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White Paper

Monocyte Activation Test (MAT)

The *in vitro* test for pyrogen detection

Pyrogens...a hot story

Adverse reactions to parenteral preparations have been described as early as the late 19th century, frequently termed **"injection fever"**. The first fever-causing agents, "pyrogens", were identified in 1912 by Hort and Penfold, who were also the first to design a pyrogen test based on injection of material into rabbits. At that time, the pyrogenic agent was identified as endotoxins included in preparations of Gram-negative bacteria. Interestingly, it was shown that live and dead microorganisms presented the same pyrogenic potential.

In the following years, it became more and more clear that **sterility is not necessarily equal to apyrogenicity**, which led to the **inclusion of a pyrogen test in the 12th edition of the United States Pharmacopoeia (USP)** in 1942.

Due to their stability, endotoxins can be very difficult to remove by classical bactericidal procedures such as heating or filtration. This made control of the whole production process necessary, especially for the water used, as this **water was frequently found as source of pyrogenic contaminations**.

The high number of pyrogen tests on rabbits and the variable sensitivity of that test system (e.g. by development of pyrogen tolerance in rabbits after repeated injections) made development of **alternative tests necessary**. The first and most successful of these new tests was the bacterial endotoxin test based on the lysate of amoebocytes from the blood of horseshoe crabs, which became commercially available in the 1970s and has been widely used as a replacement for the rabbit pyrogen test.

Today's qualified water systems no longer present such a high risk of endotoxin contamination, with more than 99% of our tests for various production sites showing contamination of much less than the specification of 0.25 EU/mL.

On the other hand, quality control for the presence of pyrogens is getting more and more complicated, as production processes (e.g. biotechnology and cell therapy products) bring new risks of **various contaminants (i.e. Non-Endotoxin Pyrogens)** entering the final product, like viruses from animal-based raw materials or Gram-positive bacteria from contaminations. Non-Endotoxin Pyrogens (NEPs) are **undetectable by the bacterial endotoxin test**, and there is therefore a risk of overlooking a NEP contamination.

In 2016, due to the increase in production of more and more complex products, **the general chapter for endotoxin testing in the European Pharmacopoeia (chapter 5.1.10)** introduced the necessity for an evaluation of the product, production process and raw materials with respect to the risk for pyrogens that are non-detectable by the bacterial endotoxin test.

In this context, the *in vitro* pyrogen test based on human cells offers a valuable alternative to the rabbit pyrogen test. **Since January 2010, the Monocyte Activation Test has been described as a compendial method for Pyrogen Detection in the European Pharmacopeia (chapter 2.6.30) and since the 2016 revision, recommendations have been given to replace tests on rabbits with the Monocyte Activation Test, wherever possible and after product specific validation (EP 2.6.8, Rev. July 2016).**

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Contents

1. Pyrogens, a broad range of contaminants threatening patient safety

1. What is a pyrogen?

A pyrogen is, by definition, a substance that produces a rise in temperature in a human or animal.

Pyrogens are differentiated into exogenous and endogenous pyrogens:

- Exogenous pyrogens are substances that induce fever reactions after parenteral administration;
- Endogenous pyrogens such as IL-1, IL-6, IL-12 or TNF-α are produced by the body itself as a reaction to contact with exogenous pyrogens.

The determination of the pyrogenic load of parenteral administered pharmaceutics is of great importance regarding patient safety and is regulated by several standards from organizations such as Food and Drug Administration (FDA), United States Pharmacopeia (USP) or European Pharmacopeia (EP).

Pyrogen contamination can occur during production or administration of pharmaceuticals, biotherapeutics and medical devices, but the presence of pyrogens can also be an inherent characteristic of the product:

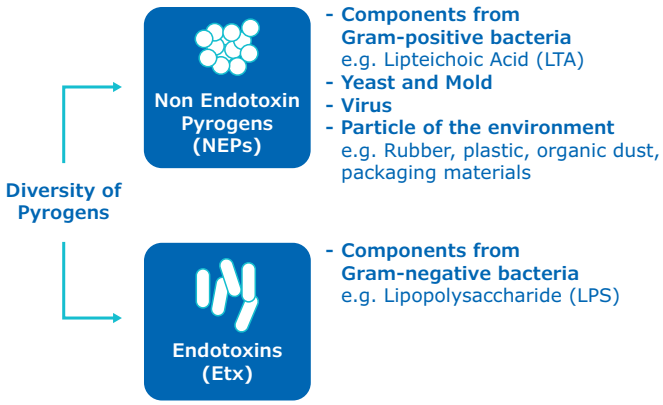
- Some adjuvants in vaccines
- Synthetic Lipopeptides

2. The broad range of pyrogens

A variety of exogenous pyrogens have been identified and characterized according to their origin[1]:

- **Endotoxins** from Gram-negative bacteria, in particular lipopolysaccharides (LPS) from bacterial cell wall, which are highly resistant against heat
- Components of Gram-positive bacteria such as **peptidoglycan, lipoteichoic acids and bacterial lipoproteins**[2]
- Viral pyrogens, in particular **virion components** from myxoviruses such as influenza
- Pyrogens from yeast and fungi[3] like **capsular polysaccharide**
- Pyrogens from non-biological sources such as **rubber particles, microscopic plastic particles or metal compounds in elastomers.**

Pyrogens can be classified into two groups: **Endotoxins** and **Non-Endotoxin Pyrogens (NEPs)**:



3. Mode of action: activation of the human immune system through TLRs

Pyrogens trigger fever through the activation of the innate immune system

Monocytes are white blood cells involved in innate immunity. They recognize antigens thanks to cell-surface receptors called Pattern Recognition Receptors (PRRs) which activate an immune response through production of endogenous pyrogens such as cytokines.

Cytokines have a direct effect on temperature regulation in the hypothalamus.

