World Pharmacopeias Are Ready to Adopt Non-animal In Vitro Replacement Tests for Detection of Pyrogens. Are You?

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Introduction

Compliance to regulatory requirements requires testing of parenteral preparations and medical devices for pyrogenic substances that may induce life threatening fever reactions in a patient. Globally harmonized tests to detect pyrogenicity include the animal-based rabbit pyrogen test (RPT) as well as the bacterial endotoxin test (BET) to detect the most potent pyrogen, bacterial endotoxin. BET is critically dependent on a natural resource, the blood amebocyte lysate from the Atlantic horseshoe crab, *Limulus polyphemus* (LAL), and from the Asian horseshoe crab, *Tachypleus tridentatus* (TAL). The world's increasing concerns with ethics of using experimental animals, and efforts to protect natural resources led regulatory agencies and pharmaceutical companies to acknowledge *in vitro* test systems minimizing such dependencies. In this article, we review suitable *in vitro* replacement tests for pyrogen testing that are acknowledged by world's pharmacopeia to ensure patient safety.

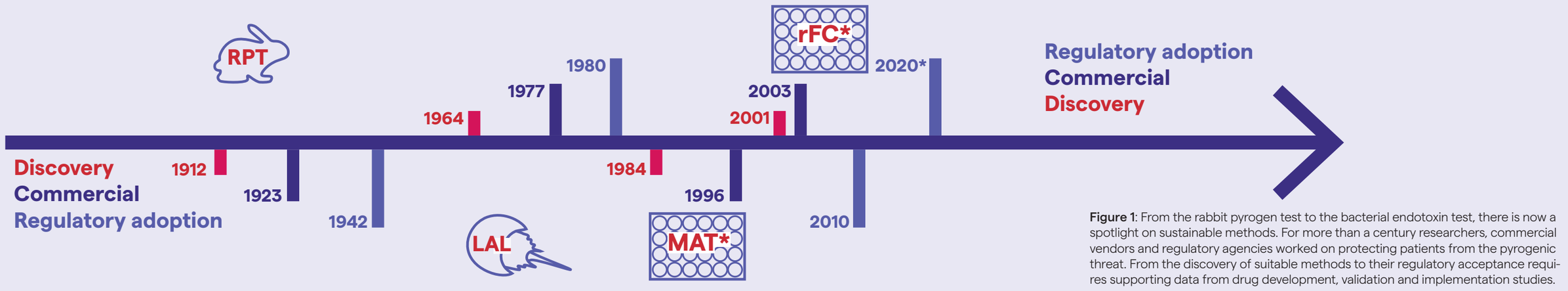


Figure 1: From the rabbit pyrogen test to the bacterial endotoxin test, there is now a spotlight on sustainable methods. For more than a century researchers, commercial vendors and regulatory agencies worked on protecting patients from the pyrogenic threat. From the discovery of suitable methods to their regulatory acceptance requires supporting data from drug development, validation and implementation studies.

The Journey to Increased Sustainability

From rabbits to amebocyte lysate tests and now recombinant methods

Early in the history of injections, patients often developed "injection fever" from unknown contaminants in the medicines. In 1926 Siebert developed a test using rabbits to detect deadly pyrogenic substances in injectable products. This assay, the RPT, became General Chapter <151> in the United States Pharmacopeia in 1941; other compendia soon followed. In 1956 Frederick Bang of the Woods Hole Institute discovered that it was the endotoxin derived from the outer membrane of gram-negative bacteria that caused the horseshoe crab's blood to clot. In 1964, Bang and hematologist Jack Levin determined the enzymatic cascade that results in the clot. This seminal discovery led to the Limulus or Tachypleus amebocyte lysate assay (LAL/TAL) as a potential test for the detection of endotoxin in injectable pharmaceuticals (BET). The BET proved to be more sensitive and accurate than the RPT assay, much less costly to perform, provide much faster time to results, and it removed live animals from use in testing. These factors alone made the LAL test superior in detecting endotoxins in parenteral preparations. The FDA licensed this technology in 1973 as a biologic, and it was added as Chapter <85> Bacterial Endotoxins Test in the USP in 1980. Since then the BET test principle has been continuously refined to adapt to ever changing requirements such as increasingly complex products, data integrity compliance, high-throughput and automation. Recently, a recombinant method – the recombinant Factor C (rFC) assay – added to the range of BET methods and was first adopted by the European Pharmacopeia in July 2020 (Test for bacterial endotoxins using recombinant factor C, General Chapter 2.6.32)[1]. Other world pharmacopeia such as the United States Pharmacopeia (Use of Recombinant Reagents in the Bacterial Endotoxins Test, <1085.1>) and the Japanese Pharmacopeia announced informational chapters for 2021 (Alternative method using recombinant protein, <G4-4-180>). The goal of the rFC assay is to remove the horseshoe crab as the source of a critical raw material that has protected the human population for over fifty years. Using recombinant technology coupled to the use of a fluorophore allows the rFC assay to be as sensitive as the LAL assay, without the remaining amplification steps. In addition, the rFC assay shows higher specificity than the natural resource since non-specific steps in the cascade, i.e. the factor G path, are removed. Finally, the increased use of recombinant technology may support recovery of the endangered Asian species of the Tachypleus genus[2].

