LAL UPDATE®

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Dear LAL User,

Although there was a lot of snow on Cape Cod this winter (and still some on the ground as I write), we have managed to move forward on two building projects. The first, a new training and technical services wing, is now complete and our February training class will be the first to benefit from this facility. The second project, a new filling/lyophilization suite, is scheduled for completion sometime late this summer. This new facility is designed to handle our rapidly increasing demand for turbidimetric and chromogenic LAL. More information (with pictures!) will appear in subsequent issues of the LAL UPDATE. Of course, if you want to see our operation for yourself, and benefit from our free training, call our office and enroll in one of our upcoming courses.

The subject of this LAL UPDATE, "Preliminary Testing", was also the subject of one of the very first issues (Vol. 2. No. 3 Supplement). Although this original treatment of the subject is somewhat out-of-date and no longer in print, it was one of the most widely distributed and translated issues of the LAL UPDATE. The article yields all the information needed to reliably test a sample with the gel-clot test. In our Contract Test Service, preliminary testing on new or unknown samples is still routinely used for rapid characterization. The information gleaned from preliminary testing sets the course for the actual sample test and ends up saving time and reagent (fewer repeated tests). The article in this LAL UPDATE expands on the original concept to include its use in the turbidimetric and chromogenic assays.

Sincerely,

Thomas J. Novitsky, Ph.D.
Editor

Preliminary Testing

by Michael E. Dawson, Ph.D.

Validation of an LAL method for testing a product requires the performance of inhibition or enhancement studies. It is therefore prudent to determine both the endotoxin concentration and the dilution at which the inhibition or enhancement test is to be carried out on an uncharacterized sample. These determinations are referred to as **preliminary tests** and entail testing a series of product dilutions, both with and without an added endotoxin spike.

Although preliminary testing is not required by any regulatory body or Pharmacopeia, it is required by the dictates of good practice and common sense. The information from the preliminary test(s) provides the justification for the dilution selected for validation of the method and this test data should be kept on file.

The principles of preliminary testing are the same for all LAL test methods. Since the gel-clot method is read as either a clot or no clot, preliminary tests for this method can only give information about inhibition of the assay; a sample which enhances the test cannot make it clot more. By contrast, in the turbidimetric and chromogenic methods, recovery of the endotoxin spike is quantified so that both enhancement and inhibition are evident.

When testing a new product, the first consideration is the endotoxin limit[1]. This is because the limit determines the maximum valid dilution (MVD)[2] for the test. Sometimes the endotoxin limit is not known, in which case a test can be performed and the results reported, but no decisions can be made without a limit as a reference point. When an official limit is not stated in the USP, it may be possible to calculate one from the maximum dose of the product. Failing that, the minimum concentration of endotoxin that must be detectable can usually be stated. This is effectively an endotoxin limit. If detection of at least 10 EU/ml is required and the sensitivity (λ) of the gel-clot reagent is 0.125 EU/ml,

then the sample cannot be diluted by more than a factor of 80 (10 EU/ml ÷ 0.125 EU/ml). This is the MVD and there is no reason to dilute the sample further because dilution reduces the sensitivity of the test beyond the point where 10 EU/ml is detectable.

If the sensitivity of the test is increased, the MVD also increases. Thus, in the above example, if the sensitivity of the gel-clot reagent is 0.03 EU/ml, the MVD is 320 (if a sensitivity of 0.03125 EU/ml is used in the calculation[3]). If Pyrochrome® is used with a standard curve extending down to 0.005 EU/ml, the MVD becomes 2000. If Pyrotell®-T is used in the LAL-5000, with a minimum standard concentration of 0.001 EU/ml, the MVD for a sample with a limit of 10 EU/ml is 10,000.

Prior to testing, check that the pH of the **sample/LAL reaction mixture** is within the required range. For Pyrotell®, Pyrotell®-T and Pyrochrome®, the pH of the reaction mixture must be between 6 and 8. This is also the range specified in the United States Pharmacopeia (USP) Bacterial Endotoxins Test chapter (BET)[4]. The European Pharmacopoeia (EP) Bacterial Endotoxins chapter[5] specifies a pH range of 6.5 - 7.5 for the sample alone. Even if the pH of the undiluted product is out of range, it frequently meets the criteria after dilution and it is unnecessary to adjust the pH. Consider the hypothetical product with an MVD of 80 for which the pH of sample plus LAL is out of range, but is within range at a 1:8 dilution. There is no need to adjust the pH of this sample. Perform the preliminary test on dilutions from 1:8 out to the MVD. In cases where pH problems are not overcome by dilution, reconstitute Pyrotell or Pyrotell-T with Pyrosol buffer and repeat the pH measurement of the sample plus LAL reagent. (Pyrochrome is always reconstituted with Pyrochrome reconstitution buffer, so Pyrosol is not used with this reagent.) As a last resort, the sample pH may be adjusted with NaOH, HCl, Pyrosol or another buffer. In cases where a precipitate forms when acid, base or buffer are used to adjust pH, try diluting the sample before adjusting the pH.

