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LAL Non-Endotoxin Reactivity - Surprisingly Non-specific

Kevin Williams

Corresponding Author: **Kevin Williams**, Senior Scientist R&D
bioMerieux

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Nature abounds in producing a variety of molecules. This is a good thing, however, in endotoxin detection a requirement for an analytical assay is "*specificity*". Unfortunately, LAL has been found to be increasingly non-specific since the inception of its use in lieu of the rabbit pyrogen test. Fortunately, there are viable workarounds in terms of using *Limulus*-based testing where non-endotoxin reactive substance false-reactivity is problematic.

In analytical testing, just as in metazoan immune detection of potential microbial invaders, context is everything. The horseshoe crab from which LAL is derived swims and crawls on a beach interface that teems with Gram Negative bacteria (GNB), estimated by some at over 10^6 CFUs/mL. In this context, the extreme sensitivity of *Limulus* hemolymph to GNB evolved over the eons. However, it also evolved to detect many other substances relevant to the sea/shore paradigm. This includes especially glucans from fungi and algae/lichens (also widely found in terrestrial plants) and also cellulose and mannans as microbial sugars.

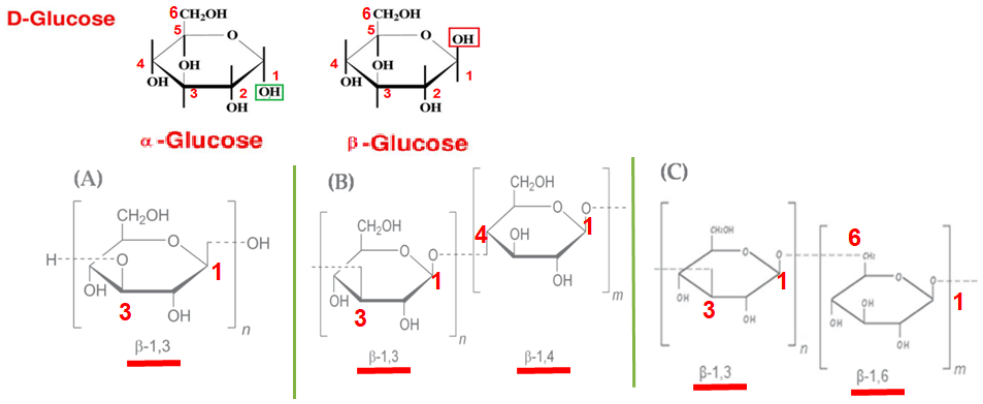
As seen above, all these decomposing biomolecule polymers drain into earth's waters (lakes, seas, rivers, etc.) as foliage dies and is broken down and recycled. The waters also contain algae, lichens, and the ocean contains seaweed and plankton. All of these things mentioned (plants and fungi etc.) produce and use β glucans and mannans and cellulose as cell wall forming molecules. These are ubiquitous molecules that predominate the earth much in the same way that gram negative bacterial endotoxin envelopes the outer layer of membrane for them and

exist then as residue in soil and water. And, to some extent, LAL is falsely reactive to all of them. Falsely reactive just means that LAL is not specific for endotoxin as it is often assumed to be.

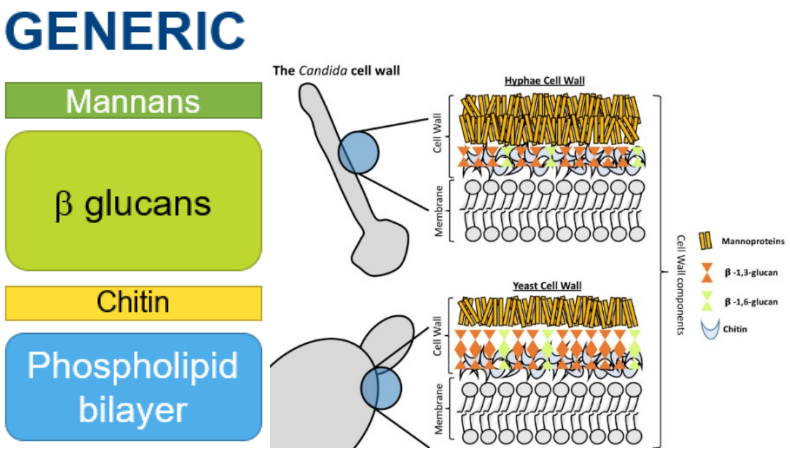
In 1981[i] It was discovered that some glucans gave false positive activation of LAL (Factor G pathway in LAL) and users have been advised by LAL manufacturers to use the "*glucan blocking buffer*" that they developed to overcome the false positive effects. The recommendation has always implied that the blocking is thoroughly effective. A second false positive revelation was found to include cellulosic materials from pharmaceutical drug filters[ii]. The two sugar polymers are described as (i) β, 1,3, D-glucan and (ii) cellulosic residues are characterized as β, 1,4 D-glucans from drug manufacturing filters. LAL manufacturers do not sell their products by elaborating on the false positive reactive substances and no new LAL reactive substances have been identified since the 1980's although older references include also polynucleotides, proteins[iii] and mannans[iv]. Before looking at some data generated using LAL-reactive substances, the structures of glucans, an overview of water systems, and LAL-rFC comparison studies are briefly discussed.