TLRs: the monocyte PRRs that recognize pyrogens

PRRs recognize highly conserved structural motifs known as PAMPs (Pathogen Associated Microbial Patterns) which are expressed by microbial pathogens, or DAMPs (Danger Associated Molecular Patterns) which are endogenous molecules released from necrotic or dying cells. Recognition of microbial pathogens by PRRs is an essential step for initiation of the innate immune response such as inflammation.

Pyrogens are recognized by a specific type of PRR called Toll-Like Receptors (TLRs) expressed by the monocytes. Toll-like receptors were the first PRRs identified.[4,5]

TLR Signaling Pathways

Stimulation of TLRs by the corresponding PAMPs or DAMPs initiates signaling cascades that trigger specific immunological responses.[6]

Most commonly, MyD88 (myeloid differentiation primary-response protein 88) is a universal adapter protein used by most of the TLRs as one of the first proteins in the reaction cascade which, at the end, leads to the activation of the transcription factor NF-κB. Between MyD88 and NF-κB, there are several phosphorylation steps and ubiquitylation steps, which leads to dissociation of previous complexes and formation of new reaction complexes. As a last step, NF-κB dissociates from a cytoplasmic complex and translocates to the nucleus where the corresponding target genes are expressed (**Figure 1**).

TLRs and their specific ligands

Bacterial cell wall components are broadly recognized by cell surface TLRs, whereas nucleic acids are recognized by intracellular TLRs.

The diversity of the TLR family and the specificity of individual TLRs for the detection of different ligands support the hypothesis that the human fever reaction can be provoked not only by LPS, but also by many other substances originating from Gram-negative and Gram-positive bacteria, fungi, yeast, viruses, and parasites.[7]

