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Studies to demonstrate the robustness and sensitivity of the PyroMAT® system for the detection of endotoxin and non-endotoxin pyrogens

Application Notes:

- Detection of **non-endotoxin pyrogens (NEP)** by Monocyte Activation Test (MAT) using the PyroMAT® system
- Quantification of pyrogen in **Hormone** with the PyroMAT® system
- Quantification of pyrogen in **Vaccine** with the PyroMAT® system
- Quantification of pyrogen in **FBS** with the PyroMAT® system
- Quantification of pyrogens in **Albumine** with the PyroMAT® system
- Comparison of **Reference Standard Endotoxins** (RSE)

White Paper:

- Monocyte Activation Test (MAT): **the *in vitro* test for pyrogen detection**
- Monocyte Activation Test: **statistical analysis**

Datasheet:

- **Validation of a cell line-based Monocyte Activation Test** method according to **USP <1225>** Validation of compendial procedures guideline

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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]

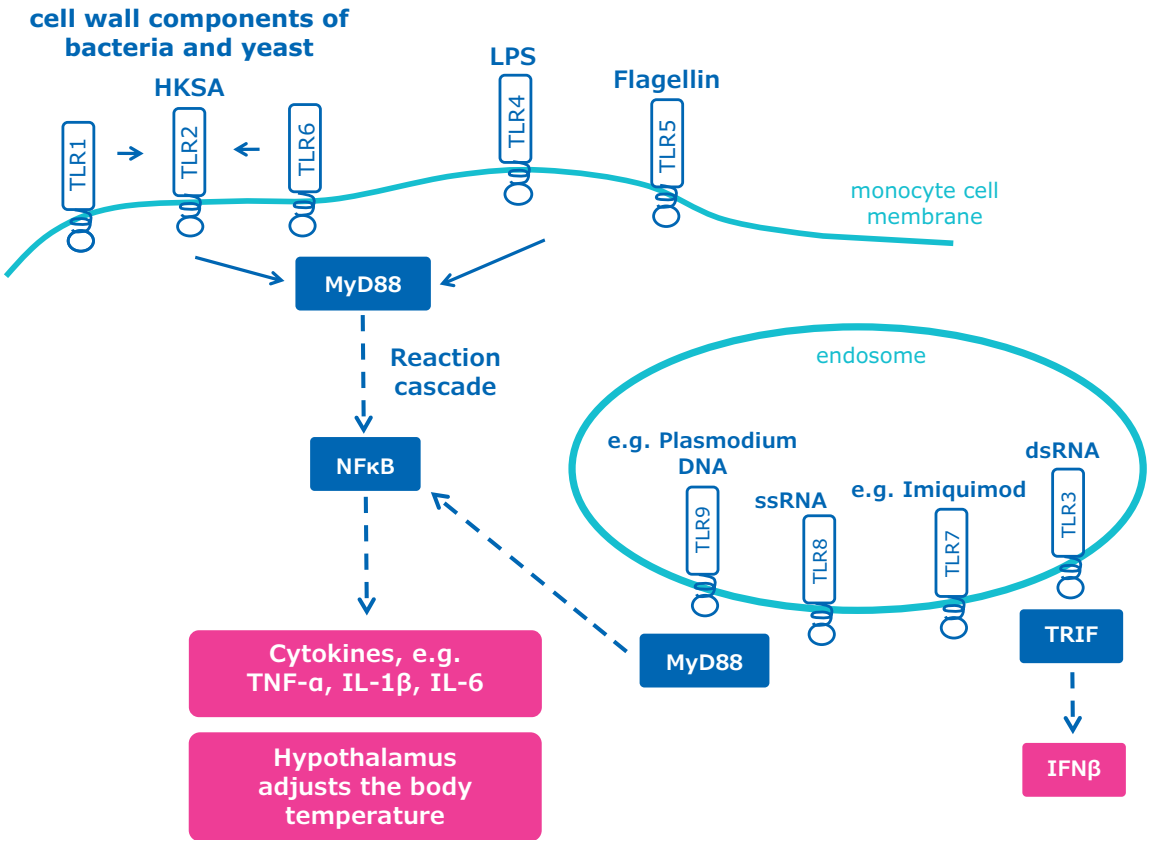


Figure 1. TLR signaling pathways

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

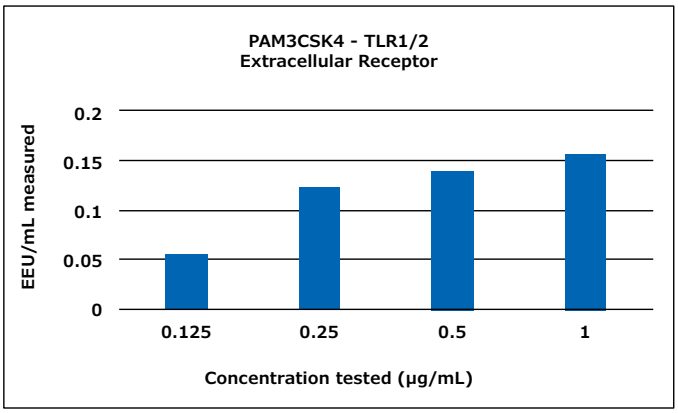
* Endotoxin concentration is given for the undiluted product (after correction of the dilution factor)

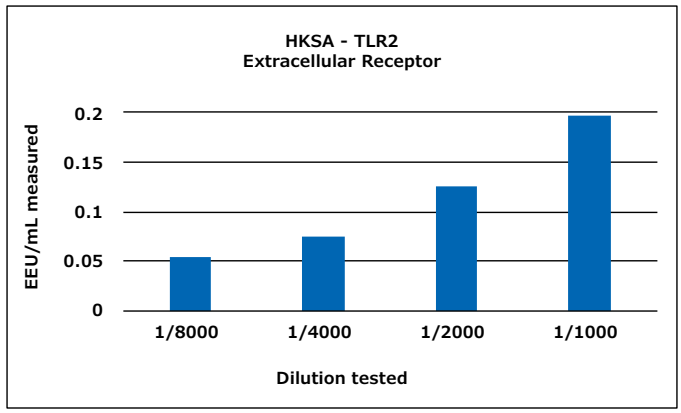
** synthetic NEPs were not tested for endotoxin contamination

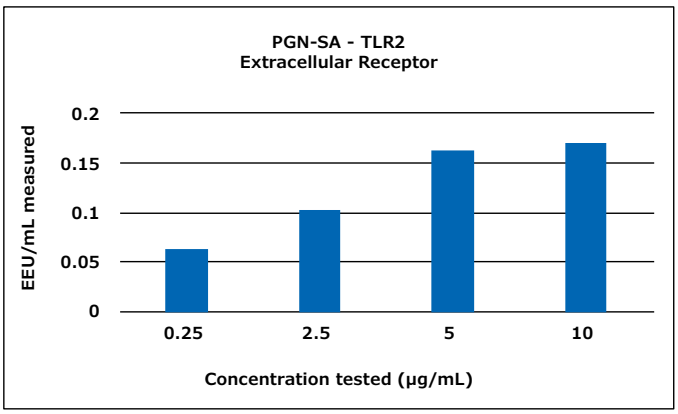
It has to be noted that the material available as non-endotoxin pyrogen is not standardized and the concentration ranges required for a reaction might differ between batches. During this study, the appropriate range was determined by dose-screening using one batch of PyroMAT® cells, followed by verification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

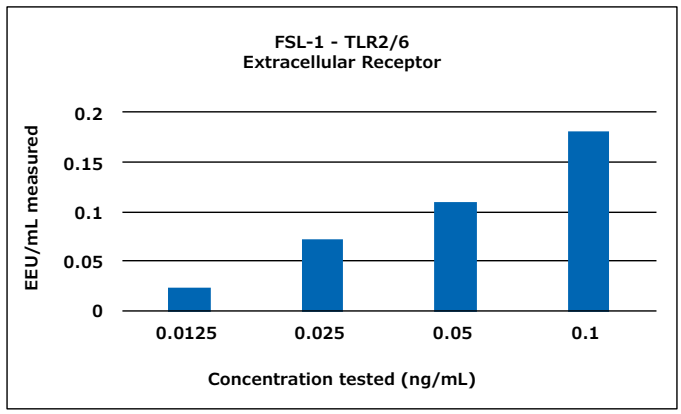
Determination of limit of detection (LOD) for the different non-endotoxin pyrogens (NEPs)

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:









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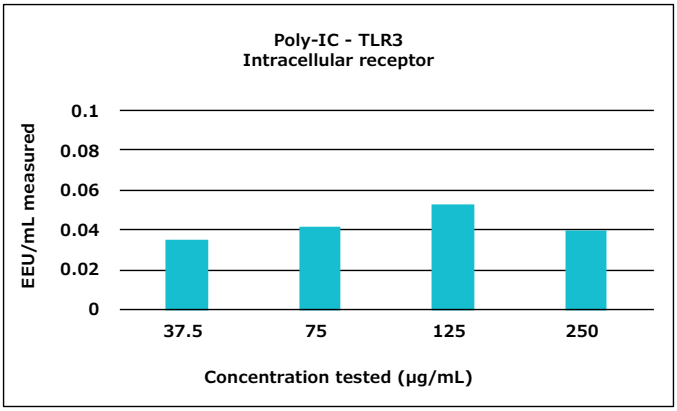
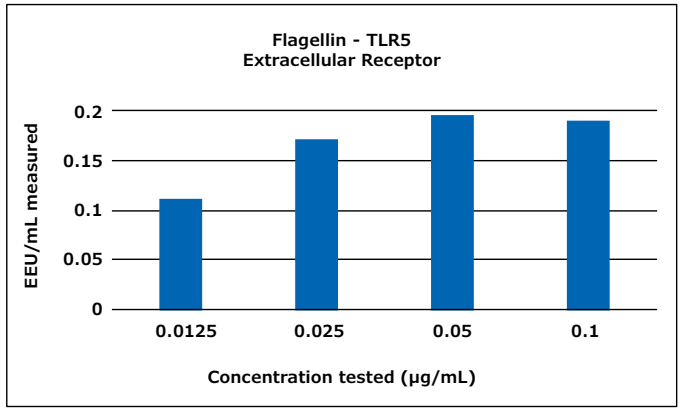
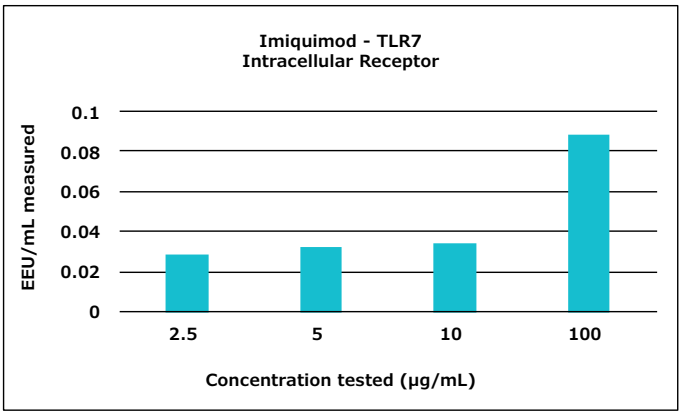
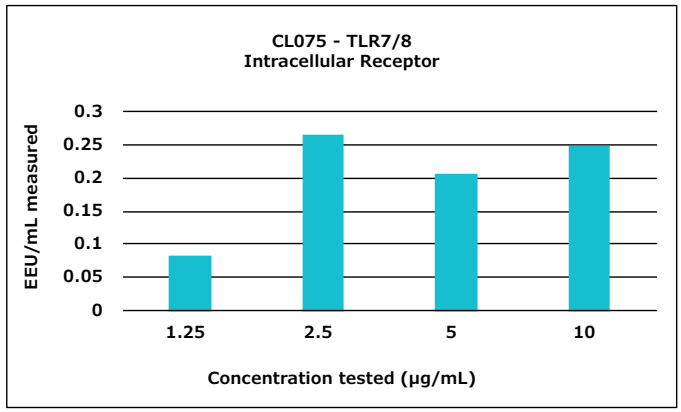
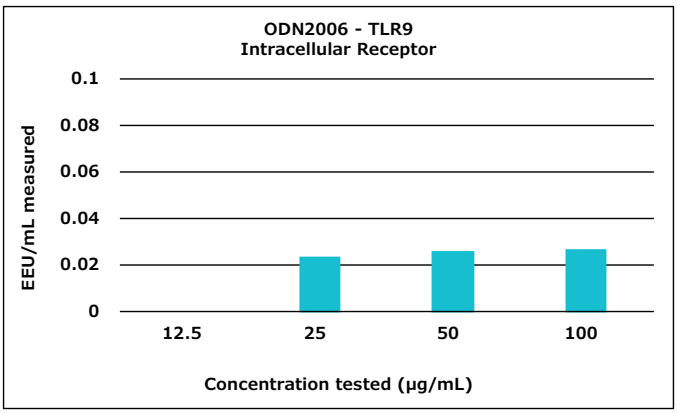
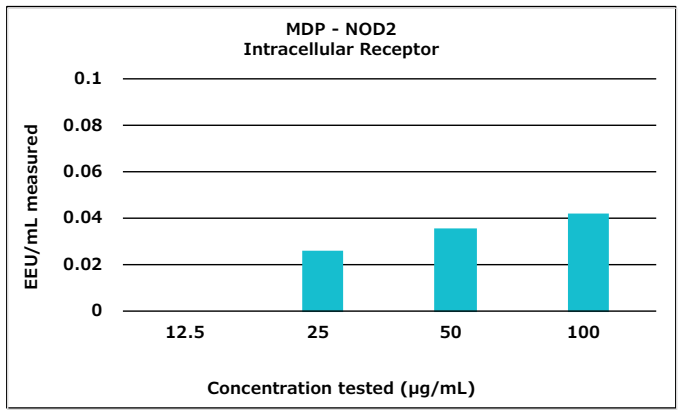












* dark blue: cell surface receptors, cyan: intracellular receptors

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:

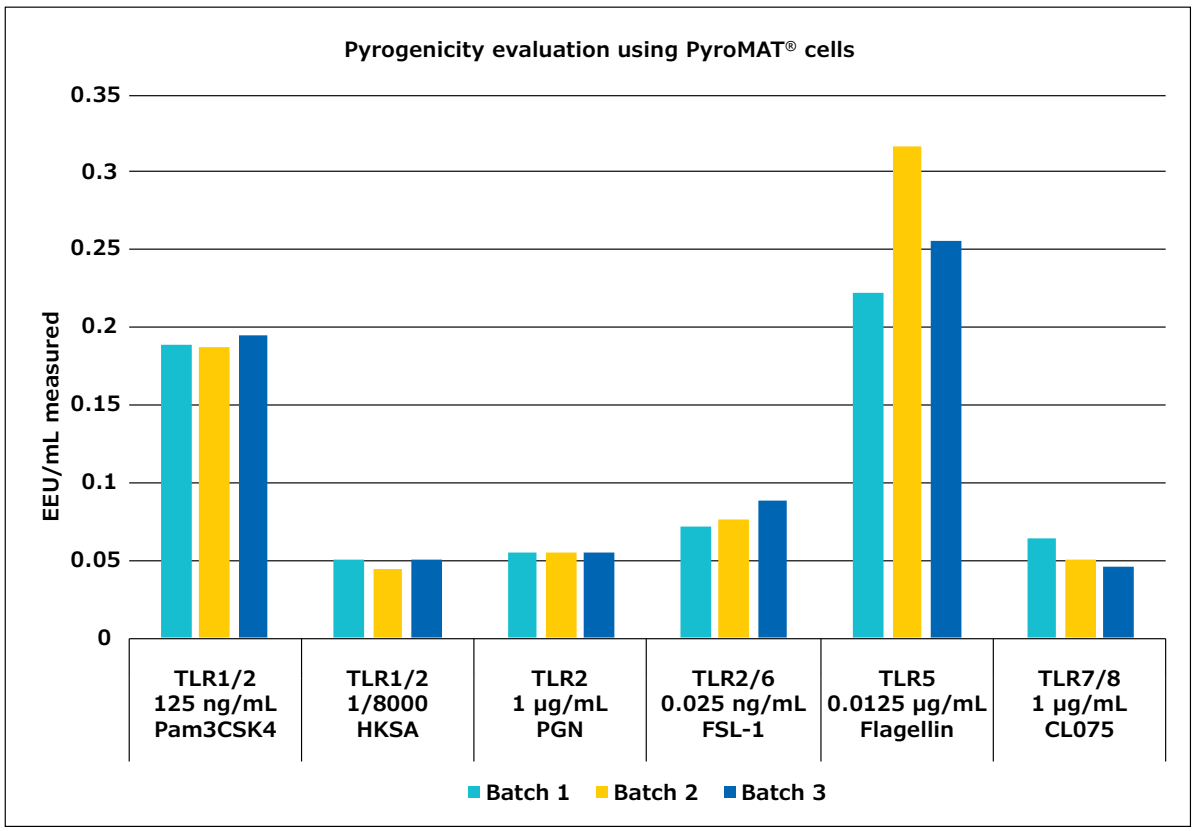
Sample	NEP	TLR	Limit of detection
1	Pam3CSK4	1/2	0.125 µg/mL
2	HKSA	2	Dilution 1/8000
3	PGN	2	1.25 µg/mL
4	FSL-1	2/6	0.01 ng/mL
5	Poly-IC	3	250 µg/mL*
6	Flagellin	5	0.0125 µg/mL
7	Imiquimod	7	100 µg/mL
8	CL075	7/8	1.25 µg/mL
9	ODN2006	9	100 µg/mL*
10	MDP	NOD2	100 µg/mL*

* 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:

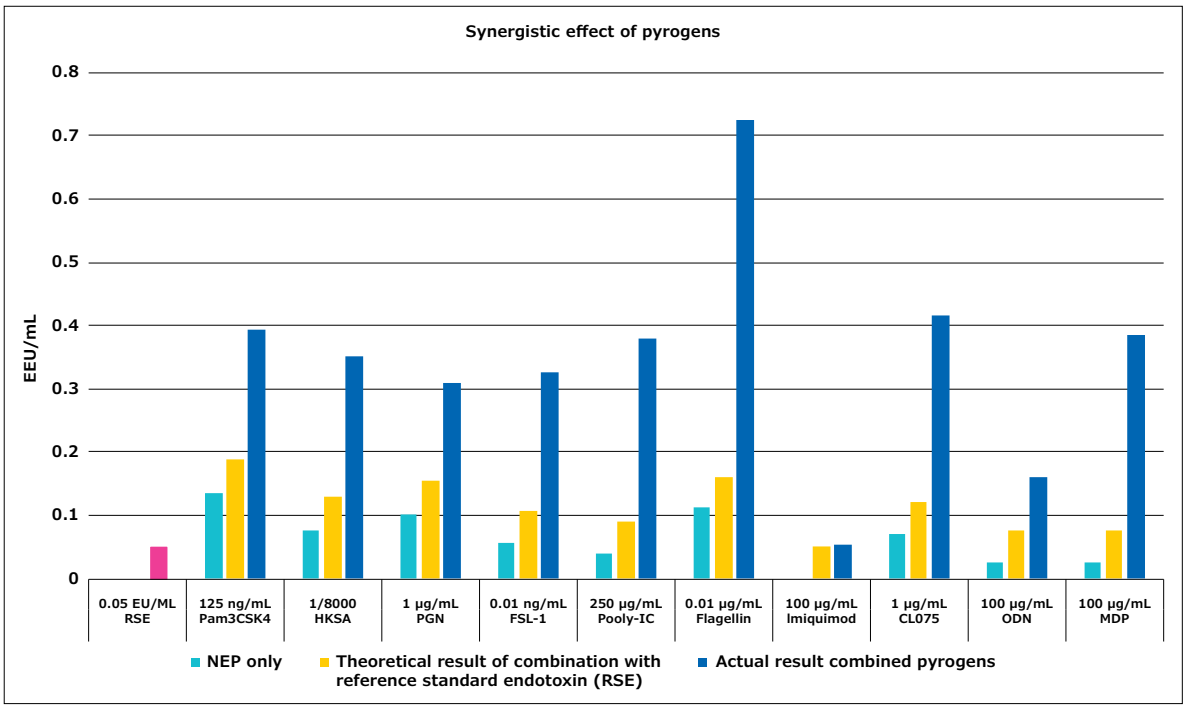


The different batches show the same reactivity for pyrogens, confirming the standardized reactivity of the Mono-Mac-6 cell line.

