

# Low Endotoxin Recovery (LER)

## Frequently Asked Questions

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Low Endotoxin Recovery (LER) is a controversial topic that has been circulating throughout the endotoxin detection community since 2013. This phenomenon is hypothetically described as a “masking effect” manifested in the biophysical formation of a complex that blocks the ability of Factor C, the main component in LAL detection, to bind endotoxin.<sup>1</sup>

LER is the masking of endotoxin in undiluted materials, thought to be attributable to combinations of specific excipients. This differs from the inhibition or interference of endotoxin tests caused by pH, high divalent ion concentrations, chelators, serine proteases and glucan, which can usually be overcome using a pre-treatment such as dilution.

Two common drug excipients, polysorbate and citrate, have been identified as probable causes of the masking effect more commonly referred to as LER. These substances are estimated to be used in more than 70% of protein formulations.<sup>2</sup> There is also some evidence that phosphate-containing formulations may also be affected by LER. However, the LER effect has only been observed in combination formulations of the aforementioned excipients, and not in individual raw materials.



## Why is LER so Important to the Manufacture of Biologics?

Biologics have been the main target for LER evaluation, primarily because of the requirement that a Rabbit Pyrogen Test (RPT) is performed to determine the drug's safety when administered to a patient. In lieu of the RPT, a bacterial endotoxins test like LAL can be used if the chosen BET method is deemed equivalent to the rabbit pyrogen test (21 CFR 610.9). It is important to note that some drug manufacturers have had to resort back to using the RPT for release of products shown to be affected by LER when using the LAL method.

To determine whether LER *in vitro* corresponds to non-pyrogenicity *in vivo*, biologics manufacturers are typically requested to conduct studies in which the product is spiked with an endotoxin standard, held, and then tested at various time points by LAL and RPT methods in parallel.

## What is the FDA's Current Position on LER?

LER is mainly associated with certain biological drug products, either monoclonal antibodies or therapeutic proteins. Results from spiking studies indicate that endotoxin recovery is affected not only by the formulation excipients, but protein concentration, BET method, reagent supplier, hold temperature, and time may also contribute to the LER effect. The greatest effects on spiked endotoxin recoverability have been observed with certain formulation excipients and proteins; in addition, the type of spike material (RSE, CSE, or Naturally Occurring Endotoxin [NOE]) has also influenced the outcome of the spiking studies.<sup>3</sup>

## What are Some Considerations for Conducting a Proper Hold-time Study?

- a. Directly spike into **undiluted** drug product, drug substance or in-process solution at a level  $\leq$  the specification.
- b. Hold product before testing according to pre-determined parameters (temperature, container type, # days).
- c. Product should be tested at specific time-points throughout hold period (ex. 4 hours, 24 hours, 72 hours, Day 5, Day 7 – note: Day 7 is 8 days).
  - LER is time-dependent; it can occur over the course of 1 week or very rapidly (within 4 hours) in the presence of citrate or phosphate plus polysorbate. More rapid LER effects have been reported when holding at 20 to 25°C than at 2 to 8°C.
- d. Determine recovery (%)<sup>\*</sup> of spike compared to amount of endotoxin originally added to sample.  
<sup>\*</sup>Products exhibiting LER typically result in declining ( $\leq 50\%$ ) recovery over time. Inability to recover spike CSE may occur in less than 4 hours.

## Does the FDA have any recommendations for performing hold-time spiking studies?

Current FDA recommendations for performing hold-time studies – in summary:

- I. Test at least three different lots
- II. Spike close to the drug specification (at or below)
- III. Test for at least 8 days (finished product)
- IV. Use Control Standard Endotoxin (worst-case scenario and FDA recommendation)

## What are the Differences Between Naturally Occurring Endotoxin (NOE) and LPS Standard Formulations?

Naturally Occurring Endotoxins (NOEs) are crude preparations of endotoxin extracted from a growing culture of gram-negative bacteria. NOEs have not undergone a purification step, unlike the reference standard or control standard endotoxins. Therefore, it is assumed that the presence of protein and other cell debris allows the LPS to be more robust under LER conditions.

In a real environment, a contamination event would not occur by a purified endotoxin standard but rather an endotoxin excreted by gram-negative bacteria. This rationale is the basis for why some researchers believe that NOEs are a more relevant analyte for hold-time studies than the control standard endotoxin or reference standard endotoxin.