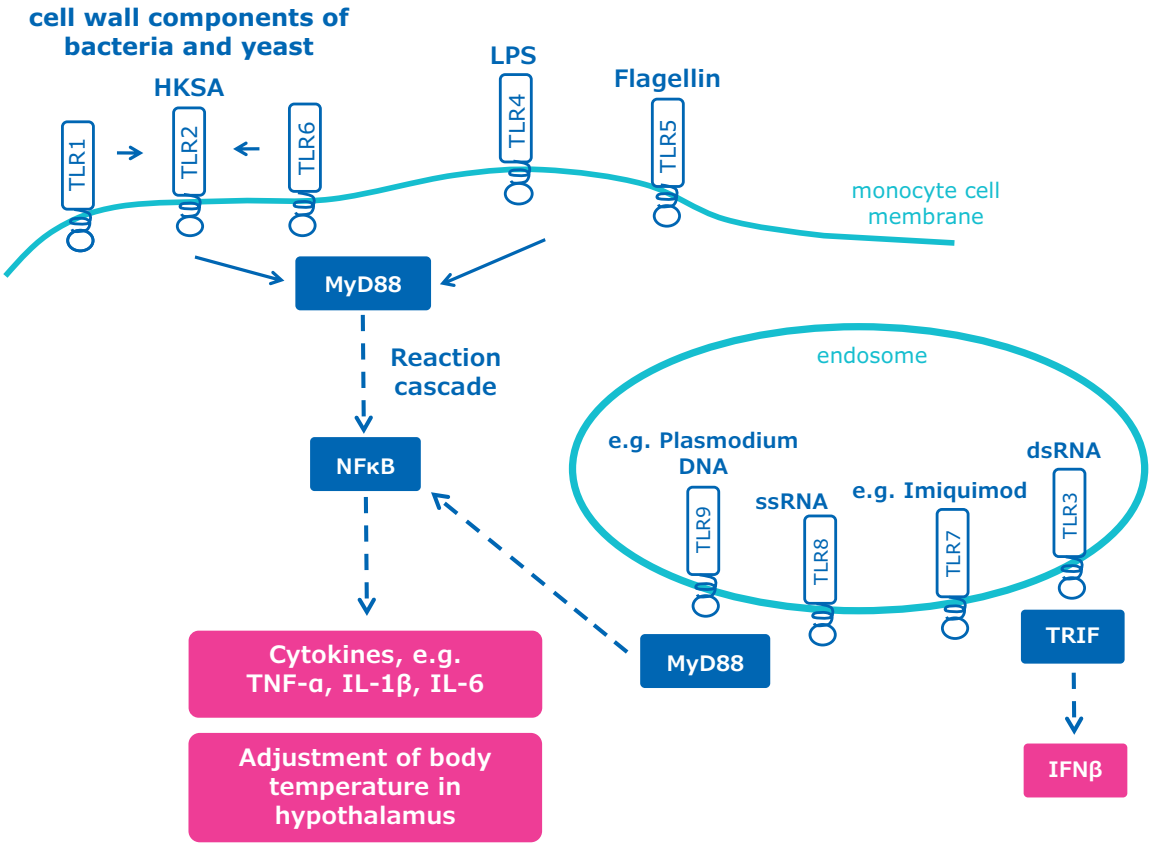


Figure 1. TLR signaling pathways

Receptor	Ligand	Origin of Ligand	References
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria	8
	Soluble factors	*Neisseria meningitidis*	9
TLR2	Lipoprotein/lipopeptides	Various pathogens	10
	Peptidoglycan	Gram-positive bacteria	11,12
	Lipoteichoic acid	Gram-positive bacteria	13
	Lipoarabinomannan	Mycobacteria	14
	Phenol-soluble modulin	*Staphylococcus epidermidis*	15
	Glyco-inositol-phospholipids	*Trypanosoma cruzi*	16
	Glycolipids	*Treponema maltophilum*	17
	Porins	*Neisseria*	18
	Atypical lipopolysaccharide	*Leptospira interrogans*	19
	Atypical lipopolysaccharide	*Porphyromonas gingivalis*	20
	Zymosan	Fungi	21
	Heat-shock protein 70*	Host	22
TLR3	Double-stranded RNA	Viruses	23
TLR4	Lipopolysaccharide	Gram-negative bacteria	24
	Taxol	Plants	25
	Fusion protein	Respiratory syncytial virus	26
	Envelope protein	Mouse mammary-tumour virus	27
	Heat-shock protein 60*	*Chlamydia pneumoniae*	28, 29
	Heat-shock protein 70*	Host	30
	Type III repeat extra domain A of fibronectin*	Host	31
	Oligosaccharides of hyaluronic acid*	Host	32
	Polysaccharide fragments of heparan sulphate*	Host	33
	Fibrinogen*	Host	34
TLR5	Flagellin	Bacteria	35
TLR6	Diacyl lipopeptides	*Mycoplasma*	36
	Lipoteichoic acid	Gram-positive bacteria	37
	Zymosan	Fungi	38
TLR7	Imidazoquinoline	Synthetic compounds	39
	Loxoribine	Synthetic compounds	40
	Bropirimine	Synthetic compounds	41
	Single-stranded RNA	Viruses	42, 43
TLR8	Imidazoquinoline	Synthetic compounds	44
	Single-stranded RNA	Viruses	45
TLR9	CpG-containing DNA	Bacteria and viruses	46
TLR10	N.D.	N.D.	–
TLR11	N.D.	Uropathogenic bacteria	47
TLR1/TLR2 heterodimer	Triacylated lipoproteins	–	48
TLR2/TLR6 heterodimer	Diacylated lipoproteins	–	49

Table 1. Toll-like receptors and their ligands. *It is possible that these ligand preparations, particularly those of endogenous origin, were contaminated with lipopolysaccharide and/or other potent microbial components, so more-precise analysis is required to conclude that TLRs recognize these endogenous ligands. N.D., not determined; TLR, Toll-like receptor.

4. Pyrogen detection in pharmaceuticals, a requirement to ensure patient safety

Why conduct a pyrogen test?

Drugs that are purported to be sterile must also be free from pyrogens to prevent patients from febrile reactions. (e.g. European GMP – Annex 1; FDA Guidance for industry – Sterile Drug Product produced by aseptic Processing – Current Good Manufacturing Practice).

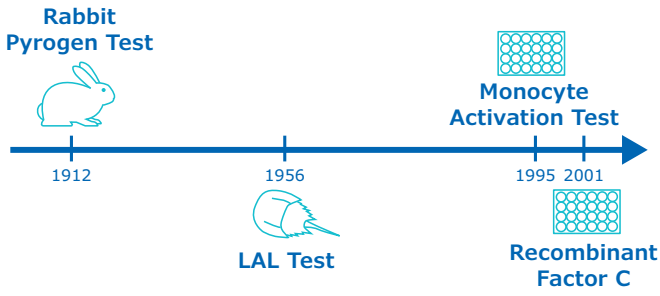
Parenteral preparations must be “pyrogen-free” because administration of pyrogens may lead to life-threatening fever in some patients.

The severity of the adverse reaction depends on the concentration and biological activity of the respective pyrogen. It is therefore necessary to test these products for the full range of pyrogens to ensure patient safety.

A sterile product does not mean “pyrogen-free” product. The Pyrogen Test is designed to limit the risks of febrile reaction to an acceptable level in the patient from the administration of a parenteral drug.

2. Methods for pyrogen & endotoxin detection

1. The rising need for pyrogen testing



With the development of injectable pharmaceutical solutions in the early 1900s, a problem called “injection fever” arose. The link with presence of microorganisms was assumed, and the first rabbit pyrogen test was developed in 1912.[50] However, its relevancy was largely overlooked until the publication of the research work of Florence Seibert.[51,52]

Additional studies during the next 2 decades finally led to the development of **the first official rabbit pyrogen test (RPT), incorporated into the USP in 1942** due to the increasing need for pyrogen-free injection solutions during world war II and several incidences with injectable solutions.

The next step in endotoxin detection was the discovery by Fred Bang in 1956 that the blood of the horseshoe crab coagulates to a gel when exposed to Gram-negative bacteria or their lysates.[53] Further studies together with the hematologist Jack Levin led to the basis of the Limulus Amebocyte Lysate (LAL) test using extracts of amebocytes from limulus blood to test for endotoxin by clotting technique.[54] **The very specific and sensitive reaction of LAL**, as well as the ease of use in comparison to RPT led to a **fast development and standardization of the test and finally its acceptance into USP**, despite knowing about its weakness to only detect endotoxins.[55]

Due to the **inability of the LAL test to detect non-endotoxin pyrogens or potentiating effects of additional contaminants like peptidoglycan**, the rabbit pyrogen test remained the standard pyrogen detection method for many decades, regardless of its intense animal consumption, low sensitivity compared to LAL test, and qualitative nature only allowing a pass/fail interpretation.

This started to change after the monocyte activation test was developed.[56,57,58,59] **Using the production of cytokines from monocytes to mimic the human reaction to pyrogens,** this ***in vitro*** **method** was soon recognized as **an alternative to the rabbit pyrogen test and included into EP as a compendial method (2010) and USP as an alternative method (2012).**[60,61]

2. Methods available for pyrogen & endotoxin detection

There are four methods that can currently be described for pyrogen and endotoxin detection. They are differentiated by:

- Their target: either pyrogens (i.e. endotoxins and non-endotoxin pyrogens) or endotoxins only
- The use or not of animals.

Endotoxin tests can detect contamination of Gram-negative bacteria, but when performing an endotoxin test, the pyrogenic activity of a preparation in humans may be underestimated due to non-endotoxin contaminants. Therefore, endotoxin tests may mostly be used for raw materials, production water and in-process testing.

On the other hand, pyrogen tests detect the whole range of pyrogens (including both endotoxins and NEPs). They are designed to predict the pyrogen activity of a preparation in human and are therefore used as quality control for final products.

