The Myth of Testing Colored Samples: Debunked

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We often hear from end users who are in the process of designing a suitability study for their new sample, "My sample is yellow, so my only choice of reagent is the turbidimetric test". But is that really the case?

Know Your Enemy: An In-depth Look at Bacterial Endotoxins

Bacterial endotoxins can be nasty little pests! As non-infectious particles found within the cell walls of every Gram-negative bacteria, endotoxins can induce immune responses leading to fever, inflammation, septic shock, and even death in severe cases. Contamination of pharmaceutical and healthcare products with endotoxins, therefore, poses serious risks. Rigorous in-house programs for endotoxin testing are imperative to ensure the safety and quality of pharmaceutical products and medical devices. As such, BET is a regulatory requirement and a critical step in safeguarding public health.

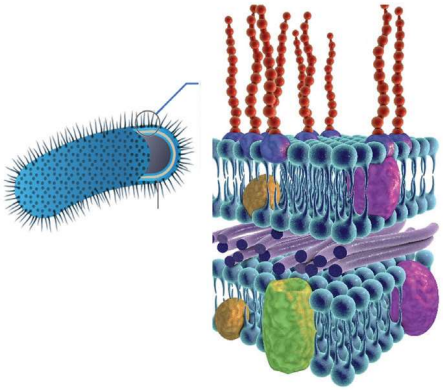


Figure 1. Diagram of cell envelope of a Gram-negative bacterial cell depicting the endotoxin structures in the outer membrane of the bacteria.

Color Me Curious: The Science Behind Chromogenic Testing, BET

Have you ever wondered how kinetic chromogenic testing works? Next, we'll walk you through the science behind this pharmacopeial technique, exploring how chromogenic tests

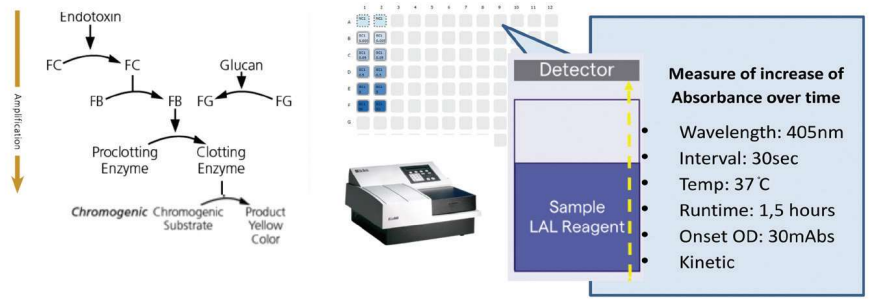


Figure 2. Depiction of the LAL cascade mechanism and the method principle of a kinetic chromogenic test.

measure the color change in a reaction to determine the presence of bacterial endotoxins.

The magic of chromogenic testing lies in its simplicity, linearity and accuracy. In addition to the LAL enzymes of the cascade mechanism (Factor C, Factor B and Preclotting enzyme), this test uses a chromogenic substrate.

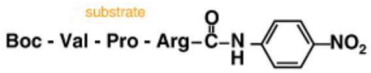


Figure 3. Example of Chromogenic substrate used in ACC chromogenic reagents.

The chromogenic substrate, which is colorless initially, is known to react with an activated Clotting Enzyme – as a result of Factor C activation by endotoxin. As Clotting enzyme cleaves the Arginine – CO bond in the chromogenic substrate, it releases a chromophore called para-nitroaniline (pNA) - a particle that absorbs light (with the absorption maxima close to the visible wavelength of 405nm) - and causes a change in color to yellow (as visible to the naked eye).

The resulting color change is then measured using an absorbance spectrophotometer. It was documented in the past that the intensity of the developing color is proportional to the amount of endotoxin present in the sample, allowing for a quantitative analysis of endotoxin in the sample.

