Pharma&Biotech

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PyroGene™ Recombinant Factor C Assay

Endotoxin Testing: It's Time to Embrace the 'Alternative'

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Introduction

Bacterial endotoxin testing (BET) enables the detection of endotoxins, which are the pyrogenic component found in the outer membrane of Gram-negative bacteria. They can elicit an adverse immune response if accidentally administered to a patient's bloodstream via contaminated parenteral drugs or medical tools such as intravenous infusion devices.

The Tachypleus and Limulus Amebocyte Lysate (TAL/LAL) assays are the most commonly used tests at this moment in time. The supply of lysate for these tests is dependent on three horseshoe crab species, namely *Limulus polyphemus* found in North America, as well as *Tachypleus gigas* and *Tachypleus tridentatus*, which live in various regions in Asia. While the natural sources of lysate have provided a powerful tool to date, there are a number of reasons the industry can no longer afford to rely on these partially declining species. As such, the need for an alternative and sustainable means of endotoxin detection has reached a crucial point.

There are several reasons for this. Firstly, there is the need to meet the growing demand exhibited by the vaccine development and production industry. If the growth in vaccine production continues to progress as it has done in recent years (for example, due to the maturing Asia-Pacific market) this could place significant strain on lysate resources. Furthermore, the rise of personalized medicine, where each treatment is produced specifically to treat a single patient, could mean each product will need to be tested individually on a 'per use' basis. This could lead to an increasing amount of testing and put further pressure on the supply of lysate-based assays.

Protecting a Finite Natural Resource

The increase in the amount of BET required is cause for concern, as the horseshoe crabs are a potentially finite resource, if their use is not managed properly. In countries such as the US, where crab populations are carefully managed, their supply is tightly regulated and conservation efforts are being carried out to ensure this continues. However, in other areas of the world, regulation is more complex or non-existent, with the populations spread across multiple different countries. In Asia-Pacific regions, declining crab populations could ultimately put the world's supply at risk. If Asian horseshoe crab populations were totally depleted, the industry would have to become solely reliant upon the resources in the Atlantic coast regions of North America.

Alternative methods usually perform comparably or sometimes even better than TAL/LAL-based methods depending on the application, and increased usage would decrease the demand on a limited natural resource. Furthermore, an alternative that does not require an animal source would meet initiatives to reduce animal use in testing.

Lastly, the amebocyte lysate is a naturally-occurring biological product. Accordingly, some lot-to-lot variability may occur between different batches of the product when obtained from different crabs and at different sampling times. Synthetic, recombinant alternatives are much more consistent and could help to reduce the variability between assay batches.

What's the Alternative?

Fortunately, there are alternative assays that can circumvent these drawbacks, helping to protect lysate supply and potentially even negate the need for animal use entirely. For example, recombinant Factor C (rFC) is the synthetic version of Factor C, an element found in the blood cells of horseshoe crabs that is the essential biological component of natural BET assays. When either the natural or synthetic form of Factor C binds to endotoxins, a clotting cascade is activated (Figure 1). Lonza's PyroGene™ Recombinant Factor C Assay is an end-point fluorescence test that uses rFC to cleave a fluorogenic substrate and subsequently reveal a measurable fluorescent signal (Figure 2).

The PyroGene™ Assay delivers comparable results to natural BET assays yet offers several benefits over these methods. These include improved lot-to-lot consistency, enhanced endotoxin specificity and statistically more robust spike recovery. In addition, these tests are easy to use, have a sensitivity range of 0.005 – 5 EU/ml or greater, and are not reliant on animal resources.

However, pharmacopeial guidelines currently list rFC as an 'alternative method'. As such, it requires additional validation steps to be carried out when used for endotoxin testing, which is why some manufacturers have been reluctant to employ these tests. However, it is much easier than most people realize to add these extra validation steps to the workflow, especially as the Lonza team has already developed protocols for easy adaption and submission. Lonza also provides additional regulatory support services, making it simple to submit the details needed to meet pharmacopeial requirements.

LAL

Endotoxin
FC
FC̄
Glucan
FB
FB̄
FḠ
FG
Proclotting Enzyme
Clotting Enzyme
Turbidimetric
Coagulogen
Coagulin *Turbidity*
Chromogenic
Chromogenic Substrate
Product *Yellow Color*

Recombinant Factor C

Endotoxin
rFC
rFC̄
Fluorogenic Substrate
Product *Fluorescence*

Figure 1

Natural Factor C initiates a clotting cascade measured using turbidimetric and chromogenic assays. Lonza's synthetic PyroGene™ Recombinant Factor C Assay triggers a similar reaction, but the output is measured using a simpler fluorogenic assay that does not rely on an amplification cascade.

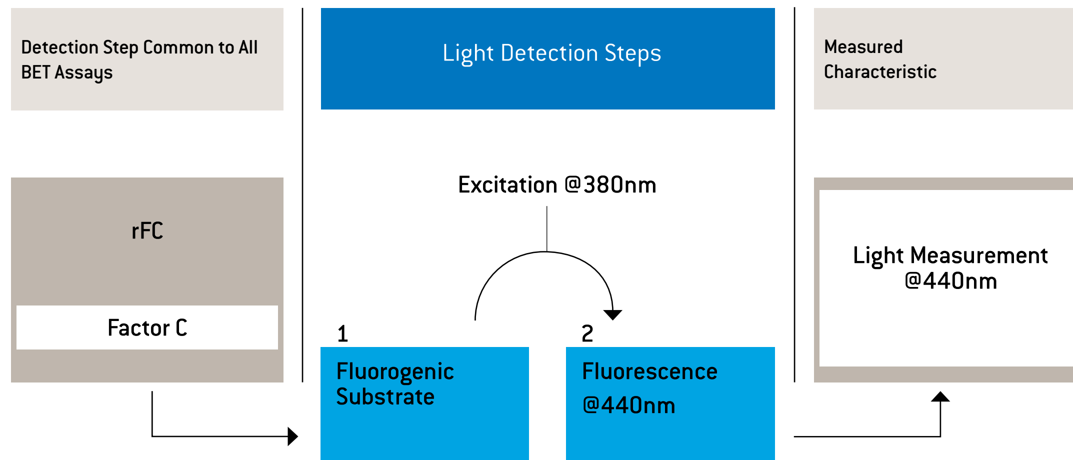

Figure 2
The PyroGene™ Recombinant Factor C Assay.