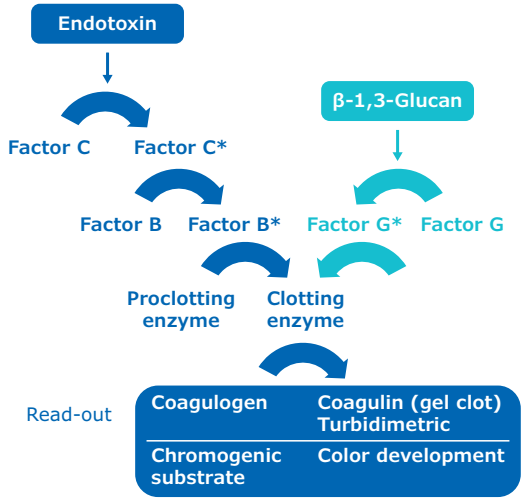
	Test Type	Animal based?
Endotoxins	**Bacterial Endotoxin Tests (BET) or Limulus Amebocyte Lysate (LAL)** EP 2.6.14, USP 85 **Principle:** use of immune response of the horseshoe crab against invasion of Gram negative bacteria	Yes
Endotoxins	**Recombinant Factor C (rFC)** In July 2016 in the EP, FDA Q&A June 2012 **Principle:** based on a rFC, genetically engineered protein, which is activated by endotoxin to produce a fluorescent end product which is quantifiable.	No
Pyrogens	**Pyrogen Test (Rabbit Pyrogen Test: RPT)** EP 2.6.8, USP151 **Principle:** rectal measurement of the body temperature after injection of the product	Yes
Pyrogens	**Monocyte-activation Tests (MAT)** EP 2.6.30, FDA Q&A June 2012 **Principle:** Monocytes activated by pyrogens produce cytokines/interleukins (IL) that are detected in an immunological assay (ELISA)	No

Endotoxin detection methods:

• The Bacterial Endotoxin Test (BET) or Limulus Amoebocyte Lysate (LAL) Test

Principle: The Bacterial Endotoxin Test (BET), also called the Limulus Amebocyte Lysate (LAL) test, refers to a number of methodologies that detect endotoxins from Gram-negative bacteria based on the clotting reaction of hemolymph in the horseshoe crab.

There are three basic methodologies for the LAL test: gel-clot, turbidimetric, and chromogenic.



Advantages	Disadvantages
- Simple and easy to perform - High sensitivity - Cost-effective	- Endotoxin detection only: failure to detect non-endotoxin pyrogens - Susceptibility to interference depending on conditions: pH, ionic strength, enzyme activity, endotoxin masking / low endotoxin recovery (LER) - The LAL test cannot be used to test some products such as blood products, cellular products, proteins, lipids, aluminium hydroxide adjuvants (common in vaccines), glucans (false positives) - Animal consumption: the mortality rate of animals used to produce LAL is estimated to be about 15%, as they are released back into the wild after a draw of 20% of circulating (aristocratic) blood: threat to the horseshoe crab population.

• The Recombinant Factor C (rFC)

Principle: based on recombinant Factor C: a genetically engineered protein which is activated by endotoxin to produce a fluorescent end product which is quantifiable.

Advantages	Disadvantages
- Same advantages as LAL test - *In vitro* assay not based on animal consumption	- Same disavantages as LAL test except for glucans

Pyrogen detection methods:

- **The Rabbit Pyrogen Test (RPT): the *in vivo* assay for pyrogen detection:**

Principle: The rabbit pyrogen test is designed to limit the risks of febrile reaction to an acceptable level in the patient after the administration by injection of the product concerned. The test involves measuring the rise in temperature of 3 rabbits following the intravenous injection of a test solution, and is designed for products that can be tolerated by the test rabbit at a dose that does not exceed 10 mL per kg injected intravenously within a period of no more than 10 minutes.

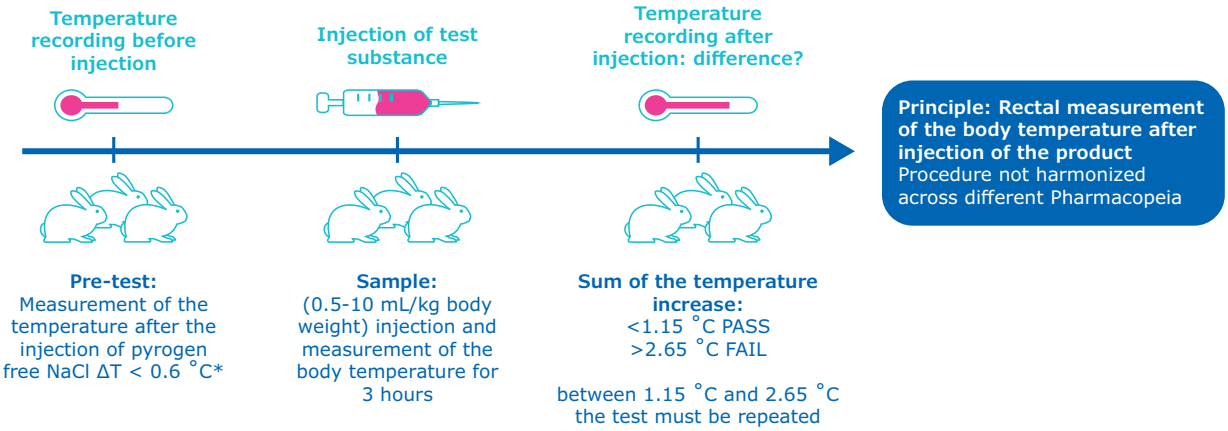
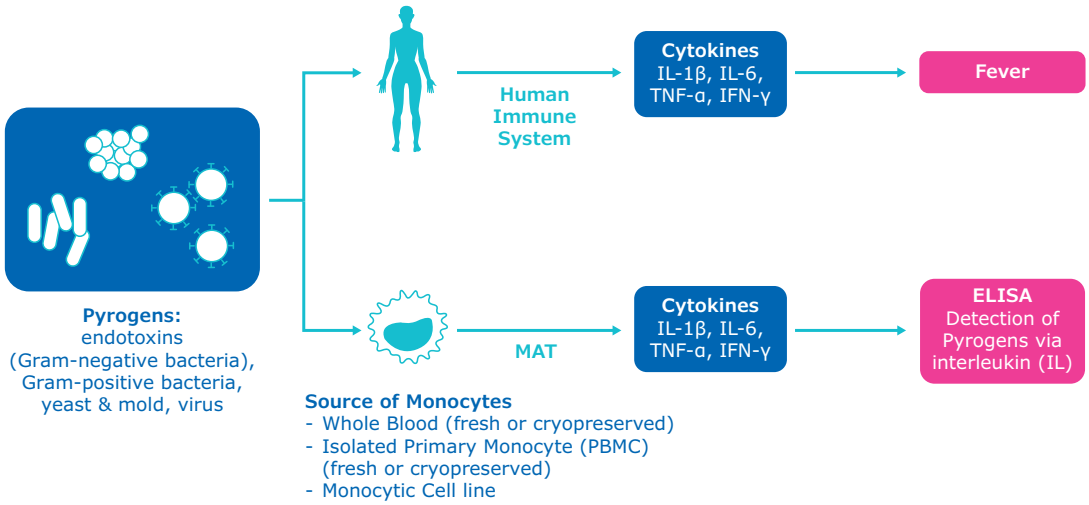


