National Pharmaceutical Control Bureau
MINISTRY OF HEALTH MALAYSIA

World Health Organization
WHO Collaborating Centre for Regulatory Control of Pharmaceuticals

PIC/S
Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme

QUALITY SYSTEM
SIRIM
Certified to ISO 9001:2000
Cert. No: AR 2293

STANDARDS MALAYSIA
ACCREDITED LABORATORY
MS ISO/IEC 17025:2005
NO: [illegible]

BACTERIAL ENDOTOXIN TEST

Centre for Quality Control

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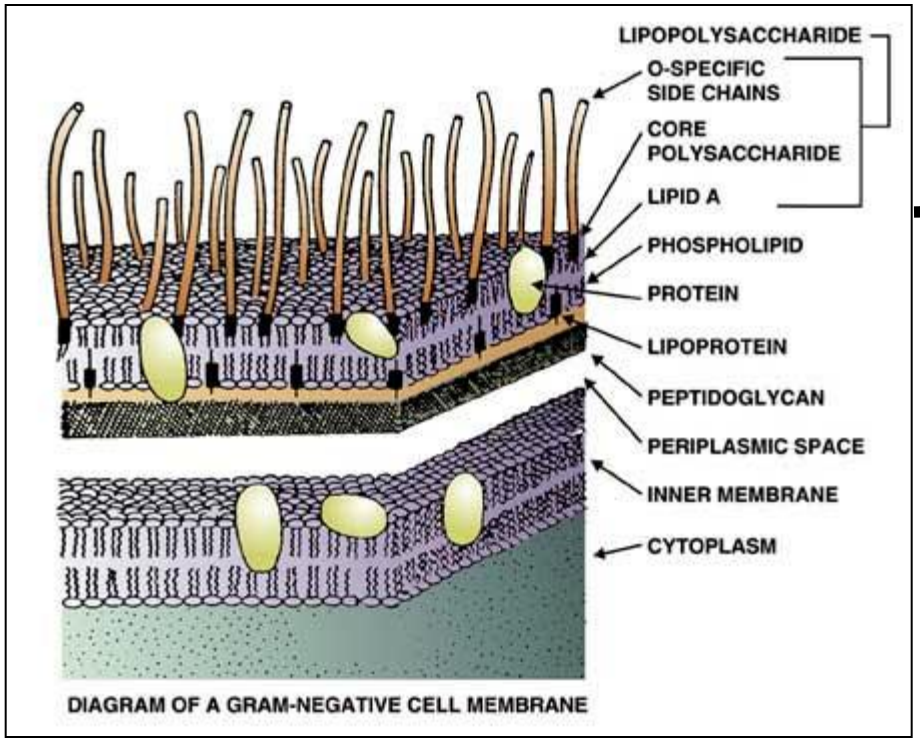
Overview of presentation

- Introduction
  - Bacteria endotoxin definition, effects of contamination
- Bacteria Endotoxin Test (BET)/LAL Test
- Types of BET / LAL test
- Documents for submission
  a) Gel-clot Method
  b) Photometric Method : Chromogenic Method

Introduction

- **Endotoxin :**
  - Endotoxin (a.k.a lypopolysaccharide), is a pyrogenic substance that is found in the cell wall of Gram-negative bacteria
  - Pyrogenic substance (or pyrogen) can induce fever when injected into the blood or cerebrospinal fluid
- It is associated with **injectable products**
- Sterile production procedures are needed
- Sterilization does not remove the endotoxin
- It is heat stable

Diagram of a gram negative cell membrane



Endotoxin

Consequences of endotoxin cotamination:

- Fever
- Headache
- Chills
- Nausea/Vomiting
- Hypotension
- Acute lung injury
- Miscarriage
- Death

Bacteria endotoxin test

- Bacterial endotoxin test (aka LAL test):

  To detect or quantify endotoxin of gram negative bacterial origin using amoebocyte lysate from horseshoe crab (Limulus polyphemus or Tachypleus tridentatus)

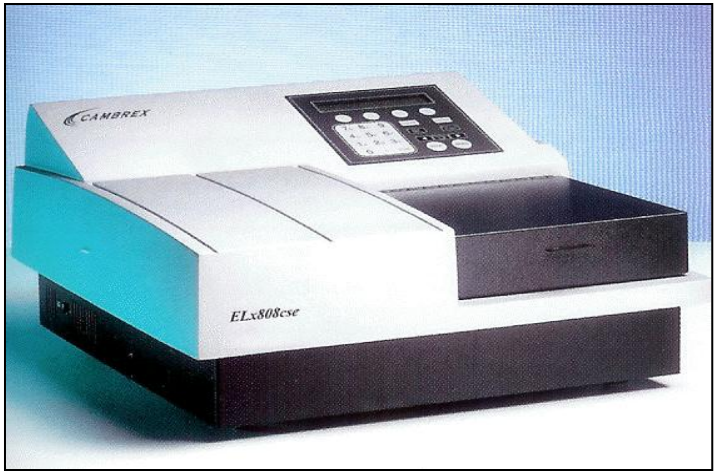
Horseshoe Crab

Types of lal test

Methods:-

i. **Gel clot**
   a) Gel clot (Limit test)
   b) Gel clot (Semi-quantitative test)

ii. **Photometric**
   a) Chromogenic (Kinetic)
   b) Turbidimetric (Kinetic)
   c) Chromogenic (End-point)
   d) Turbidimetric (End-Point)



