STANDARD OPERATING PROCEDURE	
Department: Microbiology	**SOP No.:**
Title: Preparation and Testing of Endotoxin Indicator Vials	**Effective Date:**
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1.0 OBJECTIVE

1.1 To lay down the Procedure for preparation and testing of endotoxin indicator vials.

2.0 SCOPE

2.1 This procedure is applicable for Microbiology Laboratory.

3.0 RESPONSIBILITY

3.1 Microbiologist is responsible for preparation and testing of endotoxin indicator vials.

4.0 ACCOUNTABILITY

4.1 Head Microbiology

5.0 EHS CONSIDERATIONS

5.1 Handle the endotoxin indicator vial very carefully.

5.2 Use endotoxin free glass pipettes or micropipettes with endotoxin free tips for dispensing of solutions.

6.0 PROCEDURE

6.1 Preparation of Endotoxin Indicator Spiked Vials

6.1.1 Take Endotoxin indicator vial containing 100000 EU.

6.1.2 Reconstitute each vial with 1 ml LAL reagent water (or as per supplier or COA recommendation) and vortex for 30 minutes.

6.1.3 Dispense 0.1 ml aliquots of reconstituted endotoxin indicator in to 09 depyrogenated vials, so that each vial will contain 10000 EU.

6.1.4 Keep all the spiked vials open under LAF over night.

6.1.5 Visually observe for drying and label the vials with spiked concentration, date of preparation and sign & preserve in refrigerator.

6.1.6 Close the vials with depyrogenated aluminum foil, until they are required for exposing to a validation cycle.

6.1.7 Record the preparation details as per format "Report for estimation of endotoxin log reduction".

6.2 **Endotoxin recovery and estimation of log reduction**

6.2.1 **Re-constitution and dilution of un-processed endotoxin indicator spiked vials (positive control)**

6.2.1.1 Re-constitute one un-processed (un-exposed) endotoxin indicator vial containing 10000 EU/ vial with 4 ml of LRW.

6.2.1.2 Vortex for a minimum of 10 minutes.

6.2.1.3 After reconstitution the assumed concentration of endotoxin will be 2,500 EU / ml in the vial and the dilution will be 1:4.

6.2.1.4 Make further dilutions as per Table 1 and vortex each dilution for at least 1 minute. Use 0.125 EU/ml lysate for testing.

Table 1

Tube No.	Volume of endotoxin indicator solution	Volume of LRW added	Concentration after dilution	Total dilution
1.	0.1 ml from re-constituted vial	0.9 ml	250 EU/ml	1:40
2.	0.1 ml from Tube No.1	0.9 ml	25 EU/ml	1:400
3.	0.1 ml from Tube No.2	0.9 ml	2.5 EU/ml	1:4000
4.	0.2 ml from Tube No.3	1.8 ml	0.25 EU/ml (2λ)	1:40000
5.	1.0 ml from Tube No.4	1.0 ml	0.125 EU/ml (λ)	1:80000
6.	1.0 ml from Tube No.5	1.0 ml	0.06 EU/ml (0.5λ)	1:160000
7.	1.0 ml from Tube No.6	1.0 ml	0.03 EU/ml (0.25λ)	1:320000

6.2.1.5 Test the solutions of Tube No. 4, 5, 6 and 7 in duplicate as per the recovery test.

6.2.2 **Re-constitution and dilution of processed endotoxin indicator spiked vials (test):**

6.2.2.1 Assuming ≥ 3 log reduction (so that each vial may contain ≤ 10 EU), re-constitute each processed (exposed) endotoxin indicator spiked vial with 1.0 ml of LAL reagent water and vortexing for 02 minutes initially and then for 01 minute every 10 minutes for one half hour.

6.2.2.2 Assuming the endotoxin concentration of the solution in the constituted vial as 0.125 EU / ml, perform the test with 0.125 EU/ml lysate.

6.2.2.3 Use the reconstitute as such without dilutions, but if test fails at 0.125EU/ml and then perform the dilutions as per Table 2 test the previous dilution like 0.25EU/ml, 0.5EU/ml, 1 EU/ml, 10 EU/ml one by one until test passes.