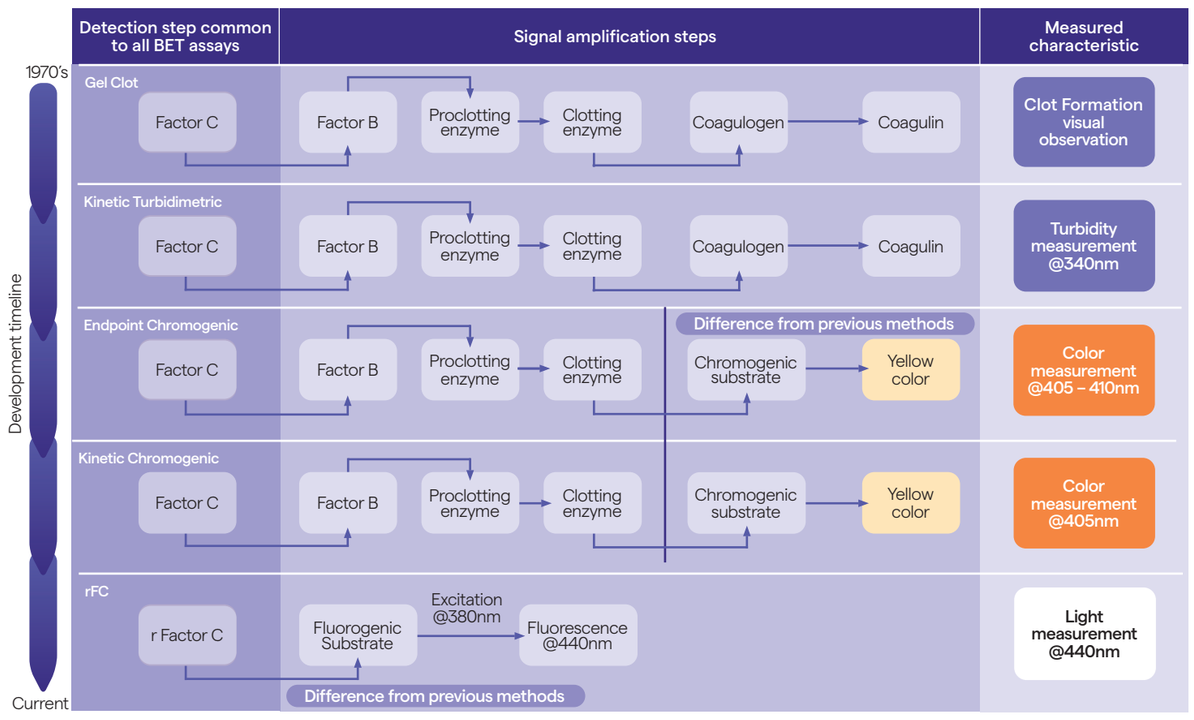


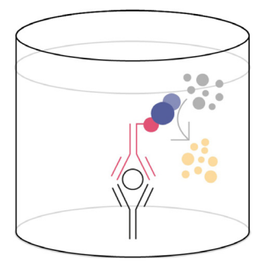
Figure 2: Development of the BET assay from 1970's until today.