How Is the Alternative Assay Validated?

Validating an alternative assay requires one change to the standard BET system and can be accomplished in as little as 1–3 days. The procedure stated in the United States Pharmacopeia (USP) <1225> and International Council for Harmonisation (ICH) Q2B includes four steps, as discussed below.

Step 1: Initial Qualification

At the initial qualification stage, it should be tested whether the endotoxin detection system is operating properly. Based on this, an assessment of reagents and equipment should be carried out to ensure it is working as expected as well.

Step 2: Inhibition and Enhancement Testing

At this stage, an assay is designed to determine the level of dilution at which product inhibition or enhancement can be overcome. In order to achieve this, interference should be assessed across a range of different dilutions. The appropriate steps need to be carried out in order to ensure any inhibition or enhancement can be resolved.

If possible, it is best to work with dilutions that are not close to the maximum valid dilution (MVD), as this will provide a more accurate determination of the endotoxin content in a sample. An acceptable Positive Product Control (PPC) recovery result can be anywhere between 50–200%. However, ideally it should be between 70–160% in order to ensure more predictable recovery values and avoid any assay variability errors that might otherwise cause problems further down the line.

Step 3: Validation of an Alternative Method

This is the additional step required to show that the results obtained using the rFC assay are comparable to those achieved with the current TAL/LAL methods. Submissions should contain enough information for experts to evaluate the comparability of the proposed alternative to the compendial methods. As such, the rationale for using an alternative should include the need to select a test that is not reliant on animals, while providing enhanced endotoxin specificity and assay reproducibility. It should also highlight the most critical element of natural TAL/LAL assays is the binding of endotoxin to Factor C, and that the alternative analytical procedure uses a recombinant form of this protein and follows the same pathway as other BET reactions. In addition, it is necessary to confirm that the assay is photometric (i.e. the measurement of the intensity of light, or relative illuminating power). It should also state that the minimum detection limit for most of the currently available BETs is 0.005 EU/ml and that this is within the sensitivity range of the PyroGene™ Assay (0.005 to 5 EU/ml).

The analytical procedure for the determination of impurities is a category II analytical method. Due to this fact, the data elements required for assay validation include assessing specificity, precision, accuracy, linearity, detection limit, quantification limit, range and robustness. Accuracy is calculated as the percentage recovery of a known amount of 'spiked-in' analyte in the sample and involves comparing the new procedure results with those achieved using the old method. The new results should be within two-fold of the known sample endotoxin concentration (50–200%) and within ± 25% of the expected value.

Precision is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample. It is measured by analyzing the standard deviation and coefficient of variation (% CV) of multiple measurements. Linearity necessitates that standard curves generated from different lots of rFC must show consistency in order to ensure more reproducible results and less variable recovery values for PPCs. Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. The range and limit of detection for the analyte in the product should be sufficient to detect at least the Endotoxin Release Limit (ERL) of the product being validated.

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Step 4: Product Validation
Product validation must show that the new method (rFC) is capable of achieving consistent results with the chosen dilution. This usually requires testing of three production lots to show that any inherent interference has been overcome and the formulation of the final product is consistent.

It is important to note that the validation scheme for an alternative method is virtually identical to that which would be needed for any TAL/LAL-based method with just the addition of one extra step, i.e. Step 3: Validation of an Alternative Method. The validation of an alternative method is required to assess the comparability between the current BET method and the rFC method. This is demonstrated by testing three production lots of a particular product with both the current BET method and the rFC method, according to the procedures identified in the Inhibition/Enhancement assay. Validation of one product using the rFC method can be conducted in as little as 3–5 days, assuming that the product has already been validated with a quantitative LAL method and taking into consideration data previously obtained using the current BET method. For example, Initial Qualification and Inhibition/Enhancement studies conducted on the current BET method would not need to be repeated for the validation of the alternative method. For your convenience, users can request a full Validation Protocol that can be followed, which outlines the steps required for validation of the rFC method in full detail.

Closing Remarks

There is a need for TAL/LAL assay alternatives due to growing demand and a risky supply line (at least, in some geographies). Fortunately, alternative tests based on synthetic factors are available, which overcome many of the drawbacks of the natural TAL/LAL assay. These alternative tests do not require a lot of extra time or effort to validate, as the rFC assay is not really an alternative method in many respects, but rather a fluorescence-based synthetic version of the existing TAL/LAL assay. Hence, companies do not need to be put off adopting these methods simply because it is classified as an alternative.The validation procedure is much easier to perform than is commonly realized, especially if following a well-structured protocol and supported by the manufacturer of the test. Lonza has already helped a number of companies take these steps and can also provide 'ready-to-go' documentation to support any regulatory submissions. As such, many of the barriers have been removed from the validation process and new adopters are in a stronger position than ever before to start using the recombinant technology within their testing workflows.

To find out more about the PyroGene™ Recombinant Factor C Assay or to learn how Lonza can help with the validation procedure, contact Lonza's Scientific Support.

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