

# What Is Limulus Amebocyte Lysate (LAL) and Its Applicability in Endotoxin Quantification of Pharma Products

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## Abstract

Limulus amebocyte lysate (LAL) is an aqueous extract of blood cells (amebocytes) from the horseshoe crab, *Limulus polyphemus*. LAL reagent reacts with bacterial endotoxin and lipopolysaccharide (LPS), which is a membrane constituent of Gram-negative bacteria. This reaction is the base on the LAL reagent, which is then used for the finding and quantification of bacterial endotoxins. The Gel Clot LAL test provides very simple positive or negative result and is most often mentioned in international pharmacopeia monographs as the official test. Gel Clot assay is a qualitative LAL test for detection of Gram-negative bacteria endotoxins. The Gel Clot assay is run in tubes that are placed in a water bath or in dry heated oven at 37°C. After a one-hour incubation period, the tubes are flipped 180°. A firm clot that stays in the bottom of the tube indicates a positive reaction. If the liquid flows down the side of the tube, the result is negative for endotoxins.

**Keywords:** Limulus amebocyte lysate, lipopolysaccharide, endotoxin, blood, bacteria, detection, horseshoe crab, pharmacopeias, delta, toxin, gel, chromogenic, acetic acid, Gram-negative

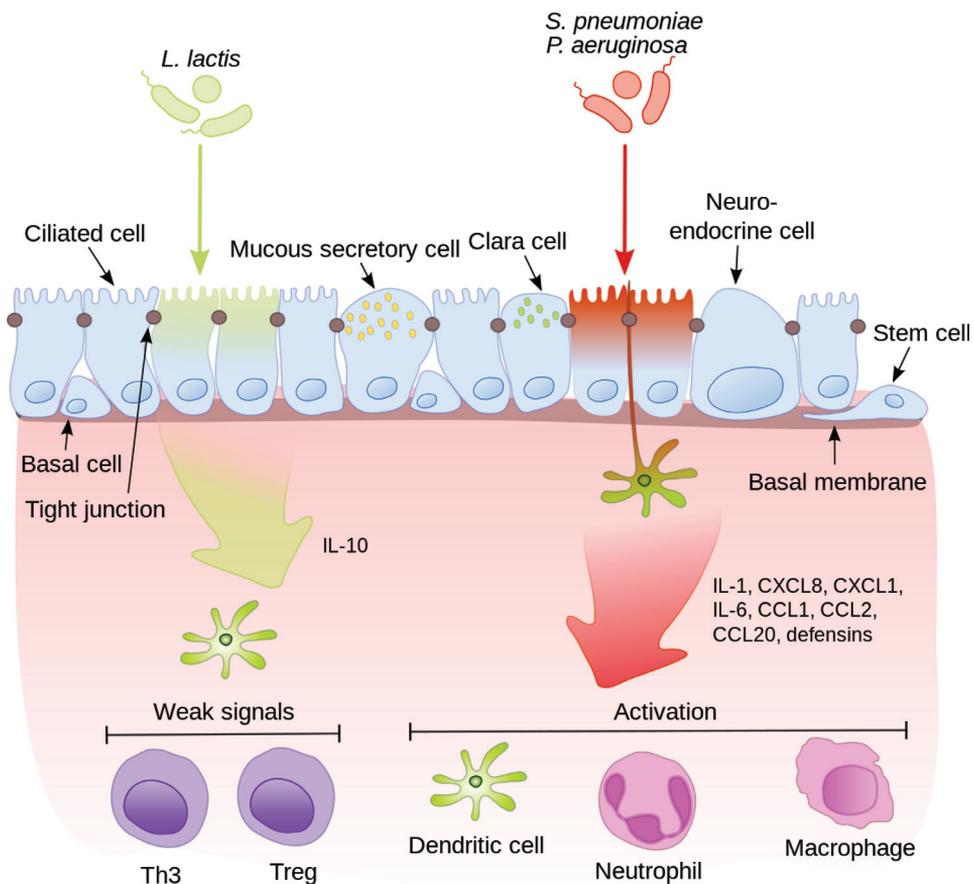
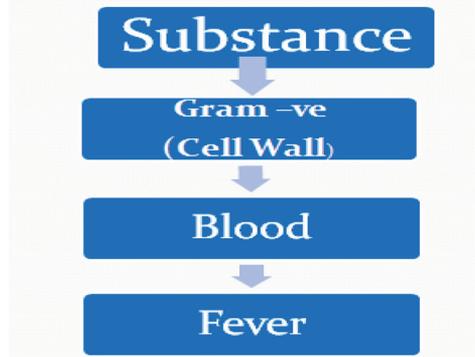
## 1. Introduction

