Low Endotoxin Recovery case studies

This case study discusses the Low Endotoxin Recovery (LER) phenomenon in the drug testing and mitigation approach using the ENDO-RS® kit and ENDOLISA® assay for detecting endotoxin interference.

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SINCE CHEN AND VINTHER first described a unique drug formulation phenomenon that renders the detection of endotoxin using *Limulus*-based methods ineffective, the debate has raged as to how the phenomenon should best be mitigated.[1] The phenomenon was labelled "Low Endotoxin Recovery" or LER. It should be noted that LER can be complicated by the presence of protein masking owing to the differential of the isoelectric points of endotoxin and many proteins.[2]

Some methods of mitigation used have included:

a) the addition of simple surfactants eg, magnesium chloride
b) and/or a pH change to mitigate the dissociation of endotoxin aggregates in quality control (QC) samples
c) and/or the use of the ENDO-RS® sample preparation kit to essentially reaggregate dissociated endotoxin in QC tests.

The removal of activity from endotoxin in QC samples (via LER) may initially seem like a good thing; however, some have shown that dissociated endotoxin remains biologically active from an immunological (non-pyrogen) perspective. Schwarz *et al*[3] found that LER dissociated endotoxin-activated key immune cell reactions. Additionally, detoxified LPS is used as an adjuvant in vaccines to stimulate immune reactions.[4] Today, many important drugs, especially biologics, act via immune-modulating properties such as suppressing autoimmune reactions or activating cancer immune response activities.

Pharmaceutical companies and the US Food and Drug Administration (FDA) have published papers on the use of the ENDO-RS kit to overcome LER (see further background paper references below). The ENDO-RS kit (**Figure 1**) was created as a means of testing samples for endotoxin with difficult-to-test matrices. A case study is presented here.

Reagent use overview

When the right ENDO-RS components in the right concentrations are added to the spiked samples with masked endotoxin, the endotoxin can be released, stabilised and re-configurated to a detectable structure (**Figure 1**). Given there are multiple endotoxin-masking conditions and mechanisms, a specific ENDO-RS sample preparation protocol must be developed for each product.

A demasking study can be divided into the following steps (see also **Figure 2**):

a) Endotoxin spike and hold to create LER sample
b) ENDO-RS component screening
c) Optimisation of the demasking system
d) Lowering of the endotoxin concentration (increasing the endotoxin test detection level)
e) Optimisation of the demasking system at the specification limit.

Spike samples for test

The method for preparing a test sample with LER is the same method (spike and hold for seven days) used for hold time studies (HTS), as required by FDA when a company submits a biologics license application (BLA) for drug approval.

The HTS answers the basic question: "Do we have LER?" This forms the starting point for experimentation in overcoming LER using ENDO-RS and ENDOLISA®.

Initial screening experiments

These tests should be done with relatively high endotoxin spike concentrations, such as 50 or 100 EU/mL. In the first step, a known LER triggering buffer (citrate and polysorbate 20) was spiked with 50 EU/mL (Control Standard Endotoxin, CSE) and held at room

Table 1: Determining a starting point for overcoming LER in citrate/polysorbate 20 buffer using ENDO-RS components A-E.

Demasking approach	#	Comp. A	Comp. B	Comp. C	Comp. D1	Comp. D2	Comp. E	% recovery after demasking	
1	1	150 µL	–	–	–	–	100 µL (undiluted)	25	
	2	150 µL	–	–	–	–	100 µL (dil. 1:10)	10	
	3	150 µL	–	–	–	–	100 µL (dil. 1:100)	5	
2	4	150 µL	–	100 µL	–	–	100 µL (undiluted)	50	
	5	150 µL	–	100 µL	–	–	100 µL (dil. 1:10)	40	
	6	150 µL	–	100 µL	–	–	100 µL (dil. 1:100)	10	
3	7	150 µL	–	100 µL	100 µL	–	100 µL (undiluted)	60	→ Optimise component concentration
	8	150 µL	–	100 µL	100 µL	–	100 µL (dil. 1:10)	50	
	9	150 µL	–	100 µL	100 µL	–	100 µL (dil. 1:100)	20	
4	10	150 µL	100 µL	100 µL	–	100 µL	100 µL (undiluted)	50	
	11	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:10)	70	
	12	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:100)	120	→ Lower the endotoxin level
Masking Control	13	–	–	–	–	–	–	5	
Endotoxin Control	14	–	–	–	-	–	–	100	