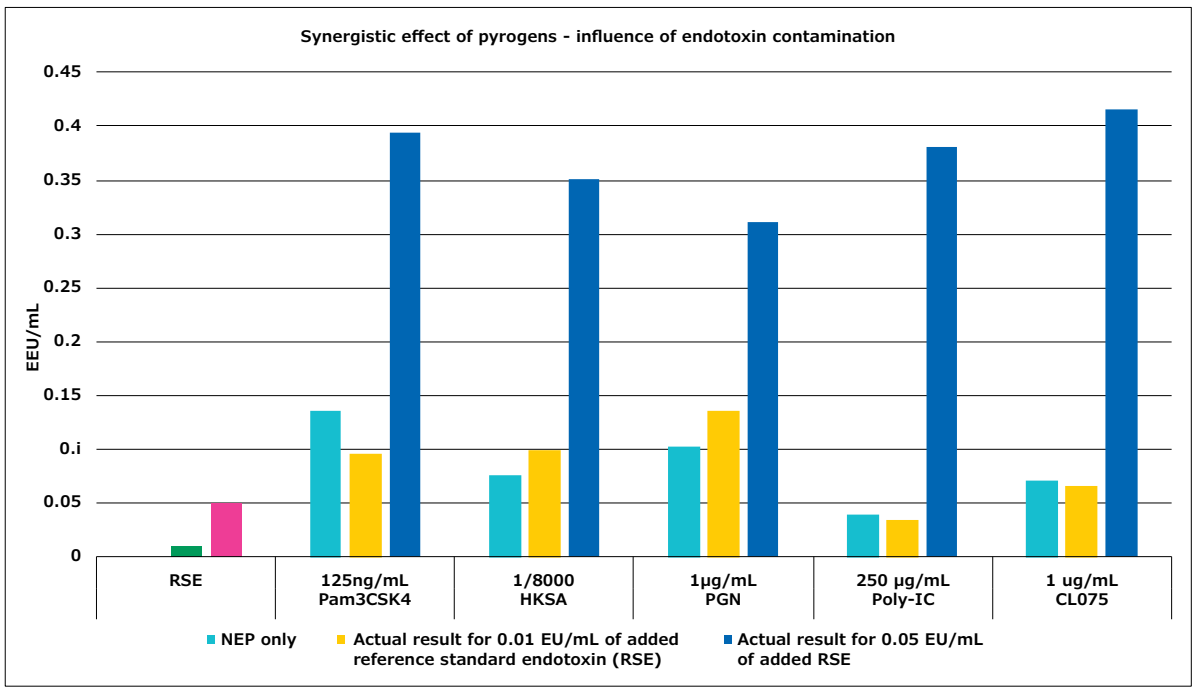
Synergistic effects of pyrogens in mixtures

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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- Quantification of pyrogen in **Hormone** with the PyroMAT® system
- Quantification of pyrogen in **Vaccine** with the PyroMAT® system
- Quantification of pyrogen in **FBS** with the PyroMAT® system
- Quantification of pyrogens in **Albumine** with the PyroMAT® system
- Comparison of **Reference Standard Endotoxins** (RSE)

White Paper:

- Monocyte Activation Test (MAT): **the *in vitro* test for pyrogen detection**
- Monocyte Activation Test: **statistical analysis**

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- **Validation of a cell line-based Monocyte Activation Test** method according to **USP <1225>** Validation of compendial procedures guideline

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Application Note

Detection of pyrogens in hormone-based drugs with 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 is the endotoxin (LPS = Lipopolysaccharide), which is produced by Gram-negative bacteria. Other microbial substances include those derived from Gram-positive bacteria such as lipoteichoic acid (LTA) or, 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 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 are required to ensure the absence of pyrogenic substances in injectable drugs. Currently, the most frequently used tests 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 and is not capable of giving any information about interactions and synergetic effects or the pyrogenic activity of the found endotoxin. Additionally, both methods are based on animals or animal products and therefore counter the principles of the 3Rs (Replacement, Reduction and Refinement) regarding animal welfare.

The purpose of the 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), in order to 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 also by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in 2008.[1]

It has been among the compendial methods for pyrogen detection in the European Pharmacopeia since 2010 (Chapter 2.6.30).[2]

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 which should be validated according to USP <1225>[3,4] 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.”[5,6]

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).[7,8]

By putting the product to be tested in contact with human monocytic cells, it mimics what happens in the human body: in presence of pyrogens, the monocytes are activated and produce cytokines such as Interleukin-1 and Interleukin-6.[9]

The cytokines are then detected using an immunological assay (ELISA) involving specific antibodies and an enzymatic color reaction.[10]

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.

Quantification of pyrogens with the MAT

For the quantification of the pyrogenic load of a sample, method A in accordance with the European Pharmacopeia 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 should be less than the CLC (Contaminant Limit Concentration) to pass the test.

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

- The criteria for the endotoxin standard curve are satisfied
- 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

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 µL – 100 µL; 100 µL – 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

Product Specific Validation (PSV) for hormone drugs

Prerequisites – European pharmacopeia, Chapter 2.6.30

Before routine testing of a pharmaceutical product with MAT, a product specific validation (PSV) must be performed according to the method chosen for routine testing to ensure the validity of the criteria for the endotoxin standard curve, the detectability of endotoxin and non-endotoxin contaminants in the sample and that the sample does not interfere with the test or detection system.

Interferences with the test can be removed by diluting the product up to a certain limit, referred to as the maximum valid dilution (MVD).

The MVD is the maximum dilution factor at which it is still possible to detect the pyrogen limit (i.e., the CLC). It is directly linked to the limit of detection (LOD) of the system.

More sensitive the system is, more the product can be diluted to remove interferences.

The MVD of a test solution is calculated using the following formula:

$$MVD = \frac{CLC \times C}{LOD}$$

CLC = Contaminant Limit Concentration (EU/mg or EU/mL)

C = Concentration of the test solution (mg/mL or mL/mL)

LOD = Limit of Detection (EU/mL).

The CLC is the acceptance criterion for the pass/fail decision, expressed in endotoxin equivalents per milligram or milliliter or per unit of the biological activity of the product.

It is calculated by the following expression:

$$CLC = \frac{K}{M}$$

K= threshold pyrogenic dose per kilogram of body mass (EU/kg)

M= maximum recommended bolus dose of product per kilogram of body mass (mg/kg or mL/kg).

When the product is injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

When testing for interfering factors, dilutions of the preparation being examined with geometric steps not exceeding the MVD should be performed. The same dilutions spiked with endotoxin at a justified concentration (in case of method A, a concentration near the estimated middle of the endotoxin standard curve) should then be performed.

These dilutions must be tested in parallel in the same experiment and together with an endotoxin standard curve, which is used to calculate the concentration of endotoxin-equivalents in each solution.

The mean recovery of the added endotoxin spike is then calculated for each dilution. The test is considered free of interference when recovery of the added endotoxin is within the range of between 50 and 200%.

Where practicable, interference testing should be performed on at least 3 different lots of the preparation being examined to investigate possible batch-to-batch variation. If the interference cannot be removed by dilution or specific sample preparation of the product within the MVD range, Method C is preferred over Method A and B.

For validation of the detection of non-endotoxin contaminants, historical batches that have been found to be contaminated with non-endotoxin contaminant causing positive responses in the rabbit pyrogens test or adverse drug reactions in man can be used. Where those batches are not available, validation should be done including at least 2 non-endotoxin ligands such as Flagellin or Heat Killed *Staphylococcus aureus* (HKSA) for toll-like receptors, one of which is to be spiked into the preparation being examined.

Once the optimum dilution of the preparation being examined has been identified, this dilution needs to be tested for interference in the detection system. The agreement between a dilution series in presence and absence for the preparation being examined is to be within ± 20% of optical density.

Sample specifications: hormone-based drugs

For definition a hormone is any signaling molecule produced by glands in multicellular organisms that are transported by the circulatory system that target distant organs to regulate physiology and behavior[11]. Hormones are used to communicate between organs and tissues for physiological regulation and behavioral activities, such as digestion, metabolism, respiration, tissue function, sensory perception, sleep, excretion, lactation, stress, growth and development, movement, reproduction, and mood.[12]

Selected hormones, their function and the effect of their deficiency in the human body are listed below. There are several pathologies characterized by hormone deficiency and consequently, related hormone drug therapies (HT) have been developed to treat these gland malfunctions.[13]

Human Growth hormone (hGH)

Human Growth hormone (hGH) stimulates growth and cell reproduction and regeneration. hGH is naturally released by the anterior pituitary gland, a pea-sized gland located at the base of the skull. Adequate level of hGH, is one of the essential factors for growth in children. Children with Growth Hormone Deficiency (GHD) do not produce enough of hGH, which has a high impact on the natural physical development. Symptoms include failure to meet height and weight growth standards. Consequently, hGH and its synthetic version, called somatropin, are used to treat this kind of disorder. Injections of somatropin can help to increase the growth rates.[14]

Human Chorionic Gonadotropin (hCG)

Human chorionic gonadotropin (hCG) hormone supports the normal development of an egg in a woman's ovary to stimulate the egg release during ovulation.[15] This hormone is produced by the placenta after implantation. The presence of hCG is detected in some pregnancy tests. hCG interacts with the LHCG receptor of the ovary and promotes the maintenance of the corpus luteum during the beginning of pregnancy. This allows the corpus luteum to secrete the hormone progesterone during the first trimester. Progesterone enriches the uterus with a thick lining of blood vessels and capillaries so that it can sustain the growing fetus. Recombinant Human Chorionic Gonadotropin (r-hCG) is the synthetic version of hCG hormone administered to stimulate ovulation, treat infertility in women and increase sperm count in men. Moreover, in case of pituitary gland disorder, r-hCG is also used in young boys when their testicles have not dropped down into the scrotum normally.

Human Follicle-Stimulating hormone (hFSH)

Follicle-stimulating hormone (FSH), also known as follitropin, is a glycoprotein gonadotropin secreted by the anterior pituitary in response to gonadotropin-releasing hormone released by the hypothalamus. hFSH regulates ovulation, the growth and development of eggs in a woman's ovaries.

In males hFSH stimulates primary spermatocytes to form secondary spermatocytes.[16] Low level of FSH secretion can result in failure of gonadal function (hypogonadism). This condition is typically manifested in males as failure in production of normal numbers of spermatozoon, and in females as cessation of reproductive cycles. The recombinant and synthetic form of hFSH (r-hFSH) is used to treat infertility in women who cannot ovulate and to stimulate sperm production in men. r-hFHS is often used in combination with another hormone, called human chorionic gonadotropin (hCG).[17]

Human Luteinizing hormone (hLH)

Luteinizing hormone (LH), also known as lutropin, is a hormone produced by gonadotropic cells in the anterior pituitary gland.[18] In females, an acute rise of LH triggers ovulation and development of the corpus luteum. In males LH stimulates Leydig cell production of testosterone.

LH acts synergistically with FSH. LH deficiency frequently occurs in conjunction with follicle-stimulating hormone (FSH) deficiency because LH and FSH are secreted by the same pituitary gonadotrope cells. LH deficiency can manifest in females or males as delayed puberty, hypogonadism at any age, or reproductive abnormalities that can be dramatic or subtle. Lutropin alpha (hLH) is used as fertility medication to help follicles (eggs) in the ovaries to develop and mature. It is used in combination with follitropin alpha (hFSH) when a women's pituitary gland does not produce enough hLH.[19]

In the present study all the suitability tests for pyrogen detection performed with the PyroMAT™ System on a selection of injectable hormone drugs are described. The hormone drugs selected are summarized below:

Name	Abbreviation	Tissue	Effect
Growth hormone	hGH	Anterior pituitary gland	Stimulates growth and cell reproduction
Human chorionic gonadotropin	hCG	Placenta	Promotes maintenance of corpus luteum during beginning of pregnancy
Follicle-stimulating hormone	hFSH	Anterior pituitary gland	In female: stimulates maturation of Graafian follicles in the ovary
Luteinizing hormone	hLH	Anterior pituitary gland	In female: ovulation In male: stimulates Leydig cell

Low Endotoxin Recovery (LER) phenomenon is well known in the Bacterial Endotoxin test (BET) as the inability of the assay to detect lipopolysaccharide (LPS), due to a “masking effect” caused by chelators or detergents commonly used in buffer formulations for medical products and recombinant proteins.[20] In the presence of LER effect, the masked LPS is considered a potential danger, as it may pose a health threat in pharmaceutical products or compromise experimental results.[21,22, 23]

Consequently, with the aim to verify that in the final drug formulation, in addition to the active ingredient, the excipients (such as proteins, surfactants, essential amino acids, salts and preservatives) do not interfere with the pyrogen detection giving the LER effect[24] endotoxin, a group of drug substances (active ingredients) and drug products (final formulation containing excipients) were tested using the PyroMAT™ System.

The drug substances and drug products tested in the present study were:

- hGH drug substance
- hCG drug substance
- hCG drug product
- hFSH drug substance
- hFSH drug product
- hLH drug substance
- hLH drug product

PSV plate layout

All the Product Specific Validation tests have been processed using the following plate layout scheme. The data analysis was performed with the Gen5™ ver3.03 software commercialized by BioTek, and the PyroMAT_PSV_I_II_III_A protocol that can be downloaded for free on our website.

The tested dilutions are specific for each drug and are described in the following sections.

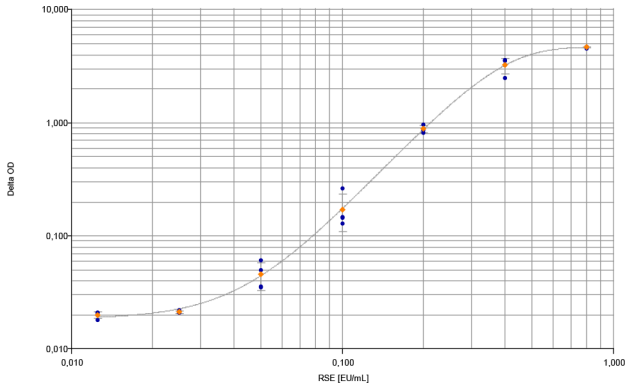
	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK	BLK	BLK	BLK	SPL1:1	SPL1:1	SPL1:1	SPL1:1	SPL1:3	SPL1:3	SPL1:3	SPL1:3	Well ID
					100	100	100	100	400	400	400	400	Conc/Dil
B	STD1	STD1	STD1	STD1	SPL1_RSE:1	SPL1_RSE:1	SPL1_RSE:1	SPL1_RSE:1	SPL1_RSE:3	SPL1_RSE:3	SPL1_RSE:3	SPL1_RSE:3	Well ID
	0,0125	0,0125	0,0125	0,0125	100	100	100	100	400	400	400	400	Conc/Dil
C	STD2	STD2	STD2	STD2	SPL1_NEP1:1	SPL1_NEP1:1	SPL1_NEP1:1	SPL1_NEP1:1	SPL1_NEP1:3	SPL1_NEP1:3	SPL1_NEP1:3	SPL1_NEP1:3	Well ID
	0,025	0,025	0,025	0,025	100	100	100	100	400	400	400	400	Conc/Dil
D	STD3	STD3	STD3	STD3	SPL1:2	SPL1:2	SPL1:2	SPL1:2	SPL1:4	SPL1:4	SPL1:4	SPL1:4	Well ID
	0,05	0,05	0,05	0,05	200	200	200	200	800	800	800	800	Conc/Dil
E	STD4	STD4	STD4	STD4	SPL1_RSE:2	SPL1_RSE:2	SPL1_RSE:2	SPL1_RSE:2	SPL1_RSE:4	SPL1_RSE:4	SPL1_RSE:4	SPL1_RSE:4	Well ID
	0,1	0,1	0,1	0,1	200	200	200	200	800	800	800	800	Conc/Dil
F	STD5	STD5	STD5	STD5	SPL1_NEP1:2	SPL1_NEP1:2	SPL1_NEP1:2	SPL1_NEP1:2	SPL1_NEP1:4	SPL1_NEP1:4	SPL1_NEP1:4	SPL1_NEP1:4	Well ID
	0,2	0,2	0,2	0,2	200	200	200	200	800	800	800	800	Conc/Dil
G	STD6	STD6	STD6	STD6	NEP1	NEP1	NEP1	NEP1					Well ID
	0,4	0,4	0,4	0,4	1	1	1	1					Conc/Dil
H	STD7	STD7	STD7	STD7	NEP2	NEP2	NEP2	NEP2					Well ID
	0,8	0,8	0,8	0,8	1	1	1	1					Conc/Dil

Figure 1. Example of plate layout for PSV Method A

Assurance of the criteria for the endotoxin standard curve:

For all the following PSV tests a standard curve using Reference Standard Endotoxin (RSE) was performed to verify that the criteria for the endotoxin standard curve were valid.

- The regression of response on log dose was statistically significant ($p < 0.01$)
- The regression of response on log dose did not deviate significantly from linearity ($p > 0.05$)



RSE standard curve validity crieria	Effect of Dose	Goodness of fit
	Valid	Valid
	Blank delta OD	**LOD**
	Valid	Valid

Figure 2. Example of Endotoxin Standard Curve obtained with the PyroMAT™ System

Test for interfering factors and method validation for detection of non-endotoxin pyrogens

A test for interfering factors and method validation for non-endotoxin pyrogens (NEPs) according to EP was performed, comparing endotoxin-spiked sample dilutions as well as NEP-spiked sample dilutions with the same unspiked sample dilutions.

PSV for hGH drug product

The hGH drug product Maximum Valid Dilution (MVD) calculation is reported in the table below.

Sample	Molecular Name	CLC (EU/mL)	PyroMAT™ Limit of Detection (EU/mL)	MVD
hGH Drug Product	Somatropin	≤40	0.05	800

The experiment on hGH drug product was carried out using the dilutions 1:100, 1:200, 1:400 and 1:800. In order to demonstrate the method robustness, tests were carried out using two different PyroMAT™ Cell lots.

The tested dilution range showed valid NEP control detection and RSE spike recoveries.

		Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve Assay 1 – 2 – 3		Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system Assay 1 - 2 -3		Valid			
		Dil 1:100	**Dil 1:200**	**Dil 1:400**	**Dil 1:800**
Results EEU* / mL Assay 1 – 2 – 3		< 5.00	<10.00	<20.00	<40.00
RSE Spike Recovery %	Assay 1	92.3%	68.5%	77.0%	92.2%
	Assay 2	83.2%	72.5%	78.5%	87.5%
	Assay 3	106.0%	88.9%	93.0%	93.4%
HKSA Detection in the sample Assay 1 – 2 – 3		Valid	Valid	Valid	Valid

*Endotoxin Equivalent Units

In summary, all the results coming from the three PSV experiments were consistent and valid. To verify if the pure sample could give interference with the PyroMAT™ test, a fourth PSV experiment was carried out using a series of lower dilutions (1:1, 1:25, 1:50 and 1:100).

Data results are showed below:

Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Dil. Factor <= MVD	Spike Rec. (%)	Nep Detection	Conclusion
hGH -DP	1	800	0.009	0.009	6.8	40	<	0.05	Valid	0	Invalid	Invalid
	1		0.009									
	1		0.008									
	1		0.008									
	25	800	0.016	0.017	9	40	<	1.25	Valid	73.6	Valid	Valid
	25		0.018									
	25		0.018									
	25		0.015									
	50	800	0.016	0.016	6.5	40	<	2.5	Valid	97.3	Valid	Valid
	50		0.016									
	50		0.016									
	50		0.014									
	100	800	0.015	0.021	53.7	40	<	5	Valid	86.6	Valid	Valid
	100		0.015									
	100		0.037									
	100		0.015									

The undiluted product (1:1) showed interference with the endotoxin spike (RSE) detection:

- Spike Recovery % = 0

Moreover, the non-endotoxin pyrogen (NEP) control was not detectable in the undiluted product:

- NEP Detection = INVALID

From 1:25 dilution to the MVD, all dilutions showed again normal NEP and RSE controls detection.

In the end, to confirm the lowest working dilution, a fifth experiment was carried out using the following dilution series: 1:10, 1:25, 1:50 and 1:100.

Data and results are shown below:

Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Dil. Factor <= MVD	Spike Rec. (%)	Nep Detection	Conclusion
hGH -DP	10	800	0.015	0.015	3.4	40	<	0.5	Valid	74.5	Valid	Valid
	10		0.015									
	10		0.015									
	10		0.014									
	25	800	0.016	0.015	6.9	40	<	1.25	Valid	97.1	Valid	Valid
	25		0.014									
	25		0.014									
	25		0.014									
	50	800	0.014	0.014	0	40	<	2.5	Valid	77.7	Valid	Valid
	50		0.014									
	50		0.014									
	50		0.014									
	100	800	0.015	0.015	6.9	40	<	5	Valid	74.5	Valid	Valid
	100		0.015									
	100		0.013									
	100		0.015									

In the last experiment, the 1:10 dilution gave valid results for both endotoxin spike recovery and non-endotoxin pyrogen (NEP) control detection.

Test for interference in the detection system:

The defined optimum dilution (Dil 1:10) was forwarded to a test for interference in the detection system (ELISA). A dilution series of IL-6 control was tested in absence and presence of the sample dilution. All IL-6 control dilutions showed less than 20% difference between dilution in absence and presence of the product, therefore no interference with the detection system could be found.

Conclusion:

In conclusion, a total of six different experiments were carried out on hGH drug product. The results were valid and in accordance to the defined acceptance criteria. Moreover the tests results showed that the PyroMAT™ system is applicable for detection of pyrogens in hGH drug product.

All the tested dilutions are reported in the table below:

Dilution Tested								
DP	1:1	1:10	1:25	1:50	1:100	1:200	1:400	1:800
	INVALID	VALID	VALID	VALID	VALID	VALID	VALID	VALID

Assurance of criteria for the standard curve	Valid
Test for interfering factors	Valid
Detection of NEP contaminants	Valid
Interference in the detection system	Valid

All acceptance criteria for the product specific validation were fulfilled and the dilution 1:10 of the drug product was chosen as the first valid dilution to be tested in routine with Method A.

