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Application Note

Detection of non-endotoxin pyrogens by Monocyte Activation Test (MAT) using the PyroMAT® system.

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Introduction

What is a pyrogen?

A pyrogen is, by definition, a substance that produces a rise in temperature in a human or animal. Pyrogens constitute a heterogeneous group of contaminants comprising microbial and non-microbial substances.
The most widely known pyrogen are lipopolysaccharides (LPS), also known as endotoxins, which are produced by Gram-negative bacteria. Other microbial substances include those derived from Gram-positive bacteria like lipoteichoic acid (LTA), particles from viruses and pyrogens originating from yeasts and fungi. Non-microbial pyrogenic substances can be rubber particles, microscopic plastic particles or metal compounds in elastomers.

Why conduct a pyrogen test?

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

The purpose of the test is to prove that the amount of pyrogens contained in the product will not exceed a certain threshold, known as the contaminant limit concentration (CLC), that will guarantee patient safety.

The monocyte activation test (MAT) method was qualified and validated for the detection of pyrogens 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.

It has been among the compendial methods for pyrogen detection in the European Pharmacopeia since 2010 (Chapter 2.6.30) [1].

The MAT is also mentioned by the FDA "Guidance For Industry – Pyrogen and Endotoxins testing: Questions and Answers" as an alternative to the rabbit pyrogen test and should be validated according to USP <1225>. Additionally, the 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."

Principle of the MAT

The monocyte activation test (MAT) is the human *in vitro* alternative to the rabbit pyrogen test, and allows the detection of the full range of pyrogens, including endotoxins and non-endotoxin pyrogens (NEPs).

When the product to be tested is put in contact with human monocytic cells, the MAT mimics what happens in the human body: in the presence of pyrogens, the monocytes are activated and produce cytokines such as interleukin-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 the 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 MM6 cells into the supernatant during the incubation phase are transferred to the ELISA plate, and 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 molecules, 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 measured with an absorbance reader.

Quantification of pyrogens with the MAT

For the quantification of the pyrogenic load of a sample, method A in accordance with the European Pharmacopeia[1] can be conducted.

Method A involves a comparison of the preparation being examined with a standard endotoxin dose-response curve. The contaminant concentration of the preparation being examined must be lower than the CLC (Contaminant Limit Concentration) to pass the test.

To ensure both the precision and validity of the test, preparatory tests need to be conducted to assure that:

- The criteria for the endotoxin standard curve are fulfilled
- The solution does not interfere with the test
- The test detects endotoxin and non-endotoxin contaminants
- The solution does not interfere with the detection system

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 an 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.[2,3]

TLR signaling pathways

Stimulation of TLRs by the corresponding PAMPs or DAMPs initiates signaling cascades that trigger specific immunological responses.[4]

MyD88 (myeloid differentiation primary-response protein 88) is a universal adapter protein and typically 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 lead to the dissociation of previous complexes and the 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, yeasts, viruses, and parasites.[5]