Acid or base solutions which are free of detectable endotoxin can be prepared from NaOH pellets or concentrated HCl using LAL reagent water (LRW). The volume of acid or base added should be not more than 5% (10% for the gel-clot method) of the sample volume to prevent significant dilution. It is often necessary to perform preliminary titrations to determine the volume and concentration of the addition.

Preliminary Test Procedure

Before preliminary testing begins, the technician, laboratory, and LAL reagent should be qualified. Also, if a control standard endotoxin (CSE) is used, a certificate of analysis must be in hand or the potency of the CSE must be determined[6].

The first step of the preliminary test is to decide on a series of product dilutions that includes the MVD. The dilutions can be arranged so that the final dilution is the MVD. Usually a serial twofold dilution is made unless the MVD exceeds approximately 500. In the Contract Test Service at Associates of Cape Cod, our technicians routinely prepare nine twofold dilutions (out to 1:512) for the gel-clot method. If the MVD is very large, as it can be in the more sensitive test methods, it may be convenient to test a series of four- or tenfold dilutions. Then a second test can be performed using twofold dilutions to more precisely determine the dilution at which interference is overcome.

There are a number of ways of preparing the series of spiked and unspiked dilutions, three of which are described in the box on page 5. The aim is the same in each case: to produce two parallel series of decreasing concentrations of product, one of which is spiked with a given concentration of endotoxin (positive product control). The product concentration decreases down the series but the spike concentration remains constant. The actual concentration of the spike depends on the LAL test method.

Endotoxin Spike Concentrations

For the gel-clot method, the spike concentration used in the preliminary test must be double the label claim sensitivity of the reagent. This is referred to as a 2λ spike, where λ is the reagent sensitivity. When testing drugs and biologicals by the kinetic turbidimetric or chromogenic methods, according to the FDA Interim Guidance,[7] spikes should be between 0.1 - 0.5 EU/ml if the endotoxin limit in the dilution of product being tested is less than or equal to 1.0 EU/ml, and 1.0 - 5.0 EU/ml if the limit is greater than 1.0 EU/ml. Alternatively, the product can be spiked at 4λ, where λ is the lowest point on the standard curve (i.e. the detection limit for the test). The 4λ spike is given in the 1987 Guideline[8] and is the only one given by the FDA for endpoint methods and for medical device testing. However, Guideline and Guidance notwithstanding, it is recommended that the spike be equal to the concentration of a standard from the middle of the standard curve for all quantitative methods. This is specified in the product insert for both Pyrotell-T and Pyrochrome.

Results and their Interpretation

For the gel-clot method, the spiked sample should clot; if it does not, the sample is inhibiting the clotting reaction. At some dilution the spiked sample will clot, though that dilution may not be less than the MVD, or even included in the series tested. If unspiked sample clots, there is endotoxin

in the sample and the test is clearly not inhibited. If the unspiked sample clots but the spiked sample does not, a technical error has probably been made and the test should be repeated.

In the quantitative test methods, the spike should be quantified within ±50%[7] or ±25%[8] of the known spike concentration, after subtraction of any endotoxin detected in the unspiked sample. Greater or lesser spike recoveries indicate enhancement or inhibition respectively.

The first dilution in which no interference is evident has been called the non-inhibitory concentration (NIC). Thus, if a product is inhibitory at 1:10, but not at 1:20, the NIC is 1:20. However, because turbidimetric and chromogenic methods will detect enhancement as well as inhibition, the term non-interfering concentration is preferred.

Examples of preliminary tests (all run in duplicate)

1. Gel-clot

Assume label claim (λ) of 0.125 EU/ml and a product MVD of 1:100 (which could be extended to 1:400 by changing to an LAL sensitivity of 0.03 EU/ml)

Standards	**0.25**	**0.125**	**0.06**	**0.03**	**neg. ctl.**
(EU/ml)	+	+	-	-	-
	+	+	-	-	-

Sample	**undil.**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**
unspiked	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
spiked	-	-	-	+	+	+	+	+
(0.25EU/ml)	-	-	-	+	+	+	+	+

Interpretation

The sample is inhibitory up to and including the 1:4 dilution and contains no detectable endotoxin. Because the spike is detected only at the 1:8 dilution, the result must be reported as <1.0 EU/ml (<8 x 0.125 EU/ml).

