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1.0 OBJECTIVE:

To lay down a procedure for Bacterial Endotoxins Test.

2.0 SCOPE:

This SOP is applicable for Bacterial Endotoxins Test in Microbiology Department.

3.0 RESPONSIBILITY:

Operating Person: Microbiologist

4.0 ACCOUNTABILITY:

Head QC

5.0 ABBREVIATIONS:

µl	Micro Liter
CSE	Control Standard Endotoxin
LAL	Limulus Amaebocyte Lysate
LRW	LAL Reagent Water
Ltd.	Limited
ml	Mille Liter
No.	Number
NPC	Negative Product Control
NWC	Negative Water Control
PPC	Positive Product Control
PWC	Positive Water Control
QA	Quality Assurance
QC	Quality Control
SOP	Standard Operating Procedure
USP	United State Pharmacopeia
DHS	Dry heat sterilizer

6.0 PROCEDURE:

6.1 The Bacterial Endotoxin Test (BET) is a test to detect or quantify endotoxins from gram –negative bacteria using amaebocyte lysate from the horseshoe crab (Limulus polyphemus or Tachypleus tridentatus).There are three techniques for this test: The Gel clot technique, which is based on gel formation, The turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate, and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex.

6.2 Receive the LAL Reagent through supplier and make an entry of receipt of reagent in Titled **"Lysate Receipt and Consumption Record"** in Respective **Annexure-III**,

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6.3 Prerequisite for Bacterial Endotoxins Test:

S.No.	Requirements
1.	Sample for Bacterial Endotoxins Test
2.	Calibrated Micropipette 20-200 Micro liter & 100-1000 Micro liter
3.	Depyrogenated Micropipette Tips 20-200 Micro liter & 100-1000 Micro liter
4.	Depyrogenated Test Tube-10x75 mm
5.	Depyrogenated Vial/ Depyrogenated Test Tube-12x75 mm
6.	Depyrogenated Vial Opener
7.	Vortex
8.	Calibrated Heating Block
9.	Lal Reagent Water
10.	Control Standard Endotoxins
11.	Limulus Amaebocyte Lysate
12.	Beaker

6.4 Switch **"ON"** the Heating Block and set the Temperature at 37°C.

6.5 Preparation of Control Standard Endotoxin (CSE):

6.5.1 Receive the Control Standard Endotoxin (CSE) through supplier and make an entry of receipt in Titled **"Control Standard Endotoxin (CSE) Receipt and Consumption Record"** in Respective **Annexure–VII,** and Reconstitute CSE in LRW as per Manufacturer's Instruction.

6.5.2 Use Reconstituted CSE as per Manufacturer's Instruction after Reconstitutions; store Reconstituted CSE at 2°C – 8°C Temperature or as per Manufacturer's Instructions.

S.No.	Endotoxin	LRW	Endotoxin Concentration (EU/ml)
1.	100 EU/vial	5 ml	20 EU/ml
2.	0.1 ml of 20 EU/ml	0.9 ml	2 EU/ml
3.	0.5 ml of 2 EU/ml	0.5 ml	1 EU/ml (8λ)
4.	0.5 ml of 1 EU/ml	0.5 ml	0.5 EU/ml (4λ)
5.	0.5 ml of 0.5 EU/ml	0.5 ml	0.25 EU/ml (2λ)
6.	0.5 ml of 0.25 EU/ml	0.5 ml	0.125 EU/ml (λ)

7.	0.5 ml of 0.125 EU/ml	0.5 ml	0.06 EU/ml (λ/2)
8.	0.5 ml of 0.06 EU/ml	0.5 ml	0.03 EU/ml (λ/4)
9.	0.5 ml of 0.03 EU/ml	0.5 ml	0.015 EU/ ml (λ/8)
10.	0.5 ml of 0.015 EU/ml	0.5 ml	0.007 EU/ ml (λ/16)

6.5.3 Prepare further dilutions as given below (in case of 100 EU/vial).

6.5.4 The supplied COA is specific for the lysate lot and CSE lot. So use the same lot for testing.

6.5.5 After end of CSE solution should be deactivated by the depyrogenation of CSE vial at 250˚C for 01 hour in dry heat sterilizer/Depyrogenator.