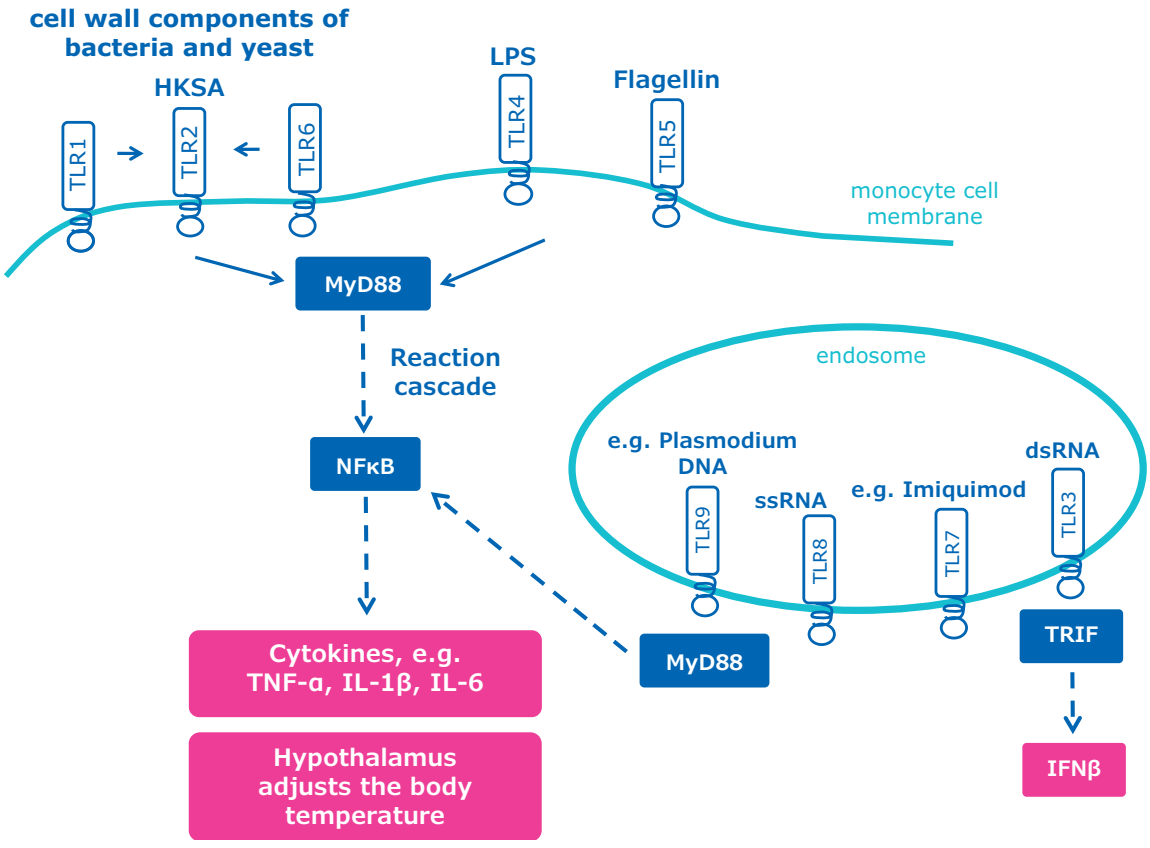


Figure 1. TLR signaling pathways

Receptor	Ligand	Origin of ligand	References
TLR1	Triacyl lipopeptides Soluble factors	Bacteria and mycobacteria *Neisseria meningitidis*	6 7
TLR2	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Lipoarabinomannan Phenol-soluble modulin Glyco-inositol-phospholipids Glycolipids Porins Atypical lipopolysaccharide Atypical lipopolysaccharide Zymosan Heat-shock protein 70*	Various pathogens Gram-positive bacteria Gram-positive bacteria Mycobacteria *Staphylococcus epidermidis* *Trypanosoma cruzi* *Treponema maltophilum* *Neisseria* *Leptospira interrogans* *Porphyromonas gingivalis* Fungi Host	8 9,10 11 12 13 14 15 16 17 18 19 20
TLR3	Double-stranded RNA	Viruses	21
TLR4	Lipopolysaccharide Taxol Fusion protein Envelope protein Heat-shock protein 60* Heat-shock protein 70* Type III repeat extra domain A of fibronectin* Oligosaccharides of hyaluronic acid* Polysaccharide fragments of heparan sulphate* Fibrinogen*	Gram-negative bacteria Plants Respiratory syncytial virus Mouse mammary-tumor virus *Chlamydia pneumoniae* Host Host Host Host Host	22 23 24 25 26, 27 28 29 30 31 32
TLR5	Flagellin	Bacteria	33
TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan	*Mycoplasma* Gram-positive bacteria Fungi	34 35 36
TLR7	Imidazoquinoline Loxoribine Bropirimine Single-stranded RNA	Synthetic compounds Synthetic compounds Synthetic compounds Viruses	37 38 39 40, 41
TLR8	Imidazoquinoline Single-stranded RNA	Synthetic compounds Viruses	42 43
TLR9	CpG-containing DNA	Bacteria and viruses	44
TLR10	N.D.	N.D.	–
TLR11	N.D.	Uropathogenic bacteria	45
TLR1/TLR2 heterodimer	Triacylated lipoproteins	–	46
TLR2/TLR6 heterodimer	Diacylated lipoproteins	–	47

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.