Endotoxins, a type of pyrogen, are natural compounds found in the outer cell membrane of Gram-negative bacteria and can impact over 30 biological activities. Endotoxin can lead to cell death by initiating complement activation. The Limulus amebocyte lysate (LAL) test was commercially introduced in the 1970s. LAL is derived from the blood cells, or amebocytes, of the horseshoe crab, *Limulus polyphemus*. Frederick Bang and Jack Levin observed that blood cells from horseshoe crabs were found to clot in the presence of endotoxin, and this technology was used in the development of endotoxin detection assays. Today, endotoxin tests are performed on raw and in-process materials, and for the final release of products in the pharmaceutical and medical device industries.

Limulus amebocyte lysate test is an aqueous extract of blood cells (amebocytes) which obtain from the horseshoe crab (*Limulus polyphemus*). LAL reagent reacts with the bacterial endotoxins or lipopolysaccharide (LPS). LAL test is recommended in all international pharmacopeias as the method for finding

bacterial endotoxins. Gram-negative bacteria produce endotoxins (pyrogen). Exceptionally *Bacillus thuringiensis*, a Gram-positive bacteria produce delta toxin as endotoxins [1] (Figures 1 and 2).

# Endotoxins



**Figure 1.** Activation of inflammation in body [1]. Note: LPS, lipoglycan. LAL test used according to the U.S. Food and Drug Administration (FDA) [2] guidelines Substituted for the U.S. Pharmacopeia (USP) pyrogen test (rabbit fever test) European Pharmacopeia (EP) Japanese Pharmacopeia (JP) [3]. LAL is used for human injectable drugs, animal injectable drug, medical devices, raw materials used in production, in process quality control.



**Figure 2.**  
*Horseshoe crab* [1].

## 2. Applications of the LAL test in the pharmaceutical industry

Among the most well-known and important applications of the LAL test are the ones related to the pharmaceutical industry. It can be said that the most common pyrogens in pharmaceutical products are endotoxins, which is why the pyrogen tests on rabbits have been replaced by the LAL test according to the recommendations of the international pharmacopeia. One of the reasons that has made the LAL test prevail in the pharmaceutical industry is the careful avoidance by the LAL manufacturers of bringing harm to live animals during both production and testing. It is important to clarify that the crabs, from which part of the hemolymph used for the LAL test was extracted, are returned to alive to their natural habitat with no lasting problems after the extraction.

### 2.1 *Limulus amebocyte lysate (LAL)* test types

*Gel Clot technique*: based on gel formation [4].

## 3. Methods to determine the pyrogen in pharma products

*Chromogenic method*: based on the producing color after cleavage of a synthetic peptide-chromogen complex.

*Turbidimetric method*: based on forming turbidity after cleavage of an endogenous substrate.

*End point method*: 0.005 endotoxins units (EU) per ml.

*Kinetic method*: 0.001 endotoxins units (EU) per ml.

*Kinetic method*: time taken to reach a specific absorbance at 405 nm (onset time) is determined. The assay requires specialized instrumentation. Take optical density readings at regular intervals. The greatest sensitivity,  $\lambda$ , of lysate is 0.001 EU/ml.

