Dear Reader,

I hope you have had the opportunity to learn about the advanced lysates developed by Charles River Laboratories. This information about lysates would go a long way in enabling you to overcome interferences and address testability issues of your products.

In the present context we wish to treat the subject of dilution in a different manner and bring out the attributes of dilution as a technique and a tool. A clear understanding of these attributes of ‘dilution’ will empower the analyst to address many a difficult situations encountered during testing.

We have attempted to illustrate with suitable examples the impact of dilutions on LAL testing, which I am sure would catch the attention of both an experienced as well as an in-experienced analyst.

It is my pleasure to announce the launch of our website: www.criverindia.com with the advent of the new fiscal year. The website is designed to provide an easy, yet effective access to the entire portfolio of Charles River’s products and services in ‘accelerating drug development. exactly’.

I welcome you to visit us at www.criverindia.com and look forward to a meaningful and continued interaction through our website.

Sincerely,

Dr. P. K. Chitnis
Director

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Dilution: A Tool and A Technique For LAL Assays
Dr. K. Nagarajan and Dr. P. K. Chitnis

Introduction
The Limulus Amebocyte Lysate (LAL) test is the assay of choice for bacterial endotoxins because of its specificity and sensitivity. An obstacle to the early acceptance of LAL test by the FDA was centered around the fact that very few parenteral products could be directly tested without encountering significant Interference (Inhibition or Enhancement) to the test. An FDA survey on LAL test compatibility with human drugs documented the extent of inhibitory conditions when it reported that only 30% of the drug entities studied could be tested without modification. Fortunately, 97% of inhibition problems were successfully resolved by simple pretreatment such as dilution, vindicating the fact that inhibition was usually concentration dependent.

Maximum Valid Dilution (MVD)
Maximum valid dilution is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. It applies to injections or to solution for parenteral administration in the form of constituted or diluted for administration, or, where applicable, to the amount of drug by weight if the volume of the dosage form for administrated could be varied. The general formula to determine MVD is:

\[
MVD = \frac{\text{Potency of Product} \times \text{Endotoxin Limit}}{\text{Labeled Lysate Sensivity (})}
\]

Example
Product name: Gentamycin Sulphate
Concentration: 40 mg/mL
Endotoxin Limit: 0.71 EU/mg
Lysate Sensitivity (\(l\)) : 0.125 EU/mL

\[
MVD = \frac{40 \text{ mg/mL} \times 0.71 \text{ EU/mg}}{0.125 \text{ EU/mL}} = 1.227
\]

(Extent of permissible dilution)

Minimum Valid Concentration (MVC)
Minimum Valid Concentration is the minimum concentration of a sample at which the endotoxin limit can be determined. The MVC applies to injectable drugs that are dosed by mg or unit. The general formula to determine MVC is:

\[
MVC = \frac{\text{Labeled Lysate Sensivity (})}{\text{Endotoxin Limit}}
\]

# Inhibition - Endotoxin recovery less than expected
Enhancement - Endotoxin recovery is more than expected
Example
Product name: Tazobactum Sodium
Endotoxin Limit: 0.2 EU/mg (Internal Limit)
Lysate Sensitivity (I) : 0.125 EU/mL

MVC = \frac{0.125 \text{ EU/mL}}{0.2 \text{ EU/mg}} = 0.625 \text{ mg/mL}

Endotoxin Limit
The endotoxin limit for active substances administered parenterally, define on the basis of dose (effects of endotoxin are related to the amount of endotoxin in the product dose administrated to a patient). Because the dose varies from product to product, the endotoxin limit is calculated using the given formula:

\text{Endotoxin Limit} = \frac{K}{M}

Where, 
K = 5.0 \text{ EU/kg for parenteral drugs and 0.2 EU/kg for Intrathecal drugs}
M = Rabbit Dose or Maximum Dosage/kg of body weight that would be administered in a single one hour period, whichever is larger.

For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time.

Use 70 kg as the weight of the average human when calculating the maximum human dose per kg.

Also, if the pediatric dose/kg is higher than the adult dose then it shall be the dose used in the formula.

Center for Devices and Radiological Health (CDRH) has recommending the following guidelines for medical devices:

A. Validation of the LAL test

1. **Sensitivity**
   Data demonstrating the sensitivity and reproducibility of the LAL test.

2. **Inhibition/Enhancement Testing**
   Each product line of devices utilizing different materials or methods of manufacture should be checked for inhibition or enhancement of the LAL test.

