Millipore®
Preparation, Separation,
Filtration & Monitoring Products

Application Note

Comparison between Rabbit Pyrogen Test (RPT) and Monocyte Activation Test (MAT) for the detection of Non-Endotoxin Pyrogens (NEP)

Author: Anne-Claire ERBA, Millipore SAS, Molsheim, France

Summary

This application note describes the methodology of the comparison study Rabbit Pyrogen Test (RPT) and Monocyte Activation Test (MAT) conducted by CMIC Pharma Science Co., Ltd. – CMIC Bioresearch Center* in collaboration with JaCVAM* (Peer review panel for pyrogen test) between November 2019 and October 2021 and the resulting conclusion. The comparison study was based on the detection of two selected non-endotoxin pyrogens (NEPs) Flagellin and HKSA. The results described on this paper show the pyrogenicity effect of Flagellin and HKSA tested at relevant concentrations with both RPT and MAT.

This study demonstrates the comparability of the results and the detection at a lower concentration for the two NEPs, Flagellin and HKSA, with MAT method versus the RPT demonstrating a higher sensitivity.

Introduction

What is a pyrogen?

A pyrogen is, by definition, a substance that induces a rise in temperature in a human or animal body. Pyrogens constitute a heterogeneous group of contaminants comprising microbial and non-microbial substances. The most widely known pyrogen is the endotoxin (LPS = Lipopolysaccharide), which comes from Gram-negative bacteria.

Other substances (e.g.: microbial substances from bacteria, particles from viruses and pyrogens originating from yeasts and fungi or non-microbial pyrogenic substances as rubber particles, microscopic plastic particles or metal compounds in elastomers) are considered as non-endotoxin pyrogens (NEP).

Why to carry out a pyrogen test?

Pyrogenic substances in pharmaceutical products can induce life-threatening fever reactions after injection into the human body. It is therefore a regulatory requirement to test such products for pyrogens to ensure product quality and patient safety.

For health and safety reasons, health authority agencies require to ensure the absence of pyrogenic substances in injectable drugs. The oldest methods used are the Rabbit Pyrogen Test (RPT) and/or the Bacterial Endotoxin Test (BET). However, both tests have their disadvantages. The RPT is only able to give a qualitative result, while the BET does not detect non-endotoxin pyrogens.

Additionally, both methods are based on animals or animals derived products and therefore counter the principles of the 3Rs (Replacement, Reduction and Refinement) regarding animal welfare.

The purpose of the pyrogen test is to prove that the quantity of pyrogens contained in the product will not exceed a certain threshold, known as the Contaminant Limit Concentration (CLC), and specified by the regulatory bodies, in order to guarantee patient safety.

The Monocyte Activation Test (MAT) method was qualified, validated and integrated for the detection of pyrogens as a compendial methods for pyrogen detection in the European Pharmacopoeia since 2010 (Chapter 2.6.30 from Ph. Eur. 07/2017).

*CIMIC Pharma Science: Non-clinical CRO (contract research organization) in Yamanashi Pref., Japan