*Endpoint chromogenic method* [5]: the released amount of pNA can be calculated after a fixed incubation period. A standard curve, consisting of measured optical density plotted against known standard endotoxin concentration. Later used to determine concentrations in the product. The greatest sensitivity,  $\lambda$ , is 0.005 EU/ml UV/visible spectrophotometers.

## 4. Test performance

Add volume of lysate to a volume of product dilution. Incubating the reaction mixture at 37.5°C. Endotoxin in the reaction would activate the LAL reagent. Cleave

small chromogenic peptides and liberates pNA. pNA, color is yellow and absorbs light at 405 nm. For samples that absorb at 405–410 nm, Diazo-coupling agent modification may be used. In this method, pNA reacted with nitrite in hydrochloric acid, ammonium sulfamate and N-(1-naphthyl)-ethylenediamine (NEDA). Absorbs at a range between 540 and 550 nm. A standard curve is used to establish concentrations in product specimens.

## **5. Materials and equipment**

10 × 75 mm fully depyrogenated borosilicate glass culture tubes (Associates of Cape Cod, Inc. catalog numbers TB050).

Optical reader is capable of reading at 405 nm, or at 540–550 nm for the diazo method. Incubator is able to maintaining  $37 \pm 1^\circ\text{C}$ . A water bath can be used for the endpoint test tube method. Both devices should have a uniform heat distribution. Test tube racks to hold the tubes and/or incubate dilution and reaction tubes. Micro-pipettes or disposable pipette tips free of interfering endotoxins and glucans are recommended. Vortex-type mixer, Para film (American National Can™) and hot-air oven with the capacity to heat to at least  $250^\circ\text{C}$  for depyrogenation of glassware.

## **6. Chemicals and reagents**

Limulus amebocyte lysate (LAL), LAL reconstitution buffer, control standard endotoxins (CSE), solution 1 (nitrite), solution 1A (0.1 N hydrochloric acid), solution 2 (ammonium sulfamate), solution 3 (N-(1-naphthyl)-ethylenediamine (NEDA)), LRW.

The endotoxins limit for USP/BP sterile WFI is only 0.25 EU/ml; therefore, sterile WFI may contain detectable endotoxins and be unsuitable for use. Use certified LRW to make dilutions of standards, and to prepare positive controls.

## **7. Quality control steps or test procedure**

### **7.1 Specimen collection and preparation**

Collect aseptically containers that are free of detectable endotoxins in depyrogenated glassware apparatus.

### **7.2 pH of the specimen**

The pH must be 6–8. Adjust the pH of the product specimen with dilute HCl, NaOH, or buffer (free of endotoxins). Dilute concentrated HCl or NaOH with LRW. Use a volume that will not lead to significant dilution of the test specimen. Dilution (LRW) alone can overcome the issue sometimes.

### **7.3 Method of lysate reconstitution**

Gently tap the vial of lysate. Loose material fall to the bottom. Break the vacuum by lifting the gray stopper. Do not contaminate the mouth of the vial. Remove and discard the stopper. Start the reconstituted lysate with 3.2 ml buffer. Avoid vigorous mixing that may cause excessive foaming and a loss of sensitivity. Wrap the vials with parafilm and store in a cold place ( $2\text{--}8^\circ\text{C}$ ) when not in use and use within 8 h of reconstitution.

## 7.4 Lysate storage conditions

### 7.4.1 Lyophilized lysate

This is relatively well stable and, if stored properly, will retain full activity through the expiration date on the label. Store the product at 2–8°C. Excess temperature over 37°C cause rapid deterioration, loss of sensitivity and distinct yellowing.

## 7.5 Control standard endotoxins (CSE)

Each vial of control standard endotoxins (CSE) contains 10 ng of endotoxins. Reconstitute CSE with the volume mentioned on the Certificate of Analysis (CA, which gives the potency of the CSE). Gently knock the vial of control standard endotoxins (CSE) to cause loose material to fall to the bottom. Break the vacuum by lifting the gray stopper. Do not contaminate the mouth of the vial. Remove the stopper and place it in a cold place aseptically for reuse.