Documents for submission

I. Certificate of analysis
II. CoA for reagents
III. Protocol of analysis
IV. Calculation (MVD and ELC)
V. Validation data
VI. Routine tests result

I. Coa for finished products

- Local manufacturer – CoA for 1 batch of finished products
- Oversea manufacturer – CoA for 3 batches of finished products
- Must contain (in relation to LAL test):
  - Product name and strength
  - Batch number
  - Specification for BET
  - Results for BET
  - Appearance
  - Ph
  - Name, signature and date of approval

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CERTIFICATE OF ANALYSIS FOR FINISHED PRODUCT

PRODUCT: Vaxcel Omeprazole 40 mg Injection	CONTENT (S): Each vial contains: Omeprazole Sodium equivalent to Omeprazole 40 mg
PRODUCT CODE: VXC 47	
BATCH NO: T0809032A	MANUFACTURING DATE: September 08 EXPIRY DATE: September 10

NO	TEST	SPECIFICATIONS	REFERENCE	RESULT
1	Description	A white, hygroscopic powder	Manufacturer	Complies
2	Completeness and clarity of solution	Meets the requirement.	USP30	Complies
3	pH	9.0 – 12.0	Manufacturer	11.0
4	Water Content	Not more than 15.0%	Manufacturer	3.42%
5	Identification • HPLC	The retention time of test solution should be concordant with that of the reference solution.	Manufacturer	Complies
6	Related Substance	Individual impurity : Not more than 1.0% Total impurities : Not more than 2.0%	Manufacturer	Unknown 1 : 0.16% Unknown 2 : 0.83% Total : 0.99%
7	Assay	26.0– 36.0% of stated amount	Manufacturer	35.5%
8	Particulate Matter	≥10µm; ≤ 6000 cts/vial ≥ 25µm; ≤ 600 cts/vial	USP30	1295 6
9	Sterility	No growth	USP30	Pass
10	Bacterial Endotoxin	Not more than 2.0 EU/mg	Manufacturer	0.0375

Prepared by:	Approved by:
NAME: DESIGNATION: DATE: 19/9/08	NAME: DESIGNATION: DATE: 200409

Product name & strength

Batch number

Physical appearance

pH

Limit for BET

Results

Signature, name & date of approval

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II. Coa for reagents

CERTIFICATE OF ANALYSIS

VIAL CONTENTS: Endosafe® Control Standard Endotoxin is prepared from *E. coli* strain 055:B5. Each vial contains 10 ng of purified Lipopolysaccharide, freeze dried in a stabilized matrix.

RSE/CSE RATIO: The potency of this standard in Endotoxin units, (EU) has been determined to be 20 EU/ng by the method described in Appendix C (***Gel-clot Technique***) of the GUIDELINE ON VALIDATION OF THE LIMULUS AMEBOCYTE LYSATE TEST AS AN END PRODUCT ENDOTOXIN TEST FOR HUMAN AND ANIMAL PARENTERAL DRUGS, BIOLOGICAL PRODUCTS, AND MEDICAL DEVICES, published by the U.S. Food and Drug Administration.

CSE Lot: EX83372 LAL Reagent Lot: A2252L RSE Lot: EC-6-3

RSE/CSE Ratio: 20 EU/ng Vial contents: 200 EU/vial

Geometric Mean Sensitivity with RSE: 0.03 EU/mL

Lysate & control standard endotoxin (CSE)

IS/CSE RATIO: The Expert Committee on Biological Standardization of WHO has assigned a potency of the IS as 10,000 IU per vial of IS, so that 1 IU = 1 EU. The potency of this endotoxin standard in International (Endotoxin) Units, IU, has been designated as 20 IU/ng.

DIRECTIONS FOR USE: Reconstitute the lyophilized material with 5.0 mL of LAL reagent grade water to obtain 40 EU/mL or 40 IU/mL. Vortex mix vigorously for at least 5 minutes after rehydration, and for at least 1 minute immediately prior to each use.

STORAGE: Store rehydrated material at 2-8°C for up to 4 weeks. Store lyophilized material at controlled room temperature or refrigerated as preferred. Diluted endotoxin should not be stored except under validated conditions.

CAUTION: DO NOT FREEZE ENDOTOXIN SOLUTIONS

Signature: [redacted] Date: 11/05/2009

Recived by P.S.P

[redacted]

Iii. Protocol of analysis

A complete protocol of analysis contains:-

A. List of equipments, glassware and reagents used

B. Directions of use for reagents – LAL reagent and CSE

C. Preparation of endotoxin standards

D. Preparation of samples

E. Test methods (how the test is performed)

✓ Standard operating procedure is acceptable except for sample preparation

a. List of equipments, glassware and reagents

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FOR RESTRICTED CIRCULATION

[redacted] STANDARD TEST PROCEDURE

STP No.: ST624-04

TEST [redacted]

REFERENCE : USP			EFFECTIVE DATE : 19.04.2010		
SUPERSEDES : ST624-03			PAGE No. : 1 of 8		
	PREPARED BY	REVIEWED BY	APPROVED BY		
DEPT	QC	DRA	R & D	QC	QA
SIGN	[redacted]				
NAME					
DATE					

1. **BACTERIAL ENDOTOXINS TEST**

1.1 **Introduction:**

This is a very sensitive test and hence requires great care. Depyrogenate all the glassware by thoroughly cleaning and rinsing 10 to 15 times with freshly collected hot water for injection, wrap in aluminum foil and depyrogenate in the hot air oven as per the validated cycle time.