PSV for hCG drug substance and drug product

The hCG drug substance and drug product Maximum Valid Dilutions (MVD) calculation to be tested are reported in the table below.

Sample	Molecular Name	CLC (EU/mL)	PyroMAT™ Limit of Detection (EU/mL)	MVD
hCG drug substance	Choriogonadotropin alfa	≤ 28	0.05	560
hCG drug products	Choriogonadotropin alfa	≤ 30	0.05	600

For hCG drug substance, the experiment was carried out using the dilutions 1:10, 1:20, 1:40 and 1:400.

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:10	**Dil 1:20**	**Dil 1:40**	**Dil 1:400**
Results EEU / mL	<0.50	<1.00	<2.00	<20.00
RSE Spike Recovery %	106.0%	103.4%	87.2%	81.0%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

All tested dilutions were valid with detection of NEP controls and RSE spike recoveries within the range of 50-200%, as defined in the acceptance criteria.

With the aim to verify that the excipients present in the final drug formulation do not interfere with the pyrogen detection, the related hCG drug product was also tested, using dilutions 1:8, 1:16, 1:32 and 1:600.

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:8	**Dil 1:16**	**Dil 1:32**	**Dil 1:600**
Results EEU / mL	<0.40	<0.80	<1.60	<30.00
RSE Spike Recovery %	93.7%	98.3%	82.7%	80.0%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

The experiment performed on the drug product confirmed that all tested dilutions, from 1:8 dilution to 1:600, were valid and NEP and RSE controls were correctly detected.

Conclusion:

In conclusion, the tests performed on the hCG drug substance and drug product were valid and in accordance with the defined acceptance criteria. Moreover the test results showed that the PyroMAT™ system is applicable to detect pyrogens in hCG drug substance (DS) and drug product (DP).

Dilution Tested				
DP	1:8	1:16	1:32	1:600
	VALID	VALID	VALID	VALID
DS	1:10	1:20	1:40	1:400
	VALID	VALID	VALID	VALID

Assurance of criteria for the standard curve	Valid
Test for interfering factors	Valid
Detection of NEP contaminants	Valid
Interference in the detection system	Valid

All acceptance criteria for the product specific validation were fulfilled and the dilution 1:8 of the drug product was chosen as the first valid dilution to be tested in routine with Method A.

PSV for hFSH drug substance and drug product

The hFSH drug substance and drug product maximum valid dilution (MVD) calculation is reported in the table below.

Sample	Molecular Name	CLC (EU/mL)	PyroMAT™ Limit of Detection (EU/mL)	MVD
hFSH drug substance	Follitropin alpha	≤1	0.05	20
hFSH drug products	Follitropin alpha	≤12	0.05	240

The experiment on hFSH drug substance was carried out using the dilutions 1:1, 1:2, 1:10 and 1:20. In order to demonstrate the method robustness, tests were carried out using two different PyroMAT™ Cell lots.

The tested dilution range showed valid NEP control detection and RSE spike recoveries.

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:1	**Dil 1:2**	**Dil 1:10**	**Dil 1:20**
Results EEU / mL	<0.05	<0.10	<0.50	<1.00
RSE Spike Recovery %	90.3%	93.1%	89.5%	95.3%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

From the pure drug substance (1:1) to the MVD, all dilutions showed valid NEP and RSE controls detection.

With the aim to verify that the excipients present in the final drug formulation do not interfere with the pyrogen detection, the related hFSH drug product was tested using dilutions 1:8, 1:16, 1:32 and 1:240.

		Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve Assay 1 – 2 – 3		Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system Assay 1 - 2 -3		Valid			
		Dil 1:8	**Dil 1:16**	**Dil 1:32**	**Dil 1:240**
Results EEU* / mL Assay 1 – 2 – 3		<0.40	<0.80	<1.60	<12.0
RSE Spike Recovery %	**Assay 1**	68.2%	73.5%	83.0%	87.5%
	Assay 2	73.8%	80.0%	63.2%	69.5%
	Assay 3	75.3%	85.2%	73.3%	76.8%
HKSA Detection in the sample Assay 1 – 2 – 3		Valid	Valid	Valid	Valid

Summarizing, all the results coming from the three PSV experiments were consistent and valid. The experiment performed on the pure hFSH drug substance (1:1) showed valid results for the endotoxin spike recovery and for the non-endotoxin pyrogen (NEP) controls detection.

For hFSH drug product experiments, starting from 1:8 dilution up to the MVD, all conditions showed valid NEP and RSE controls results.

Test for interference in the detection system:

The defined optimum dilution (Dil 1:8) was forwarded to a test for interference in the detection system. A dilution series of IL-6 control was tested in absence and presence of the sample dilution. All IL-6 control dilutions showed less than 20% difference between dilution in absence and presence of the product, therefore no interference with the detection system could be found.

Conclusion:

In conclusion, a total of four different PSV tests were performed on hFSH drug substance and drug product. The results were valid and in accordance with the defined acceptance criteria. Moreover the test results show that the PyroMAT™ System is applicable to detect pyrogens in hFSH drug substance (DS) and drug product (DP).

The tested dilutions are reported in the table below:

Dilution Tested				
DP	1:8	1:16	1:32	1:240
	VALID	VALID	VALID	VALID
DS	1:1	1:2	1:10	1:20
	VALID	VALID	VALID	VALID

Assurance of criteria for the standard curve	Valid
Test for interfering factors	Valid
Detection of NEP contaminants	Valid
Interference in the detection system	Valid

All acceptance criteria for the product specific validation were fulfilled and the dilution 1:8 of the drug product was chosen as the first valid dilution to be tested in routine with Method A.

PSV for hLH drug substance and drug product

The hLH drug substance and drug product maximum valid dilutions (MVD) calculation are reported in the table below.

Sample	Molecular Name	CLC (EU/mL)	PyroMAT™ Limit of Detection (EU/mL)	MVD
hLH drug substance	Lutropin Alfa	≤ 100	0.05	2000
hLH drug products	Lutropin Alfa	≤ 8	0.05	160

The experiment on hLH drug substance was carried out using the dilutions 1:10, 1:20, 1:40 and 1:160.

The tested dilution range showed valid NEP control detection and RSE spike recoveries.

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:10	**Dil 1:20**	**Dil 1:40**	**Dil 1:160**
Results EEU / mL	<0.50	<1.00	<2.00	<8.00
RSE Spike Recovery %	85.4%	96.8%	101.3%	105.5%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

All tested dilutions were valid and able to detect NEP and RSE controls within the range of 50-200%, as defined in the acceptance criteria.

With the aim to verify that the excipients present in the final drug formulation, do not interfere with the pyrogen detection the related hLH drug product was tested, using the same experimental conditions (Dilutions 1:10, 1:20, 1:40 and 1:160).

The hLH drug product results are showed below:

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:10	**Dil 1:20**	**Dil 1:40**	**Dil 1:160**
Results EEU / mL	<0.50	<1.00	<2.00	<8.00
RSE Spike Recovery %	100.3%	95.2%	111.4%	101.0%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

All tested dilutions (1:10, 1:20, 1:40 and 1:160) were valid and showed normal NEP control detection and RSE spike recoveries.

Conclusion:

In conclusion, the performed tests on hLH drug substance and drug product were valid and in accordance to the defined acceptance criteria. Moreover the test results showed that the PyroMAT™ System is applicable for detection of pyrogens in hLH drug substance (DS) and drug product (DP).

Dilution Tested				
DS/DP	1:10	1:20	1:40	1:160
	VALID	VALID	VALID	VALID

Assurance of criteria for the standard curve	Valid
Test for interfering factors	Valid
Detection of NEP contaminants	Valid
Interference in the detection system	Valid

All acceptance criteria for the product specific validation were fulfilled and the dilution 1:10 of the drug product was chosen as the first valid dilution to be tested with Method A.

Quantitative Pyrogen Test with Method A according to EP

Planning Test Execution

Once a valid dilution has been identified through the Product Specific Validation, Method A (quantitative test) can be performed to assess the pyrogenicity of samples from the same drug product.

The lowest dilution / highest concentration of the sample, that was found free of interference in the PSV is to be chosen as initial dilution and 2-fold serial dilutions are tested.

For quantification, the middle section of the dose-response curve of the endotoxin standard is preferred, giving the most exact results, while quantification within the upper plateau of the standard curve can lead to inaccuracy, this is due to reaching the endpoint of the reaction and is therefore not recommended.

For the most exact results, we therefore recommend analyzing sample dilutions which do not exceed the measuring range of 0.05 to 0.4 EU/mL.

Testing of r-hFSH and r-hGH drug products with method A

The test setup was performed according to the user guide of the PyroMAT™ system.

An endotoxin standard curve was performed for the test.

Three dilutions of r-hFSH and r-hGH drug products were tested according to method A described in Eu. Ph. 2.6.30.

The dilutions 1:8, 1:16 and 1:32 of r-hFSH drug product and dilutions 1:10, 1:20 and 1:40 of r-hGH drug product were all tested with and without endotoxin spike. A quantification of the found endotoxin equivalents for all dilutions of the unspiked and spiked sample was carried out using the endotoxin standard curve. The endotoxin spike recovery for all three sample dilutions was calculated.

Heat killed *Staphylococcus aureus* (HKSA) was used as additional control for detection of non-endotoxin pyrogens within the sample, spiked in the highest concentration of the product to be examined.

Data interpretation

The data analysis was performed with Gen5™ software version 3.03 and the PyroMAT™ Software Method A available on our website. Information related to the sample were completed directly on the software: sample name, CLC.

For interpretation, the layout for method A was filled with the appropriate dilution factors for this sample matrix.

					r-hFSH DP				r-hGH DP				
	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK	BLK	BLK	BLK	SPL1:1 8	SPL1:1 8	SPL1:1 8	SPL1:1 8	SPL2:1 10	SPL2:1 10	SPL2:1 10	SPL2:1 10	Well ID Conc/Dil
B	STD1 0,0125	STD1 0,0125	STD1 0,0125	STD1 0,0125	SPL1_RSE:1 8	SPL1_RSE:1 8	SPL1_RSE:1 8	SPL1_RSE:1 8	SPL2_RSE:1 10	SPL2_RSE:1 10	SPL2_RSE:1 10	SPL2_RSE:1 10	Well ID Conc/Dil
C	STD2 0,025	STD2 0,025	STD2 0,025	STD2 0,025	SPL1:2 16	SPL1:2 16	SPL1:2 16	SPL1:2 16	SPL2:2 20	SPL2:2 20	SPL2:2 20	SPL2:2 20	Well ID Conc/Dil
D	STD3 0,05	STD3 0,05	STD3 0,05	STD3 0,05	SPL1_RSE:2 16	SPL1_RSE:2 16	SPL1_RSE:2 16	SPL1_RSE:2 16	SPL2_RSE:2 20	SPL2_RSE:2 20	SPL2_RSE:2 20	SPL2_RSE:2 20	Well ID Conc/Dil
E	STD4 0,1	STD4 0,1	STD4 0,1	STD4 0,1	SPL1:3 32	SPL1:3 32	SPL1:3 32	SPL1:3 32	SPL2:3 40	SPL2:3 40	SPL2:3 40	SPL2:3 40	Well ID Conc/Dil
F	STD5 0,2	STD5 0,2	STD5 0,2	STD5 0,2	SPL1_RSE:3 32	SPL1_RSE:3 32	SPL1_RSE:3 32	SPL1_RSE:3 32	SPL2_RSE:3 40	SPL2_RSE:3 40	SPL2_RSE:3 40	SPL2_RSE:3 40	Well ID Conc/Dil
G	STD6 0,4	STD6 0,4	STD6 0,4	STD6 0,4	SPL1_NEP 8	SPL1_NEP 8	SPL1_NEP 8	SPL1_NEP 8	SPL2_NEP 10	SPL2_NEP 10	SPL2_NEP 10	SPL2_NEP 10	Well ID Conc/Dil
H	STD7 0,8	STD7 0,8	STD7 0,8	STD7 0,8	NEP 1	NEP 1	NEP 1	NEP 1					Well ID Conc/Dil

After reading the plate, the data interpretation was performed with the software.

The standard curve was valid for all the criteria.

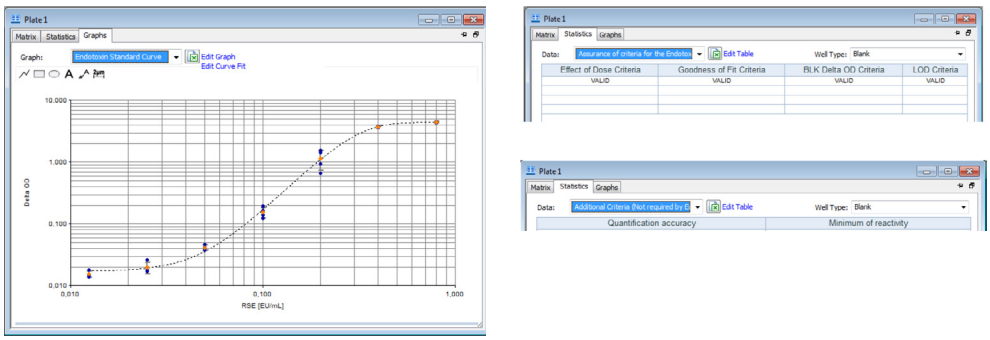


Figure 3. Standard curve obtained with PyroMAT™ Software - Method A

The NEP-control confirmed detection of non-endotoxin pyrogens in the system and in the sample.

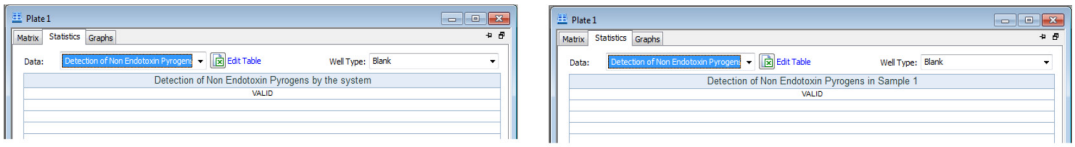


Figure 4. Result of NEP control detection in water and spike in the sample obtained with PyroMAT™ Software - Method A

The chosen sample dilutions were appropriate for both samples with all tested dilutions being within the MVD and spike recovery within 50-200%.

The data analysis showed validity of the test being fulfilled for all sample dilutions.

The r-hFSH sample itself showed a pyrogenicity <0.4 EU/mL that is below the CLC (12 EU/mL) and therefore being considered “not pyrogenic”.

Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Spike Rec. (%)	Conclusion
r-hFSH DP	8	240	0.014	0.013	30.1	12	<	0.4	64.2	PASS
	8		0.018							
	8		0.009							
	8		0.011							
	16	240	0.013	0.015	15	12	<	0.8	66.3	PASS
	16		0.018							
	16		0.014							
	16		0.014							
	32	240	0.013	0.013	4.6	12	<	1.6	70.3	PASS
	32		0.013							
	32		0.012							
	32		0.012							

The r-hGH sample itself showed a pyrogenicity <0.5 EU/mL that is below the CLC (40 EU/mL) and therefore being considered “not pyrogenic”.

Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Spike Rec. (%)	Conclusion
r-hGH DP	10	800	0.014	0.014	11.2	40	<	0.5	70.5	PASS
	10		0.012							
	10		*0.159*							
	10		0.015							
	20	800	*0.047*	0.014	7.1	40	<	1	82.5	PASS
	16		0.014							
	16		0.013							
	16		0.015							
	32	800	0.015	0.023	49.8	40	<	2	75	PASS
	32		0.03							
	32		0.012							
	32		0.036							

NOTE: The numeric values reported among asterisks *nnn* were considered outlier and not taken into account for the final calculation.

Conclusion

The capability of PyroMAT™ System to detect pyrogens in hormone drug substances and drug products was shown and is comparable to the results from the MAT system evaluation on drugs from other laboratories.[25,26,27]

The data shows that the PyroMAT™ System is suitable for detecting both endotoxin and non-endotoxin pyrogenic contaminations in drug products. Moreover, plate repetitions carried out using two different PyroMAT™ cell lots, increasing experiment variability, demonstrate that the test results are cell batch independent.

The examined recombinant hormones (drug products) occasionally led to inhibition of the monocyte reaction in the undiluted sample (see hGH drug product experiments), but this could be overcome by dilution within the authorized dilution range (not exceeding the MVD).

In conclusion, the PyroMAT™ System is a valid system for pyrogens detection in hormonal pharmaceutical products.

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Studies to demonstrate the robustness and sensitivity of the PyroMAT® system for the detection of endotoxin and non-endotoxin pyrogens

Application Notes:

- Detection of **non-endotoxin pyrogens (NEP)** by Monocyte Activation Test (MAT) using the PyroMAT® system
- Quantification of pyrogen in **Hormone** with the PyroMAT® system
- Quantification of pyrogen in **Vaccine** with the PyroMAT® system
- Quantification of pyrogen in **FBS** with the PyroMAT® system
- Quantification of pyrogens in **Albumine** with the PyroMAT® system
- Comparison of **Reference Standard Endotoxins** (RSE)

White Paper:

- Monocyte Activation Test (MAT): **the *in vitro* test for pyrogen detection**
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- **Validation of a cell line-based Monocyte Activation Test** method according to **USP <1225>** Validation of compendial procedures guideline

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Application Note

Detection of pyrogens in a vaccine preparation with the PyroMAT™ System

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 is the endotoxin (LPS = Lipo-Polysaccharide), which is 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 to 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. 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 the patient safety.

The monocyte activation test (MAT) method has been 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 which 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).

By putting the product to be tested in contact with human monocytic cells, it will mimic what happens in the human body: in 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 a source of monocytes.

The response to pyrogenic substances is determined by measurement of Interleukin-6 (IL-6) produced by the MonoMac 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 supernatant during incubation phase are transferred in 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 is to be less 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 satisfied
- 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

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 µL – 100 µL; 100 µL – 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

The PyroMAT™ Kit

REQUEST INFORMATION

Quantification of pyrogens with the PyroMAT™ system

Preparatory tests – Product Specific Validation (PSV)

European pharmacopeia, chapter 2.6.30

Before routine testing of a pharmaceutical product with MAT, a product specific validation (PSV) must be performed according to the method chosen for routine testing to ensure the validity of the criteria for the endotoxin standard curve, the detectability of endotoxin and non-endotoxin contaminants in the sample and that the sample does not interfere with the test or detection system.

Interferences with the test can be removed by diluting the product up to a certain limit, referred to as the maximum valid dilution (MVD).

The MVD is the maximum dilution factor at which it is still possible to detect the pyrogen limit (i.e., the CLC). It is directly linked to the limit of detection (LOD) of the system.

The more sensitive the system is, the more the product can be diluted to remove interferences.

The MVD of a test solution can be calculated using the following formula:

$$MVD = \frac{CLC \times C}{LOD}$$

CLC = Contaminant Limit Concentration (EU/mg or EU/mL)
C = Concentration of the test solution (mg/mL or mL/mL)
LOD = Limit of Detection (EU/mL).

The CLC is the acceptance criterion for the pass/fail decision, expressed in endotoxin equivalents per milligram or milliliter (EEU/mg or EEU/ml) or per unit of the biological activity of the product.

It is calculated by the following expression:

$$CLC = \frac{K}{M}$$

K = threshold pyrogenic dose per kilogram of body mass (EU/kg)

M = maximum recommended bolus dose of product per kilogram of body mass (mg/kg or mL/kg).

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

When testing for interfering factors, dilutions of the preparation being examined with geometric steps not exceeding the MVD should be performed. The same dilutions spiked with endotoxin at a justified concentration (in case of method A, a concentration near the estimated middle of the endotoxin standard curve) should then be performed.

These dilutions must be tested in parallel in the same experiment and together with an endotoxin standard curve, which shall be used to calculate the concentration of endotoxin-equivalents in each solution.

The mean recovery of the added endotoxin spike is then calculated for each dilution. The test is considered free of interference when recovery of the added endotoxin is within the range of between 50 and 200%.

Where practicable, interference testing should be performed on at least 3 different lots of the preparation being examined to investigate on possible batch-to-batch variation. If the interference cannot be removed by dilution or specific sample preparation of the product within the MVD range, Method C is preferred over Method A and B.

For validation of the detection of non-endotoxin contaminants, historical batches that have been found to be contaminated with non-endotoxin contaminants causing positive responses in the rabbit pyrogens test or adverse drug reaction in man can be used. Where those batches are not available, validation should be done including at least 2 non-endotoxin ligands for toll-like receptors, one of which is to be spiked into the preparation being examined.

Once the optimum dilution of the preparation being examined has been identified, this dilution needs to be tested for interference in the detection system. The agreement between a dilution series in presence and absence of the preparation being examined is to be within ± 20% of optical density.

Sample specifications: Meningococcal group C conjugate vaccine

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe, its toxins or one of its surface proteins. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of the microorganisms associated with that agent that it later encounters.

Vaccines include a variety of ingredients including antigens, stabilizers, adjuvants, antibiotics, and preservatives. They may also contain residual by-products from the production process. Knowing precisely what is in each vaccine can be helpful when investigating and minimizing adverse events following immunization.