Structures

Some background is in order regarding the structure of glucans. Glucan molecular variants affect function in several ways including: (a) type of monomers which appear as α or β monomers (determined by the placement of the hydroxyl on carbon number 1 and whether it is on the same side or the other side of the number 6 carbon group as shown below) that are used to form the polymers (b) the polymer backbone type (1>3, 1>4, 1>6 etc.) and (c) the branching (location and composition) of attachments on the linear backbone. Plants and algae also contain cellulose, 1>3 and 1>4 β glucans as well as mannans.[v] Mannans are polymers of mannose rather than glucose.



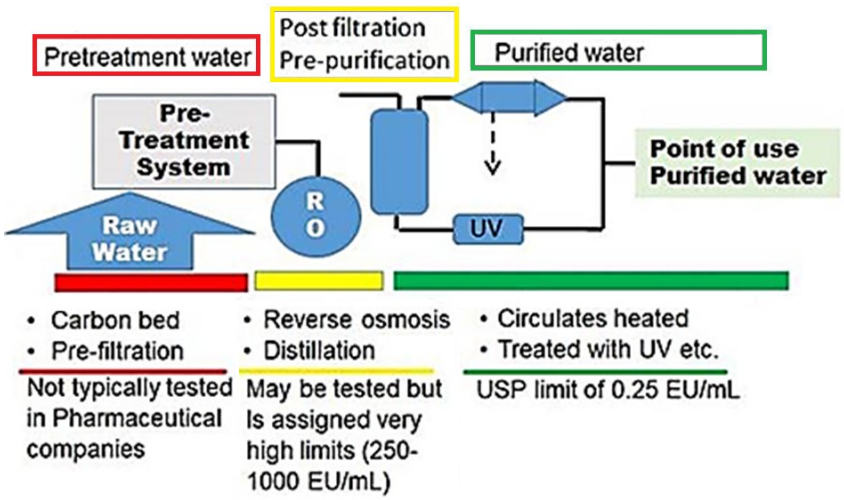
Various glucan structures



Graphical representations of the common structure of Candida (yeast) cell wall. In nature the structures are highly variable depending upon growth conditions, growth stage, polymer degradation state, etc. Figure from Garcia-Rubio et al.[vi] Generic bars at left added.

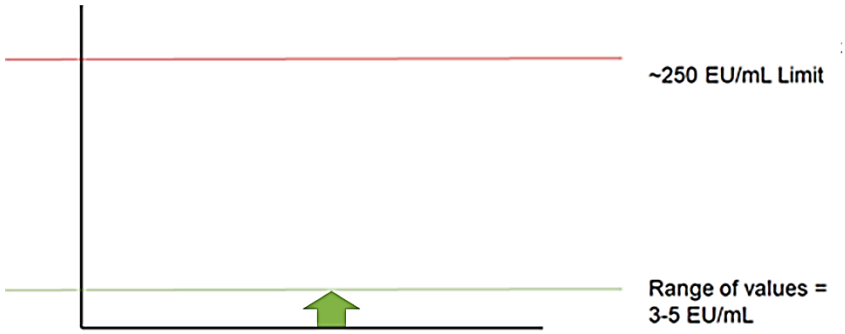
Comparison studies and pharmaceutical water systems

LAL-rFC comparison studies relevant to the pharmaceutical test environment (post filtration water, purified water, drugs, raw materials) have shown very close correlation of results (Kikuchi[vii], Bolden[viii], Mozier[ix], Marius[x], etc.). However, there is (only) one study[xi] that demonstrated greater recovery using LAL (only one type of LAL was used-kinetic chromogenic) versus rFC, but this water was pre-filtration water where one would expect β glucans, cellulosic residues, mannans etc. Industry has debated whether or not this was a fair comparison. The lack of specificity of LAL should detract from LAL rather than to be used in attempts to disqualify endotoxin specific reagents.