Wear gloves and mask while performing the BET test. Perform the test under LAF.

1.2 **Equipments and Reagents Required**

1) Vortex Mixer
2) BET incubator (Validated)
3) Glass Pipettes depyrogenated
4) i) Test tubes (for Assay) depyrogenated : 10 x 75mm
   ii) Test tubes (for Dilutions) depyrogenated : 20 x 150mm
5) Aluminium Foil depyrogenated
6) Rubber Bulb
7) Test Tube Stands
8) Parafilm
9) Micropipette (calibrated)
10) LAL reagent
11) LAL reagent water
12) Control standard endotoxin (CSE)
13) Timer (Calibrated)

Note: Do not use plastic test tubes and pipettes.

The glassware must be depyrogenated
Any plastic apparatus must be pyrogen-free

b. Directions of use for reagents

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Annexure XVI SOP QC174

FOR RESTRICTED CIRCULATION

[redacted]

STANDARD TEST PROCEDURE

STP No.: ST624-04

TEST	: [redacted]				
REFERENCE : USP			EFFECTIVE DATE	: 19.04.2010	
SUPERSEDES : ST624-03			PAGE No.	: 2 of 8	
	PREPARED BY	REVIEWED BY	APPROVED BY		
DEPT	QC	DRA	R & D	QC	QA
SIGN	[redacted]				
NAME					
DATE					

Volume of LRW used for reconstitution

1.3 Directions for use of the LAL Reagent:

Tap the lyophilized vial gently for the powder to collect at the bottom.

Detach the aluminium crimp.

Remove the stopper of the lyophilized vial carefully without spurting the lyophilized reagent. While placing the stopper on the clean surface of the table, keep the stopper plunger upwards.

Reconstitute the lysate with appropriate volume of LRW (5.2 mL for a 50 test vial)

Apply the stopper and gently swirl the contents.

Note: Lysate should not be vortexed.

1.4 Control Standard Endotoxin (CSE):

The CSE has a predetermined amount of endotoxin, as described in the certificate of analysis (COA), which is standardized against the U.S reference standard Endotoxin. The CSE is specific to a lysate lot No. The COA must be verified for matching lot numbers of CSE and LAL reagent and maintained in a file.

Tap the lyophilized vial gently for the powder to collect at the bottom.

Detach the aluminum crimp.

Remove the stopper of the lyophilized vial carefully without spurting the lyophilized reagent. While placing the stopper on the clean surface of the table, keep the stopper plunger upwards.

Reconstitute the CSE by adding the appropriate volume (as per COA) of LRW using a depyrogenated pipette. Vortex the reconstituted CSE for 5 min. This is the stock solution.

While making further dilutions from the stock solution, the solution should be vortex for a minimum of 30 seconds.

1.5 Verification of Label Claim Sensitivity

The labeled sensitivity must be confirmed for each new lot of the LAL reagent received as per SOP QC099 and use the lot, which conforms to the requirements.

c. Preparation o endotoxin standards

- **European Pharmacopeia 5.0, 2.6.14 Bacterial endotoxins: 1. Preparatory Testing (i) Confirmation of labeled lysate sensitivity**

Gel clot method : min of 4 standards, 2λ, λ, 0.5 λ, 0.25 λ, 4 replicates of each

(i) Confirmation of the labelled lysate sensitivity

Confirm in 4 replicates the labelled sensitivity λ, expressed in IU/ml, of the lysate solution prior to use in the test. Confirmation of the lysate sensitivity is carried out when a new batch of lysate is used or when there is any change in the experimental conditions which may affect the outcome of the test.

Prepare standard solutions of at least 4 concentrations equivalent to 2λ, λ, 0.5λ and 0.25λ by diluting the standard endotoxin stock solution with water for BET.

- **European Pharmacopeia 5.0, 2.6.14 Bacterial endotoxins: Photometric Techniques 3. Preparatory Testing (i) Assurance criteria for the standard curve 1**

Chromogenic method: min of 3 standards, 3 replicates of each

(i) Assurance of criteria for the standard curve

Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations to generate the standard curve. Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the lysate manufacturer (volume ratios, incubation time, temperature, pH, etc.).

d. Preparation of samples

- Samples preparation must be specific to the product.
- If there are modifications, please include
  - E.g. : pH modification, addition of endotoxin dispersing agent, ultra filtration, surfactant,
- Serial dilution of the product

* If pH modification is done, please include the pH test results in the validation data

[redacted]

PRODUCT SUMMARY REPORT		Page 5 of 8
TITLE: [redacted]		DATE 14/10/15
REFERENCE NO: BET/MISC/V0	PVP REFERENCE NO: SAL-SPD/PVP/01	VMP REF NO: SAL-SPD/VMP/R5

3.0 PRODUCT PREPARATION

This method is used when there is an official pharmacoeplal limit.