Due to the production process and the composition, vaccines may contain free endotoxin or other pyrogenic substances. Endotoxin may serve as an adjuvant and support the stimulation of the immune system or the antigen may have a pyrogenic effect itself [2,3]. For the release of some vaccines the rabbit pyrogen test (RPT) is still in use while for other products the bacterial endotoxin test (BET) was implemented.

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 and is not capable to give any information about interactions and synergetic effects or the pyrogenic activity of the found endotoxin. Additionally, both methods base on animals or animal product and therefore counter the principles of the 3Rs (Replacement, Reduction and Refinement) regarding animal welfare.

The suitability of the MAT test for detection of pyrogens in vaccine products was shown in other studies before. [3,4,5,6,7]

The examined meningococcal group C conjugate vaccine is a liquid preparation of purified capsular polysaccharide derived from Neisseria meningitidis Group C, linked to tetanus toxoid as a carrier and adsorbed to aluminium hydroxide as adjuvants.

For the estimation of the MVD of the product to be tested, the threshold for bacterial endotoxins for the product is 25 IU, corresponding to a pyrogenic load of 25 EU, per single human dose [8].

The single bolus dose of the product is a volume of 0.5 mL. Therefore, the CLC of this product was calculated as

$$CLC = \frac{K}{M} = \frac{25\ EU/dose}{\frac{0.5\ mL}{dose}} = \frac{25\ EU}{0.5\ mL} = 50\ EU/mL$$

For the PyroMAT™ system, the LOD is 0.05 EU/mL, so

$$MVD = \frac{50\ EU/mL\ x\ 1}{0.05\ EU/mL} = 1000$$

Product specific validation for testing meningococcal group C conjugate vaccine with the PyroMAT™ system

Assurance of the criteria for the endotoxin standard curve:

A standard curve using Reference Standard Endotoxin was performed to verify that the criteria for endotoxin standard curve were valid.

- The regression of response on log dose was statistically significant ($p < 0.01$)
- The regression of response on log dose did not deviate significantly from linearity ($p > 0.05$)

Test for interfering factors and method validation for detection of non-endotoxin contaminants:

A dilution series from undiluted product up to the MVD was prepared and a test for interfering factors and method validation for non-endotoxin monocyte-activating contaminants (NEPs) according to EP was performed, comparing endotoxin-spiked sample dilutions as well as NEPs-spiked sample dilutions with the same unspiked sample dilutions.

Tests results showed non-endotoxin pyrogen (NEP) control was not detectable in the undiluted product. From an 1:5 dilution to the MVD all dilutions showed detection of the NEP control.

The undiluted product was found to show interference with the detection of the endotoxin spike, the reaction was fully inhibited. From an 1:30 dilution to the MVD, the product reproducibly showed both detection of the NEP and an endotoxin spike recovery within the range 50-200%, allowing to rule out interference with the test.

Test for interference in the detection system:

The so found optimum dilution was forwarded to a test for interference in the detection system. A dilution series of IL-6 control was tested in absence and presence of the sample dilution. All IL-6 control dilutions showed less than 20% difference between dilution in absence and presence of the product, therefore no interference with the detection system could be found.

Conclusion

Assurance of criteria for the standard curve	**Valid**
Test for interfering factors	**Valid**
Detection of NEP contaminants	**Valid**
Interference in the detection system	**Valid**

All criteria of the product specific validation were fulfilled and the dilution 1:30 was chosen as the first valid dilution for the Method A.

Quantitative method A according to EP

Planning Test Execution

Once a valid dilution has been identified through the Product Specific Validation, Method A (quantitative test) can be performed to assess the pyrogenicity of samples from this product.

The lowest dilution / highest concentration of the sample, that was found free of interference in the PSV is to be chosen as initial dilution and 2-fold serial dilutions are tested.

For quantification, the middle section of the dose-response curve of the endotoxin standard is to prefer, giving the most exact results, while quantification within the upper plateau of the standard curve can lead to inaccuracy due to reaching the endpoint of the reaction and therefore is not recommended.

For most exact results, we therefore recommend to analyze sample dilutions which do not exceed the measuring range of 0.05 to 0.4 EU/mL.

Testing of meningococcal group C conjugate vaccine with method A

The test setup was performed according to the user guide of the PyroMAT™ system.

An endotoxin standard curve was performed for the test.

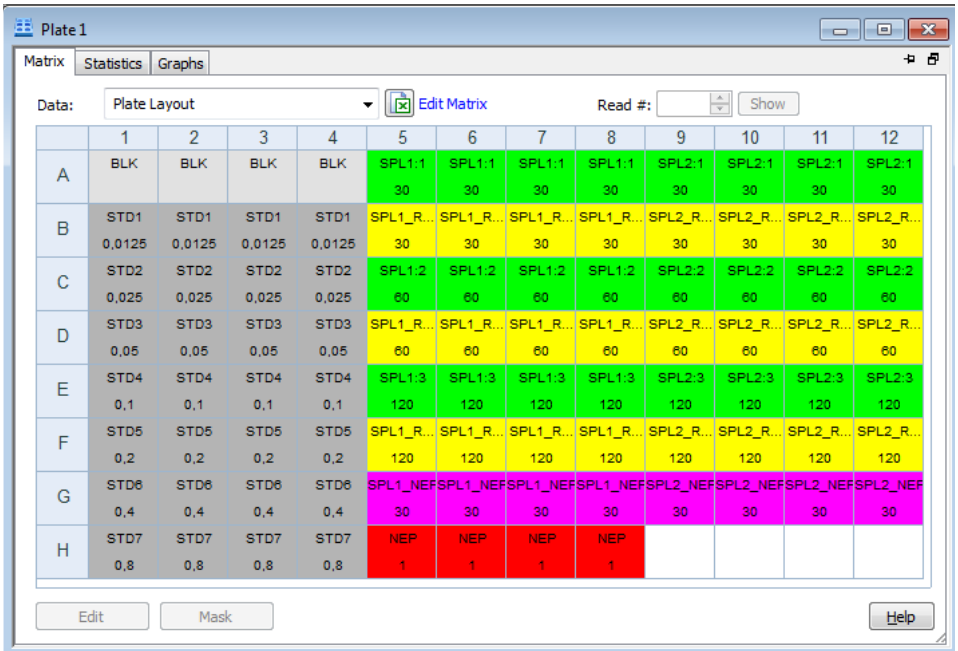
Three dilutions of the product were tested according to method A described in EP: The dilutions 1:30, 1:60 and 1:120 of the sample were all tested with and without endotoxin spike. A quantification of the found endotoxin equivalents for all dilutions of the unspiked and spiked sample was done using the endotoxin standard curve. The endotoxin spike recovery for all three sample dilutions was calculated.

Heat killed Staphylococcus aureus (HKSA) was used as additional control for detection of non-endotoxin pyrogens within the sample, tested with the highest concentration of the product to be examined.

Data interpretation

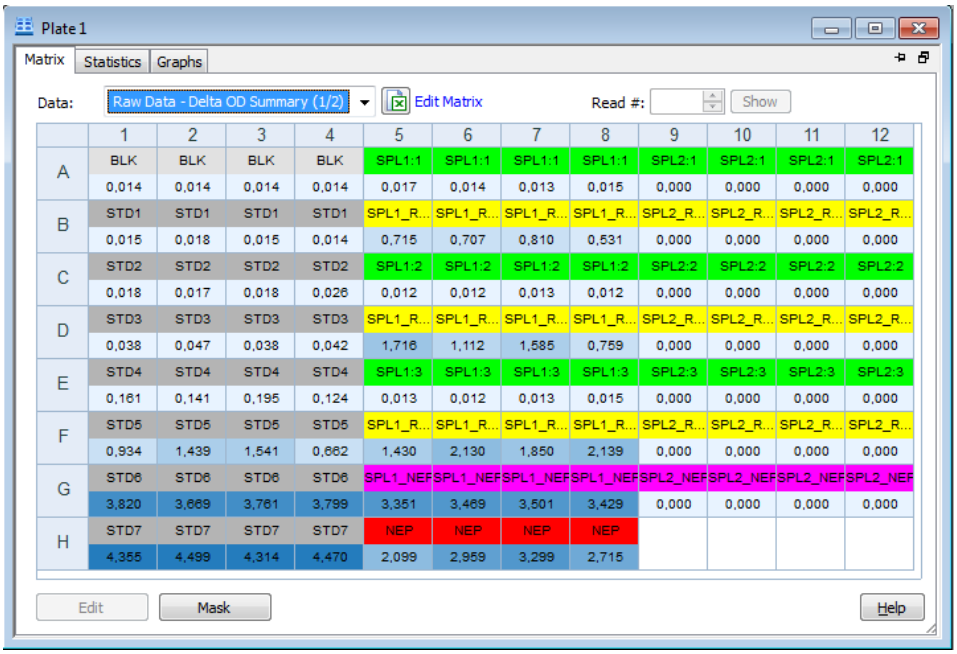
The data analysis was performed with Gen5 software and the PyroMAT™ Method A protocol available on our website. Information related to the sample were completed directly on the software: sample name, CLC.

For interpretation, the layout for method A was modified with the appropriate dilution factors for this sample matrix.



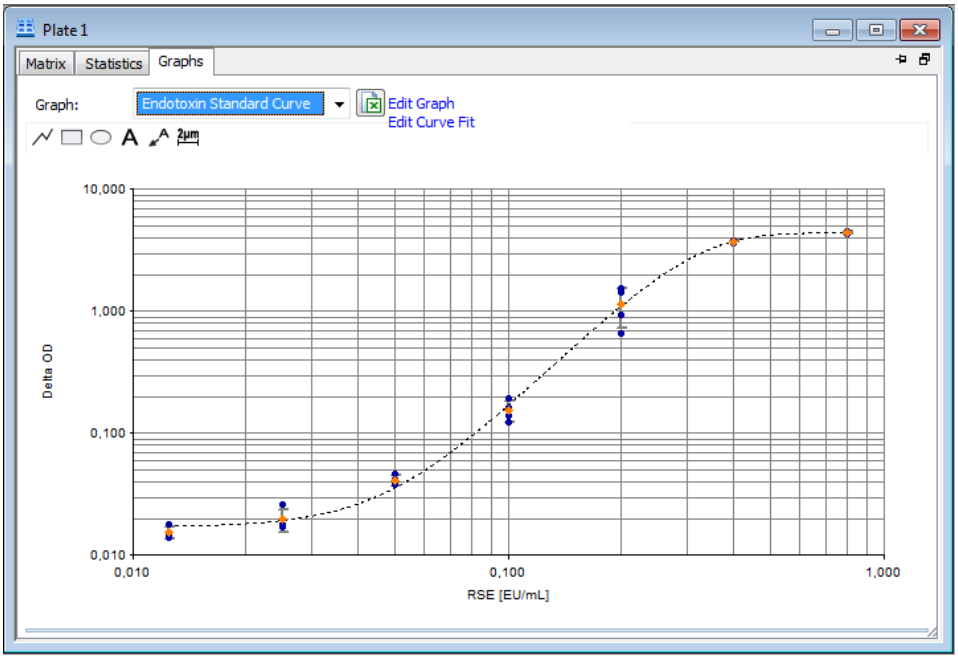
	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	BLK	SPL1:1 30	SPL1:1 30	SPL1:1 30	SPL1:1 30	SPL2:1 30	SPL2:1 30	SPL2:1 30	SPL2:1 30
B	STD1 0,0125	STD1 0,0125	STD1 0,0125	STD1 0,0125	SPL1_R... 30	SPL1_R... 30	SPL1_R... 30	SPL1_R... 30	SPL2_R... 30	SPL2_R... 30	SPL2_R... 30	SPL2_R... 30
C	STD2 0,025	STD2 0,025	STD2 0,025	STD2 0,025	SPL1:2 60	SPL1:2 60	SPL1:2 60	SPL1:2 60	SPL2:2 60	SPL2:2 60	SPL2:2 60	SPL2:2 60
D	STD3 0,05	STD3 0,05	STD3 0,05	STD3 0,05	SPL1_R... 60	SPL1_R... 60	SPL1_R... 60	SPL1_R... 60	SPL2_R... 60	SPL2_R... 60	SPL2_R... 60	SPL2_R... 60
E	STD4 0,1	STD4 0,1	STD4 0,1	STD4 0,1	SPL1:3 120	SPL1:3 120	SPL1:3 120	SPL1:3 120	SPL2:3 120	SPL2:3 120	SPL2:3 120	SPL2:3 120
F	STD5 0,2	STD5 0,2	STD5 0,2	STD5 0,2	SPL1_R... 120	SPL1_R... 120	SPL1_R... 120	SPL1_R... 120	SPL2_R... 120	SPL2_R... 120	SPL2_R... 120	SPL2_R... 120
G	STD6 0,4	STD6 0,4	STD6 0,4	STD6 0,4	SPL1_NEF 30	SPL1_NEF 30	SPL1_NEF 30	SPL1_NEF 30	SPL2_NEF 30	SPL2_NEF 30	SPL2_NEF 30	SPL2_NEF 30
H	STD7 0,8	STD7 0,8	STD7 0,8	STD7 0,8	NEP 1	NEP 1	NEP 1	NEP 1				

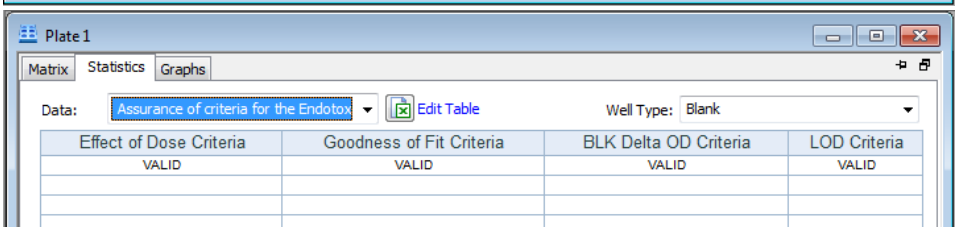
After reading the plate, the data interpretation was performed with the software.

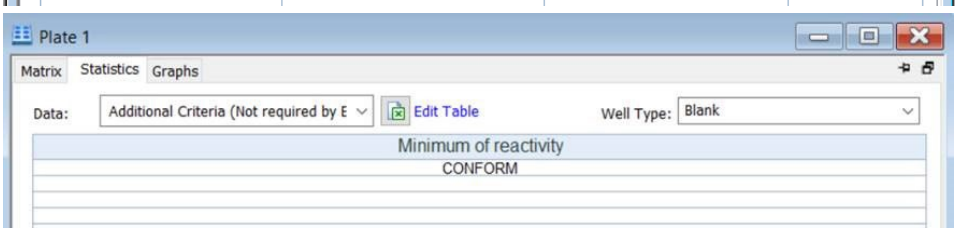


	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	BLK	SPL1:1	SPL1:1	SPL1:1	SPL1:1	SPL2:1	SPL2:1	SPL2:1	SPL2:1
	0,014	0,014	0,014	0,014	0,017	0,014	0,013	0,015	0,000	0,000	0,000	0,000
B	STD1	STD1	STD1	STD1	SPL1_R...	SPL1_R...	SPL1_R...	SPL1_R...	SPL2_R...	SPL2_R...	SPL2_R...	SPL2_R...
	0,015	0,018	0,015	0,014	0,715	0,707	0,810	0,531	0,000	0,000	0,000	0,000
C	STD2	STD2	STD2	STD2	SPL1:2	SPL1:2	SPL1:2	SPL1:2	SPL2:2	SPL2:2	SPL2:2	SPL2:2
	0,018	0,017	0,018	0,026	0,012	0,012	0,013	0,012	0,000	0,000	0,000	0,000
D	STD3	STD3	STD3	STD3	SPL1_R...	SPL1_R...	SPL1_R...	SPL1_R...	SPL2_R...	SPL2_R...	SPL2_R...	SPL2_R...
	0,038	0,047	0,038	0,042	1,716	1,112	1,585	0,759	0,000	0,000	0,000	0,000
E	STD4	STD4	STD4	STD4	SPL1:3	SPL1:3	SPL1:3	SPL1:3	SPL2:3	SPL2:3	SPL2:3	SPL2:3
	0,161	0,141	0,195	0,124	0,013	0,012	0,013	0,015	0,000	0,000	0,000	0,000
F	STD5	STD5	STD5	STD5	SPL1_R...	SPL1_R...	SPL1_R...	SPL1_R...	SPL2_R...	SPL2_R...	SPL2_R...	SPL2_R...
	0,934	1,439	1,541	0,662	1,430	2,130	1,850	2,139	0,000	0,000	0,000	0,000
G	STD6	STD6	STD6	STD6	SPL1_NEP	SPL1_NEP	SPL1_NEP	SPL1_NEP	SPL2_NEP	SPL2_NEP	SPL2_NEP	SPL2_NEP
	3,820	3,669	3,761	3,799	3,351	3,469	3,501	3,429	0,000	0,000	0,000	0,000
H	STD7	STD7	STD7	STD7	NEP	NEP	NEP	NEP				
	4,355	4,499	4,314	4,470	2,099	2,959	3,299	2,715				

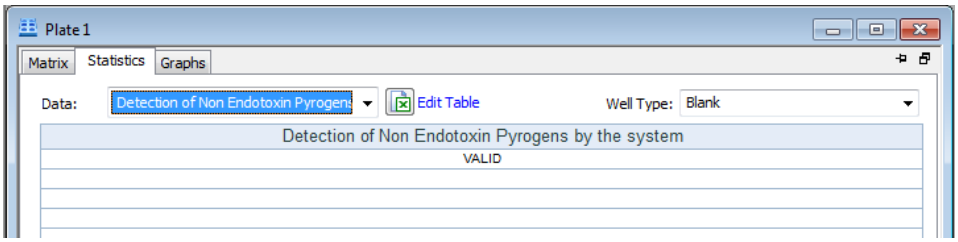
The standard curve was valid for all the criteria.

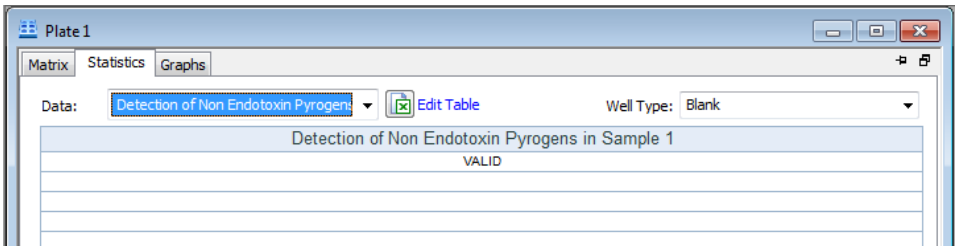






The NEP-control confirmed detection of non-endotoxin pyrogens in the system and in the sample.



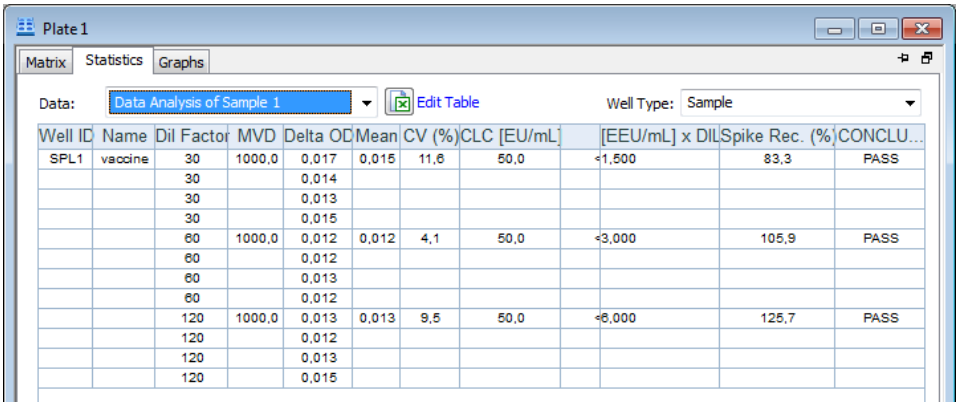


The chosen sample dilutions were appropriate for the sample with all tested dilutions being within the MVD and spike recovery within 50-200%.

The data analysis showed validity of the test being fulfilled for all sample dilutions.

The sample itself showed a pyrogenic load of <1.5 EEU*/mL which is below the CLC (50 EU/mL) and therefore being considered “not pyrogenic”.

*EEU: Endotoxin Equivalent Unit



Well ID	Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Spike Rec. (%)	CONCLU...
SPL1	vaccine	30	1000,0	0,017	0,015	11,6	50,0	<	1,500	83,3	PASS
		30		0,014							
		30		0,013							
		30		0,015							
		60	1000,0	0,012	0,012	4,1	50,0	<	3,000	105,9	PASS
		60		0,012							
		60		0,013							
		60		0,012							
		120	1000,0	0,013	0,013	9,5	50,0	<	6,000	125,7	PASS
		120		0,012							
		120		0,013							
		120		0,015							

Results

The capability of the MAT for the detection of pyrogens in the tested meningococcal group C conjugate vaccine preparation was shown and is comparable to the evaluation on the use of the MAT for testing vaccines described in other studies [4,5,6,7].