6.2.2.4 Test the solutions of all tubes as per the recovery test procedure described:

Table 2

Tube No.	Volume of Endotoxin Indicator solution	Volume of LRW added	Concentration After dilution	Total Dilution
1.	0.2 ml from reconstituted vial	1.8 ml	< 1 EU/ml	1:10
2.	1.0 ml from Tube No.1	1.0 ml	< 0.5 EU/ml	1:20
3.	1.0 ml from Tube No.2	1.0 ml	< 0.25 EU/ml (2λ)	1:40
4.	1.0 ml from Tube No.3	1.0 ml	< 0.125 EU/ml (λ)	1:80

6.2.3 **Recovery Test:**

6.2.3.1 **Test for processed vial:** Label 2 endotoxin free reaction tubes (for each dilution) as test, along with vial identification and the dilution. Add 0.10 ml of solution of the specific dilution in to each of two reaction tubes.

6.2.3.2 **Test for positive (unprocessed) vial:** Label 2 endotoxin free reaction tubes (for each dilution) as positive control, along with vial identification and the dilution. Add 0.10 ml of solution of the specific dilution in to each of two reaction tubes.

6.2.3.3 **Negative Water control:** Label 2 endotoxin free reaction tubes as NWC. Add 0.10 ml of LRW into each of 2 reaction tubes.

6.2.3.4 **Positive water Control:** Label 2 endotoxin free reaction tubes as PWC. Add 0.10 ml of 2 λ control standard endotoxin solution into each of 2 reaction tubes.

6.2.3.5 Note: One set of NWC and PWC are enough for all the samples tested at one time.

6.2.3.6 **Addition of LAL Reagent**: Add 0.10 ml of LAL reagent to all the reaction tubes quickly, starting from NWC and then Test, PWC and positive controls respectively

6.2.3.7 **Incubation:** Mix the solution and incubate the reaction mixtures in the dry bath incubator at 37 ± 1 °C for 60 ± 2 minutes. Take care to avoid vibration of dry bath incubator during incubation

6.2.3.8 **Observation:** After incubation take each tube in turn directly from the dry bath incubator and invert it through about 180 ° in one smooth motion. If a firm gel has formed that remaining in place upon inversion, record the results as positive (+ve). The result is negative (-ve) if an intact gel is not formed

6.2.3.9 Record the activity as per format ”Report for estimation of endotoxin log reduction”.

6.2.4 **Recovery Calculation:**

6.2.4.1 Reciprocal of last dilution that was positive X Sensitivity of LAL.

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6.2.5 **Log reduction calculation**

6.2.5.1 Calculate the log recovery and calculate the log reduction as per formula given below.

6.2.5.2 Log reduction = Log recovery of positive control – Log recovery of test.

6.2.5.3 In case of processed vial if all the vials are showing no clot formation then take log of 0.125 for test.

7.0 DEFINITIONS AND ABBREVIATIONS

7.1	CSE	-	Control standard endotoxin
7.2	LRW	-	LAL reagent water
7.3	NMT	-	Not more than
7.4	NWC	-	Negative water control
7.5	LAL	-	Limulus amoebocyte lysate
7.6	EU	-	Endotoxin unit
7.7	λ	-	Lysate sensitivity

8.0 REFERENCE

8.1 As Per Pievv100k-02

9.0 ANNEXURES

9.1 Annexure I: Report for estimation of endotoxin log reduction

10.0 DISTRIBUTION DETAILS

10.1 Controlled copy of this SOP shall be distributed to Quality Assurance and Microbiology.

11.0 REVISION HISTORY

Supersedes SOP No.	Change Control No.	Reason for revision
NA	NA	NA

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ANNEXURE I
REPORT FOR ESTIMATION OF ENDOTOXIN LOG REDUCTION

1. Cycle Detail:

Equipment Name		Equipment ID.	
Load Detail			
Trial No.		Start time	
Date		End time	

2. Information of spiked End toxin Indicator vials:

Endotoxin Indicator Make	
Lot No.	
Labeled Potency / Vial	
Exp. Date	
Reconstituted On	
Reconstitution volume / vial	
Concentration after reconstitution	
Vial size used for spiking	
Spiking volume in each vial	
Concentration in each spiked vial	
Prepared by	
Date of Preparation	