$$MVD = \frac{\text{Endotoxin Limit}}{\text{Sensitivity of lysate } (\lambda)} \text{ X Concentration of the Product}$$

$MVD = \frac{0.33}{0.125} \times 100 = 264$

S.NO	MVD CONCENTRATION	SAMPLE PREPARATION	Final CONC. OF PRODUCT in mg / ml
1	MVD / 32 = 8.25	0.2 ml of sample + 1.45 ml of LRW (1:8.25) (TUBE-A)	12.12 mg/ml
2	MVD/16 = 16.5	0.5 ml of Tube-A + 0.5 ml of LRW (1:2) (TUBE-B)	6.06 mg/ml
3	MVD/8 = 33	0.5 ml of Tube-B + 0.5 ml of LRW (1:2) (TUBE-C)	3.03 mg/ml
4	MVD/4 = 66	0.5 ml of Tube-C + 0.5 ml of LRW (1:2) (TUBE-D)	1.515 mg/ml
5	MVD/2 = 132	0.5 ml of Tube-D + 0.5 ml of LRW (1:2) (TUBE-E).	0.76 mg/ml

Serial dilution

11.14 Bacterial Endotoxins (method to be followed at Turbhe, India)

Principle

With a suitable preparation of limulus amoebocyte lysate (LAL), bacterial endotoxins react after incubation at 37°C by formation of a solid gel (gel-clot technique). According to EP 2.6.14., USP <85> and JP 4.01

Reagents and Equipment

According to EP 2.6.14. or USP <85>. or JP 4.01

Procedure

According to EP 2.6.14. or USP <85>. or JP 4.01

Sample preparation

Sample reconstitution

The lysate sensitivity used for this tests is 0.06 EU/mg. For testing, prepare the Endotoxin-standard solutions of 4 λ strength (0.25EU/ml)

100.0 mg – 100.3 mg of sample is dissolved in 5.0 mL of endotoxin free water to get a test solution of 20 mg/mL. From this test solution prepare a working dilution of 1:10 and 1: 20.

From the working dilution withdraw 2 unspiked samples and spike with 4 Lambda (e.g. 50 µL of 1: 10 dilution or 1: 20 dilution + 50 µL of a 4 Lambda solution) and transfer these samples into suitable assay tubes. This is Product positive control (PPC)

The negative control samples, positive control samples are also tested in duplicate.

After addition of 100 µL of lysate to all samples and incubation at 37°C for 60 min. (± 2 min.) an assessment of gel formation can be done: solid gel = positive result, no solid gel = negative result; pH (incl. lysate) : 6 - 8

Requirement

See specifications

Comment

E.U. are identical to USP endotoxin units, equivalent to I.U. (international units).

e. Test methods

- Describe how the test is performed in detail
- Gel clot method: European Pharmacopeia 5.0, 2.6.14 Bacterial endotoxins, Gel clot technique (Method A and B)
  - 1. Preparatory Testing
    - i. Confirmation of labeled lysate sensitivity
      - a) Prepare of 4 standards (2λ, λ, 0.5 λ and 0.25 λ) – 4 replicates of each conc.
      - b) Mix equal amount of Lysate (LAL) as the standard
      - c) Incubate the mixture (usually for 60 ±2 mins at 37°C)
      - d) Invert the tube (in one smooth motion)

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- ii. Test for interfering factors

- Prepare of solutions A, B, C and D (refer Table 1.1). Solution A & B: 4 replicates: solution C & D: 2 replicates
- Repeat steps b) to d) from Confirmation of labeled lysate sensitivity

Table 2.6.14.–1

Solution	Endotoxin concentration/ Solution to which endotoxin is added	Diluent	Dilution factor	Initial endotoxin concentration	Number of replicates
A	None/Test solution	-	-	-	4
B	2λ/Test solution	Test solution	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
C	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

Solution A = solution of the preparation being examined that is free of detectable endotoxins.
Solution B = test for interference.
Solution C = control of the labelled lysate sensitivity.
Solution D = negative control (water for BET).

Table 1.1

2. Limit test

- Prepare of solutions A, B, C and D (refer Table 1.2) – min 2 replicates for all solutions
- Repeat steps b) to d) from Confirmation of labeled lysate sensitivity

Solution	Endotoxin concentration/ Solution to which endotoxin is added	Number of replicates
A	None/Diluted test solution	2
B	2λ/Diluted test solution	2
C	2λ/Water for BET	2
D	None/Water for BET	2

Table 1.2

3. Semi-Quantitative test

Prepare of solutions A, B, C and D (refer Table 1.3) – 2 replicates for all solutions

- Repeat steps b) to d) from Confirmation of labeled lysate sensitivity

Solution	Endotoxin concentration/ Solution to which endotoxin is added	Diluent	Dilution factor	Initial endotoxin concentration	Number of replicates
A	None/Test solution	Water for BET	1	-	2
			2	-	2
			4	-	2
			8	-	2
B	2λ/Test solution		1	2λ	2
C	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

Solution A = test solution at the dilution, not exceeding the MVD, with which the test for interfering factors was carried out. Subsequent dilution of the test solution must not exceed the MVD. Use water for BET to make two dilution series of 1, 1/2, 1/4 and 1/8, relative to the dilution with which the test for interfering factors was carried out. Other dilutions may be used as appropriate.