ENDO-RS component:		Function:
Buffer	A	Buffer for pH adjustment of samples
Disturber	B	Destabilization of LPS-masker-complex
Adsorber	C	Surfactant adsorption
Modulator	D1	Supporting the reconfigurator
Modulator	D2	Supporting the reconfigurator
Reconfigurator	E	LPS aggregate structure formation
Endotoxin Standard	F	Spiking for Hold-Time Studies
Endotoxin-free Water	G	For reconstitution of endotoxin, adsorber and dilution of components and samples

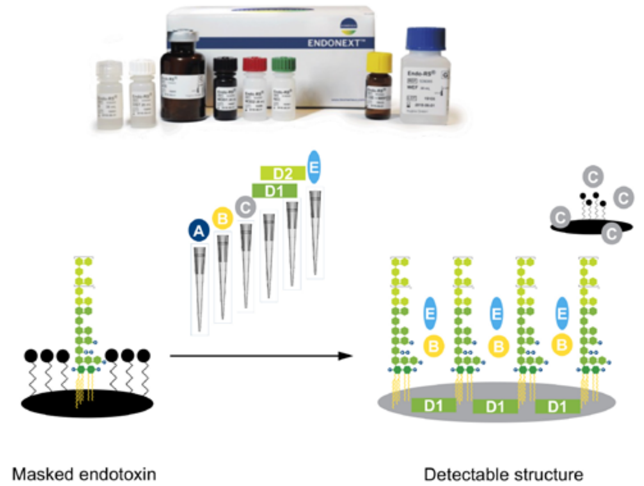

Figure 1: ENDO-RS® kit includes solutions A through E which are "mixed and matched" in an initial matrix test to gain a foothold for further experimentation. Note that solutions F and G are also included as F is standard endotoxin and G is water for BET.

As can be seen here, the surfactant and chelator combination (eg, polysorbate and citrate buffer) dissociates the active endotoxin aggregates into monomers and multimers that are subsequently thoroughly surrounded with polysorbate, which greatly outnumber the endotoxin molecules (masked endotoxin).

temperature for seven days. A series of 1mL aliquots of the spiked product were prepared in endotoxin-free glass test tubes. As a spike control endotoxin-free water was spiked and aliquoted in the same way as the sample. The first demasking experiment was performed after seven days.

The ENDO-RS components were added to the sample according to the guidance in the package insert of the sample preparation kit (**Table 1**). For a 1mL sample, 150µL of component A and 100µL each of all the other components B-E were added to the sample. After each addition, the samples were vortexed for two minutes. Following addition of the last component (normally component E), the sample was incubated without agitation at room temperature for 30 minutes before a 1:10 dilution in endotoxin-free water. The readout was performed with the recombinant Factor C (rFC) endotoxin detection assay using the ENDOLISA plate. All subsequent results were calculated including the water control (endotoxin control). For successful demasking, the recoveries should be between 50 and 200 percent.

For demasking of 50 EU/mL of the citrate/polysorbate 20 buffer, the screening was performed as shown in **Table 1**. The best recoveries of spiked endotoxin are shown in green in **Table 1**.

In the first experiment (**Table 1**), different ENDO-RS components were combined with different concentrations (pre-dilutions) of component E, which is the key component of the ENDO-RS kit. The combination of A and E as well as the combination A, C, and E (demasking approaches 1 and 2) were not very successful regarding endotoxin recoveries (<50 percent recovery after demasking). The combination of A, C, D1 and E (undiluted) gave a recovery of 60 percent »

Table 2: Endotoxin titration results in improved percentage recovery relative to the initial screening unit shown in Table 1.

Demasking approach	#	Comp. A	Comp. B	Comp. C	Comp. D1	Comp. D2	Comp. E	% recovery after demasking
4 50 EU/mL	1	150 µL	100 µL	100 µL	–	100 µL	100 µL (undiluted)	40
	2	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:10)	55
	3	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:100)	100
4 25 EU/mL	4	150 µL	100 µL	100 µL	–	100 µL	100 µL (undiluted)	30
	5	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:10)	50
	6	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:100)	80
4 12.5 EU/mL	7	150 µL	100 µL	100 µL	–	100 µL	100 µL (undiluted)	10
	8	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:10)	12
	9	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:100)	25
4 6.25 EU/mL	10	150 µL	100 µL	100 µL	–	100 µL	100 µL (undiluted)	5
	11	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:10)	7
	12	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:100)	10

→ Optimise component concentration at 12.5 EU/mL

Figure 2: Steps of an endotoxin demasking development using the ENDO-RS kit.

Table 3: Optimisation experiments using a lower endotoxin spike concentration of 12.5 EU/mL.