3. Reagent Detail:

Reagent Detail					
LAL		**CSE**		**LRW**	
Make		Make		Make	
Sensitivity (λ)		EU / ml		Lot No.	
Lot No.		Lot No.		Exp. Date	
Exp. Date		Exp. Date		Opened On	
Reconstituted On		Reconstituted On			

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4. Reconstitution and Dilution:

4.1. Processed Endotoxin vial(Test):

Assumed Log reduction after processing: Assumed concentration / vial:

Reconstitution volume / vial: Concentration after re-constitution:

No. of vials tested:

Dilution details of each Processed Endotoxin vial				
Tube No.	Volume of Endotoxin solution	Volume of LRW added	Assumed Concentration after dilution	Total dilution
01.	0.2 ml from reconstituted vial	1.8 ml	< 1 EU/ml	1:10
02.	1.0 ml from Tube No.1	1.0 ml	< 0.5 EU/ml	1:20
03.	1.0 ml from Tube No.2	1.0 ml	< 0.25 EU/ml (2λ)	1:40
04.	1.0 ml from Tube No.3	1.0 ml	< 0.125 EU/ml (λ)	1:80

4.2. Unprocessed Endotoxin vial (Positive control)

No. of vials tested: Assumed concentration / vial:

Reconstitution volume / vial: Concentration after re-constitution:

Dilution details of Unprocessed Endotoxin vial				
Tube No.	Volume of Endotoxin solution	Volume of LRW added	Assumed Concentration after dilution	Total dilution
01.	0.1 ml from re-constituted vial	0.9 ml	250 EU/ml	1:40
02.	0.1 ml from Tube No.1	0.9 ml	25 EU/ml	1:400
03.	0.1 ml from Tube No.2	0.9 ml	2.5 EU/ml	1:4000
04.	0.2 ml from Tube No.3	1.8 ml	0.25 EU/ml (2λ)	1:40000
05.	1.0 ml from Tube No.4	1.0 ml	0.125 EU/ml (λ)	1:80000
06.	1.0 ml from Tube No.5	1.0 ml	0.06 EU/ml (0.5λ)	1:160000
07.	1.0 ml from Tube No.6	1.0 ml	0.03 EU/ml (0.25λ)	1:320000

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5. Recovery:

5.1. Processed vial sample:

Test Dilution tested →										
Incubation Time										
Vial No.↓	**Rep.1**	**Rep.2**	**Rep.1**	**Rep.2**	**Rep.1**	**Rep.2**	**Rep.1**	**Rep.2**	**Rep.1**	**Rep.2**
01										
02										
03										
04										
05										
06										
07										
08										
09										
Blank										
Positive control										

5.2. Unprocessed vial sample:

Observations									
Test Dilutions →	2 λ		λ		0.5λ		0.25λ		Last dilution of the sample that was positive
Vial No. ↓	**Rep.1**	**Rep.2**	**Rep.1**	**Rep.2**	**Rep.1**	**Rep.2**	**Rep.1**	**Rep.2**	
01									

Expression of observation: '–Ve' for 'No Gel (Negative)', '+Ve' for 'Gel (Positive)'

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6. Recovery calculation:

6.1. Processed vial sample

Recovery Calculation				
Vial No.	Reciprocal of Last dilution of the sample that was tested	Sensitivity of LAL	Recovery (EU)	Log for Recovery
01	X			
02	X			
03	X			
04	X			
05	X			
06	X			
07	X			
08	X			
09	X			

6.2. Unprocessed vial sample:

Recovery Calculation				
Vial No.	Reciprocal of Last dilution of the sample that was positive	Sensitivity of LAL	Recovery (EU)	Log for Recovery
01	X			

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7. Log reduction:

* Vial No.	**Log of Recovery from Un-processed sample	Log of Recovery from processed sample	Log Reduction	Result
01	-			Pass / Fail
02	-			Pass / Fail
03	-			Pass / Fail
04	-			Pass / Fail
05	-			Pass / Fail
06	-			Pass / Fail
07	-			Pass / Fail
08	-			Pass / Fail
09	-			Pass / Fail

- This symbol indicates subtraction
* Vial numbers in this column are for processed vials

** Recovery from unprocessed sample is for one vial only

1) **Acceptance criteria:** All the spiked vials should show more than 3 log reduction.
2) **Final result: Pass/ fail.**
3) **Remarks:**_____
_____

Performed by: **Reviewed by:**

Date: **Date**