Solution B = solution A containing standard endotoxin at a concentration of 2λ (positive product control).

Solution C = 2 series of water for BET containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ and 0.25λ.

Solution D = water for BET (negative control).

Table 1.3

Common issues regarding protocol of analysis

- Protocol of analysis not given – only a reference to BP, EP or USP given

"Carry out using internationally harmonised Ph. Eur/USP/JP/LAL method"

- Too simple/not detailed/only summary given– no list of equipments & reagents, method for preparation of standards, other solutions and method of test
- Sample preparation not specific to the product
- Insufficient types of solutions
- Not enough replicates for the solutions

EXAMPLE OF AN INCOMPLETE PROTOCOL OF ANALYSIS

TEST METHOD

Cefim Injection 0.5g contains not less than 90.0 percent and not more than 115.0 percent of the labeled amount of Cefepime.

Ingredient: Each vial contains:
Cefepime Hydrochloride eq. to Cefepime Base .....500mg (potency)
(buffered with L-Arginine)

Appearance: White to pale yellow powder in vial.

Filling weight: 0.5g(potency)/vial.

Weight variation: ±7% of average weight in vial, take 10 vials for test. (JP XII, p.68)

Identification:

1) For Arginine:
The chromatogram of the *Test preparation* obtained as directed in the *Content of Arginine* exhibits a Arginine peak, the retention time of which corresponds to that exhibited in the chromatogram of the *Standard preparation 1)* obtained as directed in the *Content of Arginine*.

2) For Cefepime HCl:
The chromatogram of the *Test preparation* obtained as directed in the Assay exhibits a major peak for ingredient, the retention time of which corresponds to that exhibited in the chromatogram of the *Standard preparation* obtained as directed in the Assay.

Bacterial endotoxins:
It contains not more than 0.06 Endotoxin Unit per mg of Cefepime.

Test Procedure:
In preparing for and applying the test, observe precautions in handling the specimens in order to avoid gross microbial contamination. To quantify the amount of endotoxin in a specimen, an assay is performed on decreasing concentrations of specimens prepared by serial dilution. Select dilutions so that they correspond to a geometric series in which each step is greater than the next by a constant ratio. Include negative and positive controls, and a positive product control. Use not less than 2 replicate reaction tubes at each level of the dilution series for each specimen under test. A standard endotoxin dilution series involving not less than 2 replicate reaction tubes is conducted in parallel. A set of standard endotoxin dilution series is included for each block of tubes, which may consist of a number of racks for incubation together, provided the environmental conditions within blocks are uniform.

1) Preparation:
Since the form and amount per container of standard endotoxin and of LAL reagent may vary, constitution and/or dilution of contents should be as directed in the labeling. The pH of the test mixture of the specimen and the LAL reagent is in the range 6.0 to 8.0 unless specifically directed otherwise in the individual monograph. The pH may be adjusted by the addition of sterile, endotoxin-free sodium hydroxide or hydrochloric acid or suitable buffers to the specimen prior to testing.

2) Procedure:
A) Specimen:
Dissolve about 200mg of sample powder in 10ml of water, mix well as sample solution. Prepare a series of dilutions of the sample solution, to give concentrations 1:10 of dilutions.
B) Standard endotoxin concentration:

Written by [redacted] Date [redacted]

Prepare a series of dilutions of the CSE to give concentrations 2λ, where λ is the labeled sensitivity of the LAL reagent in Endotoxin Units per mL.

C) Positive product controls:
Positive product controls specimen, or of solution washing or extract thereof to a standardized CSE, has been added to give a concentration of 2λ, where λ is the labeled sensitivity of the LAL reagent in Endotoxin Units per mL.

D) Negative control:
Diluted solvent (pyrogen free water).

Into single test vials (STV) of pyrotell (λ is 0.03Eu/ml), dispense the specified volumes of negative controls, standard endotoxin concentrations, specimens, and positive product controls. Add appropriately constituted LAL reagent, unless single test vials are used. Mix the specimen/LAL reagent mixture and place in an incubating device such as a water bath or heating block, accurately recording the time at which the tubes are so placed. Incubate each tube, undisturbed, for 60±2 minutes at 37±1°C, and carefully remove it for observation. A positive reaction is characterized by the formation of a firm gel that remains when inverted through 180°. Record such a result as positive (+). A negative result is characterized by the absence of such a gel or by the formation of a viscous gel that does not maintain its integrity. Record such a result as negative (-). Handle the tubes with care, and avoid subjecting them to unwanted vibrations, or false negative observations may result. The test is invalid if the positive product control is negative or the endotoxin standard does not show the endpoint concentration to be within ±1 two-fold dilutions from the label claim sensitivity of the LAL reagent or if any negative control is positive. If the 1/10 dilutions test result as negative mean passed, if as positive mean unpassed.

[Note: Treat any containers or utensils employed so as to destroy extraneous surface endotoxins that may be present, such as by heating in an oven at 250℃ or above for 30 minutes]

Sterility test: Using *Membrane Filtration Method* for Sterility.

Membrane Filtration condition:
Using membrane porosity of 0.45±0.02μm, a diameter of approximately 47mm, and a flow rate of 55 to 75ml of water per minute at a pressure of 70cm of mercury.

