubation and 7 days Vero cell test.
- Sensitivity by Vero cell method is 3×10^{-5} Lf/mL. **More sensitive and Reliable**
- Results observation by Absorbance Readings. no manual intervention is required.
- No animals required.
- Complete Replacement



The **monument** commemorates the **sacrifice of the mice** in genetic research used to understand biological and physiological mechanisms for developing new drugs and curing of diseases

Thank You