From rabbits to the monocyte activation test

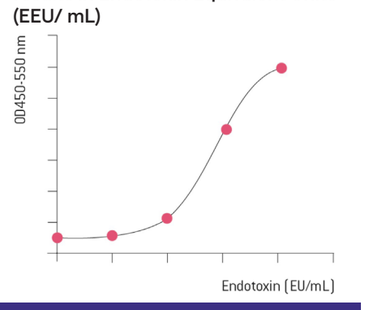
The RPT has remained fairly unchanged for nearly a century despite a number of disadvantages. Despite global commitments to the 3R principle the RPT remained a major contributor to the annual use of more than 400,000 experimental rabbits[3]. For small molecules, rabbits may be reused in the RPT after a brief time period. However, biologics consisting of large molecules may induce an immune response if the same product is tested. Here, a rabbit can only be used once to test such products, after which the animal is euthanized. Moreover, the RPT may not always elicit a response to humanized therapeutics, such as blood-based products, or substances developed with bioprocess technologies where Endotoxin alone, is not the sole pyrogenic threat.

Incubate PyroCell® pMAT Cells with a test substance for 18–24 hours in a cell culture step

Detect secreted cytokines in cell culture supernatants with an IL-6 ELISA



Calculate pyrogen content, express results in Endotoxin Equivalent Units (EEU/ mL)



The MAT Assay is capable to detect synergistic effects between "subfebrile" amounts of different contaminants

Figure 3: Principle of the MAT Test. Human monocytes are exposed to a test substance in a cell culture step. Secreted cytokines are measured and then analyzed against reference standard endotoxin.

How the monocyte activation test is supporting testing needs

Regulatory efforts to protect experimental rabbits resulted in recognition of the monocyte activation test (MAT, Fig. 3) as an *in vitro* replacement for the RPT in 2009 (European Pharmacopeia General Chapter 2.6.30, Monocyte Activation Test). Like the RPT, the MAT is capable of detecting bacterial endotoxin and non-endotoxin components that are pyrogenic to humans. Commercial MAT depend on ethically sourced, naïve primary human monocytes that release pro-inflammatory cytokines such as IL-6 upon stimulation with pyrogenic substances. The amount of released cytokines is proportional to the pyrogenic content in a drug substance. Though the MAT principle was established decades ago, its adoption for life-saving biologics, such as vaccines, bioprocessed proteins, or blood-based products, has accelerated only recently. Other advantages of the MAT include the simulation of a human response to a pyrogen (RPT: mammal response), introduction of experimental controls and cost savings. Therefore, MAT is closing the gap between patient safety and other regulatory requirements.

Identification		RPT	MAT
Pyrogen	Endotoxin	✓	✓
	Other bacteria	✓	✓
	Yeast/Funghi	✓	✓
	Virus, RNA, DNA	✓	✓
	Particles		✓
Limitation	Lipids	✓	✓
	Proteins (Bioprocess)		✓
	Blood therapeutics		✓
	Cellular therapeutics		✓
	Immunogenic biologics		✓
	Vaccines	✓	✓
Experimental controls			✓
Pyrogenicity		Mammal	Human
Experimental animals		yes	no

Figure 4: The MAT is better suited to detect pyrogens in biologics. Comparing RPT and MAT for their ability to test pyrogens as a purified substance and within a pharmaceutical preparation

Conclusion

As the need for safety testing of medical products is evident, the use of experimental animals as a human surrogate was established to predict how humans would react to the product. Over time, and as technology advanced, researchers developed more modern and sensitive assays, such as BET, then MAT and now rFC to predict any adverse events from injectable medicines on the market. Manufacturers of injectable medicines are now looking at alternatives to the older technologies that will remove animals from the testing lab and as raw material sources, all while maintaining the level of safety required to deliver these life-saving products. However, for new products in discovery phase, especially those manufactured in bioreactors, implementation of sustainable methods may not only carry significant advantages but also "future-proof" drug development for the long term commercial application.

References

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[3] Hartung, T. (2021) "Pyrogen testing revisited on occasion of the 25th anniversary of the whole blood monocyte activation test", ALTEX - Alternatives to animal experimentation, 38(1), pp. 3–19