6.6 Reconstitution of Lysate:

6.6.1 Reconstitute the lysate by opening the aluminum seal. Collect lysate powder into the bottom of the vial by tapping on a hard surface and then open the cap slowly.

6.6.2 As per manufacturer's instruction, add LRW to lysate vial by avoiding direct contact with fingers and close the cap immediately. Do not vortex lysate.

6.6.3 Reconstituted lysate shall be stored at 2°C – 8°C in refrigerator and to be used within 24 hrs. of reconstitution.

6.7 Confirmation of The Labeled Lysate Sensitivity:

6.7.1 Confirmation of the lysate sensitivity must be carried out when a new batch of lysate is use or when there is any change in the experimental conditions which may affect the outcome of the test or any alteration in test result. Confirm in four replicates the labeled sensitivityλ, expressed in EU/ml or IU/ml, of the lysate solution prior to use in the test.

6.7.2 Take 20 Depyrogenated Assay Tubes and label the tubes by numbering and arrange quadruplicate in stand and proceed the test as per mentioned below:

Tubes	CSE Dilution Used	LRW	Lysate in µl	No. of Replicates
2 λ	100 µl of 2 λ	–	100 µl	4
λ	100 µl of λ	–	100 µl	4
λ/2	100 µl of λ/2	–	100 µl	4
λ/4	100 µl of λ/4	–	100 µl	4
Negative water control (NWC)	–	100 µl	100 µl	4

6.7.3 Pipette 100 µl diluted CSE i.e. to 2λ, λ, λ/2 and λ/4 separately into depyrogenated assay tubes. For NWC use 100 µl of LRW.

6.7.4 Add 100 µl of reconstituted lysate into each tube.

6.7.5 Incubate the tubes in heating block at 37°C±1°C for 60 ± 2 minutes, avoiding vibration.

6.7.6 After incubation, take each tube and invert through approximately 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed.

6.7.7 The test is not valid unless the lowest concentration of the standard solutions shows a negative result in all replicate tests.

6.7.8 The endpoint is the last positive result in the series of decreasing concentrations of endotoxin. Calculate the mean value of the logarithms of the end-point concentrations and then the

$$\text{Geometric mean end - point concentration} = \text{antilog}\frac{\sum e}{f}$$

$\sum e$ = sum of the log end-point concentrations of the dilution series used,

f = number of replicates.

antilogarithm of the mean value using the following expression.

6.7.9 The geometric mean end-point concentration is the measured sensitivity of the lysate solution (EU/ml or IU/ml). The Lysate sensitivity should not less than λ/2 and not more than 2λ.

6.7.10 Record the Consumption Details of Lysate in Titled "Lysate Receipt And Consumption Record**"** in Respective **Annexure-III.**

6.7.11 Record the details of Lysate Sensitivity in, Titled **"Lysate Sensitivity Record"** in Respective **Annexure- IV.**

6.8 Calculation for Determining the Endotoxin Limit:

6.8.1 The Endotoxin limit for active substances administered parenterally, Define on the Basis of Dose, is equal to

Endotoxin Limit = K/M

K = Threshold pyrogenic dose of Endotoxin per kilogram of body mass in a single hour

period. 5 EU/kg body weight for parenteral drugs except those administered intrathecally 0.2 EU/kg for intrathecal drugs.

M = Maximum recommended dose of product per Kilogram of body mass in a single hour period. (For this calculation, it is assumed that the average person weight 70 kg. if pediatric dose is higher, it shall be used in the calculation)

6.8.2 The Endotoxin limit of active substances administered parenterally is specified in Unit such as IU/ml, IU/mg, IU/unit of biological activity.