Figure 2: Example of procedure of RPT according to EP

Advantages	Disadvantages
- Specificity: The RPT can detect both endotoxin and non-endotoxin pyrogens (NEPs) - Historical method for pyrogen testing in international regulations and guidelines	- Low sensitivity (0.5 EU/mL) compared to other methods - Rabbit blood is highly responsive to LPS but less responsive to Gram-positive pyrogens compared to human monocytes. - The assay is not quantitative - Lack of a positive control - Robustness: Pyrogen test limited by physiological reaction of animals: stress on the rabbit may influence results - The RPT cannot be used to test many types of pharmaceutical products, ranging from chemotherapeutics to immunosuppressive agents, and cannot be used to test human cellular preparations, such as blood components and stem cells. - Animal consumption: need for large numbers of animals to identify rare pyrogen-containing samples

- **The Monocyte Activation Test (MAT): the Humane Alternative to Pyrogen Detection**

Principle: Monocytes activated by pyrogens produce cytokines/interleukins (IL) that are detected in an immunological assay (ELISA).



Advantages	Disadvantages
- Based on the human reaction to pyrogens, it provides a better prediction of pyrogenic activity of preparations than LAL or the RPT. - Unlike the the LAL, it can detect endotoxin and non-endotoxin pyrogens and is applicable to a greater variety of products than LAL or the RPT.[62] - The method can easily be carried out in-house (no need for animals) - It has a lower limit of detection and is more accurate than the RPT. - In consideration of animal welfare, unlike the LAL or RPT, no animals are harmed.	- Lower sensitivity than LAL tests - Longer time to result than LAL

There are different variants of the MAT available depending on:

- The source of human monocytes: whole blood, isolated primary monocytes (e.g. PBMC) or monocytic cell line.
- The ELISA read-out: IL-6, IL-1β or TNF-α.

All of them mimic the human fever reaction *in vitro*.

Source of human monocytes	Whole blood cryopreserved	Peripheral Blood Mononuclear Cells (PBMC)	MonoMac 6 Cell Line
LOD	0.25 EU/mL	Around 0.01 EU/mL	0.05 EU/mL
Advantages	- Physiological reaction: closest to the human reaction: monocytes are kept in their natural environment - Commercial kit available (PyroDetect System, Merck).	- Sensitivity	- Sensitivity - Not donor dependent - Robust - No blood derived products: standardized reaction. - MonoMac 6 (MM6) cell line cited in the **international evaluation report of MAT alternative method** for pyrogen testing* - Commercial kit of qualified MM6 cells under development by Merck
Disadvantages	- Blood derived product - Biological variability (reactivity from one lot to another) - Supply depends on blood donation	- Supply availability: complex production process. - Donor dependent - Blood derived product - No commercial kit available on the market	- Monocytes are not in their natural environment

*Source: Interagency Coordinating Committee on the Validation of Alternative Methods - 2008

Test comparison

Both RPT and LAL tests are animal-based methods. LAL cannot adequately detect the full spectrum of pyrogens. Moreover, such tests cannot be used on several pharmaceutical products or for the testing of solid materials such as medical devices.

	Rabbit Pyrogen Test	Endotoxin Test	Monocyte Activation Test
Products which cannot be analyzed*	- Blood products - Cellular products - Proteins - Sedatives - Analgesics - Cytokines - Antibiotics - Chemotherapeutics	- Blood products - Cellular products - Proteins - Lipids - Aluminum hydroxide adjuvants (common in vaccines)	+/- cytotoxic drugs Other: If the product tested interferes with the detection system, the possibility of detecting pyrogens will depend on the method sensitivity
Controls	No	Yes	Yes
Animal consumption	++	+	No
Detection of	Pyrogens	Endotoxins	Pyrogens

MAT, a new lead to overcome Low Endotoxin Recovery (LER)?

LER is a phenomenon that can occur when performing LAL tests on protein formulations containing buffers like citrate or phosphate and surfactants like polysorbates. These components may cause a decreased binding of endotoxins to the component responsible for enzymatic cascade used for LAL test, leading to a complete non detectability of LPS.

LER is a main drawback of the LAL (Limulus Amebocyte Lysate) test as it can lead to false negative results, although the extent to which masking occurs in the human body remains uncertain.

MAT might be a way to overcome uncertainty of testing LER formulations, as it is a method that mimics the human reaction to pyrogens.

When LER is observed or suspected, it could be an option to perform pyrogen detection using MAT.[63]

3. The need for standards used in pyrogen tests

Pyrogen detection can be performed using a range of different methods. The use of standards as positive controls enables confirmation of the effectiveness of the method in the detection of endotoxins and NEPs.

Endotoxin standards:

There are two different types of endotoxin standards:

1. **International standard: Reference Standard Endotoxin (RSE):** RSE standards can be used without any adjustments. By definition, 1 EU (Endotoxin Unit) is equivalent to 100 pg of each of these standards.
2. **Manufacturer standard: Control Standard Endotoxin (CSE):** CSE standards in contrast are adjusted to specific lots of LAL (Limulus Amebocyte Lysate) tests. The suppliers need to reference these standards to an RSE.

