

Troubleshooting Guide PYROGENT® Gel Clot LAL Assay

Technical Tips

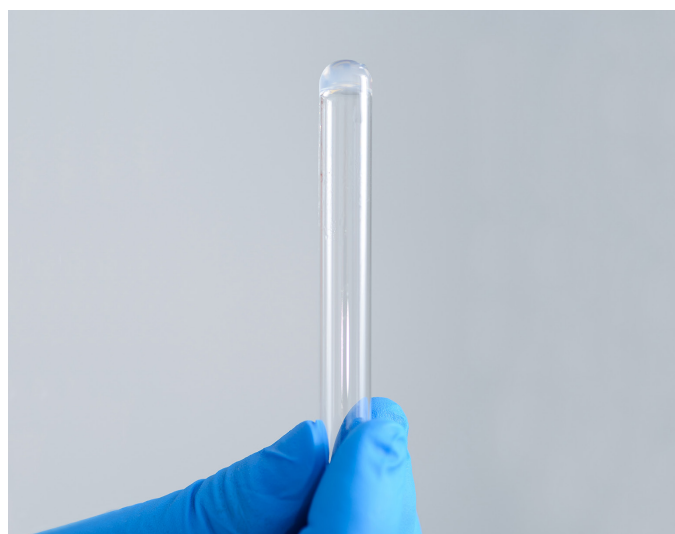
By Lonza Scientific Support, U.S.

Most problems encountered in the use of the PYROGENT® Gel Clot Limulus Amebocyte Lysate (LAL) Assay fall into two basic categories.

Category I: No firm gels are observed in reaction tubes where firm gels are expected. These types of results are sometimes called “false negatives”. Examples of these might be tubes containing endotoxin controls or endotoxin-spiked samples that have been prepared to contain endotoxin concentrations above the sensitivity level of the lysate, but a firm gel is not formed. Another example might be a sample of product that has been shown to have an endotoxin load by another test, but shows up as negative in the LAL test.

Category II: Firm gels form in reaction tubes, which are expected to yield negative results. These types of results are sometimes called “false positives”. Examples of these might be negative controls, or positive test result of a product, which has been shown to be non-pyrogenic by another test.

Proper technique is critical to the success of the LAL gel clot test, and steps performed incorrectly may contribute to problems belonging to one or both of these categories.



Category I: Problem Sources

A. Endotoxin mixing

Endotoxins that are used as controls with LAL tests are very large, highly purified molecules that have unique properties affecting their behavior in aqueous solutions. A portion of the molecule is very water-soluble and another part is not. For this reason, endotoxin molecules in solution tend to aggregate. When a solution containing endotoxin is withdrawn, such as when making serial dilutions for endotoxin testing, the endotoxin must be well dispersed or the portion withdrawn may not contain the proper potency of endotoxin. Thorough mixing of endotoxin and accurate transfer throughout a dilution series are best accomplished by observing the points noted below.

1. Make endotoxin dilutions in increments no greater than 1:10. This will increase the chance of obtaining the correct amount of endotoxin in the withdrawn portion and decrease the extent to which it will have to be dispersed when it is diluted.
2. Adhere strictly to the agitation and vortex mixing times specified for each step in the endotoxin dilution instructions. Vortex mixing should be done at the "high" speed setting in order to disperse endotoxin aggregates. The aliquot for subsequent dilution should be withdrawn immediately after mixing, and the endotoxin sample should be thoroughly mixed just prior to use.

NOTE: It is not recommended to vortex for more than 30 minutes in one 8 hour period to avoid damage to the endotoxin and loss of potency.

B. Pipetting accuracy

Pipetting accuracy is exceptionally important during every phase of LAL testing. Ensure you are adding the proper amounts of material by utilizing high quality graduated pipettes and pipette tips and the proper technique for the pipette you have chosen. Pipettes should be recalibrated annually at a minimum.

C. Conditions for incubation of LAL test tubes

Check the temperature of the water bath or heating block. The LAL reaction with endotoxin is temperature dependent and the specified standard conditions for the test require a one hour incubation at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The critical temperature is that of the material inside the reaction tube. An accurate check can be made by placing a calibrated thermometer inside a 75 x 10 mm test tube containing just enough water to cover the thermometer bulb. Place the tube and thermometer in a test tube incubation rack or a heating block and

observe the temperature over a one-hour period. Some water baths have poor heat distribution characteristics, which can be compounded by some types of test tube racks. Choose a test tube rack that will allow effective heat transfer to the tubes.

Check the location of the water bath or heating block. While the gel is forming, it is quite fragile and may be irreversibly broken by mechanical shock or vibration. Small motions, including closing a door or drawer in your work bench or a centrifuge running can cause sufficient vibration to affect the development of the clots. Generally speaking, any motion of the incubating test tubes must be regarded as a potential problem source. Agitating or circulating water baths must not be used for this reason.

D. Test results

A positive result is a firm gel that maintains its integrity through inversion to 180° . When reading test results after the one-hour incubation period, gently withdraw each tube and invert it 180° in one smooth motion. Do not stop at 45° or 90° to look before continuing inversion, and be mindful of adjacent tubes when removing them from the heat block or tube rack.