Under the Inhibition/Enhancement testing the guideline recommends

1. At least three production lots of each product type should be tested for inhibition.

2. **Testing**
   - 2 devices for lot sizes under 30,
   - 3 devices for lot sizes 30-100,
   - 3% of lots above size 100 and up to a maximum of 10 devices per lot.

**Extraction and Eluate Dilution**
The process of preparing an eluate/extract for pyrogen or inhibition/enhancement testing may vary for each device. Some medical devices can be flushed; some may have to be immersed in the non-pyrogenic rinse solution, while others may be tested by disassembling or by cutting the device into pieces prior to extraction by immersion. In general, for device being flushed, the non-pyrogenic rinse solution should be held in the fluid pathway for 60 minutes at room temperature (above 18°C) and the rinsed or flushed solutions should be combined. If a device is to undergo extraction, a minimum extraction time should be 15 minutes at 37°C, one hour at room temperature (above 18°C) or other demonstrated equivalent conditions.

Regarding rinse volumes, for unusually small or large devices, the surface area of the device which comes in contact with the patient may be used as an adjustment factor in selecting the rinsing or extracting volume. For example,

Device : Coronary Stents
No. of Devices : 3 Nos
Endotoxin Limit/Device : 20 EU/device
Rinse Volume : 60 mL
Lysate Sensitivity (I) : 0.125 EU/mL
Coronary stent system was completely filled with a total of 20 mL of LRW. The device was kept at room temperature for an hour. The rinsing solution has been collected into depyrogenated glassware.

**Dilution**

\[
\text{Dilution} = \frac{\text{Volume of the sample}}{\text{Volume of the solution}} = \frac{1.0 \text{ mL sample}}{1.0 \text{ mL sample} + 9\text{ mL water}} = \frac{1.0 \text{ mL sample}}{10 \text{ mL}} = 1:10 \text{ total}
\]

The graphical representation is given below:

Thus 1 mL of sample is diluted to 10mL.

**Serial Dilution**

Serial dilution is a dilution made of a series of smaller dilution and the total dilution can be calculated by simply multiplying each dilution factor involved in the series.

**Spiking Methods used in LAL Testing**

1. **Double Strength Method or 50:50 Method**
2. **Hot-Spike Method**

**Double Strength/Dilution Method or 50:50 Method**

1. Prepare double the strength test concentration or half of the test dilution of the sample
2. Prepare 4λ of CSE
3. Dilute 2X product (where \(X = \text{Test Sample Concentration}\)) or dilute \(\frac{1}{2}Y\) (\(Y = \text{Test Sample Dilution}\)) with equal volume of LRW in Negative Product Control (NPC)
4. Dilute 2X product (where \(X = \text{Test Sample Concentration}\)) or dilute \(\frac{1}{2}Y\) (\(Y = \text{Test Sample Dilution}\)) with equal volume of 4λ in Positive Product Control (PPC)

**Maximum Dose**

We would like to highlight some finer points* while considering maximum dose for calculation of the endotoxin limit with the help of a following example:

**Case Study**

For a drug having a labeled total dose of 1.2 gm per day with an instruction that the dosing frequency should be 4 times daily, how does one go about the calculation of maximum dose?

One might be tempted to consider the maximum dose as 1.2 gm /24 hrs = 50 mg/hr which would result in over estimation of the Endotoxin Limit. Therefore, one has to consider the actual quantum of the drug being administered ‘at one time’ for calculating the maximum dose as 1.2 gm/4 = 300 mg/hr.

Thus the dose of 300 mg/gm would result in the endotoxin limit being more conservative than while considering 50 mg/hr.

**Dilutions**

At the risk of being naive and redundant we wish to dwell on the “simple” aspect of dilutions. We have attempted to simplify the calculations regarding dilutions so as to address the common concerns.

Dilutions are expressed as the ratio of the quantity of a desired solute (i.e. sample) contained in a solvent (i.e. LAL Reagent Water). In other words,

\[
\text{Dilution} = \frac{\text{Volume of the sample (solute)}}{\text{Volume of the solution (solute + solvent)}}
\]

A 1 mL of sample is diluted to 10 mL by adding, enough diluent to the original volume to yield a final, total volume of 10 mL. Therefore, the dilution is expressed according to the following equation:

\[
\text{Dilution} = \frac{\text{Volume of the sample}}{\text{Volume of the solution}} = \frac{1.0 \text{ mL sample}}{1.0 \text{ mL sample} + 9\text{ mL water}} = \frac{1.0 \text{ mL sample}}{10 \text{ mL}} = 1:10 \text{ total}
\]

The graph below shows the graphical representation of serial dilution:

Thus 1 mL of sample is diluted to 10mL.