For Monocyte Activation Test, the RSE are used.

Non-Endotoxin Pyrogens:

Only recently, the **relevance of non-endotoxin pyrogens** (e.g. lipoteichoic acid (LTA), bacterial DNA (CpG-motifs), peptidoglycan, synthetic TLR-agonists, or endogenous pyrogens) has gained more attention, mainly as a cause of human adverse reactions (e.g. pain at the injection site, redness, shivering, and fever).

A case study concerning this matter was reported by a major pharmaceutical company[64]. The incriminated batches of a life-saving drug which had induced some complaints had passed the BET and the RPT without detectable response. There was no difference between batches that provoked adverse reactions and the “clean” batches. It became more and more clear that a so far unknown NEP contamination was disturbing human health. After introduction of the MAT as test method in accordance with FDA for batch release and the adoption of several optimization steps, reporting of adverse reaction significantly decreased.[64]

The need for Non-Endotoxin Pyrogen (NEP) standards has been raised as pyrogen tests are not limited to only endotoxin detection.[65] Yet, due to the broad range of pyrogens, and their specificity for different TLRs, there are currently no NEP standards available. However, several NEPs can be used as positive control, as long as they are endotoxin free.

3. Regulatory landscape of the Monocyte Activation Test

As the control of pyrogens is mandatory in pharmaceutical products, worldwide Pharmacopoeias describe the main methods enabling the detection and/or quantification of pyrogens.

Before the discovery and validation of the Monocyte Activation Test as an alternative to the Rabbit Pyrogen Test, the only available *ex vivo* testing method was the Bacterial Endotoxin Test, but with the limitation of being unable to detect all pyrogens. In case of any doubt of the presence of non-endotoxin contaminants, the laboratory was required to use the rabbit test.

During the last 30 years, the willingness to consider the animal pain and suffering has increased significantly and consequently the pressure to reduce animal testing has also increased.

The publication of "The Principles of Humane Experimental Technique" by W.M.S. Russel and R.L. Burch in 1959 marks the birth of the principle of the "Three Rs" (Replacement, Reduction and Refinement).

The trends in regulations due to animal testing concerns are in favor of *in vitro* methods such as MAT.

In terms of ethics, this concept has influenced regulations in many countries:

- In the USA, the Animal Welfare Act was enacted in 1966 and the FDA has been promoting initiatives to reduce animal testing (e.g. "Advancing regulatory science for public health", Oct. 2010).

- In Japan, animal experimentation is also regulated by laws, but is more based on a self-regulation system due to the combination of Buddhist and Christian assumptions.

- In Europe, the "Three Rs" have been present in EU legislation in spirit since 1986 when the first EU legislation for the protection of animals used for experimentation and other scientific purposes was adopted. Then, the Directive 2010/63/EU, described the principle of the Three Rs for the first time and made it a firm legal requirement. According to this law, if an *in vivo* test can be replaced by a validated *in vitro* test, it is an obligation to change to an *in vitro* test.

The same year, in 2010, the MAT chapter was introduced into the European Pharmacopeia as an alternative to the Rabbit Pyrogen test. Consequently, a new chapter will be adopted officially in January 2018 in EP, and is entitled “Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines.” (Chapter 5.2.14). Two other chapters related to vaccine testing (2.6.13 and 5.2.4) are being revised in order to remove or significantly reduce animal testing.

The use of MAT instead of the RPT is therefore an interesting alternative to limit the use of animal testing from an ethical and regulatory perspective. Moreover, the MAT has a lower limit of detection (LOD) (i.e. higher sensitivity) and is more accurate than the RPT, providing robust results for pyrogen testing.

1. MAT International validation

The MAT method was qualified and validated by the European Center for the Validation of Alternative Methods (ECVAM) in 2005 and by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in 2008:

- **International validation of novel pyrogen tests based on human monocytoid cells**, Journal of Immunological Methods 298, Hoffmann et al 2005,
- **International validation of pyrogen tests based on cryopreserved human primary blood cells,** Journal of Immunological Methods 316, Schindler et al. 2006,
- ICCVAM Background Review Document: **Validation Status of Five In vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and other Products**, National Institute of Environmental Health Services (NIH), May 2008.

2. Guidelines for pyrogen detection in pharmaceutical products

- USA:
  - FDA "Guidance For Industry – Pyrogen and Endotoxins testing: Questions and Answers" 2012: the possible use of Monocyte Activation Test is mentioned as an alternative to the rabbit test but should be validated according to USP <1225>;
  - USP <151> Pyrogen Test mentions that "A validated, equivalent in vitro pyrogen or bacterial endotoxin test may be used in place of the in vivo rabbit pyrogen test, where appropriate".
- Europe: the MAT was incorporated in the EP in 2010:
  - EP 2.6.8 pyrogens: recommendations to replace Rabbit Pyrogen Test by MAT (2.6.30) wherever possible (EP 2.6.8, July 2016);
  - EP 5.1.10 Guidelines for using the test for bacterial endotoxins specifies: "The Monocyte activation test (2.6.30) is a suitable method to use to rule out the presence of non-endotoxin pyrogens in substances or products" (EP 5.1.10, January 2017);
  - EP 2.6.30 Monocyte Activation Test: in the guidance notes, it is mentioned: "The monocyte activation test (MAT) is primarily intended to be used as a replacement for the rabbit pyrogen test." This chapter has been revised in 2017 to include the need to use Non-Endotoxin Pyrogens (NEPs) as positive control.

MAT has been incorporated as a compendial method for pyrogen detection in the European Pharmacopeia since 2010

- India:
  - The 8th edition of the Indian Pharmacopeia should include a new chapter on Monocyte Activation test by 2018.
- Japan:
  - In the general notice of JP XVII edition, the validation of alternative methods is possible only if the alternative method gives better accuracy & precision (General Notice 14).