6.8.3 Record the Endotoxin Limit in Titled **"Determination of Endotoxin Limit"** in Respective **Annexure- VI**.

6.9 Maximum Valid Dilution (MVD):

6.9.1 The maximum valid dilution is the maximum allowable dilution of a sample at which the Endotoxin limit can be determined. Determine the MVD using the Following Formulae.

$$MVD = \frac{\text{Endotoxin Limit X Potency of Product}}{\lambda \text{ (Lysate Sensitivity)}}$$

6.10 Test Procedure:

6.10.1 Record the Sample Receiving details in Titled **"Sample Receiving and Analysis Record For Bacterial Endotoxins Test"** in Respective **Annexure-V.** Perform the test at MVD/2 or appropriate MVD.

6.10.2 Take 8 depyrogenated tubes and label the two tubes each as product with batch number, NPC, PPC, PWC and NWC. Arrange the tube in stand and label as per mentioned below:

Solution Description	LRW in µl	Product Dilution	4λ (CSE) in µl	Lysate in µl	No. of Replicates
Negative Product Control (NPC)	50	50	-	100	2
Positive Product Control (PPC)	-	50	50	100	2
Positive Water Control (PWC)	50	-	50	100	2
Negative Water Control (NWC)	100	-	-	100	2

6.10.3 Add 50µl of LRW in product tube and PWC, and 100 µl in NWC. Immediately add 50 µl of sample, which is diluted at MVD or appropriate in sample tubes and PPC, and then add 50µl of CSE that is diluted to 4λ in a PPC and PWC.

6.10.4 Finally add 100µl of lysate in all tubes and incubate in heating block, where the temperature is maintained at 37 $\pm 1^0$C for 60 ± 2 minutes

6.10.5 Record the result of WFI /Pure Steam condensate in Titled **"Bacterial Endotoxins Test Report For WFI / Pure Steam condensate"** in Respective **Annexure-I**.

6.10.6 Record the result of Product in Titled **"Bacterial Endotoxins Test Report"** in Respective **Annexure-II**.

6.10.7 Bacterial Endotoxin Test Procedure for Rubber Bung:

6.10.7.1 Sample Preparation:-Prepare the sample solution by using LAL reagent water. If necessary, adjust the pH of the test solution so that the pH of the mixture of the lysate and the test solution falls within the pH range specified by the lysate reagent. This is usually applies to a product with a pH in the range of 6.0 to 8.0. The pH may be adjusted by the use of acid, base (prepared in LRW) or a suitable Buffer, as recommended by the lysate manufacturer. Buffer must be validated to be free of detectable Endotoxin and interfering factors.

6.10.7.2 Take required numbers of rubber bungs in a Depyrogenated Test Tube add 4 ml LRW and vortex the tube for five minutes

6.10.7.3 After vortexing performed the test as per Point No 6.10.2.

6.10.8 Bacterial Endotoxin Test Procedure for Depyrogenated Vials , Ampoule and Disposable Syringe:

6.10.8.1 Take required Vial/ ampoule / disposable syringe and take appropriate volume of LRW as per size of vial/ ampoule/disposable syringe after adding LRW vortex and composite the LRW of all vial/ ampoule/disposable syringe in a separate Depyrogenated test-tube after that vortex the test-tube for two to Five minutes

6.10.8.2 After vortexing performed the test as per Point No 6.10.2

6.11 Bacterial Endotoxins Test frequency of Water for Injections and Pure Steam condensate as per Annexure VIII and rest of the sampling and analysis of Water for Injections and Pure Steam condensate shall be performed as per water sampling schedule.

6.12 Precautions:

6.12.1 Depyrogenated assay and dilution tubes shall be used for BET analysis.

6.12.2 Rehydrated CSE shall be stored as per vender COA instructions.

6.12.3 The supplied COA is specific for Lysate Lot & CSE Lot. So use the same lot for testing.

6.12.4 Do not vortex the Lysate

6.12.5 Micropipette should be calibrated.

6.13 Results/Acceptance Criteria:

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6.13.1 After incubation each tube is interpreted as either positive or negative, positive test indicates the formation of firm gel capable of maintaining its integrity when the test tube is inverted at upside down i.e. 180°.