*JaCVAM: Japanese Center for the Validation of Alternative Methods

Principle of the RPT

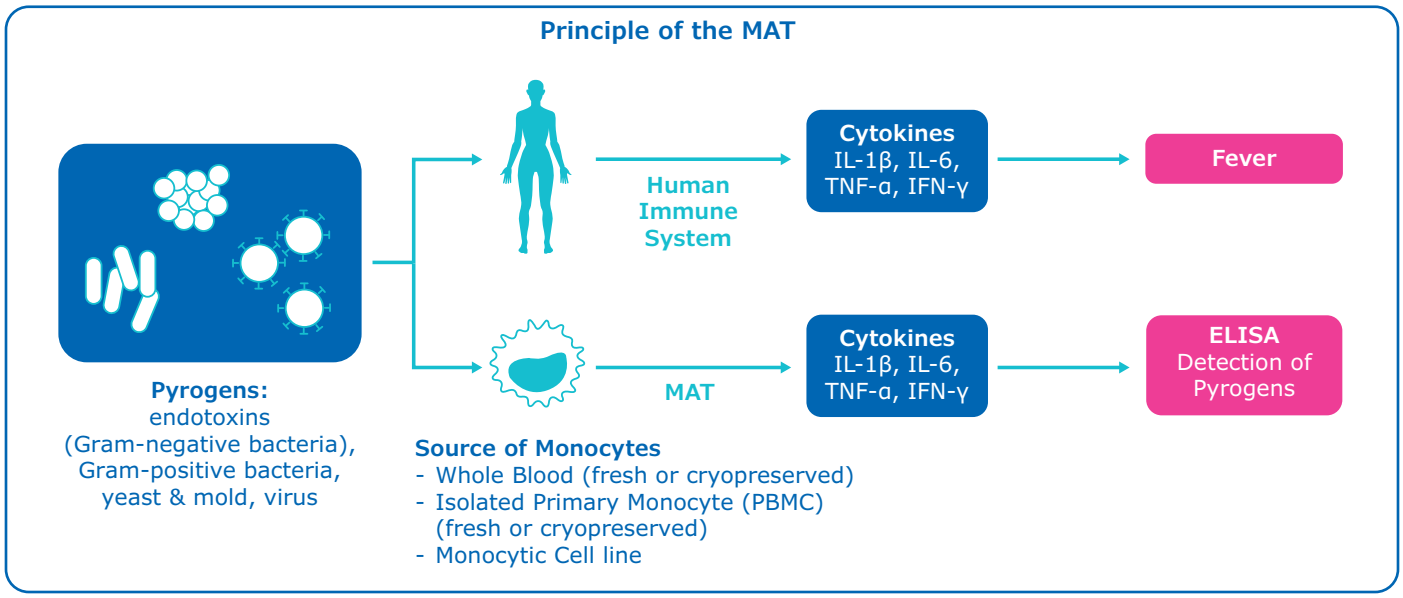
The Rabbit Pyrogen Test (RPT) is an animal test based on the injection of the product/sample in the rabbits. The principle is to measure a potential rise in temperature of the body after injection. The rabbit reacts to endotoxins as well as non-endotoxin pyrogens. The initial test is done on 3 rabbits. The result obtained is the sum of the increase in temperature of the 3 rabbits from the initial temperature considered as the base. If the test gives results between low and high threshold, retests must be completed. Up to 9 rabbits per samples could be used to obtain a final status of pyrogenic or non-pyrogenic to the tested sample.

Principle of the MAT

The Monocyte Activation Test (MAT) is the human *in vitro* test and allows the detection of the full range of pyrogens, including endotoxins and Non-Endotoxin Pyrogens (NEPs).

By putting the product/sample to be tested in contact with human monocytic cells, which are cells coming from the human immune system, it mimics what happens in the human body: in presence of pyrogens, the monocytes are activated and produce cytokines such as Interleukin-1 (IL-1β) and Interleukin-6 (IL-6).

The cytokines are then detected using an immunological assay (ELISA) involving specific antibodies and an enzymatic color reaction.



Principle of the PyroMAT® system

The PyroMAT® system uses cryo-preserved Mono-Mac-6 (MM6) human monocytic cells as a source of monocytes.

The response to pyrogenic substances is determined by measurement of Interleukin-6 (IL-6) produced by the Mono-Mac-6 cells. For this purpose, the ELISA-microplate supplied in the kit is coated with an antibody specific to IL-6.

IL-6 molecules released by the MM6 cell supernatant during the incubation phase are transferred in the ELISA plate, and are bound by the immobilized primary antibody.

A secondary antibody, linked to an enzyme, is added to form an IL-6 bound complex. After washing any unbound molecule, the IL-6 bound complex is detected in a color reaction started by the addition of an appropriate substrate.

The color development is proportional to the amount of initial IL-6 production in the supernatant and is measured with an absorbance reader.