Procedure:
From each of 20 containers, aseptically transfer about 300 mg of sample, into a sterile 500 mL conical flask, dissolve in 200 mL of ***Sterile Fluid A**, and mix to dissolve. Aseptically transfer the solution into one membrane funnel, and immediately filter with the aid of vacuum. Then wash the membrane with 3 times 200 mL of ***Sterile Fluid A** by filtering through it. Remove the membrane from the funnel, cut the membrane in half. Immerse one-half of the membrane in *Fluid Thioglycollate Medium* and the other half of the membrane in *Soybean-Casein Digest Medium*, as following conditions:

1) For Bacteria:
Kind of medium: *Fluid Thioglycollate Medium*.
Volume of medium: 100ml
Positive control of Test Microorganism: *Bacillus subtilis* (ATCC 6633).

iv. Calculation of MVD and ELC

- Maximum Valid Dilution = the maximum allowable dilution of a sample at which the endotoxin concentration can be determined
- **Detailed MVD calculation specific of the product is required in all submission**
- $MVD = \frac{\text{Endotoxin limit x Product concentration}}{\lambda}$
- E.g. MVD for azithromycin IV injection 100 mg/ml with endotoxin limit of 0.17 EU/mg, and λ = 0.03 EU/ml

$$MVD = \frac{0.17\ EU/mg \times 100\ mg/ml}{0.03\ EU/ml} = 566.667\ (566)$$

- **Detailed ELC calculation for the product is required for product with endotoxin limit not available from EP, BP, USP or JP (or in-house)**
- Endotoxin limit concentration (ELC)= K / M
  K = maximum allowable endotoxin exposure (usually 5 EU/kg/hour for a 70 kg person)

  M = maximum human dose of the product
- E.g. ELC for Enfurvitide is < 1.2 EU/mg and is not stated in any reference. Max dose of enfurvitide is 1.5 mg/kg/h. Therefore:

  ELC = 5 EU/kg/h÷ 1.5 mg/kg/h = 3.33 EU/mg.

  Value chosen is 1.2 EU/mg - which is 3 fold safety margin – this is acceptable.

IV. Validation data

- The validation data required depend on the type of test method used.

A. If gel clot method was used:-

i. Confirmation of labeled lysate sensitivity – for 1 batch of lysate

ii. Test for interfering factors a.k.a. Inhibition/Enhancement test – for 3 batches of finished products

B. If chromogenic/turbidimetric method was used:-

i. Calibration of standard curve – for 1 batch of lysate

ii. Test for interfering factors a.k.a. Inhibition/Enhancement test – for 3 batches of finished products

A. Validation for gel clot method

i. Confirmation of Labeled Lysate Sensitivity

- Requirements:-
  - Test methods – how the test is performed
  - Types of solutions used in the test:-
    a) Solution A – negative control (LRW only)
    b) Solution B – endotoxin standardssolutions – minimum 4 λ concentrations (0.25 λ to 2 λ) *
  - Results for test performed on 1 batch of lysate in raw data format **

* 4 replicates for each solution types

** Results must meet the requirements.

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Sample of presentation of results for Confirmation of Labeled Lysate Sensitivity

Replicate	Observation at different concentrations				End-point	Log 10 of end point	Observation for –ve control
	2λ (0.02 EU/ml)	λ (0.01 EU/ml)	λ/2 (0.005 EU/ml)	λ/4 (0.0025 EU/ml)			
I	+	+	-	-	0.01 EU/ml	-2	-
II	+	+	-	-	0.01 EU/ml	-2	-
III	+	+	-	-	0.01 EU/ml	-2	-
IV	+	+	-	-	0.01 EU/ml	-2	-
Mean of log 10 end point / 4						-2	
Geometric mean = antilog of mean of log 10 end point /4						0.01 EU/ml	

MUST BE IN RAW DATA FORMAT

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[redacted]

Control Curve report

Control curve report No.: [redacted]

Date: 16/07/08

C) For Lysate :

LAL Reagent (Mfg./Lot #) : Endosafe/ X28421 Exp : 04/2011 Sensitivity : 0.03 EU/mL.

LAL Reagent water (Mfg./Lot #): Endosafe/ 99732061 Exp.: 07/2010

ASSAY :

Arrange test tubes in stand and take sample solution, LRW , CSE and lysate dilution as per following table.Incubate all tubes at at 37.0 ° C ± 1.0 ° C For 60 ± 2 min

Heating block ID No. : CQCE067

Start Time : 07:30 Start Temperature : 37.0 °C

End Time : 08:30 End Temperature : 37.0 °C

Tube No.	Test details	LRW in µL	CSE in µl	Lysate in µl	Result
1 to 4	Negative Control	100	-	100	- - - -
5 to 8	2 λ	_	100 (2 λ)	100	+ + + +
9 to 12	λ	_	100 (λ)	100	+ + + +
13 to 16	λ / 2	_	100 (λ/2)	100	- - - -
17 to 20	λ / 4	_	100 (λ/4)	100	- - - -

Note :

+ : Gel clot formed	- :Gel clot not formed

Acceptance criteria: Acceptable variation is 2 λ to λ / 2 of labelled sensitivity (λ)

Conclusion: With respect to the above observations, LAL kit tested ***complies/*** ~~***doesnot comply***~~ to the inhouse specifications.