Water systems bring in raw water from nature, treat it via carbon beds and filtration feeding into reverse osmosis and distillation processes. Once purified the water is kept circulating at elevated temperatures to ward off bacterial growth. **Red:** not endotoxin tested but used in a comparison study. **Yellow:** post filtration sometimes tested with high associated limits. **Green:** purified water rarely has any endotoxin and spike studies show good comparability (LAL-rFC).

One large pharmaceutical company initiating water testing using rFC did a study (communicated but not yet published) where the limit on first filtered (prepurified) water was determined to be ~250 EU/mL (the limit that can be cleared by subsequent purification as per validation), they found that the rFC and LAL values obtained were approximately 3-5 EU/mL and therefore both tests provided a certainty of coverage for testing this water. A chart making the point is given below. Some have used scare tactics suggesting that if a result of 4 versus 4.5, for example, poses some kind of risk whereas in reality any two LAL tests will give similar variability.



Modern Data on LAL Non-Endotoxin Reactivity

"Modern" day data refers to the dated nature of most LAL non-endotoxin reactivity studies. The most recent significant study was done by Roslansky and Novitsky in 1991[xii]. They studied the major types of LAL that existed then (chloroform extracted versus that using zwittergent) and found that chloroform extracted LALs are more sensitive to β glucans and cellulosic materials. LAL manufacturers rarely reference these studies and typically suggest users just "*use the blocking buffer*" to solve any glucan associated testing issues.

Glucan determining tests are also sometimes used to demonstrate that specific samples are free (or not) of glucans. However, these tests are specific to β-1,3 D-glucans and cannot detect β-1,4 glucans or mannans and their utility to detect degradants of complex natural plant and fungal constituents has never been demonstrated. A typical description is given here as an example of one such commercially available test online (product name redacted and underline added for emphasis):

> The [redacted]® assay kit is specific for (1→3)-β-D-glucan. The assay is based upon a modification of the *Limulus* Amebocyte Lysate (LAL) pathway. The [redacted]® reagent is processed to eliminate Factor C, and is therefore specific for (1→3)-β-D-glucan. The reagent <u>does not react to other polysaccharides, including β-glucans with different glycosidic linkages</u>.

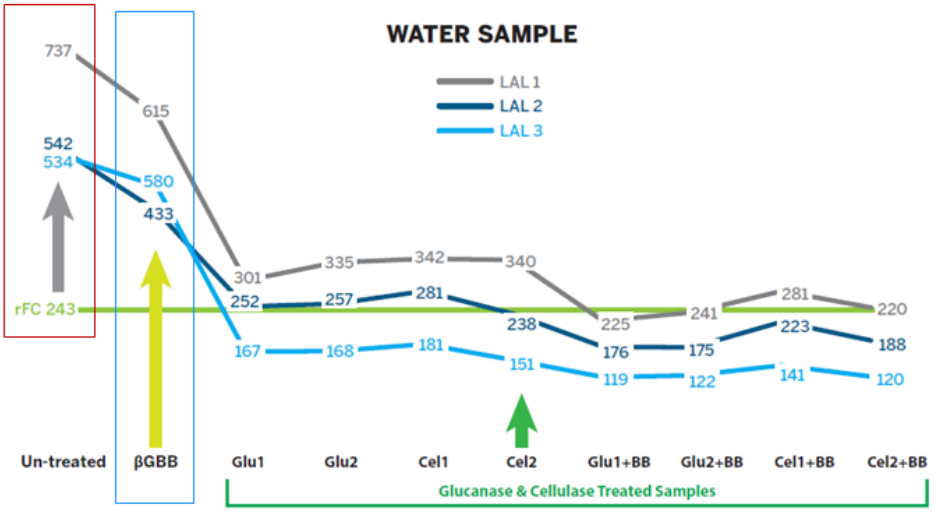
One wonders, given the horseshoe crab's split marine and terrestrial existence in the sea and on the shore, what Factor G is seeking to detect and prevent in the animal? There is some evidence that ancient algal blooms/ marine fungi have been harmful to horseshoe crabs[xiii] and could cover the carapace and invade the bloodstream so the detection via reagents that employ the LAL Factor G pathway (minus Factor C) may be geared more toward algal/marine fungal type β-glucans and thus less sensitive to terrestrial derived β-glucans that would be expected to inhabit soil (plant and fungi) and therefore fresh water from soil runoff. The picture below gives some idea of the terrestrial pressures on the horseshoe crab.