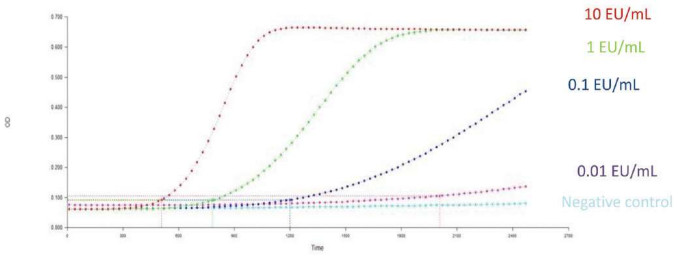


Figure 4. A typical diagram of the developing absorbance as measured at 405nm as dependent on endotoxin concentration.

It's a blend of biology and colorimetry that delivers rapid, accurate results, that made chromogenic testing a game-changer in the field of bacterial endotoxin testing back in 1990s.

How the Recombinant Chromogenic Test Further Improves the Output

The recombinant chromogenic test takes the advantages of the chromogenic test to the next level. How? Thanks to several groundbreaking features:

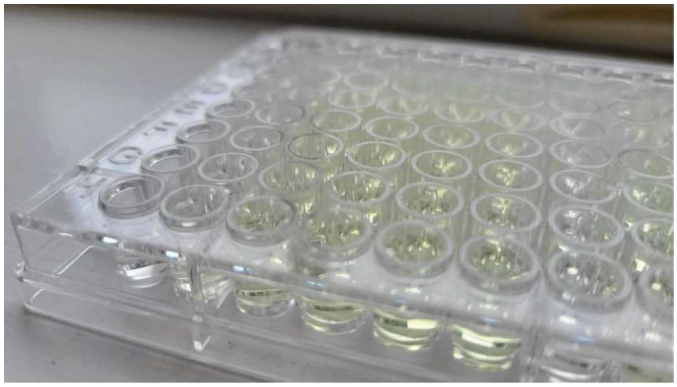


Figure 5. A typical microplate showing the developed color at the end of a kinetic chromogenic test.

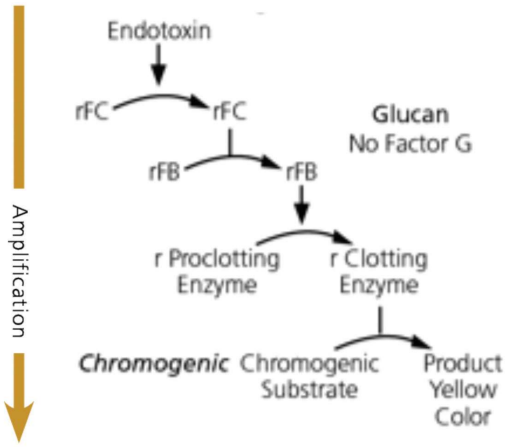


Figure 6. Mechanism of action of the recombinant cascade reagent.

- First and foremost, recombinant cascade reagent (rCR) PyroSmart NextGen® employs recombinant Factor C, Factor B and Proclotting enzyme, genetically cloned from Limulus polyphemus and expressed preparations of the cascade enzymes, thereby eliminating the need for animal-derived components and making the test eco-friendly.
- Furthermore, it is free of Factor G, a native component of the animal-derived LAL reagent, that is documented to be cause co-sensitivity to 1,3-β-glucans (common contaminants) thus reducing the risk of Out of Specification results.
- Perhaps most importantly, it has a documented lot-to-lot reproducibility of results which is a building stone towards standardization and modernization within the quality control laboratories (including automation of liquid handling).

At ACC, PyroSmart NextGen® is manufactured with consistent quality and performance under the same cGMP conditions in the same

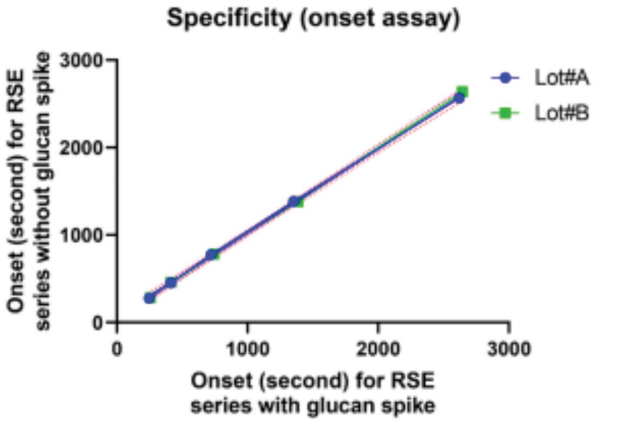


Figure 7. Linear regression of Onset Times (in seconds) for two RSE standard series with and without 1,3-β-glucan spike.