**Serial Dilution**

Serial dilution is a dilution made of a series of smaller dilution and the total dilution can be calculated by simply multiplying each dilution factor involved in the series.

To illustrate the above point consider the following example:

**Spiking Methods used in LAL Testing**

(Recommended methods only)

1. **Double Strength Method or 50:50 Method**
2. **Hot-Spike Method**

**Double Strength/Dilution Method or 50:50 Method**

1. Prepare double the strength test concentration or half of the test dilution of the sample
2. Prepare 4λ of CSE
3. Dilute 2X product (where \(X = \text{Test Sample Concentration}\)) or dilute \(\frac{1}{2}Y\) (\(Y = \text{Test Sample Dilution}\)) with equal volume of LRW in Negative Product Control (NPC)
4. Dilute 2X product (where \(X = \text{Test Sample Concentration}\)) or dilute \(\frac{1}{2}Y\) (\(Y = \text{Test Sample Dilution}\)) with equal volume of 4λ in Positive Product Control (PPC)
**Example**

Product: ACPD Solution

Endotoxin limit: NMT 5.56 EU/mL  
LAL sensitivity ($\lambda$) = 0.125 EU/mL  
MVD = 1:44

MVD/2 = 1:22

In order to test the sample at MVD or at Endotoxin Limit, prepare the solution at ½ MVD i.e. 1:22

NPC = 100 µL Sample (1:44) + 100 µL LAL  
PPC = 100 µL Sample (1:44) + 10 µL 20X CSE + 100 µL LAL

If NPC = negative and PPC = positive, then

Endotoxin content of the sample = dilution factor x Lysate Sensitivity  
= 44 x 0.125 EU/mL  
= < 5.5 EU/mL

**Hot-Spike Method**

1. Prepare test concentration or test dilution of the sample
2. Prepare 20X of CSE
3. Take X product (where X = Test Sample Concentration) or Y (Y = Test Sample Dilution) in Negative Product Control (NPC)
4. Spike test sample with minimal but accurately measurable concentrated endotoxin solution i.e. 10 µL of 20X

**Sample Preparation**

**Dilution as a Tool and a Technique**

The first step in creating a LAL test method for a product is identification of a suitable dilution or concentration for routine testing i.e. in other words the validated method defines the optimum test concentration or test dilution, preparation of samples and controls, maintenance of test conditions and acceptance criteria. Therefore, in LAL test the first step involves preparation of samples and controls i.e. it involves dilutions. Here, we are going to discuss the MVD calculation and dilution preparation of sample by taking the following example:

**Product:** Amoxicillin Sodium  
**Concentration:** 500mg/2mL  
**Endotoxin Limit:** 0.25 EU/mg  
**Lysate sensitivity ($\lambda$) = 0.125 EU/mL

**MVD** = \[
\text{Potency of Product x Endotoxin Limit} \\
\text{Labeled Lysate Sensitivity (\(\lambda\))}
\]

\[
\text{MVD} = \frac{500 \text{ mg/2 mL} \times 0.25 \text{ EU/mg}}{0.125 \text{ EU/mL}} = 1:500
\]

MVD/2 = 250 (prepared dilution)
How to approach to prepare 1:250 dilution, and thereof it will become 1:500 by Double Strength method. There are different ways to obtain 1:250 dilution and the simplest approach is as follows:

**Conclusion**

Thus ‘dilution’ qualifies as an apt tool in the hands of an analyst for effectively addressing the interferences encountered during LAL testing, while complying with the regulatory norms.

**References**

2. USP 28-NF23 Supplement 2 Chapter on <85> Bacterial Endotoxins Test.

**Events and Happenings**

**Charles River India goes online**

A website for Charles Rives India is up and running. Now you can visit us online for latest news, product line, events and downloads.

**Kinetic LAL Workshop**

Charles River India successfully conducted a workshop titled **Kinetic Methods in LAL Testing** on 15th & 16th of April 2010 at Hotel Le Meridien, New Delhi.

**What’s Next?**

The next **Kinetic LAL Workshop** is scheduled to be held at Hyderabad in August 2010. The details will be announced shortly.