The examined vaccine preparation showed inhibition of the reaction of the Monocytes in the undiluted sample that could be overcome by dilution within the authorized dilution range (not exceeding the MVD).

The data show that PyroMAT™ system is suitable for detecting both endotoxin and non-endotoxin pyrogenic contaminations in preparations of the vaccine.

References

[1] European Pharmacopoeia, chapter Monocyte activation test (2.6.30)

[2] Williams K. "The Emerging View of Endotoxin as an IIRMI" (2016) BioPharm International Volume 28, Issue 2, pg 24–31

[3] Carlin G., Viitanen E. "*In vitro* pyrogenicity of the diphtheria, tetanus and acellular pertussis components of a trivalent vaccine". Vaccine 23 (2005), 3709–3715

[4] Kaaijk P, van Straaten I. van de Waterbeemd D et al. „Preclinical safety and immunogenicity evaluation of a nonavalent PorA native outer membrane vesicle vaccine against serogroup B meningococcal disease." Vaccine 31 (2013), 1065-1071

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[6] Vipond C, Findlay L, Feavers I, Care R. "Limitations of the Rabbit Pyrogen Test for Assessing Meningococcal OMV Based Vaccines" Altex 33(1), 2016, 47-53

[7] Nordgren K. "Evaluation of the MAT for the safety testing of meningococcal B vaccine Bexsero: a collaborative study" PharmaLab Congress 2017

[8] European Pharmacopoeia, Meningococcal group C conjugate vaccine (2112)

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Studies to demonstrate the robustness and sensitivity of the PyroMAT® system for the detection of endotoxin and non-endotoxin pyrogens

Application Notes:

- Detection of **non-endotoxin pyrogens (NEP)** by Monocyte Activation Test (MAT) using the PyroMAT® system
- Quantification of pyrogen in **Hormone** with the PyroMAT® system
- Quantification of pyrogen in **Vaccine** with the PyroMAT® system
- Quantification of pyrogen in **FBS** with the PyroMAT® system
- Quantification of pyrogens in **Albumine** with the PyroMAT® system
- Comparison of **Reference Standard Endotoxins** (RSE)

White Paper:

- Monocyte Activation Test (MAT): **the *in vitro* test for pyrogen detection**
- Monocyte Activation Test: **statistical analysis**

Datasheet:

- **Validation of a cell line-based Monocyte Activation Test** method according to **USP <1225>** Validation of compendial procedures guideline

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Application Note

Detection of pyrogens in fetal bovine serum (FBS) with the PyroMAT™ System

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 is the endotoxin (LPS = Lipo-Polysaccharide), which is 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 to 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.

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 the patient safety.

The monocyte activation test (MAT) method has been 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 which 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).

By putting the product to be tested in contact with human monocytic cells, it will mimic what happens in the human body: in 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 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 MM6 cells supernatant during incubation phase are transferred in 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 is to be less 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 satisfied
- 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

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 µL – 100 µL; 100 µL – 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

The PyroMAT™ Kit

Quantification of pyrogens with the PyroMAT™ system

Preparatory tests – Product Specific Validation (PSV)

European pharmacopeia, chapter 2.6.30

Before routine testing of a pharmaceutical product with MAT, a product specific validation (PSV) must be performed according to the method chosen for routine testing to ensure the validity of the criteria for the endotoxin standard curve, the detectability of endotoxin and non-endotoxin contaminants in the sample and that the sample does not interfere with the test or detection system.

Interferences with the test can be removed by diluting the product up to a certain limit, referred to as the maximum valid dilution (MVD).

The MVD is the maximum dilution factor at which it is still possible to detect the pyrogen limit (i.e., the CLC). It is directly linked to the limit of detection (LOD) of the system.

The more sensitive the system is, the more the product can be diluted to remove interferences.

The MVD of a test solution can be calculated using the following formula:

$$MVD = \frac{CLC \times C}{LOD}$$

CLC = Contaminant Limit Concentration (EU/mg or EU/mL)
C = Concentration of the test solution (mg/mL or mL/mL)
LOD = Limit of Detection (EU/mL).

The CLC is the acceptance criterion for the pass/fail decision, expressed in endotoxin equivalents per milligram or milliliter (EEU/mg or EEU/mL) or per unit of the biological activity of the product.

It is calculated by the following expression:

$$CLC = \frac{K}{M}$$

K = threshold pyrogenic dose per kilogram of body mass (EU/kg)

M = maximum recommended bolus dose of product per kilogram of body mass (mg/kg or mL/kg).

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

When testing for interfering factors, dilutions of the preparation being examined with geometric steps not exceeding the MVD should be performed. The same dilutions spiked with endotoxin at a justified concentration (in case of method A, a concentration near the estimated middle of the endotoxin standard curve) should then be performed.

These dilutions must be tested in parallel in the same experiment and together with an endotoxin standard curve, which shall be used to calculate the concentration of endotoxin-equivalents in each solution.

The mean recovery of the added endotoxin spike is then calculated for each dilution. The test is considered free of interference when recovery of the added endotoxin is within the range of between 50 and 200%.

Where practicable, interference testing should be performed on at least 3 different lots of the preparation being examined to investigate on possible batch-to-batch variation. If the interference cannot be removed by dilution or specific sample preparation of the product within the MVD range, Method C is preferred over Method A and B.

For validation of the detection of non-endotoxin contaminants, historical batches that have been found to be contaminated with non-endotoxin contaminants causing positive responses in the rabbit pyrogens test or adverse drug reaction in man can be used. Where those batches are not available, validation should be done including at least 2 non-endotoxin ligands for toll-like receptors, one of which is to be spiked into the preparation being examined.

Once the optimum dilution of the preparation being examined has been identified, this dilution needs to be tested for interference in the detection system. The agreement between a dilution series in presence and absence of the preparation being examined is to be within ± 20% of optical density.

Sample specifications: FBS (fetal bovine serum)

FBS derived from clotted blood is the most widely used undefined supplement in eucaryotic, especially mammalian, cell culture. Though there are efforts to establish human or synthetic alternatives, fetal bovine serum still is a product that often is used in the production of vaccines and therapeutics.

Being a raw material used within the production process of injectables administered to man, FBS has to be tested for harmful substances like endotoxin or other pyrogenic substances like (1 → 3)-β-d-glucan [2,3]. Quality of FBS is also important for researchers using cell cultures as pyrogenic contaminants may create problems by affecting the bioactivity of the cultured cells and therefore influence the experimental results obtained [4].

For the examined FBS the criteria of quality was an endotoxin level of equal or smaller than 10 EU/mL. Therefore, the contaminant limit concentration of the product is:

$$CLC = 10\ EU/mL$$

For the PyroMAT™ system, the LOD is 0.05 EU/mL, so

$$MVD = \frac{10\ EU/mL \times 1}{0.05\ EU/mL} = 200$$

Product specific validation for testing FBS (fetal bovine serum) with the PyroMAT™ system

Assurance of the criteria for the endotoxin standard curve:

A standard curve using Reference Standard Endotoxin was performed to verify that the criteria for endotoxin standard curve were valid.

- The regression of response on log dose was statistically significant ($p < 0.01$)
- The regression of response on log dose did not deviate significantly from linearity ($p > 0.05$)

Test for interfering factors and method validation for detection of non-endotoxin contaminants:

A dilution series from undiluted product up to the MVD was prepared and a test for interfering factors and method validation for non-endotoxin monocyte-activating contaminants (NEPs) according to EP was performed, comparing endotoxin-spiked sample dilutions as well as NEPs-spiked sample dilutions with the same unspiked sample dilutions.

Tests results showed non-endotoxin pyrogen (NEPs) control was detectable in all dilutions of the product from undiluted sample to MVD.

The undiluted product was found to show interference with the detection of the endotoxin spike, the reaction was strongly inhibited. From an 1:10 dilution to the MVD, the product reproducibly showed both detection of the NEP and a spike recovery within the range 50-200%, allowing to rule out interference with the test.

Test for interference in the detection system:

The so found optimum dilution was forwarded to a test for interference in the detection system. A dilution series of IL-6 control was tested in absence and presence of the sample dilution. All IL-6 control dilutions showed less than 20% difference between dilution in absence and presence of the product, therefore no interference with the detection system could be found.

Conclusion

Assurance of criteria for the standard curve	**Valid**
Test for interfering factors	**Valid**
Detection of NEP contaminants	**Valid**
Interference in the detection system	**Valid**

All criteria of the product specific validation were fulfilled and the dilution 1:10 was chosen as the first valid dilution for the Method A.

Quantitative method A according to EP

Planning Test Execution

Once a valid dilution has been identified through the Product Specific Validation, Method A (quantitative test) can be performed to assess the pyrogenicity of samples from this product.

The lowest dilution / highest concentration of the sample, that was found free of interference in the PSV is to be chosen as initial dilution and 2-fold serial dilutions are tested.

For quantification, the middle section of the dose-response curve of the endotoxin standard is to prefer, giving the most exact results, while quantification within the upper plateau of the standard curve can lead to inaccuracy due to reaching the endpoint of the reaction and therefore is not recommended.

For most exact results, we therefore recommend to analyze sample dilutions which do not exceed the measuring range of 0.05 to 0.4 EU/mL.

Testing of FBS (fetal bovine serum) with method A

The test setup was performed according to the user guide of the PyroMAT™ system.

An endotoxin standard curve was performed for the test.

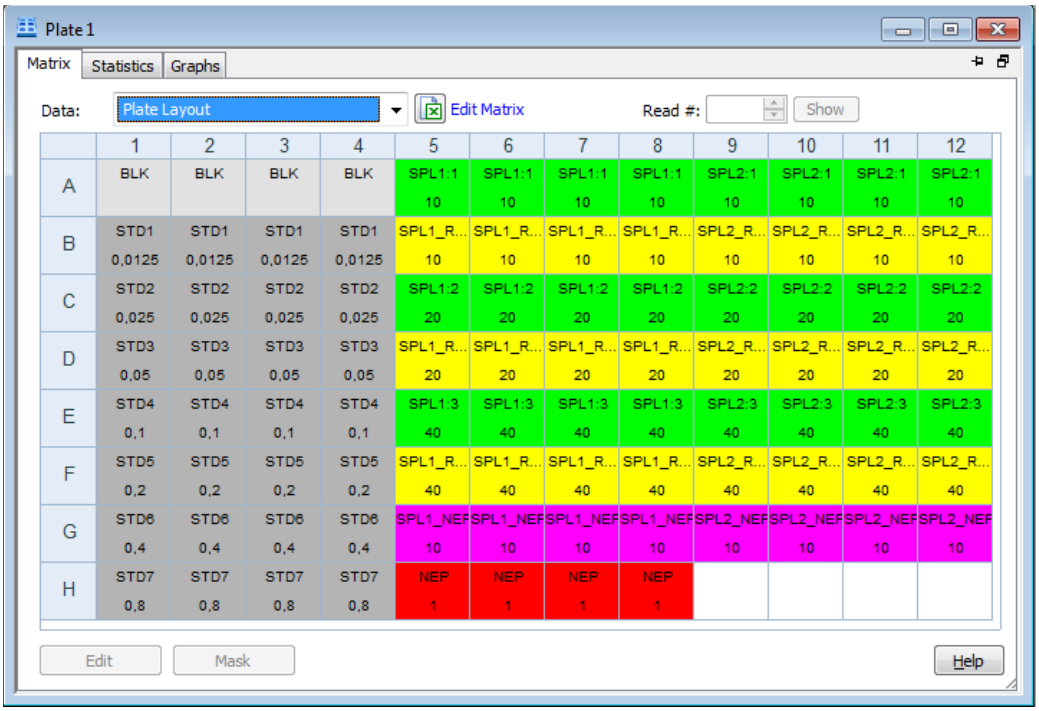
Three dilutions of the product were tested according to method A described in EP: the dilutions 1:10, 1:20 and 1:40 of the sample were all tested with and without endotoxin spike. A quantification of the found endotoxin equivalents for all dilutions of the unspiked and spiked sample was done using the endotoxin standard curve. The endotoxin spike recovery for all three sample dilutions was calculated.

Heat killed *Staphylococcus aureus* (HKSA) was used as additional control for detection of non-endotoxin pyrogens within the sample, tested with the highest concentration of the product to be examined.

Data interpretation

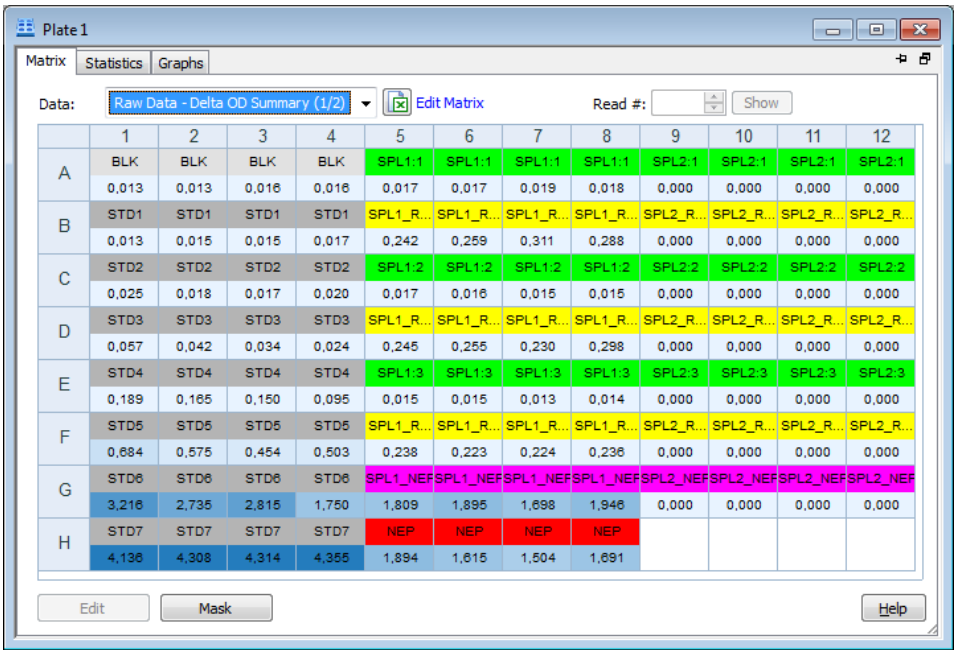
The data analysis was performed with Gen5 software and the PyroMAT™ Method A available on our website. Information related to the sample were completed directly on the software: sample name, CLC.

For interpretation, the layout for method A was modified with the appropriate dilution factors for this sample matrix.

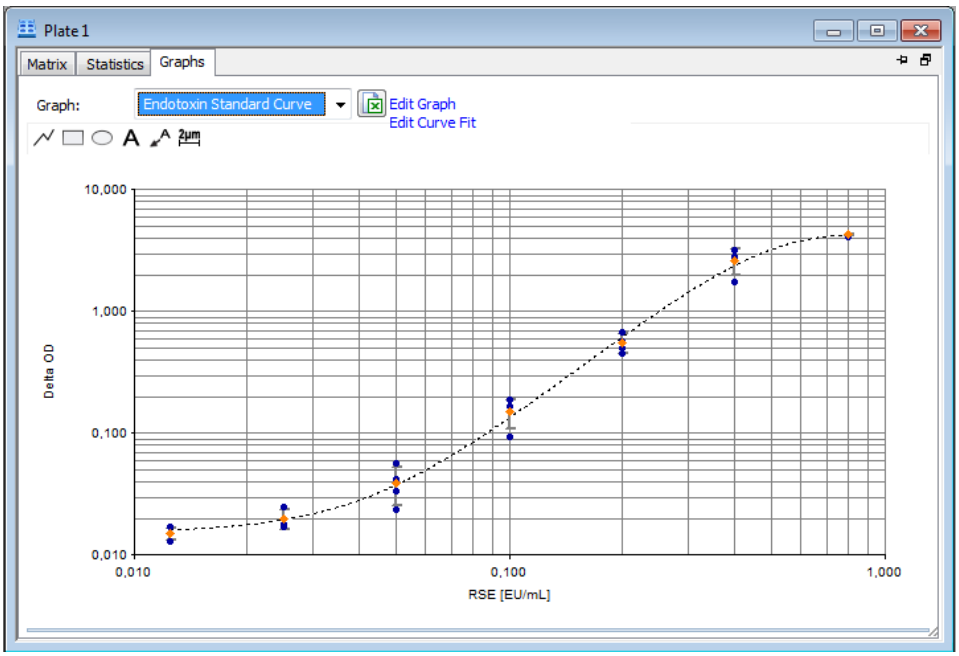


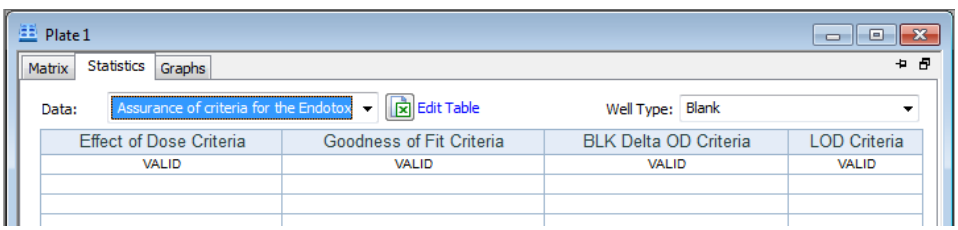
	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	BLK	SPL1:1 10	SPL1:1 10	SPL1:1 10	SPL1:1 10	SPL2:1 10	SPL2:1 10	SPL2:1 10	SPL2:1 10
B	STD1 0,0125	STD1 0,0125	STD1 0,0125	STD1 0,0125	SPL1_R... 10	SPL1_R... 10	SPL1_R... 10	SPL1_R... 10	SPL2_R... 10	SPL2_R... 10	SPL2_R... 10	SPL2_R... 10
C	STD2 0,025	STD2 0,025	STD2 0,025	STD2 0,025	SPL1:2 20	SPL1:2 20	SPL1:2 20	SPL1:2 20	SPL2:2 20	SPL2:2 20	SPL2:2 20	SPL2:2 20
D	STD3 0,05	STD3 0,05	STD3 0,05	STD3 0,05	SPL1_R... 20	SPL1_R... 20	SPL1_R... 20	SPL1_R... 20	SPL2_R... 20	SPL2_R... 20	SPL2_R... 20	SPL2_R... 20
E	STD4 0,1	STD4 0,1	STD4 0,1	STD4 0,1	SPL1:3 40	SPL1:3 40	SPL1:3 40	SPL1:3 40	SPL2:3 40	SPL2:3 40	SPL2:3 40	SPL2:3 40
F	STD5 0,2	STD5 0,2	STD5 0,2	STD5 0,2	SPL1_R... 40	SPL1_R... 40	SPL1_R... 40	SPL1_R... 40	SPL2_R... 40	SPL2_R... 40	SPL2_R... 40	SPL2_R... 40
G	STD6 0,4	STD6 0,4	STD6 0,4	STD6 0,4	SPL1_NEP 10	SPL1_NEP 10	SPL1_NEP 10	SPL1_NEP 10	SPL2_NEP 10	SPL2_NEP 10	SPL2_NEP 10	SPL2_NEP 10
H	STD7 0,8	STD7 0,8	STD7 0,8	STD7 0,8	NEP 1	NEP 1	NEP 1	NEP 1				

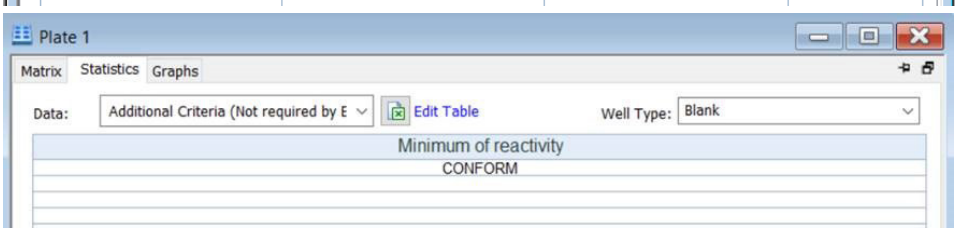
After reading the plate, the data interpretation was performed with the software.



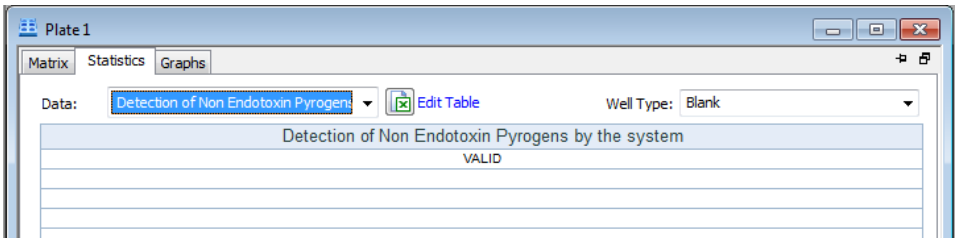
The standard curve was valid for all the criteria.