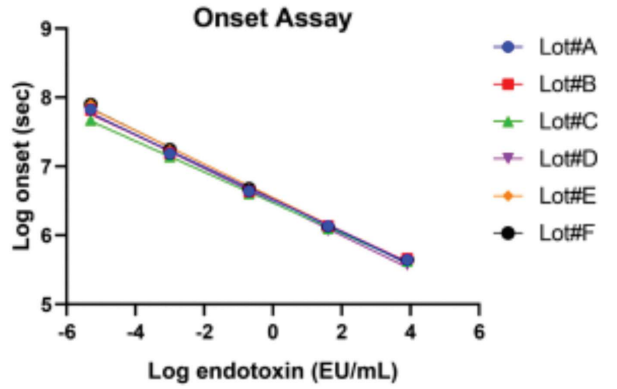


Figure 8. RSE standard curve series using six different lots of PyroSmart NextGen® demonstrating strong lot to lot reproducibility.

ISO 14385-certified facility as our FDA-licensed LAL reagent. This guarantees reliable and repeatable results, making rCR a robust and sustainable solution for endotoxin testing.

Debunking The Myth: The Data Behind Testing Colored Samples

There's a common misconception that chromogenic testing struggles when it comes to colored samples. Here, we'll set the record straight, showcasing the data that proves chromogenic testing, including the recombinant chromogenic assay, works efficiently and accurately on colored samples following a well-executed method suitability.

Per USP <85> and <1085>, method suitability testing is to be done on all samples prior to routine testing (1, 2). This allows for appropriate method development and it typically includes testing the sample in a series of dilutions (not exceeding the Maximum Valid Dilution) and evaluating the assay setup (reagent type, method type and instrumentation) for compatibility with the sample.

Fun fact #1: Most pharmaceutical sample types interfere with the BET.

Fun fact #2: A vast majority of sample interferences are overcome by simple dilution in water for BET (such as LAL Reagent Water (LRW)).

Colored samples are no different. Often in addition to the inherent color, they are likely to consist of components that interfere with the test. Based on our experience, dilution in LRW is highly likely to resolve both concerns – the optical and chemical interference - in one simple step.

In addition to dilution, there is another invaluable tool: instrumentation and software. The advent of advanced spectrophotometric methods has significantly alleviated the concern with testing colored samples. Baseline setting and zeroing play a pivotal role in this process. For example, in Pyros Kinetix Flex tube reader, where each well is individually timed and evaluated, it involves recording the initial absorbance of the sample. This is essentially measuring the absorbance by the inherent color of the sample before any reaction takes place. This baseline reading is then used as a reference point for all subsequent 10 second measurements, allowing the true color intensity increase to be accurately captured, irrespective of the color of the sample itself.

Pyros Kinetix Flex is powered by Pyros eXpress software which has built-in specifications for the raw data retrieved by each well. A sample with an intense color absorbing at 405nm will yield low transmittance values during the initial zeroing period and thus will be flagged in the software as being out of range, alarming the operator to take further action.

Case Studies: Real-World Applications of Chromogenic Methods for Colored Samples Testing

So how does this all work together? Let's examine comparability testing of MIC injection - a vitamin mix injection consisting of the primary compounds (methionine, inositol, choline) in addition to other components, e.g. Vitamin B12. Depending on the concentration of the components, the final preparation may look like this:

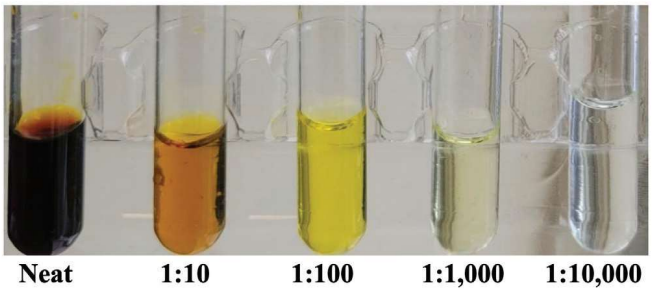


Figure 9. Dilution series of MIC injection

- Dilution series in LRW (MVD = 14,000)
- Addition of the BET reagent yields an additional dilution of the colored background.
- Testing the MIC injection using the kinetic turbidimetric assay (KTA):
  - Data collection plots for Positive control and Positive Product Controls for all dilutions tested (nominal value of 0.5 EU/mL):

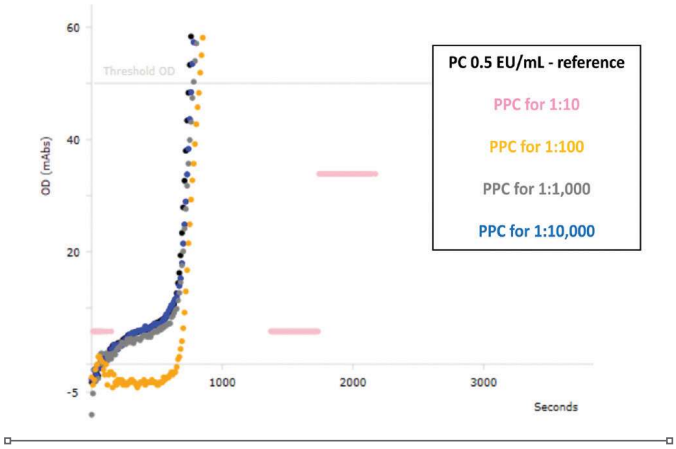


Figure 10. Data collection plots obtained for the range of MIC injection dilutions spiked with 0.5 EU/mL when tested by kinetic turbidimetric test in Pyros Kinetix® Flex tube reader.

  - Interpretation:
    - Neat – not tested. The concentrated MIC injection is off deep yellow color which absorbs non-specifically a full visible light spectrum.

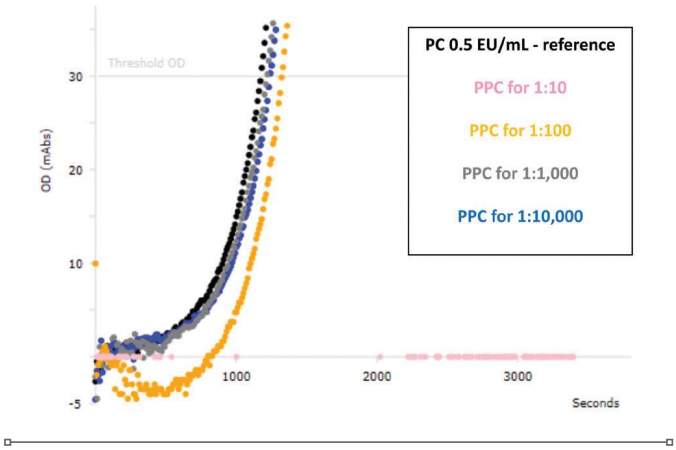


Figure 11. Data collection plots obtained for the range of MIC injection dilutions spiked with 0.5 EU/mL when tested by kinetic chromogenic test in Pyros Kinetix® Flex tube reader.

    - PPC for 1:10 – the inherent color is still too deep for the turbidimetric test, still absorbing the passing light non-specifically, Pyros eXpress notifies the user 60 seconds into the test that the transmittance specification was not met.
    - PPC for 1:100 – there is a residual optical interference between 0 to 800 seconds, which is then overcome by the increasing change in turbidity, related to the endotoxin reaction.
    - PPC for 1:1,000 – no optical interference.
    - PPC for 1:10,000 – no optical interference.