6.13.2 Negative test is characterized by the absence of gel or by the formation of a viscous mass, which does not hold when the tube is inverted at upside down i.e. 180°.

6.13.3 The test is considered when both replicates PPC & PWC are positive and NPC & NWC is negative.

6.13.4 The preparation being examined complies with the test when a negative result is found for both replicates of sample.

6.13.5 When a positive result is found for both replicates of sample, it does not comply with the test.

6.13.6 Repeat the test if a positive result is found for one replicate of sample and a negative result is found in another replicate.

6.13.7 The preparation being examined complies with the test, if a negative result is found for both replicates sample in the repeat test.

6.13.8 When positive result observed on both the tubes of Test Preparation, Investigate the cause of its Failure by checking following parameters.

6.13.9 Check Product Dilution, CSE Dilution and Lysate Dilution and Storage Condition.

6.13.10 Check Sensitivity Record of Lysate Lot and matched CSE.

6.13.11 Check Heating Block Temperature and Calibration.

6.13.12 Check Micropipette Calibration.

6.13.13 Check pH of the solution.

6.13.14 Report No. shall be recorded according to BTYYYYMMDDNNN.

For example,

BT : Bacterial Endotoxin test
YYYY : Year
MM : Month
DD : Date
NNN : Serial No. of report that day (001)
(Complete report No.: BT20170713001)

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7.0 ANNEXURES:

ANNEXURE No.	TITLE OF ANNEXURE	FORMAT No.
Annexure – I	Bacterial endotoxins test report for WFI / pure steam condensate	
Annexure - II	Bacterial endotoxins test report	
Annexure – III	Lysate receipt and consumption record	
Annexure – IV	Lysate sensitivity record	
Annexure – V	Sample receiving and analysis record for bacterial endotoxins test	
Annexure – VI	Determination of endotoxin limit	
Annexure – VII	Control standard endotoxin (CSE) receipt and consumption record	
Annexure – VIII	Bacterial endotoxins test frequency of water for injections and pure steam condensate	
Annexure – IX	Lal Reagent Water (LRW)Receipt And Consumption Record	

ENCLOSURES: SOP Training Record.

8.0 DISTRIBUTION:

- Controlled Copy No. 01 Quality Assurance
- Controlled Copy No. 02 Microbiology
- Master Copy Quality Assurance

9.0 REFERENCES:

USP-38 (<85> Bacterial Endotoxin Test)

10.0 REVISION HISTORY: CHANGE HISTORY LOG

Revision No.	Change Control No.	Details of Changes	Reason for Change	Effective Date	Updated By

PHARMA DEVILS

MICROBIOLOGY DEPARTMENT

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ANNEXURE-I
BACTERIAL ENDOTOXIN TEST REPORT FOR WFI / PURE STEAM CONDENSATE

A. R. No.						
Sampling Point No.						
Date of Sampling		**Sample Qty. at Each Point**				
Date of testing		**Date of Release**				
Shift		**Micropipette Id No.**				
Endotoxin Limit	NMT - 0.25EU/ml	**Depyrogenation Cycle No.**				
DHS ID No		**Heating Block ID No**				

Reagent Details:

Reagent Details	Lysate	CSE	LRW
Lot No.			
Sensitivity/Potency			
Date of Reconstitution /Opening			
Use Before			
Expiry Date			
Manufacturer			

MVD =			**MVD/ 2Value =**	
			MVD/4 Value =	
S.No.	**Product Dilution**	**Product**		**LRW**
S.No.	**CSE Dilution**	**CSE Concentration**	**CSE Volume**	**LRW**
Heating Block Temperature	Start time temp.	End time temp.	**Incubation Time**	60 ± 2 Minutes
Incubation Started at			**Incubation Completed at**	