Material and Equipment

To perform the MAT and a product specific validation, we recommend using:

- PyroMAT® Cells (Ref: Pyr0MATCELLS)
- PyroMAT® Kit (Ref: Pyr0MATKIT)
- Reference Standard Endotoxin (Ref: 1.44161.0001).
- NEP Control HKSA (Ref: MATHKSA)
- NEP Control Flagellin (Ref: MATFLAGELLIN)
- IL-6 Control (Ref: Pyr0MATIL6)

Additional equipment and consumables required:

- Incubator, 37 °C, humidified
- Water bath (37 °C)
- Microplate reader to measure absorbance at 450 nm and 630 nm (reference wavelength)
- Cryo-freezer (<-80 °C)
- Freezer (-20 °C)
- Refrigerator (2-8 °C)
- Vortexer
- 50 mL centrifuge
- Multichannel pipettes with suitable containers
- Adjustable pipettes: (10-100 µL; 100-1000 µL) with suitable sterile, pyrogen-free pipette tips
- Pyrogen-free glass tubes
- 2 mL endotoxin-free reaction tubes with caps
- 50 mL endotoxin-free tubes with caps

Study: Detection of various non-endotoxin pyrogens or TLR ligands with the PyroMAT® system.

For this study, the following samples were used:

Sample	NEP	TLR	Concentration range tested during study	Endotoxin contamination*
1	Pam3CSK4	1/2	1 µg/mL to 0.125 µg/mL	Not tested, synthetic**
2	HKSA	2	Dilution 1/1000 to 1/8000	0.02 EU/mL
3	PGN	2	10 µg/mL to 1.25 µg/mL	0.6 EU/mg
4	FSL-1	2/6	0.1 ng/mL to 0.0125 ng/mL	<5 EU/mg
5	Poly-IC	3	250 µg/mL to 37.5 µg/mL	0.03 EU/mg
6	Flagellin	5	0.1 µg/mL to 0.0125 µg/mL	<0.025 EU/mg
7	Imiquimod	7	100 µg/mL to 2.5 µg/mL	<0.025 EU/mg
8	CL075	7/8	10 µg/mL to 1.25 µg/mL	<0.5 EU/mg
9	ODN2006	9	100 µg/mL to 12.5 µg/mL	Not tested, synthetic**
10	MDP	NOD2	100 µg/mL to 12.5 µg/mL	<0.05 EU/mg

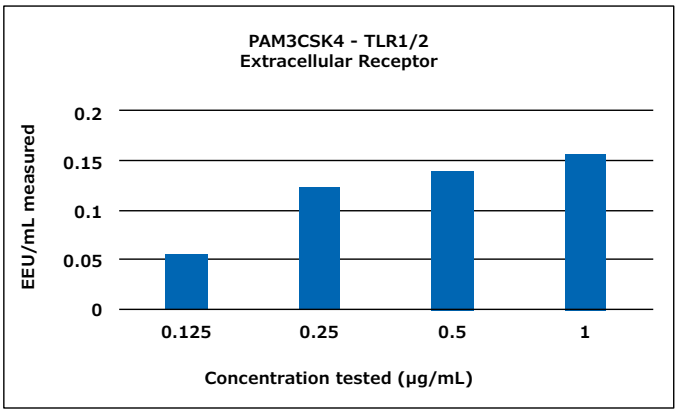
* Endotoxin concentration is given for the undiluted product (after correction of the dilution factor)

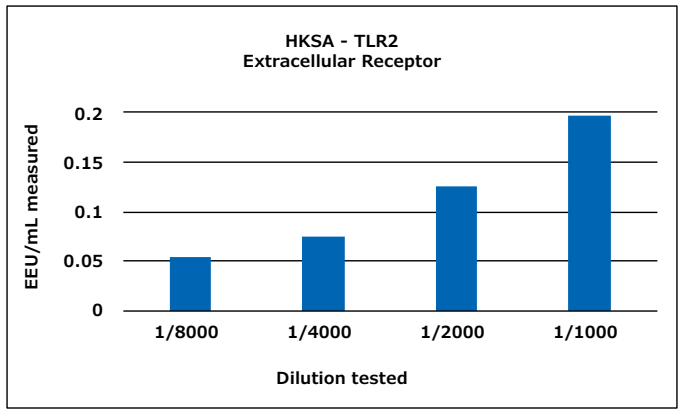
** synthetic NEPs were not tested for endotoxin contamination

