The measurement of pyrogens is an essential safety measure for pharmaceutical products with parenteral administration and for medical devices. Due to major changes in the Ph. Eur. 5.1.10, and because of the limitations of the BET / LAL test, the MAT acc. to Ph. Eur. 2.6.30 becomes an important in vitro test to assess pyrogens in your products.
What is a pyrogen?

Pyrogens comprise a chemically heterogeneous group of fever inducing compounds derived from various origins: micro-organisms (bacteria, viruses and fungi), environment, process, product. Contamination by pyrogens is considered a serious health hazard and can result in symptoms ranging from vascular alterations to shock and death.¹

General principle of fever reaction

Pyrogens can be classified in two distinct categories: Endotoxin pyrogens (LPS) and Non-Endotoxin pyrogens (NEP)

ENDOTOXINS

Endotoxins are part of the outer membrane of the cell wall of gram-negative bacteria. Although the term “endotoxin” is occasionally used to refer to any cell-associated bacterial toxin, in bacteriology it is properly reserved to refer to the lipopoly saccharide complex associated with the outer membrane of gram-negative pathogens such as Escherichia coli, Salmonella, Shigella, Pseudomonas, Neisseria, Haemophilus influenzae, Bordetella pertussis and Vibrio cholerae.²

NON-ENDOTOXIN PYROGENS (NEP)

Non-endotoxin pyrogens are all the other substances that can induce fever reaction.

Non-endotoxin pyrogenic substances¹

<table>
<thead>
<tr>
<th>Endotoxin associated compounds</th>
<th>Exotoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidoglycans (components of bacterial cell wall)</td>
<td>Lipoarabinomannans (from mycobacteria)</td>
</tr>
<tr>
<td>DNA (bacterial)</td>
<td>Fungal components for example, mannans, glucans, mannosproteins</td>
</tr>
<tr>
<td>Porins (proteins from the bacterial cell wall)</td>
<td>Parasite components (for example, phosphoinositol)</td>
</tr>
<tr>
<td>Bacterial outer surface proteins</td>
<td>Viruses</td>
</tr>
<tr>
<td>Muramyldipeptides (MDP and other subunits of peptidoglycan synergise with endotoxins)</td>
<td>Non-microbiological contaminations (for example, cytokines, media, cells, breakdown products)</td>
</tr>
<tr>
<td>Lipoteichoic acids and further gram-positive bacterial cell-wall components</td>
<td>Solid materials (for example, medical devices, plastic)</td>
</tr>
<tr>
<td>Superantigens</td>
<td>Drugs (for example steroids, bile salts, dapsone, cytokines)</td>
</tr>
</tbody>
</table>

Evolution of pyrogen detection

The first method described for the detection of pyrogens is the rabbit pyrogen test (RPT). The RPT is an in vivo test that allows detecting all substances pyrogenic for the rabbit.

Whenever possible, RPT has been replaced by the BET (Bacterial Endotoxin Test) / LAL (Limulus amoebocytolytic test), mainly relying on the fact that bacterial endotoxin is the most frequent pyrogen found in pharmaceutical products. The test is an ex vivo assay, based on the coagulation of the lysate of amoebocytes (white blood cells) from the horseshoe crab.

The MAT based on the reaction of human monocytes, now allows predicting the human response to all pyrogens (i.e. endotoxin and non-endotoxin) in vitro. The MAT can be used as alternative to animal testing, in accordance with EU legislative framework, as stated in the Ph. Eur. 2.6.30.³
**Regulation: Major changes for pyrogen detection**

- Major change of the Ph. Eur. chapter 5.1.10: “Guidelines for using the test for bacterial endotoxins”: a risk assessment is required when using the bacterial endotoxin test to evaluate the potential presence of non-endotoxin pyrogens. The MAT is recommended as a useful part of this risk assessment. When the BET appears to be not sufficient, the MAT is preferred to the rabbit pyrogen test.

- The Ph. Eur. chapter 2.6.8: “Pyrogens” mentions the MAT as possible replacement for the RPT.

- The Ph. Eur. chapter 2.6.30 includes the MAT as limit test / quantitative test and as test for batch-to-batch comparison, the choice depending on the characteristics of the targeted test item.

- USP <151>: To encourage the use of in vitro alternatives to in vivo animal tests, the new chapter allows the use of a validated, equivalent in vitro pyrogen or bacterial endotoxin test, instead of the in vivo rabbit pyrogen test, where appropriate. There is no impact on any of the monographs that refer to this chapter.

**ETHICS AND LAWS ACCORDING TO THE ANIMAL WELFARE**

- According to the European law (Directive 2010/63/EU), 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. According to this law, the MAT has to replace the RPT.

- For the moment, the LAL test is not considered as an in vivo test, although the major compound is animal blood. “The industry says that not that many of the animals die. Between 10 and 30% of the bled animals, according to varying estimates, actually die.”

- Due to human activity three of four species of the Horseshoe crab have been classified “Data Deficient.”

- “UCN SSC Horseshoe Specialist Group is currently revising the IUCN Red List Assessment for the three Asian species of horseshoe crab, (Tachypleus tridentatus, Tachypleus gigas and Carcinoscorpius rotundicauda) which are currently listed as Data Deficient.”

- Ph. Eur. 2.6.30: “In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte-activation test.”