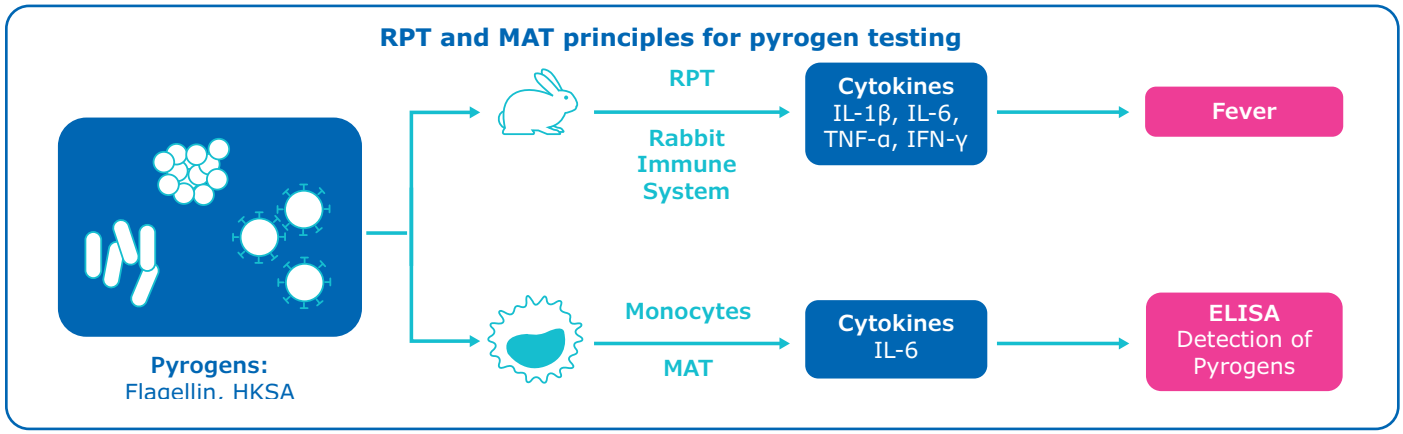
Study Design

To compare the ability of detecting pyrogens of both RPT and MAT methods, the following two NEPs have been used:

Flagellin = Lyophilized Flagellin from *Salmonella typhimurium*.

HKSA = Lyophilized cells of heat-killed *Staphylococcus aureus*.

The Japanese Pharmacopoeia was followed to test the selected NEPs with the RPT and the PyroMAT® System was used to test the same batch of NEPs according to Ph. Eur. chapter 2.6.30.



Methods

According to the CMIC Pharma Science who did the following RPT assay, the test was conducted in accordance with the Standards for Reliability of Application Data (Article 43 of Ordinance for Enforcement of the Law on Securing Quality, Efficacy and Safety of Products including Pharmaceuticals and Medical Devices).

Method for the Rabbit pyrogen test:

Healthy mature rabbits were used to perform the rabbit pyrogen tests. The control temperature of each rabbit is the mean of two temperature readings recorded for that same rabbit at an interval of around 30 min preceding the injection of the sample.

The sample injected is prewarmed at 37 °C and 10 mL per kg of body mass of the rabbit is slowly injected.

The temperature is recorded for each rabbit during a period of 3 hours after the injection. The measurements are done with a maximal interval of 30 minutes.

The difference between the control temperature and the maximum temperature of each rabbit is retained to be the rise in body temperature. The maximum temperature rises for each rabbit are summed to get the final value.

