Biopharmaceutical Development Program

Standard Operating Procedure

Title: Limulus Amebocyte Lysate (LAL) Assay for Gel Clot

SOP Number: 22182 **Revision Number: 01**

Supersedes: Revision 00 **Effective Date: SEP 27 2011**

Originator/Date: [redacted]

Approval/Date:

Approval/Date:

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1.0 Purpose

This procedure describes the methodology to determine the amount of endotoxin present in a solution using the LAL Gel Clot Assay.

2.0 Scope

This SOP applies to Process Analytics (PA) personnel that will perform this assay.

3.0 Authority and Responsibility

3.1 The Director, Process Analytics (PA) has the authority to define this procedure.

3.2 PA is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA personnel are responsible for the performance of this procedure.

3.4 PA is responsible for reviewing the data and documentation of the results of this procedure.

3.5 PA is responsible for quality oversight of this procedure

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

4.0 Endotoxin Test

4.1 Materials

4.1.1 BioWhittaker Pyrogent® Kit, Catalog No. N284, which contains Limulus Amebocyte Lysate 4 x 5.2 mL/vial (lysate sensitivity 0.125 EU/mL or 0.06 EU/mL), 50-tests/vial + 1 vial 10 ng E. coli Control Standard Endotoxin (CSE), or equivalent, BDP PN 30032.

4.1.2 LAL Reagent Water which should not cause gelation of reconstituted lysate after 24 hours incubation at 37°C ± 1°C. Commercially available Sterile Water for Injection (USP) is usually satisfactory, BDP PN 30328.

4.1.3 Pipettes and pipette tips, 5.0 mL (BDP PN 20104), 10.0 mL (BDP PN 20100, MBP200 Pipet Tips (BDP PN 20469), and 1MBP1000 Pipet Tips (BDP PN 20470), pyrogen-free.

4.1.4 10 x 75 mm glass reaction tubes, pyrogen-free, BDP PN 20277.

4.1.5 13 x 100 mm glass dilution tubes, pyrogen-free, BDP PN 20278.

4.1.6 Heating block, water bath or incubator (37°C ± 1°C).

4.1.7 Test tube racks.

4.1.8 Timer.

4.1.9 Vortex Mixer.

4.2 Reagent Preparation

4.2.1 Preparation of Limulus Amebocyte Lysate.

a) Reconstitute lyophilized lysate by adding 1.8 mL LAL Reagent Water to the 16-test vial or 5.2 mL to the 50-test vial. DO NOT SHAKE. Swirl gently but thoroughly for at least 30 seconds. Do not shake as contents will foam.

b) Reconstituted lysate can be stored for up to 24 hours at 2-8°C without loss of sensitivity. Reconstituted lysate can be divided into more convenient volumes and stored below -10°C in a non-frost-free freezer for up to four weeks. Frozen liquid lysate should be thawed immediately before use.

4.2.2 Preparation of E. coli Control Standard Endotoxin (CSE).

a) Reconstitute 10 ng vial endotoxin with 5.0 mL LAL Reagent Water.

b) Vortex the vial of Endotoxin for at least 15 minutes.

c) Dilute the Endotoxin with LAL Reagent Water to a concentration of 1 EU/mL (see Certificate of Analysis).

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4.3 Dilution Series for Use with Lysate of 0.125 EU/mL Endpoint

Tube #	Water (mL)	Volume Added to Water	Endotoxin Concentration
1	1.0	1.0 mL from 1 EU/mL	0.5 EU/mL
2	1.0	1.0 mL from Tube 1	0.25 EU/mL
3	1.0	1.0 mL from Tube 2	0.12 EU/mL
4	1.0	1.0 mL from Tube 3	0.06 EU/mL
5	1.0	1.0 mL from Tube 4	0.03 EU/mL

4.4 Test Procedure

4.4.1 Each assay should include serial two-fold dilutions of the Control Standard Endotoxin (CSE) which bracket the sensitivity value on the label, and dilutions of the test sample. LAL Reagent Water serves as a negative control.

4.4.2 To avoid microbial or pyrogen contamination, carefully transfer 0.10 mL of standard, sample or water into the appropriate 10 x 75 mm reaction tube.

4.4.3 Add 0.10 mL of the reconstituted lysate to each tube beginning with the highest concentration of endotoxin.

4.4.4 Immediately following the addition of the lysate to each tube, mix the contents thoroughly and place the tube in a 37°C ± 1°C water or dry heat bath. Follow this procedure for each dilution of the endotoxin.