2. Gel-clot

Standards	**0.25**	**0.125**	**0.06**	**0.03**	**neg. ctl.**
(EU/ml)	+	+	-	-	-
	+	+	-	-	-

Sample	**undil.**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**
unspiked	+	+	+	+	-	-	-	-
	+	+	+	+	-	-	-	-
spiked	+	+	+	+	+	+	+	+
(0.25EU/ml)	+	+	+	+	+	+	+	+

Interpretation

The sample contains endotoxin at a concentration of 1 EU/ml (8 x 0.125 EU/ml). There is no evidence of inhibition from these results.

Note: In the case of the gel-clot test, the preliminary test can be run with a spike for only the first dilution. This often saves time and reagent. However, if that spiked sample does not clot, and none of the unspiked sample dilutions clot (as in the case of example 1 above), the test must be repeated with a spike for each dilution. It would not be necessary to repeat the test if example 2 had been conducted in this manner.

3. Kinetic Test

Assume that the range of the standard curve is from 0.005 to 0.16 EU/ml so that λ=0.005. The spike concentration, taken from the middle of the standard curve, is 0.04 EU/ml. See the results in Table 1.

TABLE 1. Summary of results from a kinetic test (means of duplicates)

Standard line parameters: slope = -0.21 y intercept = 3.01 r = 0.998

	undil.	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**
unspiked sample	0.0243	0.0157	0.00826	<0.005	<0.005	<0.005	<0.005	<0.005
spiked sample 0.04 EU/ml	0.0367	0.0343	0.0412	0.0351	0.0403	0.0413	0.0392	0.0411
recovery (unsp.-sp.)	0.0124	0.0186	0.0329	0.0351	0.0403	0.0413	0.0392	0.0411
% recovery	31	47	82	88	101	103	98	103

Interpretation

The spike is recovered within ±50% at 1:4, though there is still the suggestion of inhibition at this dilution and possibly at 1:8. To calculate the endotoxin concentration of the sample, multiply the concentration measured in the first dilution to show no interference by the dilution factor: 0.00826 EU/ml x 4 = 0.330 EU/ml.

Using the Results of Preliminary Tests

Preliminary test results give the concentration of endotoxin in the sample and are used to determine the product dilution for the inhibition/enhancement test. The inhibition/enhancement test is required to validate the test for the product. As a general rule, the dilution selected for the inhibition/enhancement test should be at least one twofold dilution beyond the first dilution at which no interference or endotoxin was evident.

In example 1 above, since inhibition was evident at 1:4 but not at 1:8, it would be advisable to validate at a dilution of at least 1:16. A dilution of 1:20 would be a reasonable choice because many products are not perfectly consistent from batch to batch, so a subsequent lot might be inhibitory at 1:8, but it is unlikely to interfere at 1:20. In example 3, inhibition has technically been overcome at 1:4. However, as the results suggest possible residual inhibition even at 1:8, the conservative approach would be to validate the method at a dilution of not less than 1:16. Again, 1:20 is a convenient dilution.

When selecting the dilution at which to validate, the concentration of any natural or contaminant endotoxin in the product must be considered. The BET, which addresses the gel-clot method, states that the dilution of product selected for performing the inhibition/ enhancement test should be free of detectable endotoxin. In example 2 above, in which endotoxin was detected out to the 1:8 dilution, the inhibition/enhancement test should be performed at a dilution of at least 1:32 or greater. Alternatively, a cleaner lot of product on which to perform validation should be found. The endotoxin concentration of some products varies substantially between batches, particularly those from natural sources, and this should be taken into account when selecting the dilution for validation. It may be decided to perform inhibition/enhancement and routine testing at a greater dilution to avoid unnecessary retesting due to variable endotoxin concentrations between batches. For the quantitative methods, complete absence of detectable endotoxin in the sample is not necessary, but the concentration should ideally be less than 25% of the spike concentration.

Also, consider the MVD when choosing a dilution at which to perform the inhibition or enhancement test. If the MVD of the product is 1:1000, then the product may be validated at 1:200, even if the interference is overcome by 1:20. FDA speakers have indicated a preference for validating and testing at the dilutions less than MVD, but validation at the MVD is acceptable. The BET states that the inhibition or enhancement test should be performed "on aliquots of the specimen, or a dilution not to exceed the *Maximum Valid Dilution*", while the European Pharmacopeia specifies validation and testing at the MVD.

The FDA has stated that, if samples are pooled, they should not be tested at the MVD since the endotoxin in one contaminated vial could be diluted below the limit by the contents of clean vials. The recommendation to correct for pooled vials has been made by the FDA in written answers to questions posed by the LAL Users' Group. This implies that the greatest allowable dilution is MVD/X, where X = number of vials pooled. It is possible that the interference exhibited by the product will necessitate validation at a dilution close to the MVD, in which case vials cannot be pooled for testing. It is important to note that the reduction of the MVD reflects a more stringent endotoxin limit for pooled product. There is no requirement to correct for the effects of pooling on medical device extracts[9].