3. Guidelines for pyrogen detection in medical devices

- Revision of ISO/DTR 21852 Pyrogenicity "Principle and method for pyrogen testing of medical devices". The MAT is mentioned as a pyrogen test.
- ISO 10993-1 "Biological evaluation of medical devices – part 11: test for systemic pyrogenicity " Only the Rabbit Pyrogen Test is recommended because alternative tests were not validated – Published in 2006.

4. Overview of Pharmacopeias

Pharmacopeias	Pyrogen test	Bacterial Endotoxin test (BET)	Monocyte Activation Test (MAT)
Ph. Eur. (Europe)	EP. 2.6.8 Rev. 2016	EP 2.6.14 Rev. 2016	Compendial method EP 2.6.30 Rev 2017
USP (USA)	USP <151> Rev. 2014	USP <85> Rev. 2014	Alternative method
JP (Japan)	JP 4.04	JP 4.01	N/A
IP (India)	IP 2.28	IP 2.23	New MAT Chapter Due out in 2018 (Alternative)
CHP (China)	Vol 1 General Principles 1142 Rev 2015	Chapter (not yet translated) Rev 2015	N/A

4. Key takeaways: why should MAT be increasingly used?

- **MAT allows detection of a broad range of pyrogens**

It has been shown that human fever is provoked by all types of pyrogens. Patient safety is ensured if the full range of pyrogens is tested to ensure detection of NEPs. Like the RPT, MAT is effective for detection of both endotoxins and NEPs.

- **MAT allows testing of a wide range of product types**

The most frequently applied methods, RPT and BET, are both limited by the types of products that can be tested. The MAT offers more flexibility regarding its applications.

- **MAT is an *in vitro* method**

Unlike RPT (*in vivo* method) and LAL (*ex vivo* method), the MAT is not animal based. It therefore gives the best predictive model as it mimics the human immune reaction. In addition, it helps to reduce animal consumption.

- **MAT is supported by regulations and guidelines**

MAT is described in the international regulations and guidelines. It is in line with ethical trends of industry and regulatory authorities to decrease the use of animal based testing.

- **MAT is a robust and sensitive method**

Glossary

- BET: Bacterial Endotoxin Test
- CHP: Chinese Pharmacopeia
- CLC: Contaminant Limit Concentration
- CSE: Control Standard Endotoxin
- DAMPs: Danger Associated Molecular Patterns
- ELISA: Enzyme-Linked Immunosorbent Assay
- EP: European Pharmacopeia
- EU/mL: Endotoxin Unit per milliliter
- FDA: U.S. Food & Drug Administration
- IL: Interleukin
- IP: Indian Pharmacopeia
- JP: Japanese Pharmacopeia
- LAL: Limulus Amebocyte Lysate
- LER: low Endotoxin Recovery
- LOD: limit of detection
- LPS: lipopolysaccharide
- LTA: Lipoteichoic Acid
- MAT: Monocyte Activation Test
- MM6: MonoMac6
- MyDD8: Myeloid Differentiation primary response protein 88
- N/A: Not Applicable
- NEPs: Non-Endotoxin Pyrogens
- PAMPs: Pathogen Associated Microbial Patterns
- PBMC: Peripheral Blood Mononuclear Cell
- PRRs: Pattern Recognition Receptors
- rFC: recombinant Factor C
- RPT: Rabbit Pyrogen Test
- RSE: Reference Standard Endotoxin
- TLRs: Toll-Like Receptors
- USP: United States Pharmacopeia
- USP: United States Pharmacopeia

References

1. Milton AS. Pyretics and Antipyretics. 1982. http://www.springer.com/us/book/9783642685712
2. Rockel C, Hartung T. Systematic review of membrane components of Gram-positive bacteria responsible as pyrogens for inducing human monocyte/macrophage cytokine release. Frontiers in Pharmacology (2012);3: 56.
3. Braude AL et al. -Fever from pathogenic fungi. J. clin. Invest., (1960) 39: 1266 – 1276.
4. Akira, S.et al. 2006. Pathogen recognition and innate immunity. Cell 124, 783-801.
5. Beutler, B:A. (2009). TLRs and innate immunity. Blood 113, 1399-1407.
6. Kawai and Akira 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34, 637-650.
7. Henderson, B. and Wilson, M. 1996. Cytokine induction by bacteria: beyond lipopolysaccharide. Cytokine 8, 269-282.
8. Takeuchi, O. et al. Cutting edge: Role of Toll-like receptor 1in mediating immune response to microbial lipoproteins. J. Immunol. 169, 10–14 (2002).
9. Wyllie, D. H. et al. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. J. Immunol. 165, 7125–7132 (2002).
10. Aliprantis, A. O. et al. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor 2. Science 285, 736–739 (1999).
11. Takeuchi, O. et al. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive cell wall components. Immunity 11, 443–451 (1999).
12. Schwadner, R. et al. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. J. Biol. Chem. 274, 17406–17409 (1999).
13. Schwadner, R. et al. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. J. Biol. Chem. 274, 17406–17409 (1999).
14. Means, T. K. et al. Human Toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. J. Immunol. 163, 3920–3977 (1999).
15. Hajjar, A. M. et al. Cutting Edge: Functional interactions between Toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J. Immunol. 166, 15–19 (2001).