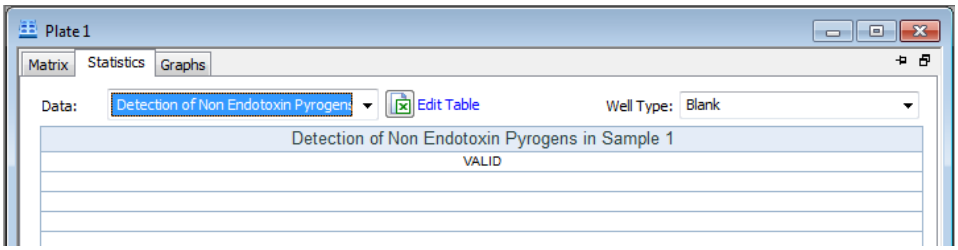






The NEP-control confirmed detection of non-endotoxin pyrogens in the system and in the sample.



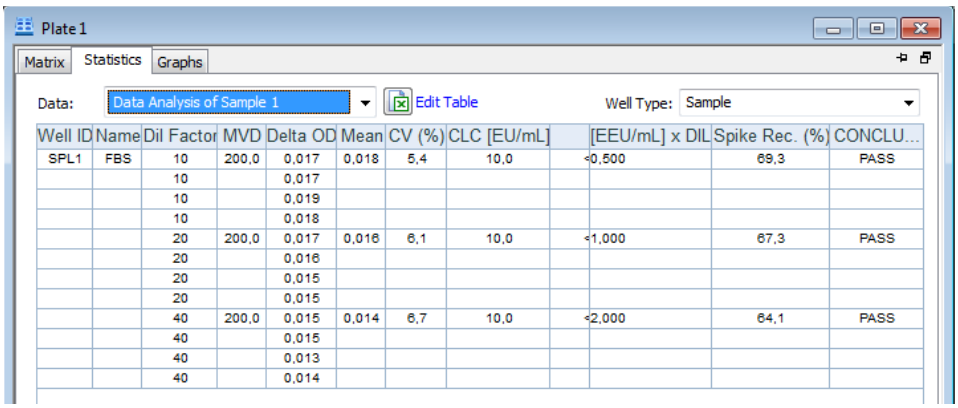


The chosen sample dilutions were appropriate for the sample with all tested dilutions being within the MVD and spike recovery within 50-200%.

The data analysis showed validity of the test being fulfilled for all sample dilutions.

The sample itself showed a pyrogenic load of <0.5 EEU*/mL which is below the CLC (10 EU/mL) and therefore being considered “not pyrogenic”.

*EEU: Endotoxin Equivalent Unit



Well ID	Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Spike Rec. (%)	CONCLU...
SPL1	FBS	10	200,0	0,017	0,018	5,4	10,0	<	0,500	69,3	PASS
		10		0,017							
		10		0,019							
		10		0,018							
		20	200,0	0,017	0,016	6,1	10,0	<	1,000	67,3	PASS
		20		0,016							
		20		0,015							
		20		0,015							
		40	200,0	0,015	0,014	6,7	10,0	<	2,000	64,1	PASS
		40		0,015							
		40		0,013							
		40		0,014							

Results

The examined FBS showed inhibition of the reaction of the Monocytes in the undiluted sample that could be overcome by dilution within the authorized dilution range (not exceeding the MVD).

The data show that PyroMAT™ system is suitable for detecting both endotoxin and non-endotoxin pyrogenic contaminations in preparations of FBS.

References

[1] European Pharmacopoeia, chapter Monocyte activation test (2.6.30)

[2] Kirikae T, Tamura H, Hashizume M, Kirikae F, Uemura Y, Tanaka S, Yokochi T, Nakano M. "Endotoxin contamination in fetal bovine serum and its influence on tumor necrosis factor production by macrophage-like cells J774.1 cultured in the presence of the serum" International Journal of Immunopharmacology Volume 19, Issue 5, May 1997, Pages 255-262

[3] Fishel S, Jackson P, Webster J, Faratian B. "Endotoxins in culture medium for human *in vitro* fertilization" Fertility and Sterility Volume 49, Issue 1, January 1988, Pages 108-111

[4] Ryan J. "Endotoxin and cell culture" Technical bulletin, www.corning.com/lifesciences

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TRUST THE DATA

Studies to demonstrate the robustness and sensitivity of the PyroMAT® system for the detection of endotoxin and non-endotoxin pyrogens

Application Notes:

- Detection of **non-endotoxin pyrogens (NEP)** by Monocyte Activation Test (MAT) using the PyroMAT® system
- Quantification of pyrogen in **Hormone** with the PyroMAT® system
- Quantification of pyrogen in **Vaccine** with the PyroMAT® system
- Quantification of pyrogen in **FBS** with the PyroMAT® system
- Quantification of pyrogens in **Albumine** with the PyroMAT® system
- Comparison of **Reference Standard Endotoxins** (RSE)

White Paper:

- Monocyte Activation Test (MAT): **the *in vitro* test for pyrogen detection**
- Monocyte Activation Test: **statistical analysis**

Datasheet:

- **Validation of a cell line-based Monocyte Activation Test** method according to **USP <1225>** Validation of compendial procedures guideline

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Application Note

Detection of pyrogens in albumin 20% solution with the PyroMAT™ System

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 is the endotoxin (LPS = Lipo-Polysaccharide), which is 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 to 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.

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 the patient safety.

The monocyte activation test (MAT) method has been 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 which 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).

By putting the product to be tested in contact with human monocytic cells, it will mimic what happens in the human body: in 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 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 MM6 cells supernatant during incubation phase are transferred in 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 is to be less 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 satisfied
- 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

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 µL – 100 µL; 100 µL – 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

The PyroMAT™ Kit

Quantification of pyrogens with the PyroMAT™ system

Preparatory tests – Product Specific Validation (PSV)

European pharmacopeia, chapter 2.6.30

Before routine testing of a pharmaceutical product with MAT, a product specific validation (PSV) must be performed according to the method chosen for routine testing to ensure the validity of the criteria for the endotoxin standard curve, the detectability of endotoxin and non-endotoxin contaminants in the sample and that the sample does not interfere with the test or detection system.

Interferences with the test can be removed by diluting the product up to a certain limit, referred to as the maximum valid dilution (MVD).

The MVD is the maximum dilution factor at which it is still possible to detect the pyrogen limit (i.e., the CLC). It is directly linked to the limit of detection (LOD) of the system.

The more sensitive the system is, the more the product can be diluted to remove interferences.

The MVD of a test solution can be calculated using the following formula:

$$MVD = \frac{CLC \times C}{LOD}$$

CLC = Contaminant Limit Concentration (EU/mg or EU/mL)
C = Concentration of the test solution (mg/mL or mL/mL)
LOD = Limit of Detection (EU/mL).

The CLC is the acceptance criterion for the pass/fail decision, expressed in endotoxin equivalents per milligram or milliliter (EEU/mg or EEU/mL) or per unit of the biological activity of the product.

It is calculated by the following expression:

$$CLC = \frac{K}{M}$$

K = threshold pyrogenic dose per kilogram of body mass (EU/kg)

M = maximum recommended bolus dose of product per kilogram of body mass (mg/kg or mL/kg).

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

When testing for interfering factors, dilutions of the preparation being examined with geometric steps not exceeding the MVD should be performed. The same dilutions spiked with endotoxin at a justified concentration (in case of method A, a concentration near the estimated middle of the endotoxin standard curve) should then be performed.

These dilutions must be tested in parallel in the same experiment and together with an endotoxin standard curve, which shall be used to calculate the concentration of endotoxin-equivalents in each solution.

The mean recovery of the added endotoxin spike is then calculated for each dilution. The test is considered free of interference when recovery of the added endotoxin is within the range of between 50 and 200%.

Where practicable, interference testing should be performed on at least 3 different lots of the preparation being examined to investigate on possible batch-to-batch variation. If the interference cannot be removed by dilution or specific sample preparation of the product within the MVD range, Method C is preferred over Method A and B.

For validation of the detection of non-endotoxin contaminants, historical batches that have been found to be contaminated with non-endotoxin contaminants causing positive responses in the rabbit pyrogens test or adverse drug reaction in man can be used. Where those batches are not available, validation should be done including at least 2 non-endotoxin ligands for toll-like receptors, one of which is to be spiked into the preparation being examined.

Once the optimum dilution of the preparation being examined has been identified, this dilution needs to be tested for interference in the detection system. The agreement between a dilution series in presence and absence of the preparation being examined is to be within ± 20% of optical density.

Sample specifications: Human Serum Albumin

Human serum albumin (HSA) 20% solution for infusion is indicated for the restoration and maintenance of circulating blood volume where volume deficiency has been demonstrated and use of a colloid is appropriate. [2].

Due to production process, albumin is known to be often contaminated with (1,3)-β- glucans origination from filter material. These (1,3)-ß-glucans are known to be pro-inflammatory molecules activating monocytes and show synergetic effects with other pyrogens like endotoxin, leading to more intensive pyrogenic reactions in man [3,4].

The suitability of the MAT test for detection of pyrogens in albumin products was shown in various studies before [5,6,7]

The administration dosage for the tested product is variable and personalized based on specific indication, patient's clinical status and response. Depending on the indication, doses of 20 – 100 g albumin 20% may be applied.

HSA 20% is a hyperoncotic solution and rapid administration can lead to rapid volume expansion and cardiac failure. It should be infused slowly to avoid this, therefore larger doses are typically given within several hours.

In the absence of acute hemorrhage, total daily albumin dosage should not exceed the theoretical amount present in total normal plasma volume (about 2 g/kg body weight), nevertheless, the product to be examined was designed to allow also higher dosage with a maximum dose of 6g/kg/day.

For the estimation of the MVD of the product to be tested, the threshold pyrogenic dose for parenteral applied solutions is 5 EU/kg (K).

The single bolus dose of the product was considered as a volume of 100 mL, corresponding to a dose of 20 g albumin. For an average adult, a body weight of 70 kg can be assumed. Therefore, the CLC of this product was calculated as

$$CLC = \frac{K}{M} = \frac{5\ EU/kg}{\frac{100\ mL}{70\ kg}} = \frac{350\ EU}{100\ mL} = 3.5\ EU/mL$$

For the PyroMAT™ system, the LOD is 0.05 EU/mL, so

$$MVD = \frac{3.5\ EU/mL \times 1}{0.05\ EU/mL} = 70$$

Product specific validation for testing albumin 20% for infusion with the PyroMAT™ system

Assurance of the criteria for the endotoxin standard curve:

A standard curve using Reference Standard Endotoxin was performed to verify that the criteria for endotoxin standard curve were valid.

- The regression of response on log dose was statistically significant ($p < 0.01$)
- The regression of response on log dose did not deviate significantly from linearity ($p > 0.05$)

Test for interfering factors and method validation for detection of non-endotoxin contaminants:

A dilution series from undiluted product up to the MVD was prepared and a test for interfering factors and method validation for non-endotoxin monocyte-activating contaminants (NEPs) according to EP was performed, comparing endotoxin-spiked sample dilutions as well as NEPs-spiked sample dilutions with the same unspiked sample dilutions.

Tests results showed non-endotoxin pyrogen (NEPs) control was detectable in all dilutions of the product from undiluted sample to MVD.

The undiluted product was found to show some interference with the detection of the endotoxin spike (in average around 200% spike recovery) so this dilution may occasionally fail validity criteria. From an 1:10 dilution to the MVD, the product reproducibly showed both detection of the NEP and a spike recovery within the range 50-200%, allowing to rule out interference with the test.

Test for interference in the detection system:

The so found optimum dilution was forwarded to a test for interference in the detection system. A dilution series of IL-6 control was tested in absence and presence of the sample dilution. All IL-6 control dilutions showed less than 20% difference between dilution in absence and presence of the product, therefore no interference with the detection system could be found.

Conclusion

Assurance of criteria for the standard curve	**Valid**
Test for interfering factors	**Valid**
Detection of NEP contaminants	**Valid**
Interference in the detection system	**Valid**

All criteria of the product specific validation were fulfilled and the dilution 1:10 was chosen as the first valid dilution for the Method A.

Quantitative method A according to EP

Planning Test Execution

Once a valid dilution has been identified through the Product Specific Validation, Method A (quantitative test) can be performed to assess the pyrogenicity of samples from this product.

The lowest dilution / highest concentration of the sample, that was found free of interference in the PSV is to be chosen as initial dilution and 2-fold serial dilutions are tested.

For quantification, the middle section of the dose-response curve of the endotoxin standard is to prefer, giving the most exact results, while quantification within the upper plateau of the standard curve can lead to inaccuracy due to reaching the endpoint of the reaction and therefore is not recommended.

For most exact results, we therefore recommend to analyze sample dilutions which do not exceed the measuring range of 0,05 to 0,4 EU/mL.

Testing of albumin 20% for infusion with method A

The test setup was performed according to the user guide of the PyroMAT™ system.

An endotoxin standard curve was performed for the test.

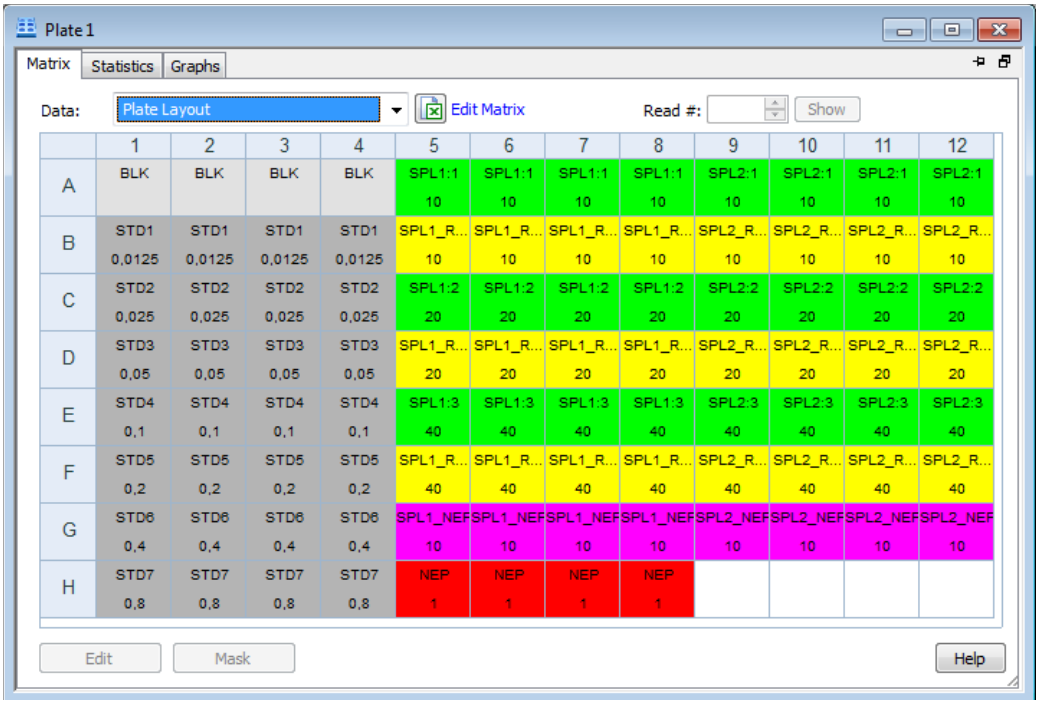
Three dilutions of the product were tested according to method A described in EP: The dilutions 1:10, 1:20 and 1:40 of the sample were all tested with and without endotoxin spike. A quantification of the found endotoxin equivalents for all dilutions of the unspiked and spiked sample was done using the endotoxin standard curve. The endotoxin spike recovery for all three sample dilutions was calculated.

Heat killed Staphylococcus aureus (HKSA) was used as additional control for detection of non-endotoxin pyrogens within the sample, tested with the highest concentration of the product to be examined.

Data interpretation

The data analysis was performed with Gen5 software and the PyroMAT™ Method A available on our website. Information related to the sample were completed directly on the software: sample name, CLC.

For interpretation, the layout for method A was modified with the appropriate dilution factors for this sample matrix.



	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	BLK	SPL1:1 10	SPL1:1 10	SPL1:1 10	SPL1:1 10	SPL2:1 10	SPL2:1 10	SPL2:1 10	SPL2:1 10
B	STD1 0,0125	STD1 0,0125	STD1 0,0125	STD1 0,0125	SPL1_R... 10	SPL1_R... 10	SPL1_R... 10	SPL1_R... 10	SPL2_R... 10	SPL2_R... 10	SPL2_R... 10	SPL2_R... 10
C	STD2 0,025	STD2 0,025	STD2 0,025	STD2 0,025	SPL1:2 20	SPL1:2 20	SPL1:2 20	SPL1:2 20	SPL2:2 20	SPL2:2 20	SPL2:2 20	SPL2:2 20
D	STD3 0,05	STD3 0,05	STD3 0,05	STD3 0,05	SPL1_R... 20	SPL1_R... 20	SPL1_R... 20	SPL1_R... 20	SPL2_R... 20	SPL2_R... 20	SPL2_R... 20	SPL2_R... 20
E	STD4 0,1	STD4 0,1	STD4 0,1	STD4 0,1	SPL1:3 40	SPL1:3 40	SPL1:3 40	SPL1:3 40	SPL2:3 40	SPL2:3 40	SPL2:3 40	SPL2:3 40
F	STD5 0,2	STD5 0,2	STD5 0,2	STD5 0,2	SPL1_R... 40	SPL1_R... 40	SPL1_R... 40	SPL1_R... 40	SPL2_R... 40	SPL2_R... 40	SPL2_R... 40	SPL2_R... 40
G	STD6 0,4	STD6 0,4	STD6 0,4	STD6 0,4	SPL1_NEP 10	SPL1_NEP 10	SPL1_NEP 10	SPL1_NEP 10	SPL2_NEP 10	SPL2_NEP 10	SPL2_NEP 10	SPL2_NEP 10
H	STD7 0,8	STD7 0,8	STD7 0,8	STD7 0,8	NEP 1	NEP 1	NEP 1	NEP 1				

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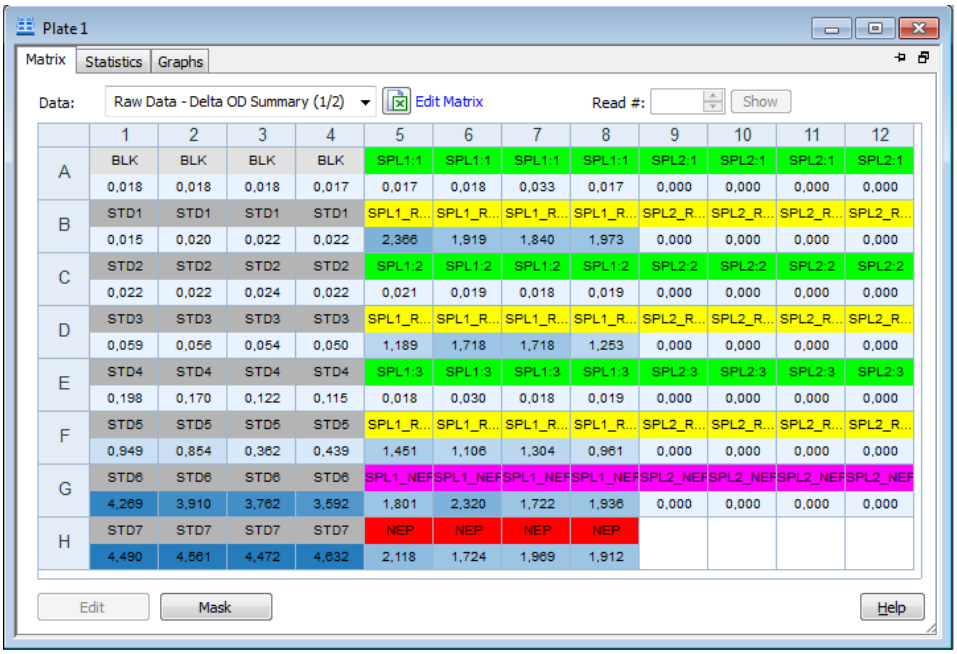
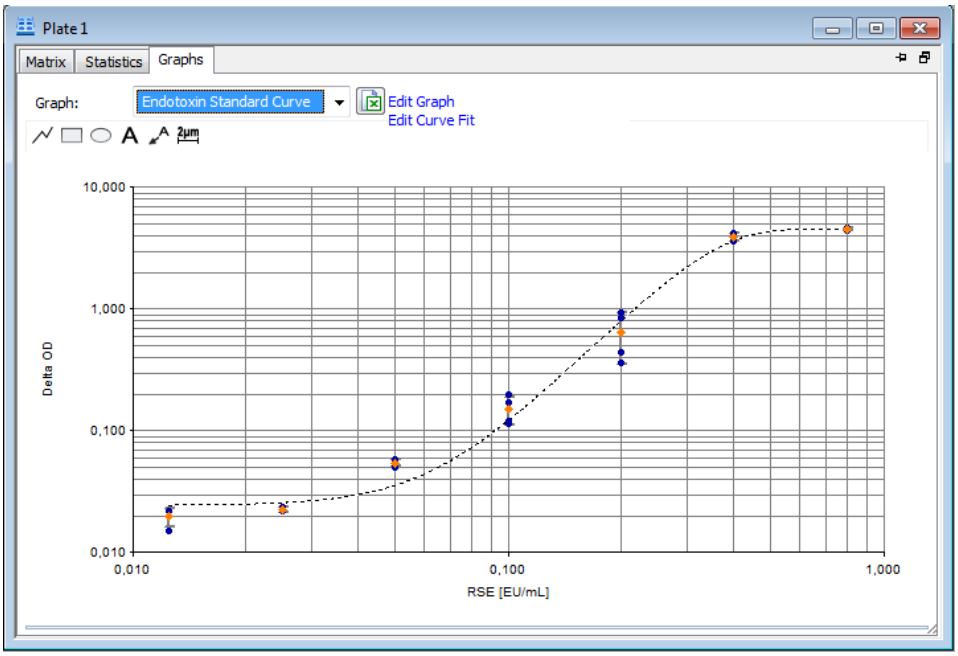
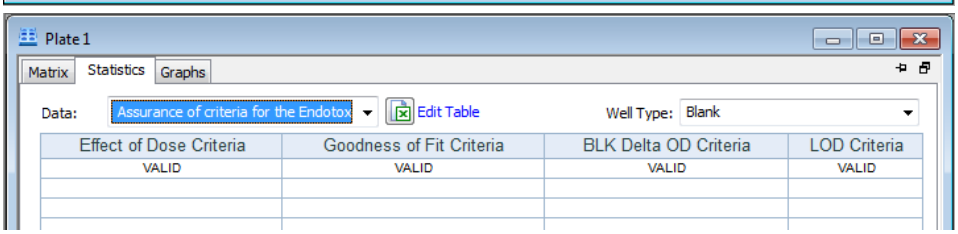
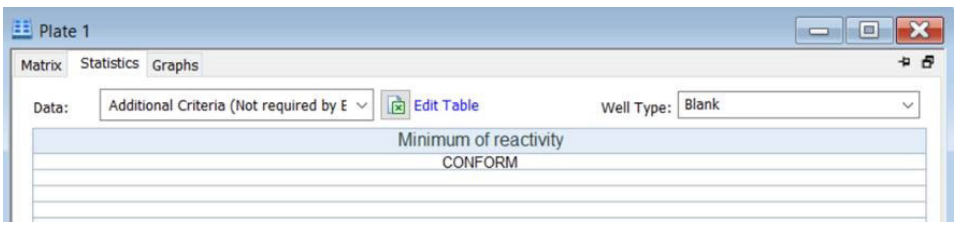
After reading the plate, the data interpretation was performed with the software.



