LAL Update

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Letter From the President

In 1986 Associates of Cape Cod, Inc. introduced the first FDA licensed kinetic turbidimetric assay. Accompanying this assay was an optical reader for running this test in glass tubes. It had been our experience, that microplate methods were ill-suited for kinetic LAL tests, regardless of whether the chromogenic or turbidimetric method was used. At the time of introduction, incubating microplate readers were just being introduced and an LAL which adequately incorporated the chromogenic substrate was still some years away. However, it was the lack of availability of "endotoxin-free" microplates that was the show-stopper. Today, reasonably "clean" plastic microplates are readily available, yet users still encounter numerous out-of-specification individual wells (hot wells). This is predictable because plastic contamination is usually particulate in nature. Plastic plates, having a static charge, tend to attract particulates and therefore are prone to exhibiting hot wells. Although the per well contamination is usually small and is therefore masked if it occurs in a well containing standard or sample with endogenous endotoxin, Murphy's Law generally dictates that a hot well will occur in a negative control, clean sample well, or will add to a low standard sufficiently to invalidate the standard curve or duplicate. To get around this problem, it has been suggested that dry heat depyrogenated glass microplates be used. Although these are expensive, with careful handling, glass microplates should be reusable many times. In addition, it has also been suggested that glass microplates might increase the sensitivity of the assay to the level (0.001 EU/mL) routinely obtained in the LAL-5000 / Pyros Kinetix readers. This Update reports on our experiments examining these issues.

Sincerely,

Thomas J. Novitsky, Ph.D.

Comparison of Glass and Plastic Microplates in Kinetic LAL Assays

by Charles Legg and Tom Novitsky

Introduction

Kinetic turbidimetric LAL methodology has been in use since ACC received approval of its license supplement in 1986. The kinetic turbidimetric method, and later the kinetic chromogenic method, became popular because of their sensitivity (0.001 and 0.005 EU/mL with the turbidimetric / LAL5000 / Pyros Kinetix and kinetic chromogenic / microplate methods respectively), expanded range (usually from 50 or 10 down to 0.005 or 0.001 EU/mL), and ease of use (i.e. single step reagents and automatic timing / data collection / calculation). There are drawbacks however, which include the necessity to use specialized equipment, i.e. the LAL5000, Pyros Kinetix, or an incubating plate reader.* When using the microplate method, plastic microplates must be pre-screened either by the manufacturer or user to determine if they contain suitably low endotoxin for reliable assays. These plates must also not adversely interfere, i.e. inhibit the test. (see "The Problem with Plastics", LAL UPDATE Vol. 6, No. 3, 1988) It is these latter issues that have led many users to consider borosilicate glass microplates.

After all, the original LAL gel clot method uses depyrogenated glass tubes and the most sensitive method of all, the kinetic turbidimetric method performed in a tube reader, also relies on depyrogenated glass tubes. Perhaps, then, glass is the key, both for reducing out-of-

specification results and to achieve the greatest sensitivity the reagent can offer.

Materials

- Depyrogenated, Class A Borosilicate 96 well microplates
- Pyroplate lot #0829802AC
- Pyrochrome lot #02020200C
- CSE lot#68
- RSE (EC-6)
- Pyrotell-T lot#500-02-136-T
- LRW lot#308-299
- Eppendorf repeating pipettor, 1.5 mL syringe
- Thermomax Plate Reader with 405 nm and 340 nm filters
- Softmax Pro Software

Procedure

***Chromogenic*:** An endotoxin standard curve of 2500 to 0.25 pg/mL was prepared using CSE in LRW with a series of 1:10 dilutions. Each standard (50µL) was added to a Pyroplate or Borosilicate microplate in triplicate. LRW (50µL), as a negative control, was added to each plate in quadruplicate. Pyrochrome, reconstituted with 3.2mL reconstitution buffer, was added to each well containing standard or negative control in aliquots of 50µL each using the repeating pipettor. The microplate was placed immediately into the plate reader and data collected using the 405 nm filter for 1.5 hours. Data was analyzed following the end of the data collection period.

***Turbidimetric*:** Endotoxin standard curves of 50 to 0.005 or 10 to 0.001 EU/mL were prepared using CSE in LRW with a series of 1:10 dilutions. Each standard (100µL) was added to a Pyroplate or Borosilicate microplate in triplicate. LRW (100µL), as a negative control, was added to each plate in quadruplicate. Pyrotell-T, reconstituted with 5mL LRW, was added to each well containing standard or negative control in aliquots of 100µL each using the repeating pipettor. The microplate was placed immediately into the plate reader and data collected using the 340 nm filter for 1.5 or 3 hours for the 50 - 0.005 EU/mL standard and the 10 to 0.001 EU/mL standard respectively. Data were analyzed following the end of the data collection periods.

Results

The chromogenic assay was found to be approximately 500 seconds faster for the low end, i.e. 0.25 pg/mL standard, in the Pyroplate compared to the Borosilicate plate. Other than this difference, the assay was comparable in both types of plates with similar curves and regression coefficients (Table 1).

The turbidimetric assay was found to be slightly faster at the high end, i.e. 50 EU/mL, in the borosilicate plate by approximately 100 seconds. As with the chromogenic assay, other attributes were similar (Table 2). It should be noted that neither assay achieved sensitivity greater than 0.005 EU/mL, even though the turbidimetric assay was run for a period of three hours (Table 3). The same lot of Pyrotell-T used in this experiment, when run in the LAL-5000, achieves onset time for the 0.001 EU/mL standard in less than 90 minutes (data not shown).

Table 1. Chromogenic Evaluation

	2500 pg/ml	0.25 pg/ml	A	B	r^2
Pyroplate	403 sec	3135 sec	3.380	-0.225	0.998
Borosilicate	453 sec	3659 sec	3.428	-0.228	1.0

Table 2. Turbidimetric Evaluation

	50 EU/ml	0.005 EU/ml	A	B	r^2
Pyroplate	746 sec	4198 sec	3.182	-0.188	0.997
Borosilicate	632 sec	4220 sec	3.114	-0.205	0.989

Table 3. Turbidimetric Evaluation – 0.001 EU/ml sensitivity

	10 EU/ml	0.001 EU/ml	A	B	r^2
Borosilicate	1003 sec	Not detected*	3.167	-0.180	0.994

**Not detected after 3 hours, 0.01 EU/ml lowest point used to construct standard.*

Conclusions

Our data suggest that borosilicate plates offer no advantage over plastic microplates with regard to increased sensitivity. With the exception of slightly different times of onset, the standard curves are surprisingly similar for the reagent used. It is possible, of course, that borosilicate plates may increase sensitivity over other brands of microplates if they cause a relative inhibition. It is interesting to note that regardless of plate type, the chromogenic assay was generally faster than the turbidimetric.

*Other types of equipment have been used mainly in Europe and Japan, namely the ATI 6000, PUR 320, and Toxinometer.

ADDITIONAL READING

1. Remillard, J.F., P.F. Roslansky, and T.J. Novitsky. Quantitation of endotoxin using the LAL kinetic turbidimetric assay in an incubating microplate reader. In, LAL UPDATE, Vol. 10, No. 3, September 1992.

2. Novitsky, T.J. Pyros Kinetix. In, LAL UPDATE, Vol. 18, No. 1, September 2000.

3. Novitsky, T.J. The problems with plastics. In, LAL UPDATE, Vol. 6, No. 3, September 1988.

4. Novitsky, T.J. Kinetic turbidimetric LAL method. In, LAL UPDATE, Vol. 4, No. 2. June 1986.

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