Template Provided By:

Cancer Imaging Program

National Cancer Institute

Standard Operating Procedure

GEN-Q114

Bacterial Endotoxins by Limulus Amebocyte Lysate (LAL) Test

1. **PURPOSE:** To describe the quality control assay utilized for detecting bacterial endotoxins that may be present in the final product solution. This assay quantifies bacterial endotoxins in sample solutions by titration to an endpoint.

2. **SCOPE:** The Limulus Amebocyte Lysate (LAL) Test will be performed at the sites manufacturing radiopharmaceuticals. This quality control procedure is one of the release tests performed on the final batch of product. The results of this test must be obtained prior to releasing the batch for human administration.

3. **RESPONSIBILITIES:**
   - **3.1.** *Manufacturing Sites*: To follow this procedure as written and to ensure that all results, control information, and raw data are documented as specified in dedicated Quality Control Laboratory Notebooks and recorded on the appropriate testing forms. Preliminary testing may be required prior to conducting the final bacterial endotoxin assay to qualify a new lot of LAL reagent or CSE, or to verify & revalidate the parameters prior to conducting the assay.

4. **REFERENCES**
   - **4.1** USP 28 ‹821› Radioactivity
   - **4.2** USP 28 ‹823› Radiopharmaceuticals For Positron Emission Tomography—Compounding
   - **4.3** **GEN-Q015**, "Investigating Out of Specification QC Test Results"
   - **4.4** FDA Draft Guidance PET Drug Products—Current Good Manufacturing Practice (CGMP)—September 2005 (CDER)
   - **4.5** USP 28 ‹85› Bacterial Endotoxins Test
   - **4.6** Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parental Drugs, Biological Products, and Medical Devices, Dec. 1987; CDER, CBER, CDRH, & CVM.

Printed Name	Signature	Date
Author:		
Regulatory Approval:		

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5. FORMS

- **Form FQC-001**, "Final Product QC Test Results"
- **Form IOS-001**, "Investigating Out of Specification (OOS) QC Test Results Form"
- **Form Q114-L1**, "Confirmation of LAL Label Claim Sensitivity" Form
- **Form Q114-L2**, "LAL Non-Interfering Concentration Form"
- **Form Q114-L3**, "GEL-CLOT ASSAY: Bacterial Endotoxins LAL Test Form"

6. DEFINITIONS

6.1 Pyrogen: A fever producing substance

6.2 Endotoxin: Toxins that can be extracted from the cell structure of a wide variety of gram-negative bacteria. Endotoxins are found in the gram-negative bacteria mostly, and are obtained subsequent to the death and autolysis of the cells.

6.3 Limulus Amoebocyte Lysate: The cell-free reagent derived from the blood cells, amebocytes, of the horseshoe crab, (LAL), that reacts to form a clot when exposed to endotoxins. Limulus is the generic name of the horseshoe crab, amebocyte is the blood cell that contains the active components of the reagent.

6.4 Positive Control: Two lambda concentration of control standard endotoxin. This should give a positive response in the assay or a gel-clot.

6.5 Negative Control: Diluent plus the Limulus Amebocyte Lysate. This should give a negative response in the assay or no gel-clot.

6.6 Control Standard Endotoxin (**CSE**): A control standard endotoxin (CSE) is an endotoxin preparation other than the RSE that has been standardized against the RSE.

6.7 Reference Standard Endotoxin (RSE): This is the Reference Standard Endotoxin obtained from the USP (RSE), and it is the standard against which all LAL reagents are validated and qualified. It has a defined potency of 10, 000 USP Endotoxin Units (EU) per vial.

7. GUIDELINES/POLICIES

7.1 There are four basic LAL test methods commercially available and currently approved by the FDA for end-product release testing: (1) the gel-clot; (2) the turbidimetric (spectrophotometric); (3) the colorimetric (Lowry protein); and (4) the chromogenic assay. The LAL reagents used in these methods must be obtained from an FDA-licensed manufacturer and must be designed specifically for the method chosen. The method described in this SOP is for the gel-clot method.