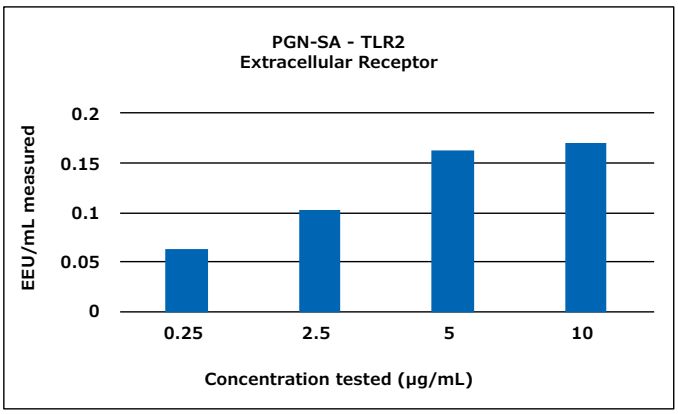
It has to be noted that the material available as non-endotoxin pyrogen is not standardized and the concentration ranges required for a reaction might differ between batches. During this study, the appropriate range was determined by dose-screening using one batch of PyroMAT® cells, followed by verification of the reaction using three batches of PyroMAT® cells.

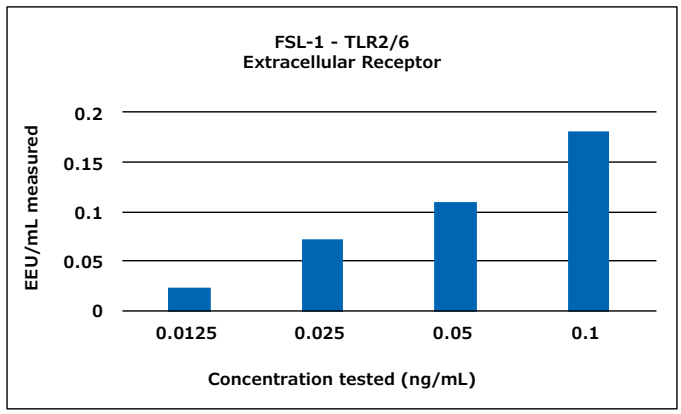
Determination of limit of detection (LOD) for the different non-endotoxin pyrogens (NEPs)

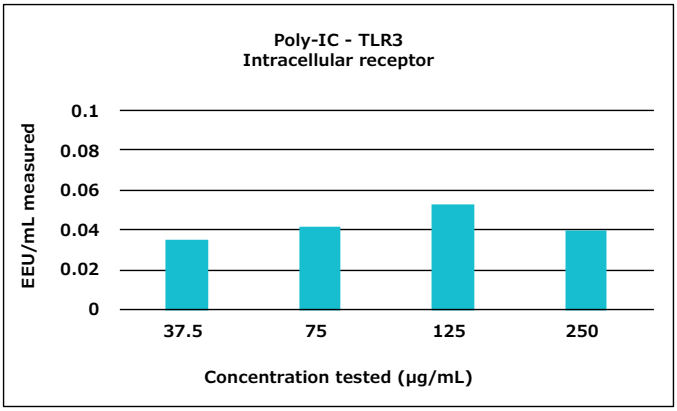
The different samples were used in a dose screening to determine the limit of detection of the monocyte activation test. For this, at least four dilutions of every NEP were run individually in the assay. The endotoxin equivalent units (EEU) measured for the pyrogenicity of each dilution are shown below:

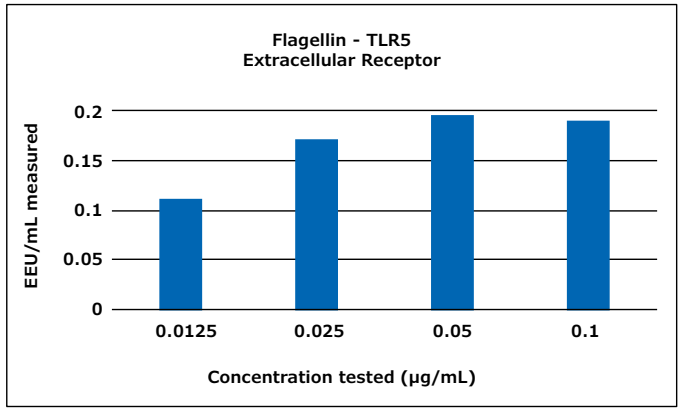


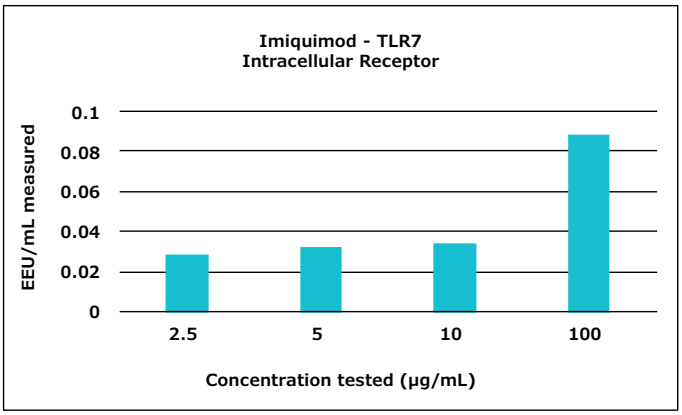


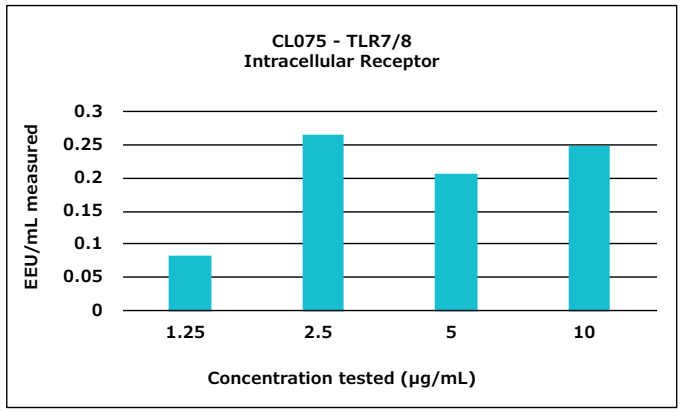


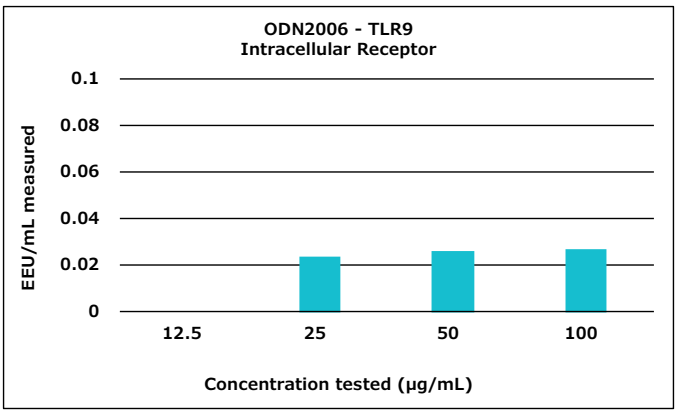


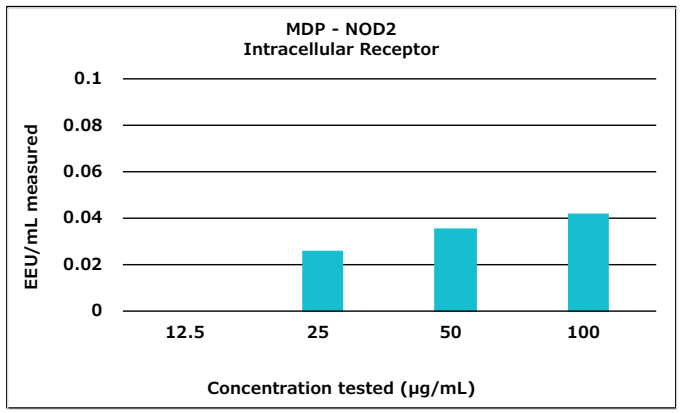












* dark blue: cell surface receptors, cyan: intracellular receptors

The non-endotoxin pyrogens tested were all detected, with values over the cut-off of the respective assay. The contaminations with MDP and ODN2006 could be detected but not quantified, as the calculated values were below the validated limit of quantification, 0.05 EU/mL.