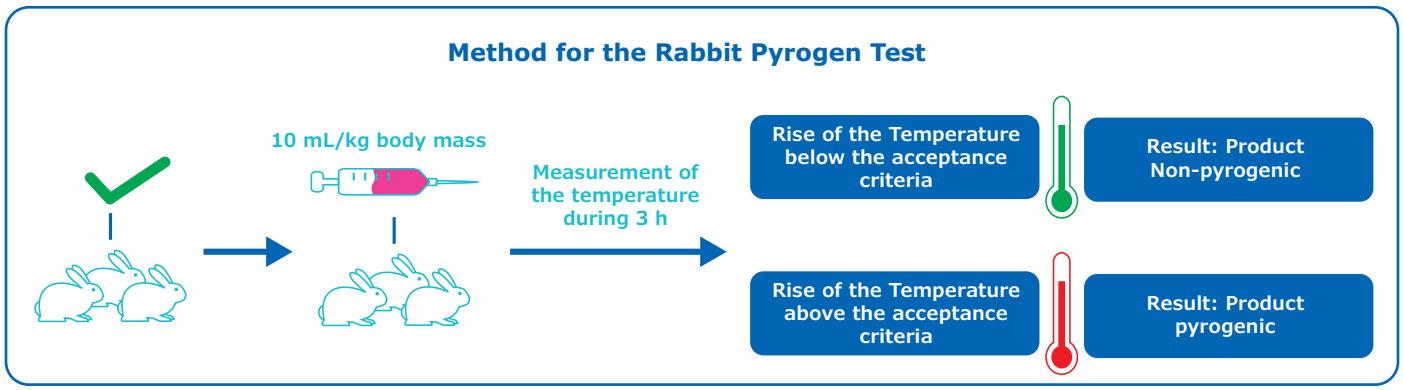


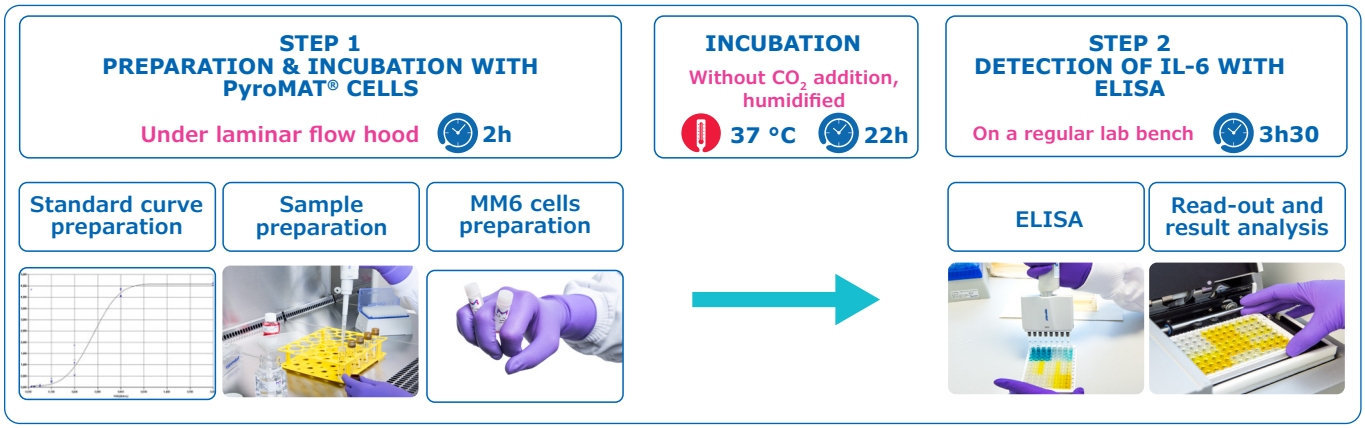
Table 1: Acceptance criteria from Japanese Pharmacopoeia 4.04 Pyrogen Test

Acceptance Criteria from the Japanese Pharmacopoeia		
Tests	**Rise of temperature**	**Results**
First test: addition of the rise of temperature for the 3 rabbits	<1.3 °C	Not Pyrogenic
	1.3<Values<2.5 °C	Retest on another 3 rabbits
	>2.5 °C	Pyrogenic
Second test: addition of the rise of temperature for the 3 rabbits of the first test + 3 rabbits of the second test	<3 °C	Not Pyrogenic
	3<Values<4.2 °C	Retest on another 3 rabbits
	>4.2 °C	Pyrogenic
Third test: addition of the rise of temperature for the 6 rabbits of the first and second tests + 3 rabbits of the third test	<5.0 °C	Not Pyrogenic
	>5.0 °C	Pyrogenic

This table reflects the acceptance criteria described in the Japanese Pharmacopoeia in the chapter 4.04 Pyrogen Test. Each test used 3 different rabbits and in the final analysis, all results obtained for each rabbit are compiled to obtain a global answer.

Method for the Monocyte Activation Test with PyroMAT® system:

For the detailed method: refer to the user guide.



Acceptance criteria for validity of the endotoxin standard curve

Method A requires a valid standard curve based on the following criteria:

- Effect of dose criteria: a statistical test that confirms a positive dose/effect response.
- Goodness of fit criteria: a statistical test that confirms the suitability of the regression model to describe the raw data. The data are modeled with a 5-parameter logistics regression model.
- Blank criteria: the mean of blank OD value should be below 0.1.
- LOD criteria: the test is valid if an LOD ≤0.05 EU/mL is reached.

Detection of non-endotoxin pyrogens

The NEP tested in water as a control is detected if the signal, expressed in EEU/mL using the endotoxin standard curve, is above the LOD = 0.05 EU/mL.

Material

Hardware and consumables

For RPT*

Product	Cat. No.
Kbl: JW rabbit, SPF (10 weeks of age, 89 male animals used)	
Water for Injection (FUSO Pharmaceutical Industries, Ltd.)	
Saline (FUSO Pharmaceutical Industries, Ltd.)	
Heat killed *Staphylococcus aureus* HKSA - TLR2 agonist	**MATHKSA**
Flagellin from *Salmonella typhimurium* TLR5 agonist	**MATFLAGELLIN**
A sensor (precision ±0.1 °C or less) (Y611-T11794, TATEYAMA KAGAKU CO., LTD.)	