7.2 There are certain products that have been known to interfere with the LAL's ability to react with endotoxin. These factors may be chemical or physical. Chemical inhibitors cause chelation (*i.e.* EDTA), protein denaturation (*i.e.* fluorescein), or pH disruption (*i.e.* pH outside range of 6.0-7.5). Physical inhibitors include adsorption of endotoxin, or product viscosity. However, most of the inhibition can be overcome by dilution of the product. Other

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factors to consider are the shape and type of glassware used in the gel-clot method—as that can affect the validity of the test. Siliconized glassware as well as plastic can inhibit gel-clot formation. It is important to determine the non-interfering concentration for routine bacterial endotoxin testing of final product initially and also anytime when changing the formulation or manufacturing processes.

7.3 Before a new lot of LAL is used, the labeled sensitivity of the lot or the performance criteria should be confirmed prior to using the LAL reagent in an assay for testing product.

7.4 The inhibition/enhancement or non-interfering concentration tests must be repeated on one unit of the product if the LAL manufacturer is changed.

7.5 When the manufacturing process, the product formulation, the source of a particular ingredient of the drug formulation, or the LAL lot is changed, the positive product control can be used to reverify the validity of the LAL test for the product.

7.6 An endotoxin standard series should be run when confirming end-product contamination.

7.7 If an endotoxin test is repeated, the repeat should consist of twice the initial number of replicates of the sample in question to examine the possibility that extrinsic contamination occurred in the initial assay procedure.

7.8 The Pass/Fail Cutoff equals the endotoxin limit of the product solution (EU/mL) times the potency of the product divided by the product dilution used for the test. For Pass/Fail Cutoffs less than or equal to 1.0 EU/mL the endotoxin spike should be between 0.1 and 0.5 EU/mL, otherwise the endotoxin spike should be between 1.0 and 5.0 EU/mL.

7.9 The administered dose of final product solution should not contain more than 175 EU/V of the injection of the maximum administered total dose.

7.10 The USP Endotoxin RS has a defined potency of 10,000 USP Endotoxin Units (EU) per vial.

8. MATERIALS AND EQUIPMENT

- Depyrogenated Flint (Soda Lime) Glass or Polystyrene Test Tubes (preferable size 10 x 75 mm)
- Depyrogenated GlassWare
- Vortex Mixer
- Limulus Amebocyte Lysate (LAL) Reagent
- LAL Reagent Water or Sterile Water for Injection (SWFI), USP
- Dry Block Incubator, Heating Block, or Non-circulating Water Bath, (37 ± 1)°C
- Timer, NIST Traceable
- Parafilm “M” (American National Can™)
- Control Standard Endotoxin (CSE) or USP RS Endotoxin (RSE)
- Automatic Pipettor(s) with tips or Pipettors
- Syringe, 1 mL
- Needle, 21G x 1.5”
- Test Tube Racks
- Sterile Pipette Tips for Automatic Pipettors

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- Polystyrene Round Bottom Tubes with Snap Caps, Depyrogenated, 14 mL
- Thermometer, 36-38°C, NIST Traceable
- Limulus Amebocyte Lysate (LAL), 0.06 EU/mL Single Test Tubes (or other suitable sensitivity)
- Final Product

9. PREPARATION OF ENDOTOXIN SOLUTIONS

9.1 Preparation of Endotoxin Stock Solutions

Control Endotoxin Standards can be obtained from the USP, EndoSafe, Cambrex, or Cape Cod. For reconstitution of the USP Reference Standard and preparation of solutions refer to 9.1.1 and 9.2.1. For reconstitution of the control reference standard (CSE) and preparation of solutions refer to 9.1.2 and 9.2.2.

9.1.1 USP Reference Standard Endotoxin (RSE)

1. Constitute the entire contents of 1 vial of the USP Reference Standard Endotoxin (RSE) with 5 mL of LAL Reagent Water (LRW) or Water For Injection (WFI).
2. Mix intermittently for approximately 30 minutes using a vortex mixer.
3. Label this concentrate with an expiration date of 14 days and preserve at 4°C after making the appropriate serial dilutions.