Conclusion

The performance of the preliminary test, and its repetition until any interference problems have been solved, is the key to successful validation and subsequent routine testing. The preliminary test procedure is also the correct approach to the assay of any uncharacterized sample that is sent to the LAL lab for testing. It is the basis for the majority of testing conducted by the Contract Test Service at Associates of Cape Cod, Inc.

References

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2. Maximum Valid Dilution and Minimum Valid Concentration, LAL UPDATE, **13**(4), December 1995.
3. LAL Label Claim Sensitivity and Its Use in Calculations, LAL UPDATE, **12**(4), December 1994.
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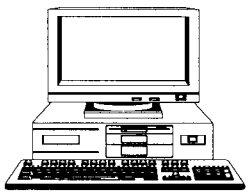
6. Endotoxin Standards and CSE Potency, LAL UPDATE, **11**(4), September 1993.
7. Interim Guidance for Human and Veterinary Drug Products and Biologicals. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration. July 15, 1991.
8. Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration, December, 1987.
9. USP Changes Medical Devices Chapter, LAL UPDATE, **12**(4), December 1994.

Preparing and Spiking Dilutions for Preliminary Testing

The aim in preparing dilutions for preliminary testing is to produce two parallel series of decreasing concentrations of product, one of which is spiked with a given concentration of endotoxin. Three approaches to preparing dilutions and spikes are presented here. The first is to make a series of dilutions in water and then to pipet an aliquot from each dilution into a parallel series of dilution tubes. To these aliquots, add a small volume of concentrated endotoxin to give the desired spike concentration without significantly diluting the sample. Generally, an addition of no more that 5% of the volume in the tube is acceptable (up to 10% for the gel-clot method). Then transfer the unspiked and spiked sample to reaction tubes. A useful variation of this method is to add the sample dilutions to reaction tubes first and then add the endotoxin spike as necessary. Since this requires very small spike volumes and pipetting precision is reduced at low volumes, this variation is only recommended for the gel-clot method. For example, if using a gel-clot reagent of 0.125 EU/ml, a spike concentration of 2λ is 0.25 EU/ml. Thus add 0.01 ml of 2.5 EU/ml endotoxin standard to 0.1 ml of sample in the reaction tube. Clearly this gives a final volume of 0.11 ml containing 0.23 EU/ml. These differences are not significant in the gel-clot test but might be for other methods.

Another approach to preparing the spiked dilutions of product is to spike the undilute sample (or the first dilution to be tested) with a small volume of concentrated endotoxin as described above. Then make dilutions with an endotoxin solution at the desired spike concentration. Dilutions of unspiked product are prepared in the usual way. This procedure can be carried out in either dilution tubes or, if twofold dilutions are required, in the reaction tubes or microplate wells to be used in the test. Making dilutions in the reaction tubes is fast because volumes are small and mixing only takes a few seconds. However, if the SOP states that the USP Bacterial Endotoxins Test is to be performed, note that the section on standard endotoxins requires mixing each dilution for 30 seconds. Making dilutions in the reaction tubes requires repeating the procedure for each replicate series, but these dilutions are so rapid that this is not a problem. If a microtiter plate is used for chromogenic or turbidimetric tests, dilutions for parallel series can be made together in the plate with a multi-channel pipette, mixing the dilutions by aspirating and expelling with the pipette.

The third method requires preparing a series of sample dilutions at twice the desired final concentration. To each tube or well that will contain unspiked sample, add LRW at half the final sample volume. For tubes/wells that will contain spiked sample, add the same volume of endotoxin solution at double the desired spike concentration. Then add the sample dilutions to the tubes/wells at half the final volume. The water in the wells for unspiked sample will dilute the sample to the desired concentration. In the spiked sample tubes/wells, the endotoxin solution dilutes the sample and the sample dilutes the spike to the correct concentrations. This is easier to do than it is to explain. Endotoxin standards can be prepared in the same way with the final twofold dilution being made in the tube or well. For turbidimetric and chromogenic methods, this has the great advantage that the spikes and the standard of the same concentration are prepared in the same way from a single 2x stock, so they are directly comparable. This is preferable to having standards prepared one way and spikes prepared another, in which case it is advisable to include a positive control consisting of LRW spiked in the same way as the product. The only disadvantage to the procedure is that undilute sample cannot be spiked in this way because the method relies upon a twofold dilution in the reaction tubes.



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