The limits of detection for the individual pyrogens are summarized below:

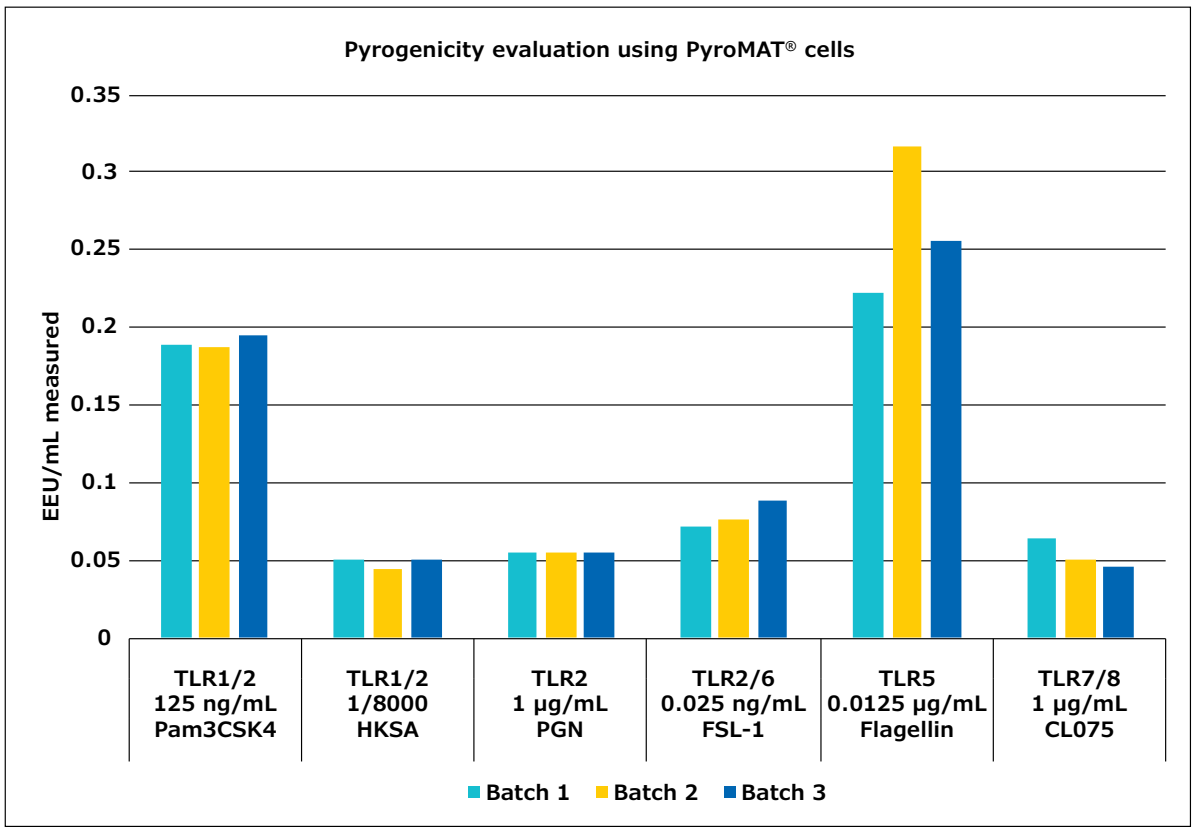
Sample	NEP	TLR	Limit of detection
1	Pam3CSK4	1/2	0.125 µg/mL
2	HKSA	2	Dilution 1/8000
3	PGN	2	1.25 µg/mL
4	FSL-1	2/6	0.01 ng/mL
5	Poly-IC	3	250 µg/mL*
6	Flagellin	5	0.0125 µg/mL
7	Imiquimod	7	100 µg/mL
8	CL075	7/8	1.25 µg/mL
9	ODN2006	9	100 µg/mL*
10	MDP	NOD2	100 µg/mL*