9.1.2 Control Standard Endotoxin (CSE)

1. When a new CSE standard series is prepared, reconstitute the control standard endotoxin (CSE) according to the manufacturer's instructions.

NOTE: CSE concentration varies with lot of CSE and individual vial, therefore, it may be necessary to perform intermediate dilutions to obtain a starting concentration of 20 EU/mL as requested in 9.1.2, #2.

2. Dilute the reconstituted CSE to 20 EU/mL in a polystyrene tube.
3. Assign a Standard Number to this stock solution, this solution expires one month after reconstitution if stored at 2-8°C.

9.2 Preparation of Endotoxin Standards

9.2.1 USP Reference Standard Endotoxin (RSE)

1. Prepare the standard solutions using the endotoxin stock solution (RSE). First mix the endotoxin stock solution vigorously, using a vortex mixer, for not less than 3 minutes before use.
2. Transfer the appropriate amount of the stock solution into the corresponding labeled vials.
3. Add the appropriate amount of LAL Reagent Water (LRW) or WFI and mix each dilution for not less than 30 seconds before proceeding to make the next dilution.

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9.2.2 Control Standard Endotoxin (CSE)

1. If the 20 EU/mL CSE stock solution is fresh proceed directly to step 9.4.2. If the stock solution has been stored at 2-8°C, check to see that it has not expired, then warm it at room temperature for approximately 30 minutes. Vortex for approximately 1 minute prior to use.
2. Prepare serial dilutions from the 20 EU/mL CSE stock solution. Refer to Table 1 below.
3. Vortex each dilution for at least 30 seconds before proceeding to the next dilution.
4. Prepare the following CSE dilutions for a LAL sensitivity of 0.06 EU/mL: 0.125 EU/mL, 0.060 EU/mL, 0.030 EU/mL, 0.015 EU/mL.
5. All tests in this procedure use example dilutions based on an LAL sensitivity of 0.06. For an LAL test of greater sensitivity the dilutions all must scale up proportionally to be 2λ, λ, 0.5λ, and 0.25λ.
6. The dilutions should be prepared fresh.
7. Label the dilutions with the CSE standard number, CSE EU/mL, the preparation date, and the expiration date. Record the Standard Number and the relevant information in the Standards Log.

TABLE 1 CSE Dilutions

LAL Reagent Water/ SWFI (mL)	Volume of CSE (mL)		Concentration of CSE (EU/mL)		Total Volume of Diluted Solution (mL)		Resultant CSE Concentration EU/mL	In Terms of λ
7.5	0.5	x	20	÷	8.0	=	1.25	20λ
9.0	1.0	x	1.25	÷	10.0	=	0.125	2λ
5.0	5.0	x	0.125	÷	10.0	=	0.0625	λ
5.0	5.0	x	0.0625	÷	10.0	=	0.03125	0.5λ
5.0	5.0	x	0.03125	÷	10.0	=	0.015625	0.25λ

9.3 Preparation of Product Samples to be Tested

9.3.1 Prepare sample solutions by diluting using LAL Reagent Water (LRW) or SWFI. The product samples should be diluted 1:19, or $^{1}/_{20th}$ their original concentration for testing. Mix well. Transfer 200 µL of the diluted product to each test tube.

10. GENERAL GEL-CLOT TEST TECHNIQUES

10.1 Set Up

10.1.1 Turn on the heating block to allow sufficient time for temperature equilibration.

10.1.2 If appropriate, assemble depyrogenated glassware at room temperature.

10.1.3 Prepare all required dilutions of the QC samples to be tested as outlined in Sections 9.0, 11.0, and 12.0 of this standard operating procedure.

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10.1.4 Label all single test LAL tubes and arrange them in a labeled test tube rack as indicated in the appropriate Table from Sections 11 and 12.

10.2 General Test Procedure

10.2.1 Add 0.2 mL of each sample to be tested to the appropriate labeled test tubes as indicated in the appropriate Tables.

10.2.2 Briefly vortex (3-5 seconds), and place each tube into the heating block.

10.2.3 Incubate the reaction mixtures for 37 ± 1°C for 60 ± 2 minutes or 20 ± 2 minutes as appropriate, avoiding vibration.