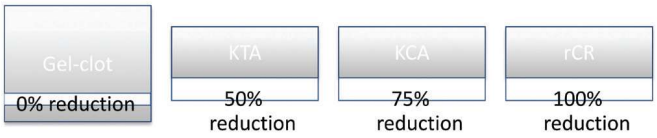
Table 1. Summary evaluation of the photometric data

	Dilutions	Mean EU/mL	PPC Recovery %	PPC CV%
KTA	1:10	Invalid	< 1%	Invalid
	1:100	< 0.5	82%	1.58
	1:1,000	<5	109%	0.39
	1:10,000	<50	109%	0.21
KCA	1:10	Invalid	< 1%	Invalid
	1:100	< 0.5	55%	0.61
	1:1,000	<5	85%	1.83
	1:10,000	<50	78%	1.42
rCR	1:500	< 2.5	90%	0.45

- In conclusion: the magnitude of optical interference observed on the turbidimetric test vs. chromogenic test was identical. Residual interference was observed at 1:100 dilution when testing by both methods. 1:1,000 dilution was free of both optical and chemical interference when testing by both LAL methods and thus could be chosen for further testing and validation.
- Additional testing was done with PyroSmart NextGen® where MIC injection was diluted 1:500 and that was sufficient enough to overcome the optical interference.

Expert Opinions: Quality Control Technicians Weigh In

Don't just take our word for it! Ask around! Leading pharma QC scientists and managers successfully validated kinetic chromogenic testing for colored samples. In some cases, they choose to go directly to the chromogenic technique, taking advantage of the wide dynamic range of the test, some started with the turbidimetric technique and then transitioned to the chromogenic one. Others, especially when implementing in-house testing for new products, go directly to the use of the recombinant chromogenic tests for colored samples. Apart from analytical performance, the photometric techniques comply with the



3R principles (Reduce, Replace, Recycle) in reducing the amount of the raw animal-derived material in the reagent with the recombinant reagent completely eliminating it:

All About Dilution: A Key to Unlocking Accurate Results

In closing, proper dilution techniques are instrumental in facilitating accurate results with colored samples (as with colorless ones), thus dismantling the misconception around chromogenic testing's capabilities. Understanding the components of the reaction and using the right instrumentation/software platforms with built-in features to report samples not meeting specifications allow the user to identify any issues shortly into the assay. With the appropriate method development, the chromogenic technique can be used for testing of colored samples with equivalent results to the turbidimetric technique.

The recombinant chromogenic method confirmed the validity of the results even at a lower dilution and has been proven suitable for colored samples as well. In the light of expert opinions, empirical data and the ethical commitment to animal welfare, it is evident that the recombinant chromogenic test is a robust, sustainable and reliable approach for endotoxin testing, regardless of sample color (3-7).

Embracing state-of-the-art methods signifies a leap forward in pharmaceutical quality control towards standardization and modernization of the procedures, while ensuring the safety and efficacy of medical products.

References

1. Bacterial Endotoxins Test <85>, United States Pharmacopeia (current revision), United States Pharmacopeial Convention, Rockville, MD.
2. Guidelines for Bacterial Endotoxins Test <1085>, United States Pharmacopeia (current revision), United States Pharmacopeial Convention, Rockville, MD.
3. Shapovalova O V et. al. New direction in the determination of bacterial endotoxins: Analysis using recombinant Factor C. Pharmaceutical chemistry journal, 56, 1133-1139, 2022.
4. Stevens I et. al. Advanced Recombinant Cascade Reagent PyroSmart NextGen® for Bacterial Endotoxins Test as Described in the Pharmacopeias BPB, Vol.5, No. 5 105-114 (2022)
5. Kelley M et. al. Evaluation of Recombinant Cascade Reagent PyroSmart NextGen® and Limulus Amebocyte Lysate Equivalency in a Plate and Tube Reader for Bacterial Endotoxins Testing, BPB, Vol.6, No. 1 11-15 (2023).
6. Kelley M et al. A Demonstration of the Validation Process for Alternative Endotoxin Testing Methods Using PyroSmart NextGen® Recombinant Cascade Reagent, BPB, Vol.6, No. 2 68-75 (2023).
7. Kikuchi Y et al. Collaborative study of bacterial endotoxins test using recombinant Factor C-based procedure for detection of lipopolysaccharides (Part 3). Pharmaceutical and Medical Device Regulatory Science, 54 (4), 341-351 (2023).