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Preparing for the Future of QC Testing

Straightforward Adoption of Sustainable Endotoxin and Pyrogen Tests

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Pyrogen testing is critical to ensure the safety of injectables and parenteral pharmaceuticals. However, manufacturers face growing pressure to move away from traditional pyrogen tests, toward more sustainable *in vitro* tests. Here, we discuss sustainable tests already available, and how to select the right test. We also explain how to implement sustainable pyrogen tests in your laboratory, showing that the process is quicker and easier than commonly believed.

The Future of Pyrogen Testing is Sustainable

Testing parenteral pharmaceuticals for pyrogens such as bacterial endotoxins is critical for their safe release to market. Such testing has historically relied on the rabbit pyrogen test (RPT), which consumes experimental rabbits, and the limulus or tachypleus amoebocyte lysate (LAL or TAL) test, which is prepared from the blood of the horseshoe crab.

Today, manufacturers face growing pressure to adopt more sustainable *in vitro* tests. For example, the 3Rs initiative is driving companies to replace, reduce, and refine animal experimentation. United Nations Sustainable Development Goals (SDGs) are incentivizing companies to reduce their reliance on finite natural resources. And, some pharmacopoeias have even pledged to remove certain animal-based tests from their chapters altogether.

Moreover, evolving regulations and the complexity of novel biologics have led to expanded pyrogen testing requirements, such as an increasing number of in-process tests. Conducting an ever-growing number of tests, however, is not always feasible with tests that rely on animals or finite natural resources.

Meeting the Needs of Today and Tomorrow: Established, Sustainable Testing Solutions, Supported by Regulations

Sustainable alternatives to the traditional RPT and LAL/TAL methods are already available and well established – namely the monocyte activation test (MAT) and the recombinant factor C (rFC) assay.

The MAT: A Sustainable and 'Complete' Replacement for the RPT

The MAT works by mimicking the human immune system's reaction to pyrogens (Figure 1), where monocytes from human blood donations respond to the presence of pyrogens, including non-endotoxin pyrogens (NEPs), by secreting pro-inflammatory cytokines, such as interleukin-6 (IL-6). These cytokines are then measured to provide a readout of pyrogenicity. Because of its ability to detect both endotoxins and NEPs, the MAT is considered a 'complete' *in vitro* pyrogen test, replacing the rabbit pyrogen test.

The MAT is already acknowledged by most Pharmacopoeia. It was introduced into the European Pharmacopeia back in 2010 as a non-animal pyrogen test suitable for replacing the RPT, with the RPT compendial chapter now set to be discontinued in Europe by 2026. The United States Pharmacopeia (USP), as well as other leading pharmacopeia, describes the MAT as a suitable alternative test method to the RPT.

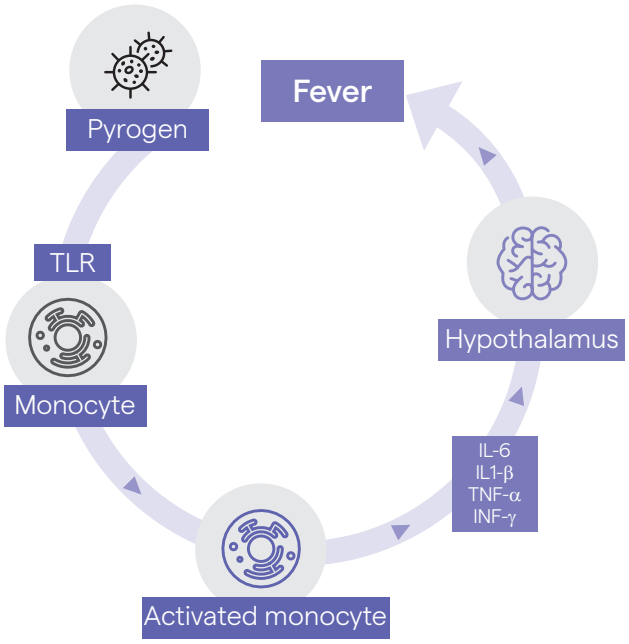

Figure 1.
How human monocytes react *in vivo* to the presence of pyrogens to induce fever.

Moreover, a wealth of scientific studies attest to the MAT's suitability as an RPT replacement, and MAT data has already been used to support product license applications.