4.4.5 Run the unknown test sample in parallel with the control standard endotoxin.

4.4.6 The assay may be conducted either as a yes/no test at a single dilution or as a quantitative test via a dilution series.

4.4.7 Determine the initial or starting time for each tube placed in the 37°C bath.

4.4.8 Do not remove assay tubes from incubation or disturb tubes prior to the time specified for reading the test.

4.4.9 After exactly one hour (± 2 minutes) of incubation, examine each tube for gelation. A positive reaction is indicated by a firm gel that remains intact momentarily when the tube is inverted 180°.

4.4.10 Record in columns the reaction in each tube as either positive or negative.

4.5 Initial Quality Control

4.5.1 Label each vial of LAL with the lysate sensitivity obtained using the CSE, and which is expressed in *Endotoxin Units* (see Certificate of Analysis).

4.5.2 As part of an initial in-house validation, each user must reverify the labeled lysate sensitivity using an endotoxin standard whose potency is known.

4.5.3 Prepare serial two-fold dilutions of the control standard endotoxin which brackets the labeled lysate sensitivity.

4.5.4 Assay each dilution, and a negative water control in quadruplicate.

4.5.5 After the one-hour incubation period (± 2 minutes), record the positive and negative results on Attachment 2.

4.5.6 Determine the endpoint dilution as the last dilution of endotoxin which still yields a positive result.

EXAMPLE

Assay Results - Gel-Clot Method							
Endotoxin Dilution (EU/mL)							
Replicate	0.50	0.25	0.12	0.06	0.03	H_2O	Endpoint
1	+	+	+	-	-	-	0.12
2	+	+	+	-	-	-	0.12
3	+	+	+	+	-	-	0.06
4	+	+	+	-	-	-	0.12

4.5.7 Calculate the lysate sensitivity by determining the geometric mean of the endpoint. Each endpoint value is converted to log 10. The individual log 10 values are averaged, and the lysate sensitivity is taken as the antilog 10 of this average log value. The acceptable variation is one-half to two times the labeled lysate sensitivity.

EXAMPLE

Calculation of Geometric Mean Endpoint	
Endpoint EU/mL	Log 10 Endpoint
0.12	-0.921
0.12	-0.921
0.06	-1.222
0.12	-0.921
Mean = -0.996 Antilog 10 Mean = 0.10 EU/mL	

4.6 Determination of Endotoxin in an Unknown

4.6.1 To determine the endotoxin concentration of an unknown solution, test serial two-fold dilutions of sample until an endpoint is reached. The unknown samples are run in duplicates.

4.6.2 Calculate the geometric mean dilution as before and multiply by the labeled lysate sensitivity. Record results on Attachment 1.

EXAMPLE

Determination of Endotoxin Concentration in an Unknown Lysate Sensitivity = 0.125 EU/mL						
	SAMPLE DILUTION					
Replicate	1/2	1/4	1/8	1/16	1/32	1/64
1	+	+	+	-	-	-
2	+	+	+	+	-	-

Endpoint Dilution	Log 10 Endpoint
1/8 (0.125)	-0.903
1/16 (0.0625)	-1.204
	Mean =-1.054
	Antilog 10 Mean = 0.088 = 1/11.3

Endotoxin Concentration = lysate sensitivity x endpoint dilution
= 0.125 EU/mL x 11.3 = 1.4 EU/mL

4.7 Product Inhibition

4.7.1 The Limulus Amebocyte Lysate reaction is enzyme mediated and has an optimal pH range and specific salt and divalent cation requirements.

Occasionally, test samples may alter these optimal conditions to an extent that the lysate is rendered insensitive to endotoxin. Negative results with samples which inhibit the LAL test do not necessarily indicate the absence of endotoxin.

Screen each type of sample for product inhibition, by preparing a series of two-fold dilutions of endotoxin in LAL Reagent Water and a similar series of endotoxin dilutions using sample as diluent.

Note: It may be necessary to use dilutions of the sample in LAL Reagent Water to get a valid assay with no product inhibition.

4.7.2 Assay each of these series in parallel using standard procedures as described in section 4.3.

4.7.3 At the end of the incubation period, record positive and negative results, and calculate the geometric mean endpoint for both series of endotoxin dilutions. Products are said to be free of product inhibition if the geometric mean endpoint of endotoxin in product is within one-half to two times the geometric mean endpoint of a similar series of endotoxin in water.

4.7.4 An LAL activity assay is valid only if it is performed at a sample dilution that is equivalent or more dilute than a dilution that shows no product inhibition.