Three questions arise that, when answered, contradict the current paradigm in using β glucan blocking buffers:

1. Can we really block all β-glucans by just using a β glucan blocking buffer (βGBB)?
2. Do chrome and turb LAL methods give the same result in the presence of β-glucans?
3. Are there other microbial polymer sugars that react with LAL?

The first question we approached in two different ways: (a) using a fairly clean natural water from two different retention ponds and testing it via LAL and rFC followed by treatment with (i) βGBB and (ii) β glucan and cellulosic enzymes that attack and break glycosidic bonds. **For the second question** we looked at simply testing purified sugars with both chromogenic and turbidimetric methods (all samples were negative via use of rFC). **For the third question** we used mannan which is not a β glucan and is a predominate sugar paired with β glucans in many fungi and plant cell wall structures. Two different mannans were used- one from plants and one from yeast.

Q1. Can we really block all β-glucans by just using a β glucan blocking buffer (βGBB)?



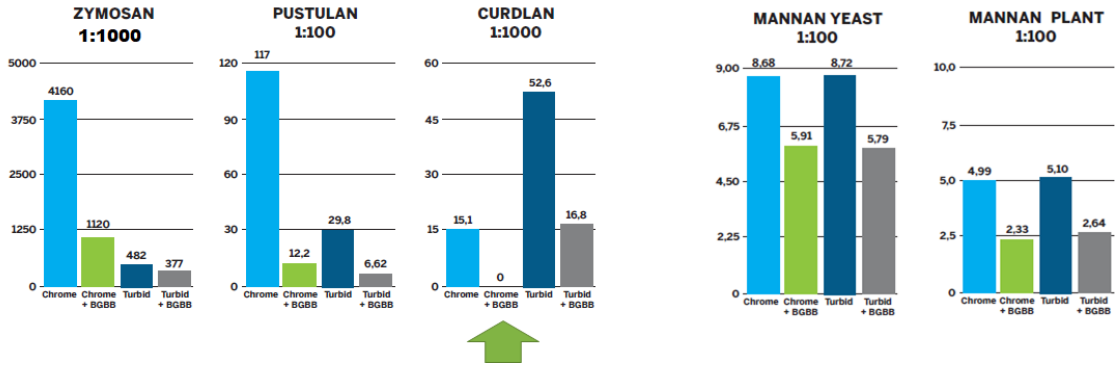
This test was repeated with nearly identical results using a second water source and has now been repeated with similar results in several independent labs. The original data was published

in Amer. Pharm. Review[xiv] and the method used is described there. The testing shows that initial values tested with LAL are much higher than rFC and values are reduced with βGBB but not much. Using the enzymes served to reduce the values to around the values originally obtained with rFC thus demonstrating the false reactivity of LAL with non-endotoxin substances in the natural water.

A more recent study (this year) was performed using specific highly purified purchased sugars to try and identify which of the constituents that may be present in natural water were more (or less) reactive with LAL. Sample commercial sugars of the highest purity available and labeled <1 EU/mL and as tested by human TLR4-expressing HEK293 cells and as tested negative with rFC. It would be impossible to look at all the different sugar types or all the polymer sugar degradation variants in nature (even if they were all known) of course but a small sampling may give us an idea of non-endotoxin specific LAL reactivity.

Various highly purified sugars were purchased and reconstituted using purified water to 1 mg/mL with heat and vortexed for an extended period. Subsequently the solutions were diluted 1:100 or 1:1000 using purified water for testing. βGBB was purchased commercially and used as per package insert instructions.

As shown below the use of βGBB does generally significantly reduce the amount of false reactivity of various sugars with LAL, however, the significant residual amounts for most of the sugars, except curdlan, suggests that the use of βGBB is insufficient to produce results suitable for LAL-rFC comparison studies. These residual values will add systematically higher recovery for LAL relative to rFC.