10.2.4 After twenty minutes or one hour, gently remove each tube from the heating block and carefully invert toward 180°. If the gel remains intact, record the result as positive (+). If the mixture remains liquid or if the gel breaks upon inversion of the tube, record the result as negative (-). All preliminary data should be recorded on **Form Q114-LX.**

11. PREPARATORY TESTING FOR THE GEL-CLOT TECHNIQUES

11.1 Test for Confirmation of Labeled LAL Reagent Sensitivity—

This test must be performed when either: *a new lot of LAL Reagent is used; an alternate LAL reagent manufacturer is utilized.* This test confirms the labeled LAL Reagent sensitivity.

11.1.1 Set Up

1. Turn on the heating block to allow sufficient time for temperature equilibration.
2. Prepare a series of two-fold dilutions of the Control Standard Endotoxin (CSE) in LAL Reagent Water (LRW) or Sterile Water for Injection (SWFI) to give concentrations of 2λ, λ, 0.5λ, and 0.25λ as outlined in table 1 Section 9.0 of this Standard Operating Procedure.
3. Label eighteen single-test LAL tubes and arrange them in a labeled test tube rack as indicated in Table 2 so each represented CSE concentration is in quadruplicate:

TABLE 2

Row of Test Tube Rack	0.125 EU/mL	0.0625 EU/mL	0.03125 EU/mL	0.015 EU/mL	SWFI/ LAL Reagent Water
A	**2λ**	**λ**	**0.5λ**	**0.25λ**	**NC**
B	**2λ**	**λ**	**0.5λ**	**0.25λ**	**NC**
C	**2λ**	**λ**	**0.5λ**	**0.25λ**	
D	**2λ**	**λ**	**0.5λ**	**0.25λ**	

11.1.2 Test Procedure

1. Add 0.2 mL of each CSE dilution to the appropriate labeled test tubes in quadruplicate as indicated in Table 2 above.

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2. Perform the Gel-Clot test. All preliminary data should be recorded on **Form Q114-L1.**
3. The test is not valid unless the lowest concentration of the standard solution shows a negative result in all replicate tests.
4. Determine the endpoint of each set of four tubes. The endpoint is the last positive test in the series of decreasing concentrations of Control Standard Endotoxin.
5. Determine the geometric mean (GM) for the replicate series using the following equation where:

e = the $\log_{10}$ of each of the four endpoints
f = the total number of endpoints (in this case, 4)

$$\mathbf{GM = Antilog\ (\sum e/\mathit{f})}$$

11.1.3 Acceptance Criteria

1. If the **geometric mean endpoint concentration is not less than 0.5λ and not more than 2λ, the labeled sensitivity is confirmed** and is used in tests performed with this lysate. For 0.06 EU/mL LAL the geometric mean of the replicate series is between 0.03 EU/mL and 0.125 EU/mL.
2. Both negative controls must not gel.

11.2 LAL Non-Interfering Concentration (NIC)

The non-interfering concentration is defined as the highest concentration of final product that will cause neither enhancement (recovery of more endotoxin than expected), nor inhibition (recovery of less endotoxin than expected) of the bacterial endotoxin test. An interference test shall be performed on three lots of final product prior to initiating routine testing. This procedure is repeated with a change in LAL reagent source or formulation of the final product.

11.2.1 Define Test Parameters

1. Determine the Endotoxin Limit of Final Product in EU/mL:

   EL = (175 EU/Dose) ÷ (Dose Volume in mL/Dose)

   Where Dose Volume is the largest volume of final product given for injection from a batch and 175 EU/Dose is defined by the USP as the *per dose* limit for radiopharmaceuticals.
2. Determine the Maximum Valid Dilution of Final Product, which is the limit to which the final product may be diluted to overcome any interference in a valid test and is represented as a ratio (e.g. 1:100).

$$MVD = EL \div \lambda$$

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Where EL is defined as the Endotoxin Limit calculated above, and λ is the labeled sensitivity of the LAL reagent (the minimum concentration of endotoxin that the LAL reagent can detect).