Demasking approach	#	Comp. A	Comp. B	Comp. C	Comp. D1	Comp. D2	Comp. E	% recovery after demasking	
4.1	1	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:50)	20	
	2	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:100)	25	
	3	150 µL	100 µL	100 µL	–	100 µL	100 µL (dil. 1:200)	15	
4.2	4	150 µL	100 µL	100 µL	–	100 µL (dil. 1:2)	100 µL (dil. 1:50)	35	
	5	150 µL	100 µL	100 µL	–	100 µL (dil. 1:2)	100 µL (dil. 1:100)	48	
	6	150 µL	100 µL	100 µL	–	100 µL (dil. 1:2)	100 µL (dil. 1:200)	20	
4.3	7	150 µL	100 µL	100 µL	–	100 µL (dil. 1:4)	100 µL (dil. 1:50)	55	
	8	150 µL	100 µL	100 µL	–	100 µL (dil. 1:4)	100 µL (dil. 1:100)	70	
	9	150 µL	100 µL	100 µL	–	100 µL (dil. 1:4)	100 µL (dil. 1:200)	35	
4.4	10	150 µL	100 µL	100 µL	–	100 µL (dil. 1:8)	100 µL (dil. 1:50)	80	→ Lower the endotoxin level
	11	150 µL	100 µL	100 µL	–	100 µL (dil. 1:8)	100 µL (dil. 1:100)	90	
	12	150 µL	100 µL	100 µL	–	100 µL (dil. 1:8)	100 µL (dil. 1:200)	45	

which would be a good start for optimisation experiments. Better results were obtained with the combination A, B, C, D2 and E (1:10 or 1:100 pre-diluted). With 70- and 120-percent endotoxin recovery of the initial 50 EU/mL spike. These are the demasking systems that should be optimised in the next step. The masking control (**Table 1, #13**) shows that the 50 EU/mL spike is masked and not detectable within 50-200 percent.

Refining the initial best recovery method

Refining the test involves the addition of progressively smaller endotoxin spikes for the HTS samples. The sample (citrate/polysorbate 20 buffer) was spiked with different amounts of endotoxin (50, 25, 12.5 and 6.25 EU/mL) to determine which concentration can be demasked with the system A, B, C, D2 and E (**Table 2**). In this manner, a new "foothold" on a method capable of demasking a smaller amount of endotoxin contamination can be achieved.

The results for the 50 EU/mL spike (**Table 2, #1-3**) were reproduced compared to the first assay (**Table 1, #10-12**). The lower the endotoxin concentration, the lower the expected effectiveness of the current demasking system. Therefore, optimisation experiments should be done for the endotoxin spike of 12.5 EU/mL (25 percent recovery, **Table 2, #9**).

The reconfigurator (E) is the most important ENDO-RS component, supported by multiple modulators (D1, D2 and D3*). During the optimisation experiments, where the concentrations of these buffers were changed, it was observed that the higher the pre-dilution of component E, the higher the endotoxin recoveries. So, for the subsequent assay pre-dilutions of up to 1:200 were tested in combination with different concentrations of D2. When using a higher diluted component

Table 4: Overcoming common interference properties in *Limulus* testing using ENDOLISA®.[5]

	Substance	Solvent	ENDOLISA	LAL assay
Bufer/pH	Acetate (pH 4.0)	100 mM NaCl	50 mM	12.5 mM
	Acetate (pH 5.0)	100 mM NaCl	100 mM[a]	12.5 mM
	MES (pH 6.0)	100 mM NaCl	100 mM[a]	5 mM
	Potassium phosphate (pH 7.2	100 mM NaCl	100 mM[a]	50 mM
	Imidazole (pH 7.4)	Water	500 mM	40 mM
	HEPES (pH 7.5)	100 mM NaCl	100 mM[a]	100 mM[a]
	Sodium borate (pH 9.0)	100 mM NaCl	100 mM[a]	50 mM
Salt	NaCl	Water	1 M	0.5 M
	KCl	Water	1 M	0.25 M
Chaotropic agent	Urea	Water	6 M	0.5 M
	Guanidinium chloride	Water	1 M	0.05 M
Organic solvent	Methanol	Water	20%[a]	5%
	Ethanol	Water	30%	0.5%
	2-Propanol	Water	20%	0.2%
	DMSO	Water	10%	2%
Detergent	SDS	Water	0.05%	0.001%
	CTAB	Wateer	0.004%	0.0001%
	Zwittergent 3-14	Water	0.02%	0.005%
	Tween 20	Water	2%	0.1%
	Triton X-100	Water	0.02%	0.005%
Chelator	EDTA (pH 8.0)	Water	0.5 mM	0.4 mM
	Citrate (pH 7.5)	Water	10 mM	10 mM
Protease inhibitor	Benzamidine	Water	100 mM [a]	0.1 mM
	PMSF	2-Propanol	5 mM	<0.05 mM
Antibiotic	Rifampicin	Methanol	3.5 mg/ml	0.04 mg/ml
	Chloramphenicol	Ethanol	3.5 mg/ml	0.1 mg/ml

[a] Highest concentration tested.