Reconstitute CSE with the volume specified on the Certificate of Analysis (CA, which gives the potency of the CSE) and as directed in the package insert. Place the stopper. Vortex the vial for 40–60 s to form a homogenous mixture. Discard solution if not used immediately, vortex the vial for 30 s prior to use.

### 7.5.1 Mixing and incubation

Read the tubes UV/visible spectrophotometers (**Table 1**).

CSE + lysate	Incubation time (min)
50 µl of 0.50 EU/ml + 50 µl	30
50 µl of 0.250 EU/ml + 50 µl	30
50 µl of 0.125 EU/ml + 50 µl	30
50 µl of 0.0625 EU/ml + 50 µl	30

**Table 1.**  
*Dilution mixing and incubation time.*

### 7.5.2 Mixing and incubation

Stop the reaction by adding 50% acetic acid. Add 0.025 ml (25 µl) read the optical density (OD) at 405 nm read the test.

Sample + lysate	Incubation (min)
50 µl of sample + 50 µl	30

## 7.6 Stop reaction solution preparation

### 7.6.1 Read the test

Reconstitute vial 1 with entire contents of vial, reconstitute vial 2 with 4 ml of water, reconstitute vial 3 with 4 ml of water. Add 0.05 ml (50 µl) of solution 1

(sodium nitrite reconstituted with dilute HCL). Add 0.05 ml (50 µl) of solution 2 (ammonium sulfamate). Add 0.05 ml (50 µl) of solution 3 (NEDA) use new pipette tip agitate the plate to mix. Full color (magenta) should develop immediately. Read the test at 540–550 nm.

### *7.6.2 Positive control*

#### *7.6.2.1 Make standard curve*

Positive control must be included to verify that it is appropriate to use the parameters of a previous (archived) standard curve to calculate endotoxin concentrations.

### *7.6.3 Negative controls*

LRW negative controls should be included with each test

1: Equation of straight line (results)

$$y = mx + c$$

$$m = \text{slop}$$

x = endotoxin concentration,

c = y-intercept and

y = mean absorbance

$$X = y - c / m$$

Example calculation

### *7.6.4 Sample preparation*

Prepare sample solutions by dissolving or diluting drugs (pH 6.0–8.0). The pH may be adjusted by the use of acid, base, or suitable buffers as recommended. Do not exceed the MVD or MCV while making dilutions and adjusting the pH.

### *7.6.5 Maximum valid dilution (MVD)*

$$\text{MVD} = (\text{endotoxin limit} \times \text{concentration of sample solution}) / (\lambda)$$

Endotoxin limit given in USP, concentration of a sample of the label,  $\lambda$ : the labeled lysate sensitivity in the gel-clot technique (IU/ml) or the lowest concentration used in the standard curve for the turbidimetric or chromogenic techniques.

### *7.6.6 Minimum valid concentration (MVC)*

$$\text{MVC} = \lambda / \text{endotoxin limit}$$

$\lambda$ : the labeled lysate sensitivity in the gel-clot technique (IU/ml) or the lowest concentration used in the standard curve for the turbidimetric or chromogenic techniques.

Sample 1

Endotoxin limit: 0.5 EU/ml

Concentration of sample: 100 mg/ml

$\lambda$ : 0.06 EU/ml

$$\text{MVD} = 0.5 \text{ EU/ml} \times 100 \text{ mg/ml} / 0.06 \text{ EU/ml}$$

$$\text{MVD} = 833$$

Add 1 ml of sample 1 in to 832 ml of LRW. Prepare sample 2 in using the same method.

### 7.6.7 Preparation of CSE dilutions

Using 10-fold and 2-fold dilution methods prepare the following dilutions of control standard endotoxins (CSE)

1. 0.5 EU/ml
2. 0.25 EU/ml
3. 0.125 EU/ml
4. 0.0625 EU/ml

Reconstitute the lysate with 3.2 ml of buffer provided with it. Follow the standard procedure for reconstitution.

#### 7.6.7.1 Mixing and incubation

Stop reaction.