*This study was approved by the Institutional Animal Care and Use Committee (IACUC) of CMIC Pharma Science Co., Ltd. (Approval No. 2019-183, IACUC-CBR-2009-007, IACUC-CBR-2109-012). This facility is accredited by AAALAC International (File No. 001182).

For MAT

Product	Cat. No.
PyroMAT® kit	**PYR0MATKIT**
PyroMAT® cells	**PYR0MATCELLS**
Heat killed *Staphylococcus aureus* HKSA - TLR2 agonist	**MATHKSA**
Flagellin from *Salmonella typhimurium* TLR5 agonist	**MATFLAGELLIN**
Reference Standard Endotoxin [3]	**1.44161**
BioTek Reader	**Elx808**
BioTek Gen5 Data Analysis Software	
PyroMAT® Method Software	**Available on SigmaAldrich.com**

- In total 89 rabbits have been used to perform this comparison study
- In total, 5 different batches of HKSA were used for comparison between RPT and MAT
- In total, 6 different batches of Flagellin were used for comparison between RPT and MAT
- The concentration of the 2 NEPs used are described in “X”. The validated concentration used in the routine MAT test for each NEPs is 1x. This 1x concentration is considered as the NEP positive control.

Table 2: Results of RPT with HKSA

HKSA Concentration	Result	Number of Rabbits used
5 X	Pyrogenic	3
2.5 X	Pyrogenic	3
1 X	Pyrogenic	6
0.5 X	Pyrogenic	3
	Pyrogenic	3
	Pyrogenic	3
	Pyrogenic	3
	Pyrogenic	3
0.2 X	Non Pyrogenic	3

This table reflects the tests done with the NEP HKSA on the rabbits. In the “number of rabbits used” column, a number 3 corresponds to 1 test, a 6 means 1 test and a retest, a 9 would mean 1 test and 2 retests. All results have been combined in one result.

The results “pyrogenic” or “non pyrogenic” is defined following the range described in the acceptance criteria of the Japanese Pharmacopoeia.

Table 3: Comparison of the results obtained with the RPT and the MAT for pyrogenicity of HKSA

HKSA concentration	RPT[1]		MAT[2]	
5 X	1 test	Pyrogenic	NA	NA
2.5 X	1 test	Pyrogenic	NA	NA
1 X	1 test	Pyrogenic	4 tests	Pyrogenic
0.5 X	5 tests	Pyrogenic	3 tests	Pyrogenic
0.25 X	1 test	Non Pyrogenic	2 tests	Pyrogenic

[1]: pyrogenic according the acceptance criteria described in the Japanese Pharmacopoeia

[2]: Pyrogenic for the MAT is considered as >LOD (0.05 EU/mL)

This table represents the results comparison of the detection of pyrogenicity for the HKSA with the RPT and the MAT.

The pyrogenicity for the RPT is according to the Japanese Pharmacopoeia and for the MAT is according to the Limit of detection of the PyroMAT® System (LOD= 0.05 EU/mL)

The pyrogenicity of HKSA is measurable from 0.5 X HKSA with the RPT and from 0.25 X with the MAT. The pyrogenic effect of HKSA is detected at a lower dose with MAT, meaning a higher sensitivity compared to RPT.

The concentration above 1 X (5 X and 2.5 X) was not tested on MAT because not relevant for the detection method.

Table 4: Results of RPT with Flagellin

Flagellin Concentration	Result	Number of Rabbits used
10 X	Pyrogenic	3
5 X	Pyrogenic	9
2.5 X	Pyrogenic	9
	Pyrogenic	9
	Pyrogenic	3
	Pyrogenic	6
	Pyrogenic	6
1 X	Pyrogenic	9
	Non Pyrogenic	3
	Non Pyrogenic	3
0.5 X	Non Pyrogenic	3

This table reflects the tests done with the NEP Flagellin on the rabbits. In the “number of rabbits used” column, a number 3 corresponds to 1 test, a 6 means 1 test and a retest, a 9 would mean 1 test and 2 retests. All results have been combined in one result.

The results “pyrogenic” or “non pyrogenic” is defined following the range described in the acceptance criteria of the Japanese Pharmacopoeia.