* highest concentration tested; detected, but quantified to be below the 0.05 EEU/mL limit

In general, non-endotoxin pyrogens recognized by a cell surface receptor did show a dose-dependent increase of pyrogenicity in the monocyte activation test, but dose-dependency was less pronounced for pyrogens recognized by intracellular receptors. This indicates that the internalization process is an important factor in the reaction and that the quantification of non-endotoxin pyrogens with intracellular receptors might be hampered by the need for the cells to internalize the pyrogens.

Stability of reaction over different cell batches

Following the assays to determine suitable concentrations of NEPs for the monocyte activation test, the stability of the reaction over several batches of PyroMAT® cells was evaluated. For this evaluation, only those pyrogens showing a dose-dependent response were used, as only for those would any differences in cell reactivity be expected to have a large influence on quantification of the contamination. The results are shown below:

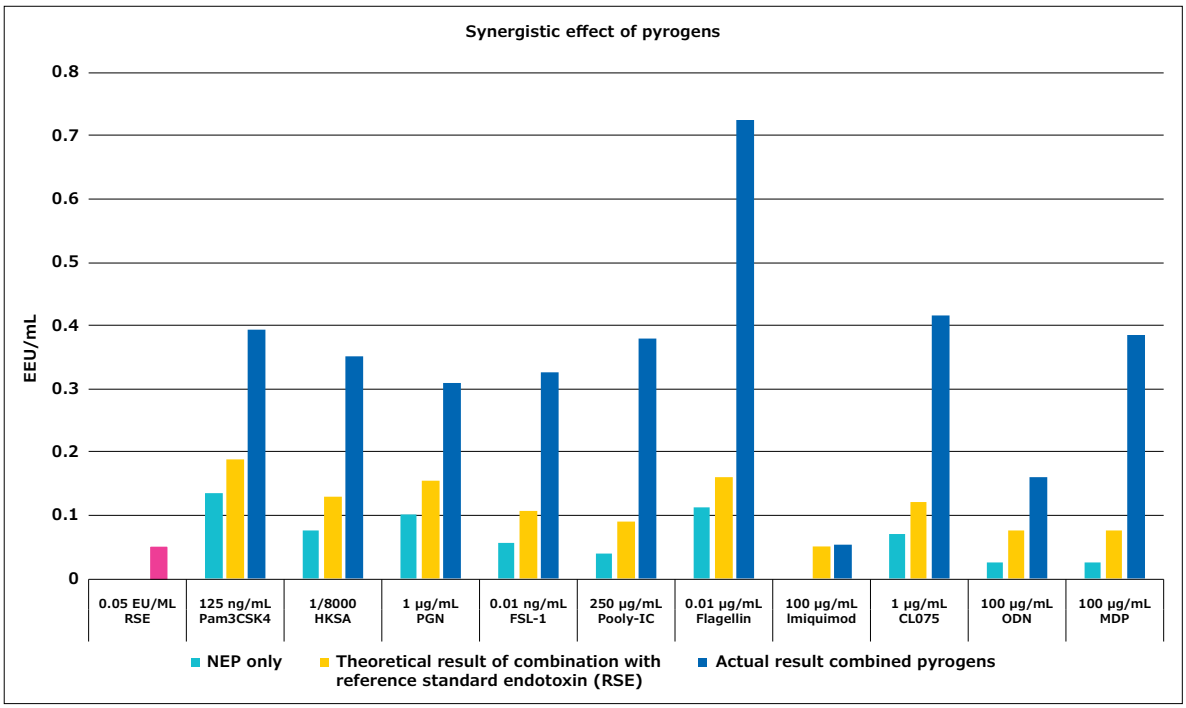


The different batches show the same reactivity for pyrogens, confirming the standardized reactivity of the Mono-Mac-6 cell line.