As an alternative to the RPT, the MAT has several advantages:

- **Sustainable:** No rabbits are required, eliminating the reliance on experimental animals
- **Safe:** The MAT mimics the innate human immune response, enables robust positive and negative controls, and eliminates variables associated with the use of experimental animals
- **More sensitive:** Earlier recognition of contaminants due to lower detection limits
- **Flexible:** With a choice of different serums, labs can tailor the MAT assay to their specific product needs
- **Cost-efficient:** The MAT is a straightforward, two-day *in vitro* test, while the RPT incurs the significant time and costs of animal housing and training

The rFC Assay: A Recombinant Version of LAL-based Methods

The rFC assay uses a recombinant form of Factor C, the endotoxin-detecting protein component of the LAL clotting cascade, which is conserved across horseshoe crab species. Once activated by endotoxins, the rFC protein cleaves a fluorogenic substrate to produce a fluorescent signal (Figure 2), eliminating the need for other enzymes for signal amplification. Unlike the MAT, this assay does not detect NEPs, and is therefore considered an endotoxin-specific test, similar to the LAL test except it does not react with glucans.

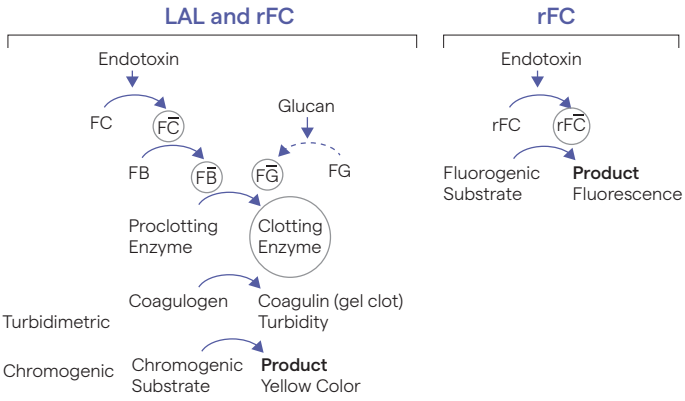

Figure 2.
Comparison of the LAL enzymatic cascade with the single enzymatic step of the rFC assay. Unlike the LAL test, the rFC test is not susceptible to glucan interference.

The rFC assay has been commercially available for almost two decades, is supported by a wealth of scientific publications addressing its suitability and comparability to LAL tests, and is either a compendial or alternative method in several pharmacopoeias. The rFC assay has also been used for release testing of several approved drugs, the first being Eli Lilly's Emgality® in 2018.

As an alternative to LAL-based tests, the rFC assay has several advantages:

- **Sustainable:** Uses recombinant proteins manufactured in a lab as an alternative to the blood of HSCs
- **Specific detection of bacterial endotoxins:** Unlike LAL-based tests, rFC assays work via a single enzymatic step which is not impacted by presence of glucans
- **Supply security and better consistency:** Use of liquid, synthetic proteins relieves supply challenges that may arise from dependence on a natural resource, provides better lot-to-lot consistency and eases reagent scaling

Two Complimentary Tests: Knowing Which to Apply and When

Due to their differing abilities to detect NEPs, the MAT and the rFC assay serve different, complementary testing purposes. Knowing which test is appropriate in any given testing situation is therefore critical.

To discern which test is most appropriate, you must conduct a risk analysis for the presence of NEPs. The MAT should be used for NEP risk assessment during new drug development and new process validation (Figure 3, left side), as only the MAT can detect NEPs. If NEPs are found to pose a risk, the MAT is recommended for routine testing. If NEP risk is ruled out, endotoxin testing alone is often sufficient for routine production (Figure 3, right side). For specific detection of endotoxins, rFC is the most suitable sustainable method, as it does not rely on a natural resource and it is quicker, easier to use, and more scalable than the MAT.

In addition, to new drug development and new process validation, the MAT is also a valuable tool to assess NEP risk for any process changes, out of specification events (OOS), and as a periodic quality control.