Analysed By : [redacted]

Date : 16/07/08

Checked By : [redacted]

Date : 16/7/08

FORMAT NO:QC009-F03-01

i. Non-Inhibitory Dilutions

- Requirements:-
  - Test methods – how the test is performed
  - Types of solutions used in the test *:-
    a) Sample only (4 concentrations)
    b) Sample + endotoxins (4 concentrations)
  - Results for test performed on 1 batch of lysate in raw data format **

* Minimum of 2 replicates for each solution types

** Results must meet the requirements.

NPCB
MOH

Sample of presentation of results for Non-Inhibitory Dilutions Test

[redacted] TITLE : VALIDATION OF BACTERIAL ENDOTOXIN TEST FOR [redacted]

Validation Protocol No.: [redacted] Page No. : 06 of 17

Tube No.	Description	LRW in µL	CSE in µL	Sample in µL	Lysate in µL	Result
1&2	LRW blank	100	--	--	100	- -
3&4	2λ		100 (2λ)	--	100	+ +
5&6	λ		100 (λ)	--	100	+ +
7&8	λ/2		100 (λ/2)	--	100	- -
9&10	λ/4		100 (λ/4)	--	100	- -
11&12	SPL (16 MVC)	50	--	50 (32 MVC)	100	+ +
13&14	PPC (16 MVC)	--	50 (4λ)	50 (32 MVC)	100	+ +
15&16	SPL (8 MVC)	50	--	50 (16 MVC)	100	+ +
17&18	PPC (8 MVC)	--	50 (4λ)	50 (16 MVC)	100	+ +
19&20	SPL (4 MVC)	50	--	50 (8 MVC)	100	- -
21&22	PPC (4 MVC)	--	50 (4λ)	50 (8 MVC)	100	+ +
23&24	SPL (2 MVC)	50	--	50 (4 MVC)	100	- -
25&26	PPC (2 MVC)	--	50 (4λ)	50 (4 MVC)	100	+ +
27&28	SPL (1 MVC)	50	--	50 (2 MVC)	100	- -
29&30	PPC (1 MVC)	--	50 (4λ)	50 (2 MVC)	100	+ +

SPL : Sample PPC : Positive product control

\+ : Gel clot formed - : Gel clot not formed

Done by : [redacted] **Checked by:** [redacted]

Date : **Date :**

Selection of concentration of Product for routine analysis :

After getting the result select the two fold before dilution where PPC is showing positive result and sample is showing negative result, as a Non-interfering dilution.

Selected concentration for routine BET is 0.48 mg/mL (2 MVC)

iii. Inhibition/Enhancement Test (Test for Interfering Factors)

- Requirements:-
  - Test methods – how the test is performed
  - Types of solutions used in the test:-
    a) Solution A – negative product control (sampleonly) **
    b) Solution B – positive product control [endotoxin + samples, minimum 4 λ concentrations (0.25 λ to 2 λ)] **
    c) Solution C – endotoxin standard solutions – minimum 4 λ concentrations (0.25 λ to 2 λ) *
    d) Solution D – negative control (LRW only) *
  - Results for test performed on 3 batches of finished products in raw data format ^

* 2 replicates for each solution types

** 4 replicates for each solution types

^ Results must meet the requirements.

NPCB
MOH

Sample of presentation of results for INHIBITION/ ENHANCEMENT TEST

REPLICATES	SOL A (sample only)	SOL B (sample + endotoxin)				SOL C (endotoxin only)				SOL D
		2λ (0.02 EU/ml)	λ (0.01 EU/ml)	λ/2 (0.005 EU/ml)	λ/4 (0.0025 EU/ml)	2λ (0.02 EU/ml)	λ (0.01 EU/ml)	λ/2 (0.005 EU/ml)	λ/4 (0.0025 EU/ml)	
I	-	+	+	-	-	+	+	-	-	-
II	-	+	+	-	-	+	+	-	-	-
III	-	+	+	-	-	+	+	-	-	-
IV	-	+	+	-	-	+	+	-	-	-
End-point		0.01 EU/ml				0.01 EU/ml				
Log end-point		- 2				- 2				

MUST BE IN RAW DATA FORMAT

NPCB
MOH

Sample of presentation of results for Inhibition/Enhancement Test

[redacted]

TITLE : VALIDATION OF BACTERIAL ENDOTOXIN TEST FOR [redacted]

Validation Protocol No.: VPR - 525 | Page No. : 07 of 17

b) PART - II Validation **Test 1**

Label and arrange test tubes(10x75mm) in test tube stand and add LRW, sample prepared control standard endotoxin and lysate asper following table.

Solution A: Solution of the product at 2 MVC (0.48 mg/mL)
Solution B: Test solution spiked with indicated CSE concentrations (Positive product control: PPC)
Solution C : Standard solution which indicated CSE concentration in LRW
Solution D : LRW (Negative control : NC)

Gel clot Incubator ID No. COCE067
Incubation: Time Start 14:50 End 15:50
Temperature: Start 37.0°C End 37.0°C.