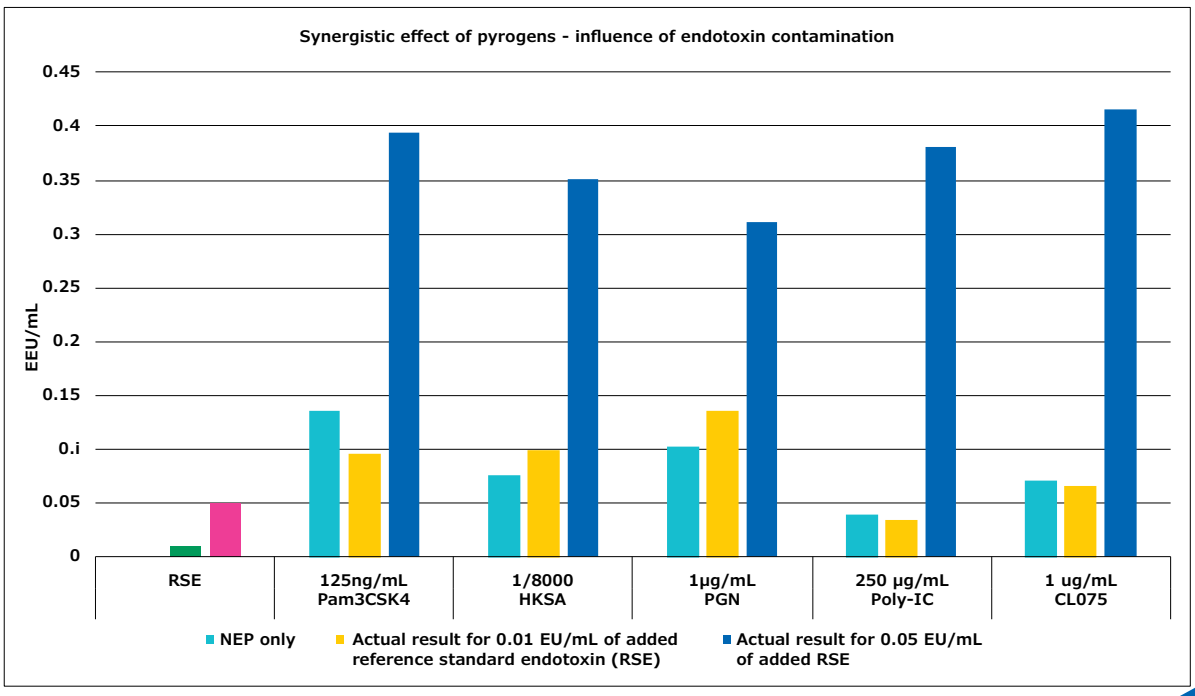
Synergistic effects of pyrogens in mixtures

Assays performed with single pyrogenic contaminants are often misleading, as contaminations in pharmaceutical products or on medical devices rarely contain just one type of pyrogen. Even in contaminations with single microorganisms, several toll-like receptors can be engaged, targeting different cell wall components or bacterial structures (e.g. in the case of flagella-bearing bacteria).

A major advantage of the monocyte activation test is its ability to show the total response of the activated monocytes, resulting in an efficient evaluation of the pyrogenicity of a mixture of pyrogens in a human test system. This was analyzed by adding endotoxin at the limit of detection of the assay to some of the above tested pyrogens at their respective limits of detection. The results reveal strong synergies for pyrogens with cell-surface and intracellular receptors, except for imiquimod.



The effect is dependent on the dose of the endotoxin present among the non-endotoxin pyrogens. This leads to a striking non-linearity of the result obtained using different dilutions of a sample contaminated with several pyrogenic entities. This highlights that the test should, wherever possible, be run with the lowest possible dilution (highest concentration) at which the sample does not interfere with the assay.



Conclusion

With this study, we have demonstrated that the Mono-Mac-6 cells used in the PyroMAT® system can detect a wide range of ligands targeting various TLRs, including intracellular ones. The MAT-based PyroMAT® system also shows a reproducible reaction to reference standard endotoxin and non-endotoxin pyrogens.

In addition, it is able to detect synergistic activation of multiple TLRs on the cell surface in the presence of e.g. endotoxin and one other non-endotoxin pyrogen. The assay is therefore capable of detecting contaminations with individual pyrogens as well as mixtures and predicting the response of the human immune system to the contamination.

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