E. Test reagent handling

Before reconstitution, the PYROGENT® Gel Clot LAL reagent is very stable but should be stored in the dark at 2 to 8°C (note expiration date). The lysate reagent, when reconstituted, is a buffered solution containing enzymes and other proteins from the *Limulus* amoebocyte. Preparation, handling and storage are critical and should be approached keeping the following points in mind:

1. The water used to rehydrate the PYROGENT® Gel Clot LAL reagent should be LAL Reagent Water (LRW) manufactured by Lonza. Water for Injection (WFI) is generally not acceptable as it is not tested to the proper endotoxin concentrations for endotoxin content nor has it been tested for assay performance compatibility. Furthermore, LRW manufactured by other endotoxin detection companies may not be compatible with the Lonza LAL gel clot reagents. If it is necessary to use reagent water other than a Lonza product, it must be thoroughly validated prior to use in testing samples.
2. Pipetting technique must be aseptic and apyrogenic. Even when rehydration water is pyrogen-free, it can easily become contaminated unless appropriate precautions are observed.

Aseptic and apyrogenic techniques consist of precautions to avoid the entry of gross amounts of bacterial and/or endotoxin contamination. A great deal of endotoxin may be present on surfaces (hands, glassware, etc.) even when few or no viable bacteria are found. For this reason, labware must be treated to remove endotoxin and handled carefully to prevent contamination.

a. Lonza recommends the use of Eppendorf Biopur® pipette tips.

A. Catalog No. 25-415 2 – 200 µL

B. Catalog No. 25-416 2 – 300 µL

C. Catalog No. 25-417 50 – 1000 µL

b. Lonza discourages the use of any manufacturer's barrier (filter) style tip. Barrier style tips have been known to cause assay interference.

3. Rehydrated PYROGENT® Gel Clot LAL reagent may be stored for as long as 24 hours, but it must be kept at 2 to 8°C. The lysate should not be pre-incubated in the LAL test tubes before adding samples to them, as this may cause sensitivity loss in the PYROGENT® Gel Clot LAL reagent.

If rehydrated lysate must be stored for a longer period (up to four weeks), it must be kept frozen at -10°C or below and checked to ensure the lysate is frozen solid in the tubes. Many frost-free freezers have surface heating cycles that may periodically warm articles in contact with the heated surfaces. Repeated freezing and thawing of the lysate will result in deterioration well before four weeks have passed.

4. Lyophilized *E. coli* endotoxin should be stored at 2 to 8°C. Endotoxin solutions are generally very stable upon storage except when highly diluted. This is related to the solubility characteristics discussed earlier. Rehydrated *E. coli* endotoxin should be stored refrigerated only at 2 to 8°C and not be frozen. The stock solution may be kept for up to four weeks. Further dilutions of *E. coli* endotoxin may begin to lose potency after a few days. The storage stability of dilute endotoxins may be related to the cleanliness of the glassware in which they are kept, as endotoxin can bind on irregular or dirty glass surfaces. Since some types of plastic have been shown to have an affinity for endotoxin, the use of plastic containers to prepare and store endotoxin must be approached with caution. The precautions for technique, water and pipettes discussed previously (for the lysate rehydration) also apply to endotoxin rehydration.

5. Diluents other than water or saline may also cause inhibition. When all water controls behave as expected in the LAL test but a specific sample fails to give positive results when prepared to contain the specified endotoxin concentrations, true inhibition has been recognized.

6. Handling of samples is as critical as the handling of reagents, and cleanliness is important to ensure the use of aseptic and apyrogenic techniques. Always check the product for inhibition as some products, especially members of the small volume parenteral family, may contain substances at great enough concentrations to inhibit the LAL gelation reaction.

In addition, the pH of the sample to be tested should be checked and should be in the range of 6.0 – 8.0. A pH outside of this range is one of the most common causes of product inhibition in the LAL test. The PYROGENT® Gel Clot LAL reagent does have a certain amount of buffering capacity, but it must be assured that a product, when added to the PYROGENT® Gel Clot LAL reagent, does not exceed that capacity. An easy way to determine whether or not a given product will require pH adjustment is simply add a volume of the product in question to an equal volume of rehydrated PYROGENT® Gel Clot LAL reagent and check the pH to determine if it falls between 6.0 and 8.0. Remember that because of PYROGENT® Gel Clot LAL reagent's buffering capacity, there is no need to adjust the pH of WFI or Saline for Injection USP. If a pH adjustment is necessary, Lonza Tris Buffer (Lonza Catalog No: S50-642) is available for this use. Tris Buffer is a convenient way to adjust pH when the buffering capacity of the sample will allow it.

Category II: Problem sources

The majority of Category II problems is caused by failure to observe proper handling technique, failure to use adequate equipment preparation procedures, and/or use of contaminated water in the test.

The extreme sensitivity of LAL to endotoxin is regarded as its greatest virtue, but this sensitivity can also be a source of problems when careless technique is used.

Care in preparation and handling of PYROGENT® Gel Clot LAL reagent and test samples is essential. Any surface or substance that could come into contact with either the LAL or the sample must be free of endotoxins. This need was discussed previously and

is re-emphasized here. Remember that endotoxin is virtually everywhere in nature.

As a rule, when testing parenteral pharmaceutical products, if a firm gel appears in the LAL test, it must be assumed that endotoxin is present. Use of a negative control as part of the test will help you determine if positive results are obtained due to endotoxin in the test sample or to inadvertent contamination.

During inhibition/enhancement testing, another indication of contamination may appear when you have prepared a series of dilutions of endotoxin in a product and another series of endotoxin in LRW. If the endpoint of the LAL assay of endotoxin in product is more than a two-fold dilution lower in concentration than the endpoint of the water-endotoxin dilution series, contamination of the product might be suspected. Further testing is necessary to exclude enhancement.

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