## Is There an Advantage in Using NOE Preps Versus CSE or RSE?

Compiled data from several BLAs submitted to the FDA show that regardless of the source of endotoxin, CSE or NOE, there are conflicting results between the LAL and RPT test. In examples where LER is observed with either CSE or NOE, a pyrogenic reaction is not detected in rabbits spiked with the same material. On the other hand, data is also showing that while an LER effect was detected with the LAL test, pyrogenic reactions were detected with the rabbit pyrogen test. The confounding results of these early applications are the basis for the FDA's reason to not support or encourage the use of NOE in the hold-time study (see Table 1). It should also be noted that each of the NOEs developed may have been prepared from different species and subjected to different growth conditions. Without a standardized process for developing an NOE, no real conclusions can be drawn by regulators as to whether NOEs actually provide a useful alternative to the purified LPS standards.

Spike Material	LAL Test	Rabbit Pyrogen Test
CSE	No LER	Pyrogenic
CSE	LER	Not Pyrogenic
CSE	LER	Pyrogenic
NOE	No LER	Not Pyrogenic
NOE	No LER	Pyrogenic

**Table 1.** Summary of review findings from various BLAs submitted to the US Food and Drug Administration comparing results of spiking studies with CSE or NOE, and the corresponding observation of recovery or pyrogenicity in the LAL test or Rabbit Pyrogen Test.<sup>4</sup>

Some researchers have been able to successfully perform their hold-time studies using NOE instead of CSE or RSE. In September 2015, a group of industry leaders, including one LAL vendor and end-users, published an article in the US Pharmacopeial Forum (PF) that provided data showing that naturally occurring endotoxins (NOE) should be used as a replacement for CSE/RSE in hold-time studies.<sup>5</sup> The basis for this argument is that NOE are more robust than the purified endotoxin standards, and would be less likely to be effected by LER.

However, many other researchers have invalidated the argument for use of NOE in hold-time, spiking studies because they were not able to successfully recover certain NOEs or CSE/RSE from their undiluted samples. Therefore, the jury is still out on whether NOEs are the solution to conducting successful hold-time studies in LER-affected matrices.

## Can You Use Dispersing Agents, i.e. PyroSpense™, to Overcome LER?

Some drug manufacturers have been able to use dispersing agents, like PyroSpense™, to overcome LER in their samples. However, as each LER case is different, dispersing agents may not always be the solution. It is recommended to try a dispersing agent like PyroSpense™ if you observe LER. If this does not work, other sample preparation methods will need to be evaluated.

## What Other Sample Prep Methods Have Been Used to Overcome LER?

Demasking solutions have been evaluated to reverse the effect of LER. Demasking involves re-assembly of the endotoxin aggregates and as such requires using sample treatments to push the equilibrium towards the aggregate state. This can include adjusting the pH to influence hydrogen bonding or adding Mg<sup>2+</sup>/Ca<sup>2+</sup> to the solution to saturate the chelating agent and prevent destabilization of the LPS aggregates. The addition of metallo-modified polyanionic dispersants, such as PyroSpense™, can also prevent the surfactant from interfering with the LPS aggregation state.

Dialysis treatment methods have also proven to be successful in overcoming LER in certain products.

## I've Heard Various Opinions About the Long-term Effects of LER, and The Potential Public Safety Issue. Are These cConcerns Valid?

The FDA is taking a conservative and cautious approach when discussing LER and its relevance to drug safety. Although there are no reports or indications to date that LER is a public safety issue, the FDA is concerned that LER could result in endotoxin not detected by the compendial USP <85> methods causing a pyrogenic effect in humans.

## What is the LAL Community Doing to Help Drug Manufacturers Who Experience LER?

The Parenteral Drug Association (PDA) LER Task Force and Biophorum Operations Group (BPOG) LER Task Force were formed to investigate the effects of LER and provide viable means of overcoming the effects of LER. These task forces are comprised of LAL suppliers and end-users, working closely with global regulators to investigate and provide recommendations for handling LER-affected products.

### References:

1. J. Chen, Low Endotoxin Recovery in Common Biologics Products, presented at the 2013 Annual PDA Meeting, Orlando, FL, April 2013.
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3. Williams, K.L. 2013. Endotoxin Test Concerns in Biologics. *American Pharmaceutical Review* 16 (Endotoxin Detection Suppl.): 4–9.
4. Hughes, P. 2014. Endotoxin Challenges – A Regulatory Perspective. *PDA 9th Annual Global Conference on Pharmaceutical Microbiology*, October 2014.
5. Bolden, et al. 2015. Stimuli to the Revision Process – The Use of Endotoxin as an Analyte in Biopharmaceutical Product Hold-Time Studies. *US Pharmacopeial Forum*, September 2015.

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