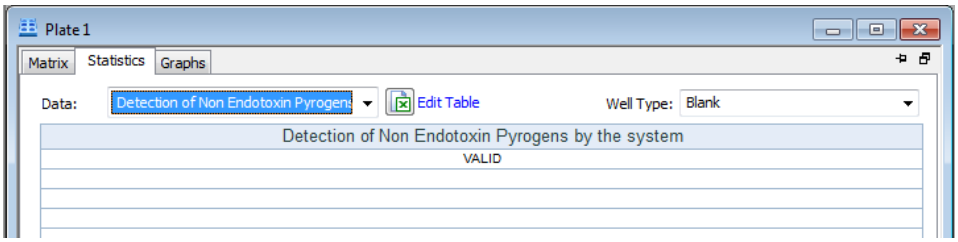
The standard curve was valid for all the criteria.

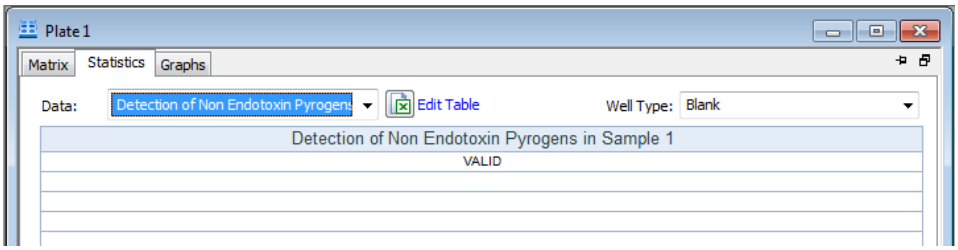






The NEP-control confirmed detection of non-endotoxin pyrogens in the system and in the sample.



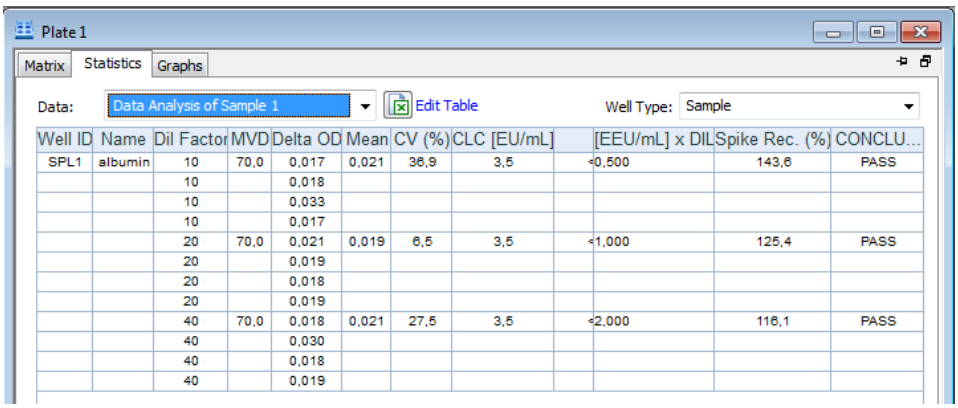


The chosen sample dilutions were appropriate for the sample with all tested dilutions being within the MVD and spike recovery within 50-200%.

The data analysis showed validity of the test being fulfilled for all sample dilutions.

The sample itself showed a pyrogenic load of <0.5 EEU*/mL which is below the CLC (3.5 EU/mL) and therefore being considered “not pyrogenic”.

*EEU: Endotoxin Equivalent Unit



Well ID	Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Spike Rec. (%)	CONCLU...
SPL1	albumin	10	70,0	0,017	0,021	36,9	3,5	<	0,500	143,6	PASS
		10		0,018							
		10		0,033							
		10		0,017							
		20	70,0	0,021	0,019	6,5	3,5	<	1,000	125,4	PASS
		20		0,019							
		20		0,018							
		20		0,019							
		40	70,0	0,018	0,021	27,5	3,5	<	2,000	116,1	PASS
		40		0,030							
		40		0,018							
		40		0,019							

Results

The capability of the MAT for the detection of Pyrogens in albumin preparations is described in several studies [5,6,7].

The examined albumin preparation showed an enhancement of the reaction of the Monocytes in the undiluted sample that could be overcome by dilution within the authorized dilution range (not exceeding the MVD).

The data show that PyroMAT™ system is suitable for detecting both endotoxin and non-endotoxin pyrogenic contaminations in preparations of albumin.

References

[1] European Pharmacopoeia, chapter Monocyte activation test (2.6.30)

[2]Tullis JL: Albumin 2.Guidelines for Clinical Use. JAMA 1977; 237:460-463

[3] Sandle T “Pharmaceutical Product Impurities: Considering Beta Glucans” (2013) americanpharmaceuticalreview

[4] Pardo-Ruiz Z1, Menéndez-Sardiñas DE2, Pacios-Michelena A1, Gabilondo-Ramírez T2, Montero-Alejo V1, Perdomo-Morales R3. “Soluble β-(1,3)-glucans enhance LPS-induced response in the monocyte activation test, but inhibit LPS-mediated febrile response in rabbits: Implications for pyrogenicity tests” Eur J Pharm Sci. 2016 Jan 1;81:18-26

[5] Spreizer I. “Advanced Pyrogen Testing” (2017) americanpharmaceuticalreview

[6] Spreizer I., Fischer M., Hartzsch K., Luderitz-Puchel U., Montag T. „Comparative study of rabbit pyrogen test and humon whole blood assay on human serum albumin.“ Altex. 19 Suppl 1/02, 73-75

[7] Perdomo-Morales R., Pardo-Ruiz Z., Spreitzer I, Lagarto A, Montag T. “Monocyte Activation Test (MAT) Reliably Detects Pyrogens in Parenteral Formulations of Human Serum Albumin” Altex 28, 3/11, 227-235.

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- Comparison of **Reference Standard Endotoxins** (RSE)

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- Monocyte Activation Test: **statistical analysis**

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- **Validation of a cell line-based Monocyte Activation Test** method according to **USP <1225>** Validation of compendial procedures guideline

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Application Note

Reference Standard Endotoxins suitable for PyroMAT™ System

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 is the endotoxin (LPS = Lipo-Polysaccharide), which is 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 to 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.

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 the patient safety.

The monocyte activation test (MAT) method has been 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). 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 which 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).

By putting the product to be tested in contact with human monocytic cells, it will mimic what happens in the human body: in 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 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 MM6 cells during incubation phase are transferred from the cells supernatant 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.

Comparison of Reference Standard Endo- to ent suppliers

Preparation of endotoxin standard solutions is needed to assess the limit of detection (LOD) of the system, to build a standard curve for quantification or to estimate the pyrogen content of a sample, depending on the MAT method used.

The use of a validated Reference Standard Endotoxin is required and such a standard can be supplied by the European Pharmacopeia (EDQM) or the United States Pharmacopeia (USP).

Control Standard Endotoxins (CSE) provided by LAL suppliers should not be used for MAT test.

The reference standard endotoxin (RSE) supplied by the USP / EDQM is a reference endotoxin preparation with a certified activity upon reconstitution. Control standard endotoxin preparations (CSEs) are qualified using the RSE, but their activity is certified only in combination with a test system, e.g. a defined preparation of limulus amoebocyte lysate used for the bacterial endotoxin test.

Using these CSEs outside their test system might lead to unexpected results and is not recommended by the respective suppliers. As there is currently no dedicated reference standard for the pyrogen test available, standardization is achieved using a strong pyrogen like the RSE whose production and standardization is not depending on the use of a specific bacterial endotoxin test.

The scope of this application note is to show the suitability of Reference Standard Endotoxins from different suppliers (USP/EDQM) for MAT with the PyroMAT™ System.

Materials

Material	Description	Cat. No.
PyroMAT™ Kit		**Pyr0MATkit**
PyroMAT™ Cells		**Pyr0MATcells**
PyroMAT™ Endotoxin Standard	European Pharmacopoeia (EP) Reference Standard Endotoxin	**1.44161.0001**
Sigma-Aldrich RSE	European Pharmacopoeia (EP) Reference Standard Endotoxin	**E0150000**
ACILA RSE	USP Reference Standard Endotoxin (USP) 10.000 E.U./Fl	**1220200**
NIBSC RSE	3rd International Standard 10,000 USP Endotoxin units Replacement I.S. for 94/580	**10/178**

Table 1: Materials used to generate standard curves

Resuspension of RSE

Lyophilized RSE were reconstituted and aliquoted according to supplier guidelines.

Dilution of Reference Standard Endotoxin aliquots

The standard endotoxin solutions were prepared from the RSE stock solution at 2000 EU/mL. Seven (7) endotoxin concentrations (0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 EU/mL) were prepared to generate the standard curve according to the following procedure:

- Thaw a 50 µL-aliquot of RSE and vortex at maximum speed during 1 min.
- Perform serial dilutions in endotoxin-free water, using endotoxin-free glass tubes, as described below. Make sure to vortex all the dilutions before using.

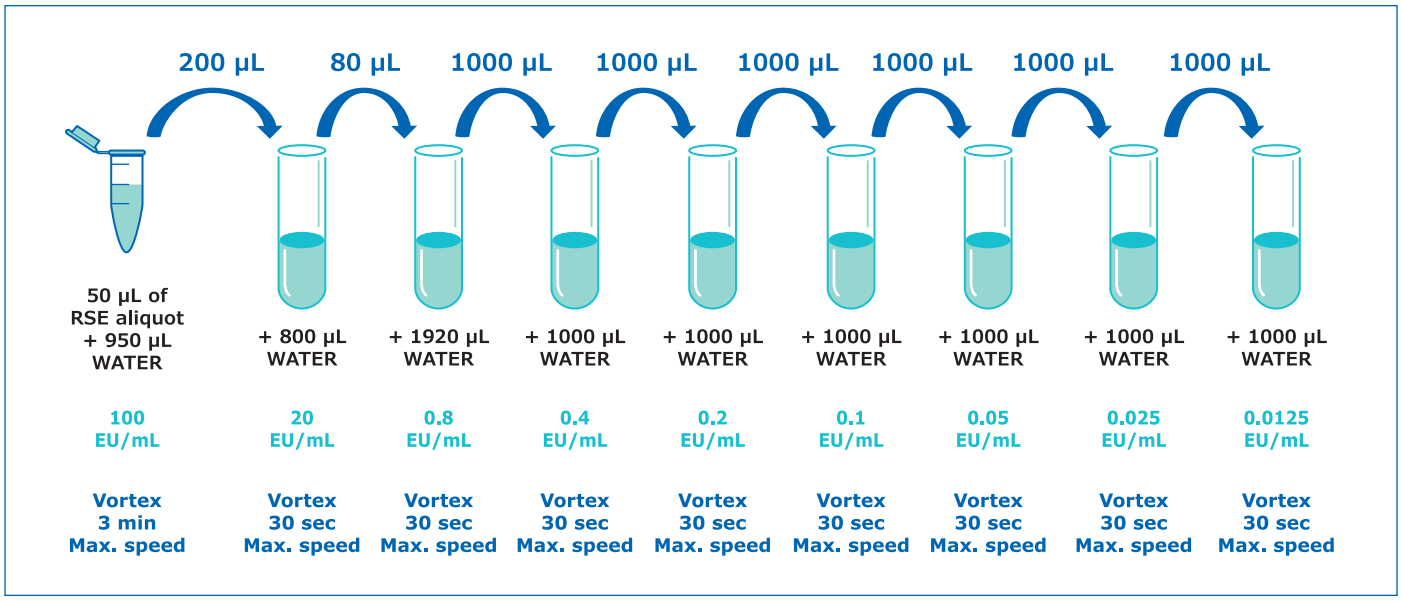


Figure 2: Materials used to generate standard curves

MAT quick procedure with PyroMAT™

Step 1: Preparation and incubation with PyroMAT™ cells

1	• **Prepare suitable endotoxin standard dilutions** • **Load the different solutions on the 96-wells cell culture plate** • **Prepare the PyroMAT™ cells and dispense in each well**	
2	• **Incubate the plate for 22 ±2 hours at 37 °C with humidified atmosphere, without CO_2**	

Step 2: Detection of IL-6 with ELISA

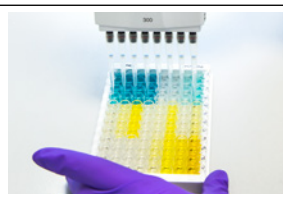
3	• **Transfer the cell supernatants into IL-6 microplate** • **Add the IL-6 conjugate to each well** • **Incubate 2 hours at room temperature**	
4	• **Remove the liquid and wash the plate 4 times** • **Prepare the substrate solution by mixing color reagent A and B and add the mixture to each well** • **Incubate 30 minutes at room temperature, in the dark**	
5	• **Add the stop solution**	
6	• **Read the plate at 450 nm and 630 nm within 30 minutes after adding the stop solution**	

Figure 3: PyroMAT™ workflow with standard ELISA procedure

Results

Endotoxin standard curves were generated using RSE from different suppliers. To be considered "VALID", the endotoxin standard curve must fulfill the following acceptance criteria described in the EP Chapter 2.6.30 :

- **f dose criteria:** a statistical test that confirms a positive dose/effect response.
- **Goodness o t:** 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.

An additional criterion was implemented in the protocol to assess the reactivity of the standard curve:

- **Minimum of reactivity:** OD of the 4 replicates of the highest standard (0.8 EU/mL) should be above 3.

It is not required by the European pharmacopeia and is given as an additional indication for the customer.

Data analysis was performed using the PyroMAT™ data analysis tool, which consists of a specific protocol developed for PyroMAT™ using Gen5 Software (Biotek).

The **Figure 4** presents the curves that were obtained with the PyroMAT™ system using Reference Standard Endotoxins (RSE) from four different suppliers as described in **table 1.**

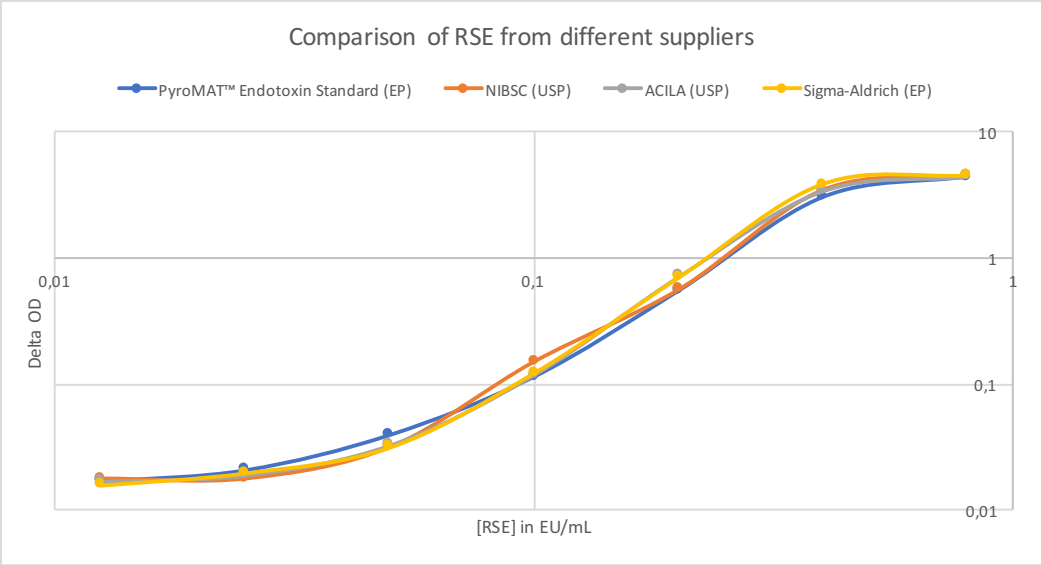


Figure 4: Comparison of standard curves generated with various Reference Standard Endotoxins

The validity of the acceptance criteria for the endotoxin standard curve was determined using the PyroMAT™ data analysis tool (protocol for Gen5 Software).

The **Figure 5** shows the results obtained and the legend used in the software for data interpretation:

Material	Effect of close	Goodness of Fit	Blank Delta OD	LOD criteria	Minimum of reactivity
PyroMAT™ Endotoxin Standard	VALID	VALID	VALID	VALID	**CONFORM**
Sigma-Aldrich RSE	VALID	VALID	VALID	VALID	**CONFORM**
ACILA RSE	VALID	VALID	VALID	VALID	**CONFORM**
NIBSC RSE	VALID	VALID	VALID	VALID	**CONFORM**

Legend

Effect of Dose Criteria: VALID: p<0.01; INVALID: p≥0.01

Goodness of Fit Criteria: VALID: p>0.05; INVALID: p≤0.05

BLK Delta OD Criteria: VALID: MEAN(BLK)<0.1; INVALID: MEAN(BLK)≥0.1

LOD Criteria — VALID: LOD ≤ 0.05 EU/mL; INVALID: LOD > 0.05 EU/mL

Additional Criteria – Minimum of reactivity — CONFORM: All replicates of Delta OD at STD7 are above 3; NOT REACHED: At least one replicate of Delta OD at STD7 is below 3

?????: Unable to Evaluate

Figure 5: Acceptance criteria for the endotoxin standard curves and legend for data interpretation

All standard curves generated with RSE from different suppliers passed the acceptance criteria of a valid standard curve according to the EP chapter 2.6.30.

Conclusion

All four different Reference Standard Endotoxins (RSE) tested led to the generation of a valid standard curve and can be used to perform MAT with PyroMAT™ system.