Table 5: Comparison of the results obtained with the RPT and the MAT for pyrogenicity of Flagellin

Flagellin concentration	RPT[1]		MAT[2]	
10 X	1 test	Pyrogenic	NA	NA
5 X	1 test	Pyrogenic	NA	NA
2.5 X	5 tests	Pyrogenic	1 test	Pyrogenic
1 X	1 test	Pyrogenic	5 tests	Pyrogenic
	2 tests	Non Pyrogenic		
0.5 X	1 tests	Non Pyrogenic	2 tests	Pyrogenic
0.25 X	NA	NA	1 test	Pyrogenic
			1 test	Non Pyrogenic

[1]: Pyrogenic according the acceptance criteria described in the Japanese Pharmacopoeia

[2]: Pyrogenic for the MAT is considered as >LOD (0,05 EU/mL)

This table represents the results comparison of the detection of pyrogenicity for the Flagellin with the RPT and the MAT.

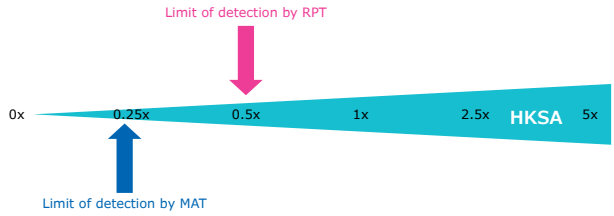
The pyrogenicity for the RPT is according to the Japanese Pharmacopoeia and for the MAT is according to the Limit of detection of the PyroMAT® System (LOD= 0,05 EU/mL)

The pyrogenicity of the Flagellin is measurable at a concentration between 1 and 2,5 X with the RPT. Indeed, at a concentration of 1 X, one RPT was positive whereas the two other tests were negative. This difference could be explained by the variability of the biological response of the rabbits towards contaminants. At 2,5 X, the detection of flagellin was always pyrogenic. To ensure an accurate and consistent detection of Flagellin with RPT, the limit of detection is therefore set at 2.5 X.

The pyrogenic concentration of Flagellin detected with the MAT is between 0,25 and 0,5 X. Indeed, at a concentration of 0.25 X, one MAT was positive whereas the other one was negative. Concentrations of 0,5 X and above are always pyrogenic. To ensure an accurate and consistent detection of Flagellin with MAT, the limit of detection is therefore set at 0.5 X.

Conclusion

Image 1: limit of detection of HKSA by 2 pyrogen detection methods

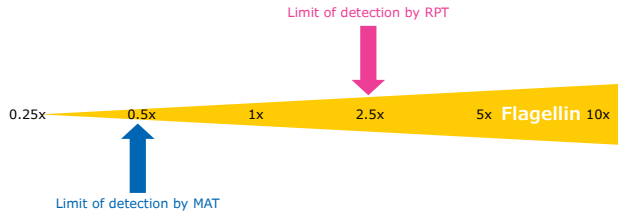


The comparison of the pyrogenicity detection for HKSA with the RPT and the MAT shows a higher sensitivity of the MAT. Indeed, HKSA is detected at 0.25 X with the MAT, whereas a concentration of 0.5 X is required for the detection with the RPT.

The pyrogenicity for the RPT is according to the Japanese Pharmacopoeia and for the MAT is according to the Limit of detection of the PyroMAT® System (LOD= 0.05 EU/mL)

MAT has the ability to detect a 2-time lower concentration of HKSA, compared to RPT.

Image 2: limit of detection of Flagellin by 2 pyrogen detection methods



The comparison of the pyrogenicity detection for Flagellin with the RPT and the MAT shows a higher sensitivity of the MAT. Indeed, Flagellin is detected at 0.5 X with the MAT, whereas a concentration of 2.5 X is required for the detection with the RPT.

The pyrogenicity for the RPT is according to the Japanese Pharmacopoeia and for the MAT is according to the Limit of detection of the PyroMAT® System (LOD= 0,05 EU/mL)

MAT has the ability to detect a 5-time lower concentration of Flagellin, compared to RPT.

REQUEST INFORMATION
SigmaAldrich.com/pyromat

To place an order or receive technical assistance

In the U.S. and Canada, call toll-free 1-800-645-5476
For other countries across Europe and the world, please visit: **SigmaAldrich.com/offices**
For Technical Service, please visit: **SigmaAldrich.com/techservice**

SigmaAldrich.com

We have built a unique collection of life science brands with unrivalled experience in supporting your scientific advancements.

Millipore® Sigma-Aldrich® Supelco® Milli-Q® SAFC® BioReliance®

Merck KGaA
Frankfurter Strasse 250
64293 Darmstadt, Germany

MK_AN14734EN Ver. 1.0
64930
10/2025