11.2.2 Determine the Non-Interfering Concentration of Final Product for Routine Bacterial Endotoxin Testing

1. Prepare serial dilutions from CSE as instructed in Section 9.0 and referenced in **Table 1**.

2. Label the dilutions as indicated in Section 9.0.

3. Prepare Final Product dilution series in polystyrene tubes.

a. To each tube add the following reagents to achieve the resultant Final Product Dilution. Vortex each dilution for 30 seconds before proceeding to the next dilution.

TABLE 3

Sterile Water (mL)	Final Product Volume (mL)	Final Product Solution Used	Resultant Final Product Dilution	Resultant Final Product Dilution Label
1.0	1.0	Undiluted	1/2	A
1.6	0.4	A	1/10	B
1.5	0.5	B	1/40	C
1.0	1.0	C	1/80	D
0.9	0.1	B	1/100	E

b. Label each tube with the resultant final product dilution ratio.

4. Perform LAL Enhancement Test

a. Label ten single-test LAL tubes with the final product dilution ratios.
b. Add 0.2 mL of each final product dilution to the appropriate tubes.
c. Run the Gel-Clot LaL test and record the results on **Form Q114-L2.**

5. Perform LAL Inhibition Test

a. Label ten single-test LAL tubes with the final product dilution ratios.
b. Add 0.2 mL of each final product dilution to the appropriate tubes.
c. Add 20 µL of 1.25 EU/mL CSE to each LAL tube and swirl to mix.
d. Run the Gel-Clot LAL Test and record the results on **Form Q114-L2.**

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6. **Perform LAL Control Test**
   a. Label sixteen single-test LAL tubes with CSE concentrations 2λ, λ, 0.5λ, 0.25λ.
   b. Add 0.2 mL of each CSE concentration of the appropriate tubes.
   c. Label two single-test LAL tubes with NC for negative control. To both tubes, add 0.2 mL of LAL reagent water.
   d. Run the Gel-Clot LAL test and record the result on **Form Q114-L2.**
   e. Determine the Endpoint for each dilution series. The endpoint is the most dilute concentration of CSE to yield a positive result. Calculate the Geometric Mean Endpoint Concentration (GMEC) of the Final Product at Non-Interfering Concentration (NIC) and record on the **Form FQC-###.**

NOTE: Do not round CSE Concentrations.

GMEC = Antilog ($\Sigma e \div f$) where endpoint concentration is in EU/mL

Where Σe is the sum of the $\log_{10}$ endpoint concentration of the dilution series, and f is the number of replicates used.

7. Determine the non-interfering concentration (NIC): the least dilute final product concentration to yield a positive result in the inhibition test and a negative result in the enhancement test is a compatible concentration. To ensure against interference, a more dilute concentration (not exceeding the Maximum Valid Dilution) may be used.

11.2.3 Validate the Non-Interfering (NIC) Concentration

1. **Perform LAL POSITIVE PRODUCT Test**
   a. In a round bottom polystyrene tube, prepare a 4 mL sample of final product at the NIC determined above. Label the tube NIC.
   b. In a round bottom polystyrene tube, add 0.4 mL of the 20λ CSE Standard to 3.6 mL of the NIC final product solution. Label this solution NIC-2λ.
   c. Prepare test solutions of CSE in final product in round bottom polystyrene tubes by serial dilution of the NIC-2λ (11.2.3,1.b.) with NIC (11.2.3,1.a).

TABLE 4

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	NIC (mL)	Resultant CSE Concentration (EU/mL) of Final Product at NIC
1 mL NIC-2λ	**0**	**0.125 (NIC-2λ)**
1 mL NIC-2λ	**1**	**0.0625 (NIC-λ)**
1 mL NIC-λ	**1**	**0.03125(NIC-0.5λ)**
1 mL NIC-0.5λ	**1**	**0.015625 (NIC-0.25λ)**

d. Label sixteen single-test LAL tubes with CSE concentration. Add 0.2 mL of each test solution to the appropriate tube in quadruplicate. Concurrently perform the LAL Control Test (11.2.2, #6).
e. Run the Gel-clot LAL test and record the results on **Form Q114-L2**.