For sample 1 and sample 2:

Stop the reaction by adding 50% acetic acid. Add 0.025 ml (25 µl) (**Tables 2 and 3**).

CSE + lysate	Incubation (min)
50 µl of 0.50 EU/ml + 50 µl	30
50 µl of 0.250 EU/ml + 50 µl	30
50 µl of 0.125 EU/ml + 50 µl	30
50 µl of 0.0625 EU/ml + 50 µl	30
50 µl of sample 1 + 50 µl	30
50 µl of sample 2 + 50 µl	30

**Table 2.**  
 Different dilution of CSE and lysate.

Sample	Absorbance at 405 nm	Mean Absorbance	Mean Absorbance – Blank Absorbance
LAL Reagent	0.080	0.082	
Water (Blank)	0.084		
0.0625 EU/ml	0.128	0.126	0.044
	0.124		
0.125 EU/ml	0.160	0.170	0.088
	0.180		
0.25 EU/ml	0.309	0.317	0.235
	0.325		
0.5 EU/ml	0.570	0.564	0.482
	0.557		
Sample 1	0.372	0.382	0.300
	0.392		
Sample 2	0.416	0.417	0.335
	0.418		

**Table 3.**  
 Make two replicates of each CSE and sample preparation to reduce any errors.

## 8. Results

Use Microsoft word for further calculations and results. Make standard curve and endotoxin concentration (**Figure 3**).

$R^2$  = coefficient of determination

R = correlation coefficient

$R \geq 0.98$

$R^2 = 0.99$

$R = \sqrt{R^2} = 0.99$

Equation of straight line

$y = mx + c$

m = slop

x = endotoxin concentration

c = y intercept

y = mean absorbance

Equation of straight line

$Y = 1.019X - 0.026$

### 8.1 Rearranging the equation

$X = Y + 0.026/1.019$

m = slop = 1.019,

C = y intercept = 0.026,

Y = mean absorbance,

X = endotoxin concentration

### 8.2 Sample 1

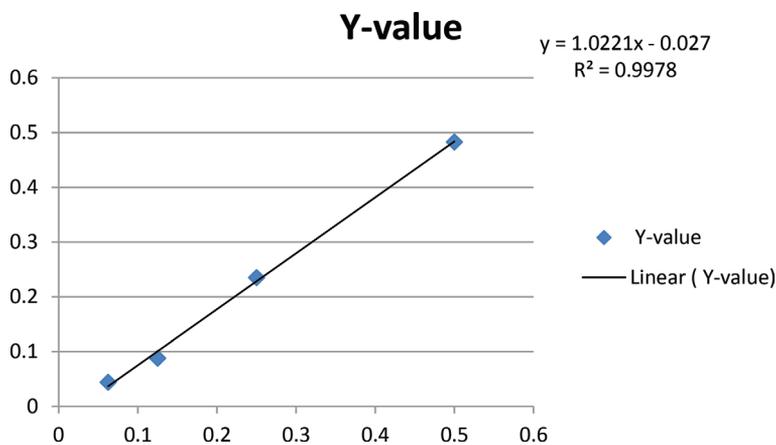
$X = Y + 0.026/1.019$

$Y = 0.300, X = 0.300 + 0.026/1.019, X = 0.319\text{EU/ml}$

### 8.3 Sample 2

$X = Y + 0.026/1.019$

$Y = 0.335, X = 0.335 + 0.026/1.019, X = 0.354\text{ EU/ml}$



**Figure 3.**  
Validation of standard curve.

## 8.4 Advantages of Gel Clot method

Gel Clot LAL provides a simple positive/negative result and is most often mentioned in pharmacopeial monographs as the official referee test.

This is very easy to perform.

This is not time consuming.

Accuracy is 100 percent.

The LAL Gel-Clot assay, gives a more quantitative measurement of endotoxin over a range of concentrations.

## 8.5 Standard operating procedure

### 8.5.1 Material

Gel Clot lysate for 20 test, Gel Clot standard 0.5 EU/Vial, LAL reagent water (LRW 50 ml).