Observation Table:

Sampling Point No.												
Observation	**Tube1**	**Tube2**	**Tube1**	**Tube2**	**Tube1**	**Tube2**	**Tube1**	**Tube2**	**Tube1**	**Tube2**	**Tube1**	**Tube2**
Negative Product Control **(NPC)**												
Positive Product control **(PPC)**												
Negative Water Control **(NWC)**												

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Positive Water Control **(PWC)**	

+ve: Gel formation -ve : No Gel formation

DILUTION TABLE:

Solution	Tube No.	LRW	Product Dilution	Control Standard Endotoxin(4λ)	LAL Reagent	Total Volume
Negative Product Control (NPC)	01	50µL	50µL	–	100µL	200µL
	02	50µL	50µL	–	100µL	200µL
Negative Control (NWC)	01	100µL	–	–	100µL	200µL
	02	100µL	–	–	100µL	200µL
Positive Control (PWC)	01	50µL	–	50µL	100µL	200µL
	02	50µL	–	50µL	100µL	200µL
Positive Product Control (PPC)	01	–	50µL	50µL	100µL	200µL
	02	–	50µL	50µL	100µL	200µL

+ve: Gel formation -ve : No Gel formation

Remark: The sample complies/ does not comply as per specification.

Microbiologist:
Date:

Checked By:
Date:

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ANNEXURE-II
BACTERIAL ENDOTOXINS TEST REPORT

Name of Product		**A.R. No.**	
Batch No.		**Sample Quantity**	
Date of Receiving		**Date of Analysis**	
Shift		**Date of Release**	
Endotoxin Limit		**Depyrogenation Cycle No.**	
DHS ID No		**Heating Block ID No**	
Micropipette ID. No			

REAGENT DETAILS:

REAGENT DETAILS	LYSATE	CSE	LRW
Lot No.			
Sensitivity/Potency			
Date of Opening			
Use Before			
Expiry Date			
Manufacturer			

MVD =			**MVD/ 2Value =**	
			MVD/4 Value =	
S.No.	**Product Dilution**	**Product**		**LRW**
S.No.	**CSE Dilution**	**CSE Concentration**	**CSE Volume**	**LRW**
Heating Block Temperature	Start time temp.	End time temp.	**Incubation Time**	60 ± 2 Minutes
Incubation Started at			**Incubation Completed at**	

DILUTION TABLE:

Solution	Tube No.	LRW	Product Dilution (……)	Control Standard Endotoxin (4 λ)	LAL Reagent	Total Volume	Observation
Negative Product Control	01	50 µL	50µL	–	100µL	200µL	
	02	50 µL	50µL	–	100µL	200µL	

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(NPC)							
Negative Water Control (NWC)	01	100µL	–	–	100µL	200µL	
	02	100µL	–	–	100µL	200µL	
Positive Water Control (PWC)	01	50 µL	–	50 µL	100µL	200µL	
	02	50 µL	–	50 µL	100µL	200µL	
Positive Product Control (PPC)	01	–	50µL	50µL	100µL	200µL	
	02	–	50µL	50µL	100µL	200µL	

+ve: Gel formation
-ve: No Gel formation

Remark: The sample complies/ does not comply as per specification.

Microbiologist:
Date:

Checked By:
Date:

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ANNEXURE-III
LYSATE RECEIPT AND CONSUMPTION RECORD

Date	Quantity Received	Lysate Lot No.	Expiry Date	Vial No./Lot Quantity	Opening Date	Balance Quantity	Used By Sign & Date	Checked By Sign &Date

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ANNEXURE-IV
LYSATE SENSITIVITY RECORD

REAGENT DETAILS	LYSATE	CSE	LRW
Lot No.			
Sensitivity/Potency			
Date of Opening			
Use Before			
Expiry Date			
Manufacturer			

Instrument Details:

Micropipette ID No		**Date of Calibration**		**Next Due Date**	
Micropipette ID No		**Date of Calibration**		**Next Due Date**	
Heating Block ID No		**Date of Calibration**		**Next Due Date**	
DHS ID No		**Date of Calibration**		**Next Due Date**	

CSE DILUTION:

S.No.	CSE Dilution	CSE Concentration	CSE Volume	LRW

Heating Block Temperature	37°C ± 1°C		**Incubation Time**	60 ± 2 Minutes
	Temperature	Start Time:		
		End Time:		
Incubation Started at			**Incubation Completed at**	

OBSERVATIONS:

Tube No.	Endotoxin Concentration						NWC	Test End Point EU/ml	Log of End Point	Geometric mean end point
1										
2										
3										
4										

+ ve = Gel Formation - ve = No Gel Formation

Remarks: The lysate lot complies / does not comply for its sensitivity as mentioned.

Microbiologist: **Checked By:**
Date: **Date:**

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ANNEXURE-V
SAMPLE RECEIVING AND ANALYSIS RECORD FOR BACTERIAL ENDOTOXIN TEST

Sample Name	Batch No.	A.R No	Sample Qty	Receiving Date	Testing Date	Release Date	Tested By Sign & Date	Report Received by Sign & Date

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ANNEXURE-VI
DETERMINATION OF ENDOTOXIN LIMIT

Product Name		**API**	
Product Concentration		**Test Concentration**	
Rout of Administration	IM/IV/ Intrathecally	**Product Safety Factor**	
Threshold Pyrogenic Dose of Endotoxin		**Maximum Recommended Dose**	

Determination of Endotoxin Limit:

Determination of Test Concentration:

Determination of Product Safety Factor:

Final Endotoxin Limit:

Remarks: The Final Endotoxin Limit of_____is _____ IU/EU/mg/ml.

Microbiologist: **Checked By:**
Date: **Date:**

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ANNEXURE-VII
CONTROL STANDARD ENDOTOXIN (CSE) RECEIPT AND CONSUMPTION RECORD

Date	Quantity Received	CSE Lot No.	Expiry Date	Used Quantity in µl /ml	Opening Date	Dehydrated Balance Quantity	Reconstituted Balance Quantity µl /ml	Used By Sign & Date	Checked By Sign & Date

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ANNEXURE-VIII
BACTERIAL ENDOTOXINS TEST FREQUENCY OF WATER FOR INJECTIONS AND PURE STEAM CODENSATE

S.No.	Location	Sampling point	Sample Point No.	Frequency
1.		After Distribution		Daily
2.		In return loop line after conductivity		Daily
3.		MCDP(WFI final out let)		Daily
4.		In return loop line after conductivity		Daily
5.		MCDP Out Let Line (WFI)		Daily
6.		After distribution pump		Daily
7.		In return loop line after conductivity		Daily
8.		Manufacturing 01		Fortnightly
9.		Manufacturing 02		Fortnightly
10.		Disinfectant Preparation		Fortnightly
11.		Equipment Washing		Fortnightly
12.		Filtration 01		Fortnightly
13.		Filtration 02		Fortnightly
14.		Filling 01		Fortnightly
15.		Filling 02		Fortnightly
16.		MCDP of 2nd Column		Fortnightly
17.		MCDP of 3rd Column		Fortnightly
18.		MCDP of 4th Column		Fortnightly
19.		MCDP of 5th Column		Fortnightly
20.		MCDP of 6th Column		Fortnightly
21.		MCDP of 7th Column		Fortnightly
22.		MCDP of 8th Column		Fortnightly
23.		Pure Steam		Daily
24.		Pure Steam		Weekly

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ANNEXURE-IX
LAL REAGENT WATER RECEIPT AND CONSUMPTION RECORD

Date of Receiving	Quantity Received	LRW Lot No.	Expiry Date	Opening Date	Balance Quantity	Opened By Sign & Date	Checked By Sign &Date