2. Determine the Endpoint for each dilution series. The endpoint is the most dilute concentration of CSE to yield a positive result. Calculate the Geometric Mean Endpoint Concentration (GMEC) of the Final Product at NIC and record on the **Form Q114-L2**.

NOTE: Do not round CSE concentrations.

GMEC = Antilog ($\Sigma e \div f$) where endpoint concentration is in EU/mL

Where Σe is the sum of the $\log_{10}$ endpoint concentration of the dilution series, and f is the number of replicates used.

11.2.4 Acceptance Criteria

1. Negative water controls must not gel.
2. CSE in water dilution series must confirm LAL Label Claim*.
3. CSE in final product must confirm LAL Label Claim*.

For 0.06 EU/mL LAL the geometric mean of the replicate series is between 0.03 EU/mL and 0.125 EU/mL.

12. GEL-CLOT ASSAY: BACTERIAL ENDOTOXINS LAL TEST

This assay quantifies bacterial endotoxins in every batch of final product by titration to an endpoint. The allowable limit must be less than 175 EU/dose.

12.1 Prepare Equipment and Reagents

NOTE: All reconstitutions, dilutions, and inoculations must be performed using supplies free of detectable endotoxin. This may include glassware depyrogenated using a validated oven cycle.

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12.2 CSE Standard Series Dilutions Method

A CSE standard series of the control standard endotoxin must be prepared fresh unless the product LAL gel clot test is run at a sensitivity of 10 times greater than the MVD. Then the CSE standard series must be run for the first 3 product runs to show the method is valid and for the repeat test when a product sample LAL test is positive, and when the test described in section 12.4 fails. Also perform the test by the CSE Standard Series Dilutions Method when fresh CSE is prepared, the method or formulation for the radiopharmaceutical is changed or when a new lot of LAL is used. Standards are prepared as stated in section 9.0.

12.2.1 Number fourteen single-test LAL tubes, #1-#7, in duplicate. If a 20-minute test is run, number an additional tube #7 and one tube #8.

12.2.2 Obtain a 0.1 mL sample of the final product and dilute to the non-interfering concentration (NIC) as determined in **Section 11.2**.

12.2.3 Add the following reagents in duplicate to tubes #1 - #7. If a 20-minute test is run, add reagent to the additional tube #7 and tube #8 (refer to **Table 5**).

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TABLE 5

Test	Tube Number	Label	Sterile Water (mL)	Final Product at NIC (mL)	Volume of CSE	Concentration of CSE (EU/mL)	λ
60-Minute Endotoxin Test	1	Negative Control	0.2				
	1	Negative Control	0.2				
	2	Positive Water Control			0.2 mL	0.015625	0.25λ
	2	Positive Water Control			0.2 mL	0.015625	0.25λ
	3	Positive Water Control			0.2 mL	0.03125	0.5λ
	3	Positive Water Control			0.2 mL	0.03125	0.5λ
	4	Positive Water Control			0.2 mL	0.0625	λ
	4	Positive Water Control			0.2 mL	0.0625	λ
	5	Positive Water Control			0.2 mL	0.125	2λ
	5	Positive Water Control			0.2 mL	0.125	2λ
	6	Positive Product Control		0.2	20 µL	1.25	2λ
	6	Positive Product Control		0.2	20 µL	1.25	2λ
	7	Final Product at NIC		0.2			
	7	Final Product at NIC		0.2			
20-Minute Endotoxin Test	7	Final Product at NIC		0.2			
	8	Positive Product Control		0.2	50 µL	20	80λ

12.2.4 Run the Gel-clot LAL test and record the results on **Form Q114-L3**.

12.3 Gel-Clot Limit Test

Use for each product test after the CSE Standard Series has been run unless a complete CSE dilution series must be run for the reasons described in section 12.2.

12.3.1 Number eight single-test LAL tubes #1 - #4 in duplicate. If a 20-minute test is run, number an additional tube #4, and one tube #5.