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EXAMPLE

Product Inhibition Testing						
Endotoxin Dilution (EU/mL)						
Endotoxin in Water		0.50	0.25	0.12	0.06	0.03
	1)	+	+	+	-	-
	2)	+	+	+	-	-
	3)	+	+	+	+	-
	4)	+	+	+	-	-
	geometric mean endpoint = 0.10 EU/mL					
in Product A	1)	+	+	-	-	-
	2)	+	+	+	-	-
	3)	+	+	+	-	-
	4)	+	+	+	-	-
In Product B	1)	+	-	-	-	-
	2)	+	-	-	-	-
	3)	+	-	-	-	-
	4)	+	-	-	-	-
geometric mean endpoint = 0.50 EU/mL inhibitory						

4.8 Operator Qualification

4.8.1 PA personnel shall be trained and qualified in this procedure prior to conducting this assay for GMP assay. This shall be done by performing assays on water samples spiked with unknown quantities of endotoxin. Submit documentation of results of this training to BQA.

5.0 Documentation

5.1 Results are recorded on Form 22182-01.

6.0 Attachments

6.1 **Attachment 1** Form 22182-01, LAL Gel Clot Assay Data Sheet

6.2 **Attachment 2** Form 22182-02, LAL Gel Clot Assay – New Lot/Technician Validation Data Sheet

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Attachment 1

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LAL Gel Clot Assay Data Sheet

LAL Reagent Lot No. _____ Exp. Date: _____
Endotoxin Lot No. _____ Exp. Date: _____
LAL Water Lot No. _____ Exp. Date: _____
Kit CAT No. _____ Exp. Date: _____
Kit Lot No. _____ Exp. Date: _____
Sample ID No.: _____ Sample Name: _____
Timer ID: _____ Calibration Due Date: _____
Incubator ID: _____ Calibration Due Date: _____
Assay Time Start: _____ Assay Time Stop: _____

1. Standard Curve:

	Endotoxin Dilution (EU/ml)				
Replicate	0.25	0.125	0.06	0.03	0.015
1					
2					

Lysate Sensitivity = _____EU/ml

Calculations:

End Point	log 10 End Point
1.	
2.	
	Mean = Anti log 10 mean =

2. Negative Control: LAL Water + Lysate:

1	2	3	4

3. Unknown Endotoxin Concentration Determination:

Neat	1:2	1:4	1:8	1:16	1:32

If further dilutions are required to reach endpoint, record in step 4.

Calculations:

End Point	log 10 End Point
1.	
2.	
	Mean = Anti log 10 mean =

Lysate sensitivity _____ X End point _____= _____EU/mL
Completed By: _____ Reviewed By: _____

Comments:_____

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Attachment 1 (Continued)

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4. Unknown Endotoxin Concentration Determination of Samples requiring further dilution to reach endpoint.

1:64	1:128	1:256	1:512	1:1024	1:2048

Comments:_____

_____

_____

5. Product Inhibition Testing:

Time Start:_____ Time End _____

Replicate	0.25	0.125	0.06	0.03	0.015
1					
2					

Calculations:

	End Point	log 10 End Point
1.		
2.		
		Mean = Anti log 10 mean =

- Sample is free of product inhibition if the geometric mean endpoint of Endotoxin in the sample is within ½ to 2 times the geometric mean endpoint of the standard curve in section 1.

Completed By: _____ Reviewed By: _____

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Attachment 2

NCI-Frederick
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LAL Gel Clot Assay – New Lot/ Technician Validation Data Sheet

LAL Reagent Lot No. _____ Exp. Date: _____
Endotoxin Lot No. _____ Exp. Date: _____
LAL Water Lot No. _____ Exp. Date: _____
Kit CAT No. _____ Exp. Date: _____
Kit Lot No. _____ Exp. Date: _____
Timer ID: _____ Calibration Due Date: _____
Incubator ID: _____ Calibration Due Date: _____
Time Start: _____ Time Stop: _____

1. Standard Curve:

Replicate	0.25	0.125	0.06	0.03	0.015
1					
2					
3					
4					

Calculations:

End Point	log 10 End Point
1.	
2.	
3.	
4.	
	Mean = Anti log 10 mean =

Lysate Sensitivity: _____

*Standard Curve is valid if the geometric mean endpoint of Endotoxin is within ½ to 2 times the labeled Lysate sensitivity.

2. Negative Control: LAL Water + Lysate:

1	2	3	4

❑ Pass ❑ Fail

Completed By: _____ Reviewed By: _____

Comments:

_____

_____

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