Tube No.	Solution		LRW in µL	CSE in µL	Sample in µL(mL)	Lysate in µL	Result
1 to 4	A	--	50	--	50	100	- - - -
5 to 8	B Product	2λ	--	50 (4λ)	50	100	+ + + +
9 to 12		λ	--	50 (2λ)	50	100	+ + + +
13 to 16		λ/2	--	50 (λ)	50	100	- - - -
17 to 20		λ/4	--	50 (λ/2)	50	100	- - - -
21 to 24	C Endotoxin	2λ	--	100 (2λ)	--	100	+ + + +
25 to 28		λ	--	100 (λ)	--	100	+ + + +
29 to 32		λ/2	--	100 (λ/2)	--	100	- - - -
33 to 36		λ/4	--	100 (λ/4)	--	100	- - - -
37 to 40	D	--	100	--	--	100	- - - -

Done by : [redacted]
Date :

Checked by: [redacted]
Date :

B. Validation for chromogenic method

i. Criteria for Standard Curve

- Requirements:-
  - Test methods
  - Types of solutions *:-
    - Solution A – negative control (LRW only)
    - Solution B – endotoxin standards (minimum 3 concentrations)
  - Test results for 1 batch of lysate **

* Minimum 4 replicates

** Results must meet specifications

Sample of presentation of results for Criteria for Standard Curve

[redacted]

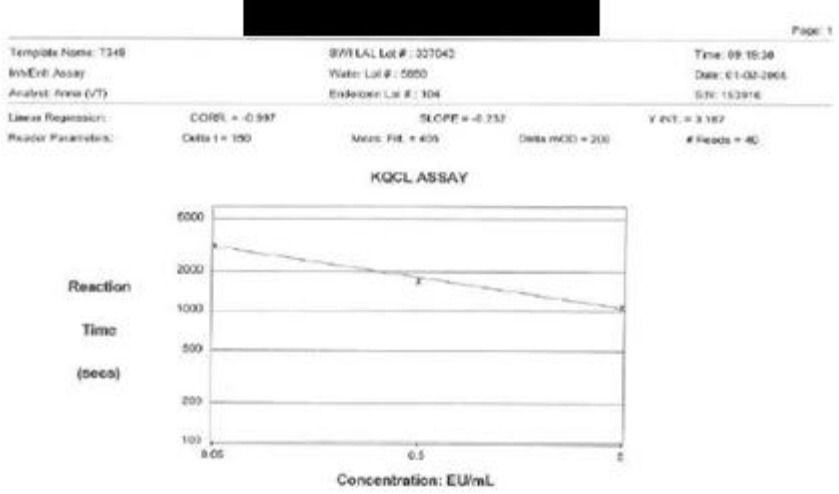
COMMON TECHNICAL DOCUMENT MODULE 3, QUALITY

[redacted]

Figure P.5.3.3.1 Inhibition / Enhancement Results, Tranexamic Acid Injection 250mg /5ml,

[redacted]

[redacted]



SUMMARY DATA

STANDARDS	CONCENTRATION	WELL	REACTION TIME (sec)	AVG. TIME	Back Prediction
Blank	Blank	A 1 B 1 C 1	---- ---- ----	----	----
Std. 1	0.05	D 1 E 1 F 1	3160 3153 3162	3158	0.0454
Std. 2	0.5	A 2 G 1 H 1	1735 1720 1725	1730	0.6059
Std. 3	5	B 2 C 2 D 2	1082 1078 1092	1084	4.542

MUST BE IN RAW DATA FORMAT

i. Inhibition/Enhancement Test (Test for Interfering Factors)

- Requirements:-
  - Test methods
  - Types of solutions *:-
    - Solution A – negative product control (sample only)
    - Solution B – positive product control (sample + endotoxin)
  - Test results for 3 batches of finished products **

* Minimum 4 replicates

** Results must meet specifications

Sample of presentation of inhibition / enhancement test

[redacted]

COMMON TECHNICAL DOCUMENT MODULE 3, QUALITY
[redacted] **INJECTION**
[redacted] **250 mg / 5 mL**

Figure P.5.3.3.1 Inhibition / Enhancement Results, [redacted] Injection 250mg /5ml, Batch A106T (3/4)

[redacted]

Page: 2

Template Name: T349 | BWI LAL Lot # : 337043 | Time: 09:19:30
Inh/Enh Assay | Water Lot # : 5880 | Date: 01-02-2008
Analyst: Anna (VT) | Endotoxin Lot # : 104 | S/N: 153916

Tranexamic Acid 250mg/5ml Ampoules
Lot# A106T

Rel. Limit : 0.016

SAMPLES	CONCENTRATION	WELL	REACTION TIME (sec)	AVG. TIME	RAW EU	Results (LR) EU/mg
S 1	1	E 2 F 2	**** ****	****	< 0.0500	< 0.0500
PPC	1	G 2 H 2	2077 2078	2077	0.2751	

PPC Value: 0.5000 (PPC - SAMPLE 1) Endotoxin Recovered : 0.2751

Comment: 250mg/5ml;1/4,1/2,1/2

Conclusion: Confirms

Reviewed By: [redacted] Date: 01/02/2008

MUST BE IN RAW DATA FORMAT

Common issues regarding validation

- Test methods not given
- Not enough solution types / replicates
- The results given are not in raw data format
- Not enough data (i.e. not neough for 3 batches)
- Results did not meet specifications
- The raw data given is in foreign language and not translated

THANK YOU