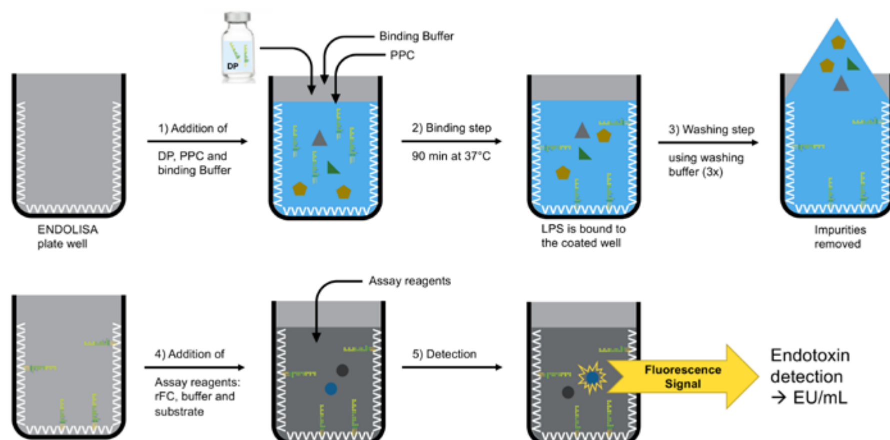

Figure 3: Endotoxin detection using ENDOLISA after ENDO-RS treatment for overcoming LER.

D2 for demasking, endotoxin recoveries of 70-90 percent were obtained (**Table 3, #8 #11**).

A demasking protocol was thus developed that could detect 12.5 EU/mL in the citrate/polysorbate 20 buffer (the mock-spiked LER sample). With A, B, C, D2 (1:8) and E (1:100), followed by a 1:10 dilution in endotoxin-free water, 90 percent of the 12.5 EU/mL CSE spike was detectable using the ENDOLISA assay.

This demasking protocol can be used to further test lower amounts of endotoxin. Ultimately, the amount lower needed to be tested will depend upon the detection limit needed for the specific product test. Further optimisation experiments could comprise testing different concentrations of components A, B and C, as well as testing different dilution factors prior to endotoxin detection. Ultimately, a reliable and stable sample preparation can be developed with the ENDO-RS kit to detect possible endotoxin contaminations in drug products.

ENDOLISA®

ENDOLISA®, a black 96-well plate that has been pre-coated with recombinant phage proteins specific for Gram-negative bacteria, is used in conjunction with ENDO-RS to overcome LER. The plate is able to 'pull out' endotoxin from samples to enable the complete removal of samples (and ENDO-RS solutions) that would otherwise greatly interfere with *Limulus* testing. After removing the liquid solutions, incubation and binding endotoxin, the seemingly empty plate (like an ELISA test) is then overlain with recombinant Factor C and tested by fluorescence method. This process is detailed in **Figure 3**.

Prior to the LER phenomenon discovery, ENDOLISA was developed to be used with many difficult-to-test samples that have common interference properties. The level of testing that can be achieved using ENDOLISA is shown below in **Table 4** for some very common solutions including specific categories: buffer/pH, salt, chaotropic, solvents, detergents, chelators, protease inhibitors, and antibiotics. Note that the level of testing achieved is shown as compared to the level achieved using traditional LAL testing. ENDOLISA shows a great ability to overcome interferences that otherwise cannot be overcome, in some cases up to 1000X as shown for a protease inhibitor, Benzamidine.

Finalising the LER test method

Once a development scheme has been achieved, the user can test this using different lots of materials (drug and test kits and reagents) and, if desired, using different analysts. Such a test, once developed, with validation performed and documented, should be no more difficult to perform routinely than any other QC bacterial endotoxin test (BET).

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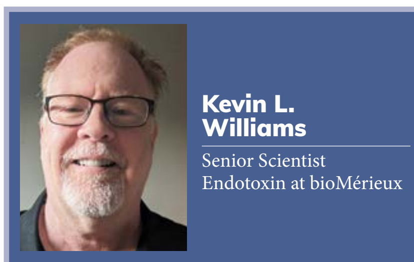

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Foootnote and References

* D3 available but not in the commercial kit

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Further background information can be gained from the literature including:

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- PharmaLab conference 2022 by Harald Meißner. LER Challenges and Their Solutions – A Case Study.
- PharmaLab conference 2022 by Gertrud Lallinger-Kube and Michael Kracklauer. Validation of a Dedicated Sample Preparation Method.