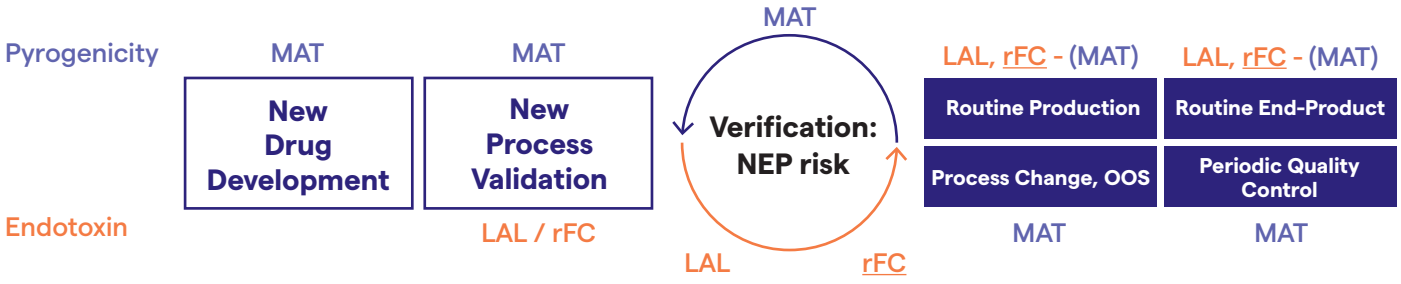

Figure 3.
Endotoxin and pyrogen testing: From drug development to routine production.

Validation of Alternative Method – A Possible Additional Step

Dependent on the feedback of your competent authority, both sustainable methods may need to be implemented as either alternative compendial methods (where only the comparability to the already established monograph method needs to be demonstrated, as noted in Step 1), or as alternative methods.

To adopt either the rFC or the MAT as a sustainable alternative method, you will need to do validation of an alternative method (for example, as per USP General Chapter <1225>), which involves demonstrating its suitability for the intended application across a range of performance characteristics, such as accuracy, precision, specificity, linearity, range, detection limit, quantitation limit, and robustness.

Importantly, the performance characteristics you need to evaluate will depend on the requirements of the relevant authorities. You may also already have the data you need to evaluate several of these characteristics from the studies conducted during Steps 1 and 2.

Adopting a Sustainable Method: A Straightforward Process

Adopting sustainable *in vitro* QC test methods in your lab is easier than commonly anticipated. And, whether using the MAT or the rFC assay, the implementation process is largely the same.

Step 1:

Adding the Analytical Method to Your Pharmaceutical Quality Management System (QMS)

As with any new analytical method, the first step to adopting a new sustainable method in your lab is to add the method to your QMS.

Dependent on your QMS set-up, this may involve several activities, including Installation Qualification (IQ), Operation Qualification (OQ) and Performance Qualification (PQ) of the analytical instruments and operating software. Adding the analytical method to your QMS may also require you to assess the comparability of the new analytical method with the compendial analytical method already established in your QMS. For the comparability study, you may use the original validation product, or a 'neutral' product where no interferences are expected.

When adopting both the rFC and the MAT, the implementation of a multimode reader might be the most efficient option, as it supports both fluorescence and absorbance reading. While the rFC assay utilizes a fluorescent reading (excitation at 380 nm, reading at 440 nm) the analysis of most commercial MAT requires an absorbance reading at a wavelength of 450 nm, as well as a reference wavelength in the range of 540 – 590 nm.

Next, an initial qualification should be performed to confirm that the entire analytical method (including equipment, reagents, and analyst) is functioning properly for its intended purpose, that it operates in line with all requirements, and does so safely and consistently.

Materials and Timings

The initial qualification typically requires two consecutive plate runs by one or two analysts to demonstrate correct preparation of the standard curve and reproducible recovery of a known endotoxin concentration on a test matrix or an example product. This can be done in as little as one day for the rFC, or just three-to-four days for the MAT (see summary Table 1).

Step 2:

Feasibility Study

Conducting a feasibility or product characterization study is the next recommended step. The feasibility study determines if (and how) your product interferes with the assay reagents and helps you ascertain how to overcome this interference (typically through sample dilution, or through sample pre-treatment using additional reagents).

To conduct a feasibility study, prepare multiple dilutions of your product, from undiluted up to the maximum valid dilution (MVD). Spike each dilution with a positive product control (PPC) and determine the dilution factor with optimal recovery (the allowable spike recovery range is between 50% and 200%). Note that, with the MAT, recovery of both endotoxin and NEP spikes must be evaluated.