16. Coelho, P. S. et al. Glycosylphosphatidylinositol-anchored mucin-like glycoproteins isolated from Trypanosoma cruzi trypomastigotes induce in vivo leukocyte recruitment dependent on MCP-1 production by IFN-γ-primedmacrophages. J. Leukoc. Biol. 71, 837–844 (2002).
17. Opitz, B. et al. Toll-like receptor-2 mediates Treponema glycolipid and lipoteichoic acid-induced NF-κB translocation. J. Biol. Chem. 276, 22041–22047 (2001).
18. Massari, P. et al. Cutting edge: Immune stimulation by Neisserial porins is Toll-like receptor 2 and MyD88 dependent. J. Immunol. 168, 1533–1537 (2002).
19. Werts, C. et al. Leptospiral liopolysaccharide activates cells through a TLR2-dependent mechanism. Nature Immunol. 2, 346–352 (2001).
20. Hirschfeld, M. et al. Signaling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect. Immun. 69, 1477–1482 (2001).
21. Underhill D. M. et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 401, 811–815 (1999).
22. Asea, A. et al. Novel signal transduction pathway utilized by extracellular HSP70: role of Toll-like receptor (TLR) 2 and TLR4. J. Biol. Chem. 277, 15028–15034 (2002).
23. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 413, 732–738 (2001).
24. Poltorak, A. et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282, 2085–2088 (1998). The first report that TLR4 is involved in the recognition of bacterial components.
25. Kawasaki, K. et al. Mouse Toll-like receptor 4–MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by taxol. J. Biol. Chem. 275, 2251–2254 (2000).
26. Kurt-Jones, E. A. et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nature Immunol. 1, 398–401 (2000).
27. Rassa, J. C. et al. Murine retroviruses activate B cells via interaction with Toll-like receptor 4. Proc. Natl Acad. Sci. USA 99, 2281–2286 (2002).
28. Bulut, Y. et al. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. J. Immunol. 168, 1435–1440 (2002).
29. Ohashi, K., Burkart, V., Flohe, S. & Kolb, H. Cutting edge: Heat shock protein 60 is a putative endogenous ligand of the Toll-like receptor-4 complex. J. Immunol. 164, 558–561 (2000).
30. Vabulas, R. M. et al. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J. Biol. Chem. 277, 15107–15112 (2002).
31. Okamura, Y. et al. The extra domain A of fibronectin activates Toll-like receptor 4. J. Biol. Chem. 276, 10229–10233 (2001).
32. Termeer, C. et al. Oligosaccharides of hyaluronan activate dendritic cells via Toll-like receptor 4. J. Exp. Med. 195, 99–111 (2002).
33. Johnson, G. B., Brunn, G. J., Kodaira, Y. & Platt, J. L. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. J. Immunol. 168, 5233–5239 (2002).
34. Smiley, S. T., King, J. A. & Hancock, W. W. Fibrinogen stimulates macrophage chemokine secretion through Toll-like receptor 4. J. Immunol. 167, 2887–2894 (2001).
35. Hayashi, F. et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor-5. Nature 410, 1099–1103 (2001).
36. Takeuchi, O. et al. Discrimination of bacterial lipopeptides by Toll-like receptor 6. Int. Immunol. 13, 933–940 (2001).
37. Schwadner, R. et al. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. J. Biol. Chem. 274, 17406–17409 (1999).
38. Ozinsky, A. et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proc. Natl Acad. Sci. USA 97, 13766–13771 (2000).
39. Hemmi, H. et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nature Immunol. 3, 196–200 (2002).
40. Heil, F. et al. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur. J. Immunol. 33, 2987–2997 (2003).
41. Heil, F. et al. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur. J. Immunol. 33, 2987–2997 (2003).
42. Heil, F. et al. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. Science 303, 1526–1529 (2004).
43. Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. & Reis e Sousa, C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529–1531 (2004).
44. Jurk, M. et al. Human TLR or TLR8 independently confer responsiveness to the antiviral compound R-848. Nature Immunol. 3, 499 (2002).
45. Heil, F. et al. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. Science 303, 1526–1529 (2004).
46. Hemmi, H. et al. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740–745 (2000).
47. Zhang, D. et al. A Toll-like receptor that prevents infection by uropathogenic bacteria. Science 303, 1522–1526 (2004).
48. Science 303. (5663):1526-9.
49. Uematsu S., Akira S. (2008) Toll-Like Receptors (TLRs) and Their Ligands. In: Bauer S., Hartmann G. (eds) Toll-Like Receptors (TLRs) and Innate Immunity. Handbook of Experimental Pharmacology, vol 183. Springer, Berlin, Heidelberg
50. Holt, Penford. Microorganisms and their relation to fever. 1912.
51. Seibert FB. Fever-producing substances found in some distilled water. Am. J. Physiol. 1923, 87: 90-104.
52. Seibert FB. The causes of many febrile reactions following intravenous injections. Am. J. Physiol. 1925, 71: 621 – 652.
53. Bang FB. A bacterial disease of Limulus polyphenus. Bull Johns Hopkins Hosp 1956; 98:325-351.
54. Levin J, Bang FB. The role of endotoxin in the extracellular coagulation of Limulus blood. Bull Johns Hopkins Hosp 1964; 115:265-274.
55. The United States Pharmacopeia, 20th Rev., Mack Publishing Co., Easton, PA, p. 888 (1980).
56. Hartung T, Wendel A. Detection of pyrogens using human whole blood. ALTEX. 1995;12(2):70-75.
57. S Fennrich et al. Evaluation and Further Development of a Pyrogenicity Assay Based on Human Whole Blood. ALTEX 15 (3), 123-128. 1998.
58. Fennrich, S., Fischer, M., Hartung, T. et al. (1999). Detection of endotoxins and other pyrogens using human whole blood. Dev. Biol. Stand 101, 131-139.
59. Schindler S, et al. "Development, validation and applications of the monocyte activation test for pyrogens based on human whole blood." Altex 26.4 (2009): 265-277.
60. European Pharmacopoeia (Ph. Eur.) chapter Monocyte activation test (2.6.30).
61. Guidance for Industry Pyrogen and Endotoxins Testing: Questions and Answers, U.S. Food and Drug Administration, June 2012.
62. Hartung T. 2015. ALTEX. 32(2):79-100).
63. A. Fritsch -Low Endotoxin Recovery (LER) is today one of authorities serious concerns regarding pyrogen testing; Avril 2017.
64. Hasiwa et al. 2013
65. Hasiwa et al. 2013. Evidence for the Detection of Non-Endotoxin Pyrogens by the Whole Blood Monocyte Activation Test.

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