### 8.5.2 Reconstitution

Lysate: add 2 mL LRW and mix it slowly. Do not shake and avoid foaming. Transfer 0.1 ml in 20 test tubes. Store it at  $-8^{\circ}\text{C}$  (in freezer).

Standard: Add 2 mL of LRW in the vial and mix it well for 15 min. Store the vial at  $2-8^{\circ}\text{C}$ . Storage life is 15 days.

### 8.5.3 Procedure

Take three test tubes and mark them as test, positive control and negative control [1].

Add your sample in test tube marked as sample. Add standard in test tube marked as Positive control. Add LRW in test tube marked as negative control. Incubate the test tubes at  $37 + 2^{\circ}\text{C}$  for 60 min. After an incubation, check for the gel by inverting the test tube. If the material remains firm in the bottom of the test tube, it means gel has formed. This positive if the material gets the flow down, it means gel has not formed. This means negative.

### 8.5.4 For water for injection

Take similarly three test tubes as above and add water for injection (WFI) in test tube marked as sample. And proceed as above. The results should be as follows (Table 4):

### 8.5.5 For product

We have to make dilution.

Sample	Positive control	Negative control	Result
-ve (gel not formed)	+ve	-ve	Pass
Sample	Positive control	Negative control	Result
+ve (gel formed)	+ve	-ve	Fail

**Table 4.**  
Results shown sample pass or not.

Example: If the product endotoxin limit is 1 EU/ml, then we have to make the dilution as follows:

Since we are using 0.25 EU/ml, this is called lambda. Divide the endotoxin limit of product with lambda

$$1/0.25 = 1:4$$

As per USP, we have to test 3 test as follows:

One test tube	1:3	The result should be positive
Second	1:4	The result may be positive or negative
Third	1:5	The result should be negative

This means the product is passed.

#### 8.5.6 LAL test reagents (*chromogenic method*)

Chromogenic lysate [2],

Respective endotoxin standard,

Diazo coupling reagent (set of four bottles).

Note: All reagents must be stored in refrigerator at 2–8°C.

#### 8.5.7 Preparation of acetic acid 0.8 m

Dissolve 45.6 ml of acetic acid in 1 liter of distilled water. The final concentration of acetic acid is 0.8 M. This solution can be stored for 3 months.

Remove the plastic cover. Wipe off with 70% alcohol around the rubber cap and top portion of every vial. Remove the aluminum cap with sterile and pyrogen free forceps and then cover with depyrogenated aluminum foil to avoid any Endotoxin contamination. (2.8 ml LAL water vial is provided with Endotoxin vial, concentration is mentioned on the label). Pour whole quantity of LAL water into the ET vial and cover with foil. Mix vigorously for at least 10 s by vortexer. During stirring solution must not touch the foil.

**Storage:** Store reconstituted Endotoxins solution at 4°C in a refrigerator for 14 days. The solution can also be stored at –20°C for a month. Avoid freezing during storage.

Note: Stir every time vigorously before use.

Toxicolor lysate

(Buffer vial 0.35 ml and LAL water are provided with Lysate. Sensitivity is mentioned on the certificate). After taking from the refrigerator, pour whole quantity of buffer and 0.35 ml LAL water into the lysate vial as soon as possible, covers with foil. Then quickly stir to dissolve. Avoid air bubbling during stirring. Place the vial in ice water bath for 2–3 min before use.

Note: Be sure that the reagent is completely dissolved. This reagent must be reconstituted just before use. The reagent is extremely sensitive and must be consumed at one time. Storage should be avoided, but can be stored at –20°C in 0.1 ml dispensed quantities in small test tubes. Use stored lysate if the color is not changed. Reconstituted lysate may only be deep frozen once.

#### 8.5.8 Diazo coupling reagent

Four bottles are provided with one set, marked as 7, 8, 9 and 10s respectively. Transfer whole quantity of bottle no. 7 s into bottle no. 8 s. Then add 12 ml distilled

water into each of bottle no. 9 s and 10s. Ultimately, we will have three bottles 8, 9, and 10 s, which are used stepwise to block the reaction.