---

**Monocyte Activation Test**

> “The MAT is used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators which have a role in the human fever response. The MAT is suitable, after product-specific validation, as a replacement for the RPT.

The MAT offers significant advantages over animal testing: based on the human fever response, it provides a more relevant prediction of pyrogenic activity than the RPT, it can detect endotoxin and non-endotoxin pyrogens and is applicable to a greater variety of products than the RPT; moreover, it is more accurate as well as more cost- and time-effective than the RPT.”

**General principle of MAT**

- **PYRROGENS**
- **HUMAN WHITE BLOOD CELLS**
- **INTERLEUKINS**
- **ELISA**
- **QUANTIFICATION (IU/ML)**

**ADVANTAGES OF THE MONOCYTES ACTIVATION TEST**

- Based on the human fever response, the MAT provides a more relevant prediction of pyrogenic activity than the RPT or BET/LAL.

- It can detect endotoxin and non-endotoxin pyrogens and is applicable to a greater variety of products than the RPT or BET/LAL (e.g., certain drugs and herbal formulations; see Hartung T. 2015. ALTEX. 32(2):79-100).

- It has a lower limit of detection and is more accurate as well as more cost- and time-effective than the RPT.

- Three variants of the MAT have been standardized and validated.

DIFFERENT MAT FORMATS
Three different formats of the MAT are internationally validated:
- Blood based
- Peripheral Blood Mononuclear Cell (PBMC) based
- Cell line based

<table>
<thead>
<tr>
<th>ORIGIN OF THE CELLS</th>
<th>BLOOD BASED</th>
<th>PBMC BASED</th>
<th>CELL LINE BASED</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANGE</td>
<td>0.2 to 1.0 EU/mL</td>
<td>0.05–5.0 EU/mL</td>
<td>0.05–5.0 EU/mL</td>
</tr>
<tr>
<td>REACTION STANDARDIZED</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>LIMITS</td>
<td>Raw material from blood banks, batch size limited</td>
<td>Raw material from blood banks, batch size limited</td>
<td>Only cell line available: Mono Mac 6</td>
</tr>
<tr>
<td>KIT AVAILABILITY</td>
<td>Yes</td>
<td>Under development</td>
<td>Yes</td>
</tr>
</tbody>
</table>

MAT WITH THE MONO MAC 6 CELL LINE
The MAT can be performed based on a cell line. Confarma has implemented the method based on the cell line Mono Mac 6. The focus of Confarma is to offer batch release testing with good sensitivity and very high reproducibility. The reproducibility of a method for batch release analysis is key to guarantee stability in batch-to-batch analysis. The MAT format chosen concurs with Confarma's vocation.

Other formats do not have the same stability and reproducibility (donor-to-donor variability) and are difficult to manage due to the risks associated with the human blood or derived human blood (HIV, viruses...).

The MAT with a cell line is able to detect all human pyrogens and has the advantage of the stability of an assay based on a cell line.

The MAT with the Mono Mac 6 cell line was included in the international MAT evaluation (Hoffmann et al., 2005).

PYROGEN | DETECTION LIMIT WITH MONO MAC 6
---------|--------------------------
Endotoxin | 0.05 EU/mL
Flagellin of B. subtilis | 0.2 µg/mL
Heat-killed S. aureus | 0.5 x 10^6 cells/mL
Pam3CSK4 | 50 ng/mL
Peptidoglycan of S. aureus | 0.1 µg/mL
Lipoteichoic acid | 0.25 µg/mL

CONFARMA SERVICES
Confarma is the first lab worldwide to be accredited ISO 17025 (COFRAC*) for MAT for pharmaceutical products and medical devices.
- MAT based on Mono Mac 6 cell line, which is a valuable, stable assay in routine
- Sensitive test system, stable low background
- First optical readout under the microscope → serves as assay control
- True in vitro test, completely standardized
- Sample preparation is simple
- Analysis of difficult samples possible
- In routine use at Confarma as investigation tool, and in the context of method development / validation
- Analysis of medical devices possible in direct contact with the cells
- Analysis of cell therapy products possible
- Analysis of non-sterile products possible
- Assessment of finished products as well as raw materials

WHY CONFARMA?
- First to use a robust MAT for QC release with a worldwide validated cell line
- More than 10 years experience with MAT
- MAT with all 3 formats available: cell line, PBMC, blood
- Experience with difficult products such as coagulation factors, cell-based therapeutics, strong antibiotics, products with low pH, gels...
- Strong expertise with endotoxin testing (all methods) as well
- Dedicated project team and flexible capacity planning to ensure the respect of the given timelines

REFERENCES
1. Today’s Online Textbook of Bacteriology, Endotoxin
3. Ph. Eur. 2.6.30. Monocyte activation test
4. Ph. Eur. 5.1.10.: Guidelines for using the test for bacterial endotoxins
7. The Blood Harvest The Atlantic, 2014
9. European Pharmacopoeia Commission adopts revised general chapter on Monocyte-activation test to facilitate reduction in testing on laboratory animals; EDOM, 2016
10. The monocyte activation test for assessing pyrogenicity, PETA, 2010

*scope available on www.cofrac.fr

Conferma France SAS
ZI rue du Canal d’Alsace, F-68490 Hombourg
info@confarma.fr | www.confarma.fr