Materials and Timings

A feasibility study for the rFC assay requires just one plate and can be conducted in as little as 0.5 days. For the MAT, the process requires only one-to-two MAT plates and one additional ELISA test, and can be completed in one-to-two weeks (see summary Table 1).

Step 3:

Product-specific Validation

Finally, you must conduct a product-specific validation (PSV) to confirm or refine the optimal dilution factor identified during the feasibility study, and to demonstrate that you can achieve consistent PPC recoveries across a number of production lots of your test product.

Materials and Timings

As with the feasibility study, the PSV is rapid and straightforward (see Table 1). An rFC assay PSV can be conducted in approximately one day using a single plate. A MAT PSV can be conducted in just three-to-four weeks (owing to the requirement for overnight culturing steps) and requires only five to six plates, with three additional ELISA tests, (if you are able to test the three product batches in parallel).

Once the PSV is complete, labs can immediately use the rFC and/or the MAT for raw materials and in-process sample testing. For drug product or device testing, however, you must follow up with the appropriate regulatory filing. (It is always recommended that you seek input from the competent authorities to confirm your product-specific testing plan.)

	Materials Required (plates)		Time Required	
	rFC*	**MAT*†**	**rFC**	**MAT‡**
Initial qualification	2 x consecutive	2 x consecutive	1 day	3 – 4 days
Feasibility study (per condition)	1 x	1 – 2 x MAT 1 x ELISA	0.5 day	1 – 2 weeks
Product validation (3 lots)	1 x	5 – 6 x MAT 3 x ELISA	1 day	3 – 4 weeks
Entire process	**4 rFC kits**	**3 – 4 MAT kits**	**2 – 3 days**	**6 – 8 weeks**

*Each product dilution requires a positive product control (PPC). Each test in duplicate
†Each test in quadruplicate
‡Includes overnight culture of MAT cells with product

Table 1.
Summary comparison of the materials and time required to conduct initial qualification, feasibility studies, and product-specific validation with the rFC and MAT assays.

Smoothing the Path to Sustainability, Preparing QC Operations for the Future

The QC laboratory of the future will have reduced dependence on traditional pyrogen tests such as the RPT and LAL. Sustainable *in vitro* methods that are equivalent to traditional pyrogen tests are already well established, as well as being supported by regulations, a wealth of scientific studies, and extensive industry use.

Furthermore, implementing and validating these sustainable pyrogen tests is not difficult, time-consuming, or expensive — a common misconception among pharmaceutical QC laboratories.

For more detailed guidance on how to adopt the rFC or the MAT in your laboratory, contact Lonza Scientific Support, download our easy-to-follow rFC validation protocol, or check out our detailed MAT validation White Paper, "Ask the Expert: How to Swifty Adopt the MAT".

Alternatively, visit our pyrogen and endotoxin testing webpage today.

Resources:

Loverock B, Simon B, Burgenson A, Baines A. A recombinant factor C procedure for the detection of Gram-negative bacterial endotoxin. Pharmacop. Forum, 36, 321–329 (2010). https://www.uspnf.com/pharmacopeial-forum/pf-legacy-pdfs-archive-28-44

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European Directorate for the Quality of Medicines (EDQM). European Pharmacopeia general chapter 2.6.32: Test for bacterial endotoxins using recombinant factor C, Edition 11.

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Der E, Marin C, da Fonseca V.G., Silva L. Validation Strategy for New Recombinant Factor C Users. American Pharmaceutical Review (February 2022). https://www.americanpharmaceuticalreview.com/Featured-Articles/583996-Validation-Strategy-for-New-Recombinant-Factor-C-Users/

Burgenson, A L. Comparison of Four Endotoxin Detection Reagents in Measuring Autochthonous Endotoxin Levels in Four Representative Parenteral Products. Pharmacopeial Forum, 49, no. 2 (2023). DocID: GUID-39D7842E-76C1-4CF2-B2F6-A10B2857B74A_10101_en-US.

Product Validation Protocol for the Lonza PyroGene® rFC Endotoxin Detection Assay. https://bioscience.lonza.com/lonza_bs/GB/en/pyrogene-validation-request-form

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