## **9. Pre-test preparations**

### **9.1 pH of the sample**

The pH of the sample is adjusted by pyrogen free 0.1 N NaOH or 0.1 N HCl. The pH of the sample should be between 6.0 and 8.0.

### **9.2 Test tubes settings**

Arrange test tubes in two stands as under; stand 1—test tubes for sample and standard dilutions; stand 2—test tubes for reaction.

## **10. Procedure**

### **10.1 Sample preparations**

Take 0.05 ml well-mixed sample into small test tubes. If required, make 1/10 dilution of the sample with Pyrogen free water as Below, Take 4.5 ml of pyrogen free water in the test tube. Then add 0.5 ml of well-mixed sample. Vortex mixing for a few seconds.

Take 0.1 ml into a small test tube for further process.

### **10.2 Standard preparation**

Make a dilution of the endotoxin (concentration 0.470 EU/ml) according to the product limit. For making 0.235 EU/ml (if the product limit is 0.25) proceed as follows;

Take 0.05 ml of the reconstituted endotoxin in the test tube after stirring. Add 0.05 ml of pyrogen free water and vortex to mix. Now the final dilution is 0.235 EU/ml.

Take 0.05 ml of step 2 into a small test tube for further process.

### **10.3 Blank preparation**

Pour 0.05 ml of pyrogen free water (being used in the test) in small test tube as a blank for further process.

Lysate addition

Place the tube stand for small test tubes (containing the tubes of blank, standard and diluted samples) in ice water bath or suitable ice water container. Add 0.05 ml of lysate to all of the tubes as soon as possible. Stir the contents of every tube soon after the addition of lysate for a few seconds. Avoid foaming.

### **10.4 Incubation**

Soon after the addition of lysate, place the test tube rack in the incubator set at  $32.5 \pm 2.5^{\circ}\text{C}$  for 30 min. The tube rack can be placed in the water container placed in the incubator.

## **10.5 Blocking the reaction**

After completion of the incubation period, place tube rack in ice water bath, then blocks the reaction immediately from one of the two methods mentioned below:

By acetic acid

Add 0.4 ml of 0.8 M acetic acid into each tube and stir to mix.

By diazo coupling reagent

Three bottles of the reagent are used as under;

Add 0.5 ml from bottle no 8 s to each tube and stir to mix.

Add 0.5 ml from bottle no 9 s to each tube and stir to mix.

Add 0.5 ml from bottle no 10s to each tube and stir to mix.

Absorbance reading (using spectrophotometer) measurement at 405 nm

If 0.8 M acetic acid is used to block the reaction, then absorbance reading is taken at 405 nm.

Note: The readings. Glass photocell is used for reading at 405 nm. Because the volume of the tube content is not sufficient, the distilled water is added to each tube and is stirred to mix.

## **10.6 Measurement at 545 nm**

When Diazo coupling reagent is used for blockage of the reaction then the reading is taken at 545 nm. Note all the readings.

Note: Distilled water is used for reference in both cases.

## **11. Results from software**

All the absorbance readings are fed in the “Software reader for window version 1.51” to collect the results.

### **11.1 Manual measurement of endotoxin**

Following Formula is used to calculate the results

$$\text{Endotoxin/ml in product} = \frac{\text{conc.of std.in solution} \times \text{sample ABS} - \text{blank ABS}}{\text{Std.ABS} - \text{blank ABS}} \times \text{DF}$$

Calculations of MVC, MVD and ELC.

$$\text{MVC} = \text{M/K}$$

Where the lowest sensitivity of lysate, M is the maximum dose/kg body weight and K is constant having value equal to 5.

$$\text{MVD} = \text{concentration of product in 1 ml/MVC}$$

$$\text{ELC} = \text{X MVD}$$

## **Conflict of interest**

The author(s) confirm that this chapter content has no conflict of interest.

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