12.3.2 Obtain a 0.1 mL sample of final product and dilute to the non-interfering concentration (NIC) as determined in **Section 11.2**.

12.3.3 Add the following reagents to tubes #1 - #4 in duplicate. If a 20-minute test is run, add reagent to the additional tube #4 and tube #5 as shown in **Table 6** below.

TABLE 6

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Test	Tube Number	Label	Sterile Water (mL)	Final Product at NIC (mL)	Volume of CSE	Concentration of CSE (EU/mL)	λ
60-Minute Endotoxin Test	1	Negative Control	0.2				
	1	Negative Control	0.2				
	2	Positive Water Control			0.2 mL	0.125	2λ
	2	Positive Water Control			0.2 mL	0.125	2λ
	3	Positive Product Control		0.2	20 µL	1.25	2λ
	3	Positive Product Control		0.2	20 µL	1.25	2λ
	4	Final Product at NIC		0.2			
	4	Final Product at NIC		0.2			
20- Minute Endotoxin Test	4	Final Product at NIC		0.2			
	5	Positive Product Control		0.2	50 µL	20	80λ

12.3.4 Run the Gel-clot LAL test and record the results on **Form Q114-L3**.

12.4 Acceptance Criteria

12.4.1 CSE Standard Series

1. The two #1 Tubes, Negative Controls, must be negative. A positive result indicates contamination of the test materials.
2. The two #2 Tubes are Positive Water Controls and they must be negative. A positive result indicates an error in preparation of CSE dilution series because the test sensitivity cannot be greater than a 0.03125 EU/mL (half the labeled sensitivity of the LAL).
3. The two #3 and two #4 tubes are positive water controls. Either the λ or the 0.5λ controls must be positive for duplicates.
4. The two #5 tubes are positive water controls and must be positive. A negative result would indicate an error in preparation of CSE dilution series because the test sensitivity cannot be less than 0.125 EU/mL (twice the labeled sensitivity of the LAL).
5. The two #6 tubes are positive product controls and must be positive. A negative result indicates interference with the proper function of the LAL reagent.
6. The #7 tubes must be negative both at 20 minutes and one hour. A positive result indicates detectable endotoxin in the final product.

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7. The #8 tube is the 20-minute positive product control, and must be positive at 20 minutes.

12.4.2 Gel-Clot Limit Test

1. The two #1 tubes are negative water controls and must be negative. A positive result indicates contamination of the test materials.
2. The two #2 tubes are positive water controls and must be positive. A negative result would indicate an error in preparation of CSE dilution series because the test sensitivity cannot be less than 0.125 EU/mL (twice the labeled sensitivity of the LAL).
3. The two #3 tubes are positive product controls and must be positive. A negative result indicates interference with the proper function of the LAL reagent.
4. The #4 tubes must be negative both at 20 minutes and one hour. A positive result indicates detectable endotoxin in the final product.
5. The #5 tube is the 20-minute positive product control and must be positive at 20 minutes.

12.4.3 If the positive product control does not gel, or the negative water control gels, repeat the test. If the above criteria are met in the repeat test, the Final Product passes the bacterial endotoxin test.

12.4.4 If the final product tests positive, repeat the test using fresh supplies. In addition to the 1:X dilution of the Final Product, test duplicate dilutions of the Final Product at the Maximum Valid Dilution. The Maximum Valid Dilution Must be negative (no gel formed) or the Final Product fails the LAL test for bacterial endotoxins.

The Maximum Valid Dilution (MVD) is calculated from the following equation:

$$175 \div (\text{Volume} \times 0.0625) = \text{MVD}$$

Where Volume = Total Maximum Volume of the Final Drug Product

12.4.5 If the 0.125 EU/mL CSE does not gel, or if the 0.015625 EU/mL CSE gels, repeat the CSE series. If results are the same, prepare new CSE dilutions and repeat the test.

12.4.6 Record the final result on **Form FQC-###**, Final Product QC Test Results" Form I.

12.4.7 If the final product has failed the test, reject the batch and initiate an out of specification investigation as per **GEN-Q015**, "Investigating Out of Specification QC Test Results."

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