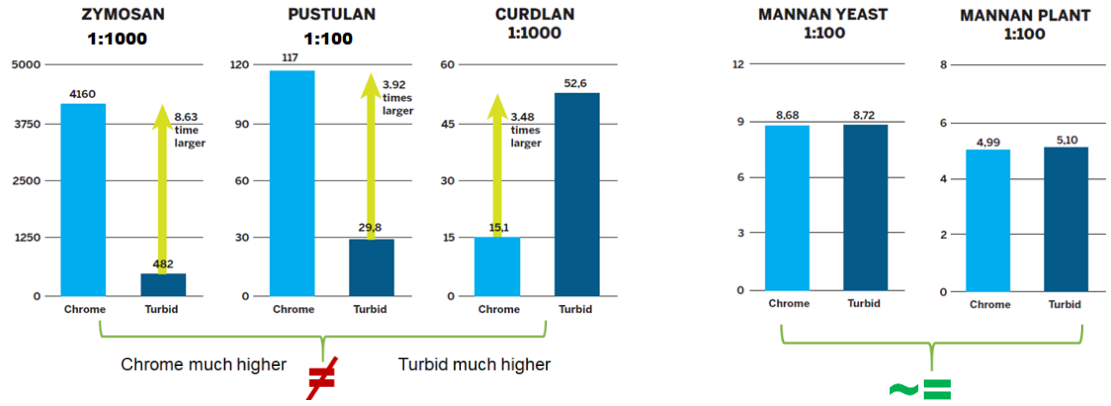


Specific purified sugars show high reactivity with LAL (no reactivity with rFC, so not shown) and some reduction with βGBB, yet a significant residual amount of reactivity with all sugars except curdlan which was well blocked. Curdlan has served as the prototypical sugar for demonstrating βGBB utility.

Q2. Do chromogenic and turbidimetric LAL methods give the same result in the presence of β-glucans?

As seen above, chromogenic and turbidimetric methods have distinctly different reactions with non-endotoxin active sugars and the use of one LAL will not necessarily demonstrate that another LAL will behave similarly. As shown below, for zymosan and pustulan chromogenic gives much higher results than turbidimetric whereas for curdlan turbidimetric gives much higher results than chromogenic. This data suggests that when non-endotoxin reactants are

present including β glucans, then LAL reagents cannot give a "gold standard" result by which rFC as an endotoxin specific reagent can be honestly compared.



Q3. Are there other microbial polymer sugars that react with LAL?

The answer to this question is already contained within the two previous graphs. Mannans, plant and yeast derived, both show significant LAL reactivity and no rFC reactivity. Oddly though, the mannans do seem to give very close reactivity with both turbidimetric and chromogenic when tested side by side.

Mannans are not β -1,3 D glucans but are rather polymers of mannose instead of glucose, then they cannot be detected with commonly used β glucan tests and cannot be blocked with βGBB. In this way a single example serves to disqualify the premise that non-glucans constituents are not as important as β glucans.

One LAL manufacturer has even explored adding β -glucans to LAL formulations in order to increase the reactivity of LAL to endotoxin (see Patent[xv]): "…the sensitivity of such amebocyte lysate preparations to endotoxins can be enhanced by the addition of exogenous (1-3 b-D-glucan." It would be disingenuous at best for a LAL manufacturer to claim "no glucans" in test solutions all the while including it as an LAL formulation constituent in a comparison study. Test users have no way of knowing if any current lysates utilise the addition of β glucans to increase sensitivity.

Conclusion

Repeatedly we hear that a specific given test solution (particularly in LAL-rFC comparison studies) is "β glucan negative" as if that disqualifies a myriad of other similar false reactants in naturally derived substances such as pre-filtration waters. This is a gross mischaracterization of a complex topic.

The simple experiments performed here can be repeated in any laboratory. Why would LAL false positive reactions from β glucans be any more or less relevant than false positives from mannans or cellulosic residues? As seen in the basic data shown here the β–GBB "fix" is imperfect and only works with *some* specific β-glucans and as paired with *some* LAL reagents.

Users may form a question: Why is it "ok" to NOT detect β glucans in drug process flows? The use of LAL has been built upon the paradigm of the detection of endotoxin and not non-endotoxin pyrogens and remains true:

- The presumption that purified water & other manufacturing process constituents are clean of non-endotoxin pyrogens and other reactants (β -glucans)
- The presumption that β -glucans and other contaminants are excluded *during process validation-not routine testing*
- Only Gram-negative bacteria can "*spring up*" quickly in purified water-not fungi or yeast or algae that has much more complex nutritional requirements
- All LAL tests give basically the same answer (this presumption is true in general but as data shows here is not true when β –glucans/ α-glucans /mannans/cellulosic residues are present)
- Glucans are not pyrogenic

As an important reminder: There is no β-glucan standard in the LAL test and therefore it can only "interfere" with a true endotoxin test result (false positive and/or enhancement of endotoxin test values). In this way rFC continues the basic pharmaceutical drug manufacturing paradigm relevant for LAL while removing confounding factors that include non-endotoxin reactivity (false positive results).

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