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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:

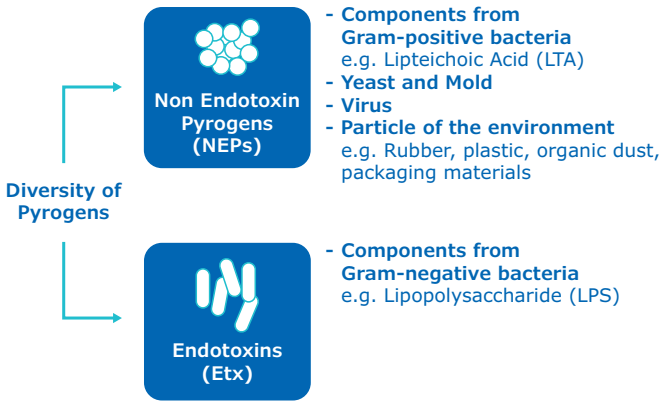
- Some adjuvants in vaccines
- Synthetic Lipopeptides

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]

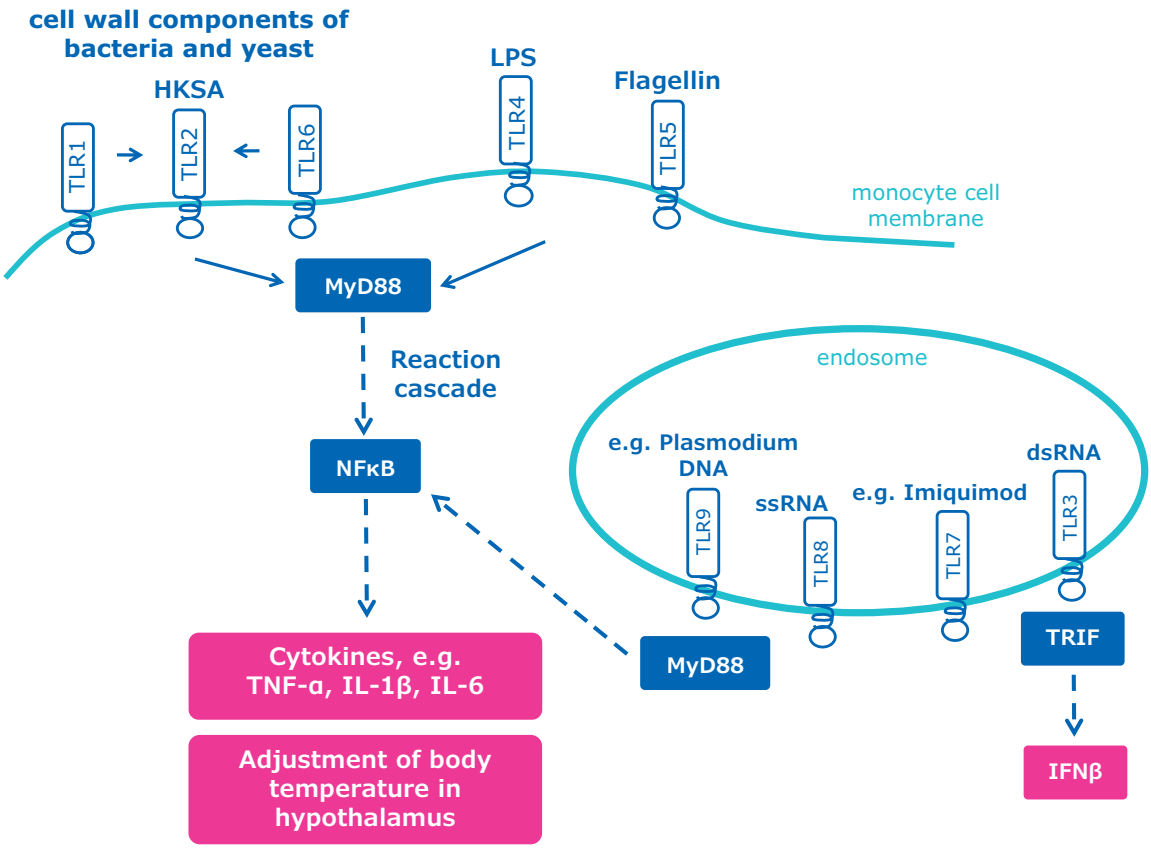


Figure 1. TLR signaling pathways

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).

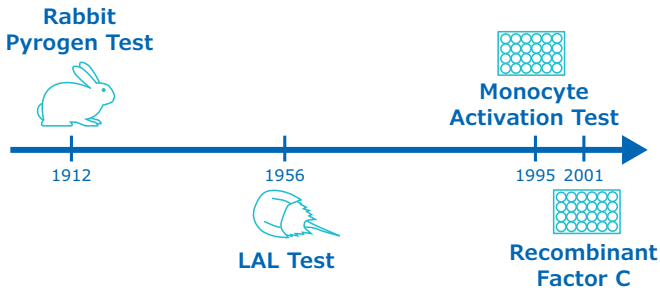
Parenteral preparations must be “pyrogen-free” because administration of pyrogens may lead to life-threatening fever in some patients.

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.

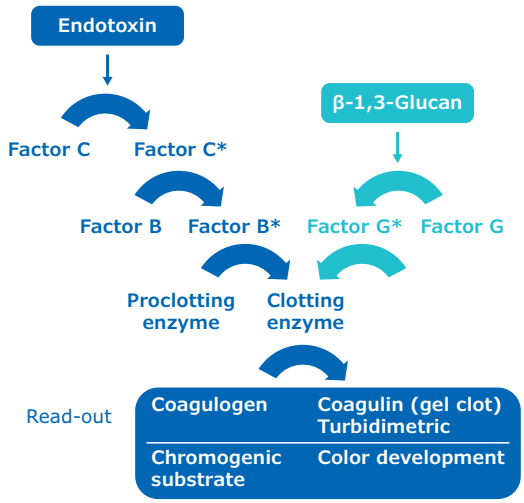
	Test Type	Animal based?
Endotoxins	**Bacterial Endotoxin Tests (BET) or Limulus Amebocyte Lysate (LAL)** EP 2.6.14, USP 85 **Principle:** use of immune response of the horseshoe crab against invasion of Gram negative bacteria	Yes
Endotoxins	**Recombinant Factor C (rFC)** In July 2016 in the EP, FDA Q&A 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.

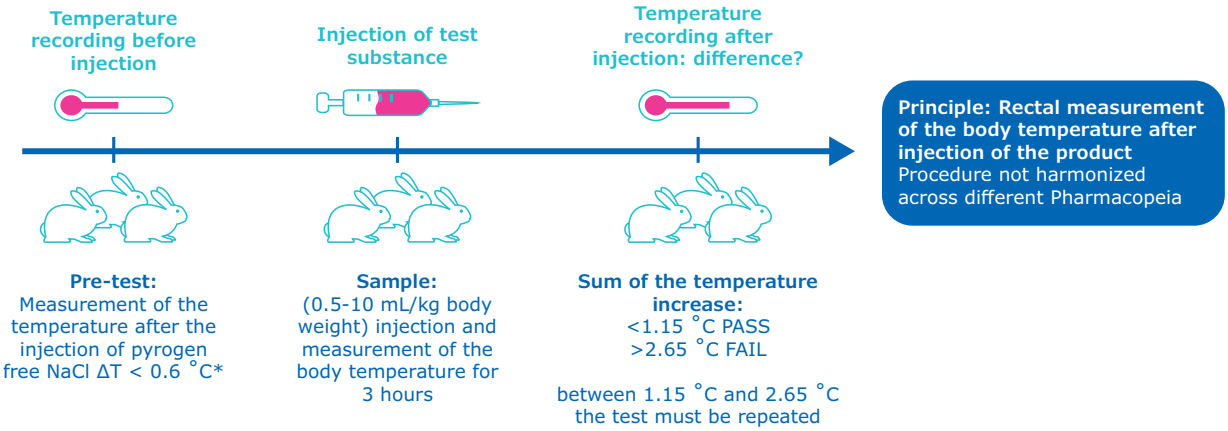
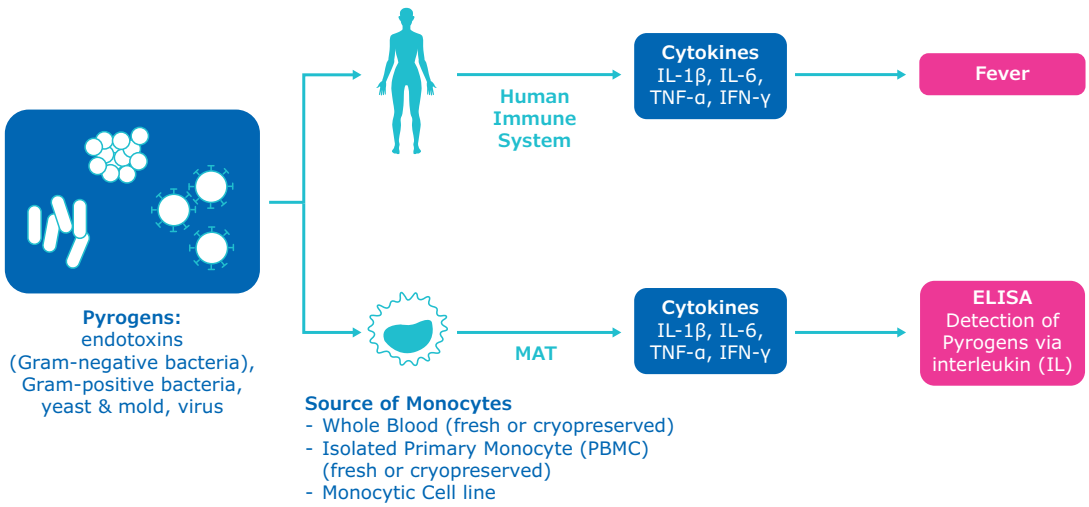


Figure 2: Example of procedure of RPT according to EP

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).



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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, MilliporeSigma).	- 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 MilliporeSigma
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.

n 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

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Studies to demonstrate the robustness and sensitivity of the PyroMAT® system for the detection of endotoxin and non-endotoxin pyrogens

Application Notes:

- Detection of **non-endotoxin pyrogens (NEP)** by Monocyte Activation Test (MAT) using the PyroMAT® system
- Quantification of pyrogen in **Hormone** with the PyroMAT® system
- Quantification of pyrogen in **Vaccine** with the PyroMAT® system
- Quantification of pyrogen in **FBS** with the PyroMAT® system
- Quantification of pyrogens in **Albumine** with the PyroMAT® system
- Comparison of **Reference Standard Endotoxins** (RSE)

White Paper:

- Monocyte Activation Test (MAT): **the *in vitro* test for pyrogen detection**
- Monocyte Activation Test: **statistical analysis**

Datasheet:

- **Validation of a cell line-based Monocyte Activation Test** method according to **USP <1225>** Validation of compendial procedures guideline

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White Paper

Monocyte Activation Test
Logistic regression model of the endotoxin standard curve and statistical tests

1. Introduction

The Monocyte Activation Test (MAT) is used to detect or quantify pyrogenic substances that activate human monocytes.

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 MAT has been described as a compendial method for pyrogen detection in the European Pharmacopeia since 2010 (Chapter 2.6.30). It is a test that mimics the human reaction to pyrogens, by using a source of monocytes, which release some interleukins upon activation by pyrogenic substances:

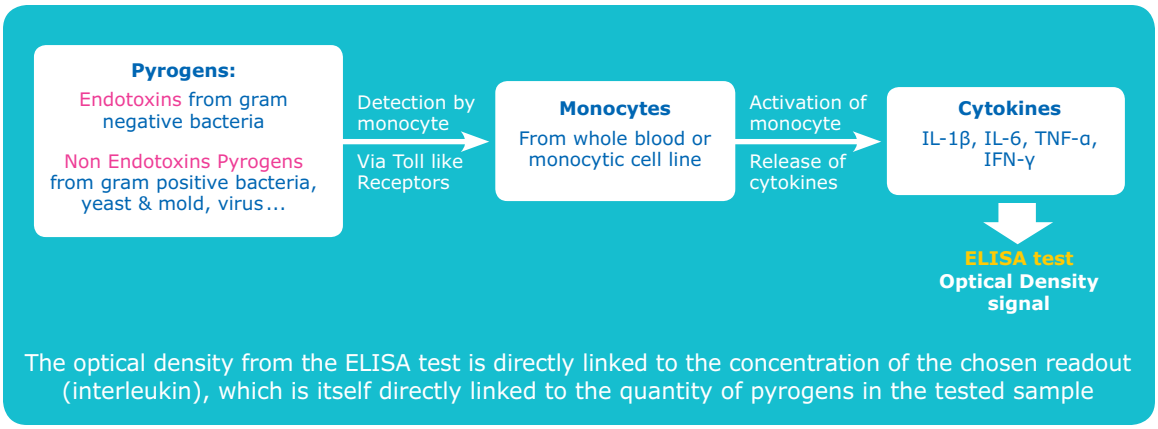

Figure 1: principle of the Monocyte Activation Test

The test requires the use of an endotoxin standard curve for Product Specific Validation (PSV) and for the Method A (quantitative test) described in the European Pharmacopeia. The Optical Density (OD) signal is the result of the biological reaction of monocytes to pyrogens and endotoxins. It is represented in function of the standard endotoxin concentration.

The endotoxin standard curve is then used to convert an OD signal into a pyrogen concentration and allows the quantification of pyrogens or endotoxin equivalent units in the tested sample.

A regression model is needed to modelize properly the response of monocytes to different Reference Standard Endotoxin (RSE) concentrations (Standard curve) in a continuous manner on the range of interest.

We currently provide two MAT solutions:

- The PyroDetect System uses cryopreserved whole blood as a source of monocytes, and the interleukin-1ß as a readout for the ELISA;
- The PyroMAT™ System uses the Mono-Mac-6 (MM6) cell line as a source of monocytes, and the interleukin-6 as a source of monocytes for the ELISA.

The use of different sources of monocytes (blood vs cell line) leads to a different reactivity profile that requires the use of different regression models for each of these solutions.

To analyze the data generated by PyroDetect or PyroMAT™ System, the Gen5™ software[1] (Biotek, 2017) is used to carry out all the modelling and statistical analysis. Gen5™ offers extensive curve fit methods for quantitative and qualitative analysis including 4- and 5-parameter logistic curve fits. We have developed specific protocols to perform automatically all the calculations and conclusions, according to Method A, B or C described in the European Pharmacopeia 07/2017:20630 (MAT Chapter).

The purpose of this white paper is to describe how the response of monocytes (either from cryoblood or MM6 cell line) to endotoxins has been modelized in order to fulfill the EP requirements. The statistical analysis performed in our data analysis tool supported by Gen5™ Software is also described. The handling of outliers is described at the end of the document. This white paper was written with the support of a statistician from the Institute of Advanced Mathematical Research (UMR 7501) of the University of Strasbourg and the Centre National de la Recherche Scientifique (CNRS), who also provided support along the development of our data analysis tool.

Design of experiments

In order to get an efficient dose-response modelling, the following design of experiments is recommended by the EP chapter 07/2017:20630.

"Using the standard endotoxin solution, prepare at least 4 endotoxin concentrations to generate the standard curve. Perform the test using at least 4 replicates of each concentration of standard endotoxin."

Both the design of experiments for PyroDetect and PyroMAT™ Systems satisfy that criterion:

- For PyroDetect System, the experiment is made of 4 replicates of 6 RSE concentrations, including a blank.
- For PyroMAT™ System, the experiment is made of 4 replicates of 8 RSE concentrations, including a blank.

What is a good curve model?

A good curve model should possess three properties[2]:

1. Curve model must do a good job at approximating the true curve. If the curve model does not do this, then whatever the number of replicates may be, the model will still be differing from the mean observed values because the model is wrongly specified: for instance, using a straight line to deal with nonlinear relationships.
2. A good curve model must be able to average out as much of the random variation in the assay data as possible to produce concentration estimates that are distorted by pure error as little as possible.
3. A good curve model must be able to predict concentrations well at points between the standard points and not just at the fitted data points.

[1] https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/features/#1

[2] The five-parameter logistic: A characterization / P.G. Gottschalk, J.R. Dunn / Anal. Biochem. 343 (2005) 54–65

What is curve fitting and how can it be performed?

Once a curve model is chosen, one still needs to select the "best" curve among all the possible curves. This is called the fitting process and it aims to adjust the free parameters of the function until these parameters approximate the assay's true curve better than any other parameter set. From a statistical point of view, this is called maximum likelihood estimation.

Once we assume that the error of the model, i.e. the residual discrepancies (or just residuals) between the predicted values ($\check{y}_i$) using the fitted curve and the actual observed values (y_i), is –approximately– Gaussian, then fitting a curve using maximum likelihood is equivalent to finding the curve whose parameters generate the smallest weighted sum of squared errors (*SSE*).

The weighted sum of squared errors (*SSE*) is the sum of all of the squares of the residuals (y_i-$\check{y}_i$) –the differences between the observed standard responses and the response predicted by the curve model–, weighted by the inverse variance (w_i=1/Var(y_i)) of the responses at that concentration[3]:

$$\text{SSE} = \sum_{i=1}^{n} w_i \, (y_i - \check{y}_i)^2$$

Why a curve would not perfectly fit the data?

In any regression, regardless of what curve model is used, there are two reasons why the curve will not fit the data perfectly:

1. Pure error: the presence of random variation in the data. It can be reduced by increasing the number of replicates of each standard concentration.
2. Lack-of-fit error: the curve model may not approximate the true curve very well. It cannot be reduced by increasing the number of standard replicates.

Two criteria for assessing dose-response modelling

According to the EP chapter 07/2017:20630 there are 2 acceptance criteria for the endotoxin standard curve: fit and effect.

1. To use a model, it is necessary to check if it correctly fits the data. For instance, for linear regression, the EP recommends that:

"The regression of responses on log dose must not deviate significantly from linearity ($p > 0.05$)."

As our model differs from linear regression, we have to adapt our goodness of fit procedure while still using a 5% level of significance.

2. Then, if the model fits the data, one wants to assess whether there is or not a significant effect of the doses on the optical densities. The EP recommends using a 1% level of significance:

"The regression of responses (appropriately transformed if necessary) on log dose shall be statistically significant ($p < 0.01$)"

[3] According to regression theory, the weights should be set equal to the inverse variance of the responses at that concentration: weighting the squared errors in this way causes the fitting procedure to adjust the curve to be tighter around those standard responses with the smallest variance (error).

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Data modelling using sigmoid dose-response curves

Figure 2 represents data generated with the PyroDetect System. The endotoxin concentrations are represented on the horizontal axis. The responses are indicated on the vertical axis. The individual responses (OD values) to each endotoxin concentrations are indicated with black dots.

As can be seen on **Figure 3,** the goodness of fit of a linear model is very poor. Monocytes do not respond in a linear way to the increasing pyrogens concentrations, that's why other models must be investigated.

According to The European Pharmacopoeia chapter 07/2016:50300 corrected 9.2, a sigmoid[4] dose-response curve is suitable for some immunoassays. Such a model, the four-parameter logistic one, is fitted to the data on **Figure 4** and seems relevant.

In the chapter 07/2016:50300 corrected 9.2 the EP provides some guidance on selected statistical questions that are raised by the use of non-linear dose-response curves and their extensions.

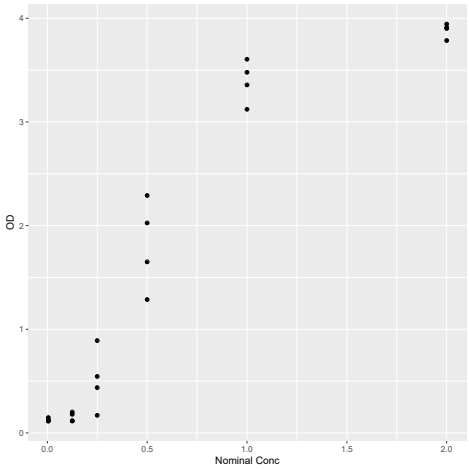

Figure 2: Raw data

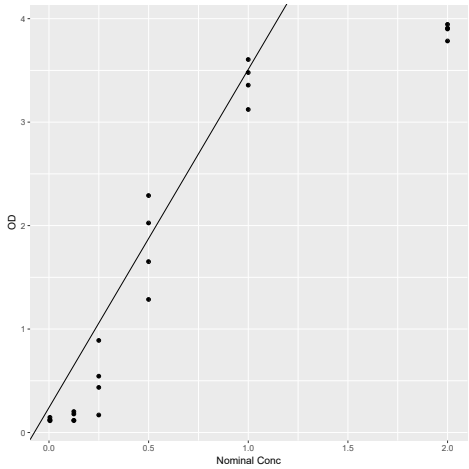

Figure 3: Linear fit

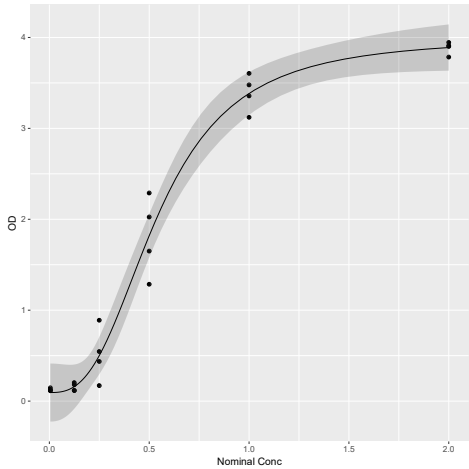

Figure 4: Sigmoid fit

1. ***[…] "However, models based on functions giving other sigmoid curves may also be used. Models incorporating additional asymmetry parameters have been suggested.***
2. ***Heterogeneity of variance is common when responses cover a wide range. If the analysis ignores the heterogeneity, interpretation of results may not be correct and estimates may be biased. Use of the reciprocal of the error variances as weights is unlikely to be reliable with limited numbers of replicates. It may be appropriate to estimate a function which relates variance to mean response.***
3. ***The statistical curve-fitting procedures may give different estimates depending on assumptions made about the homogeneity of the variance and on the range of responses used.***
4. ***In principle, equality of upper and lower response limits for the different preparations included in an assay can be directly tested in each assay. However, interpretation of the results of these tests may not be straightforward." […]***

As we will quickly review now, the PyroDetect and PyroMAT™ datasets feature some of the specificities highlighted by the EP. To provide the users with an efficient statistical methodology, a special care was devoted to address all these specificities:

1. For the PyroDetect datasets, the logistic four-parameter model will fit to most of the data. Yet, as we will show later, PyroMAT™ datasets feature some additional asymmetry and will require the use of extended non-linear dose-response curves such as the five-parameter logistic function that incorporate an additional asymmetry parameter.
2. Heterogeneity of variances is observed for PyroMAT™ dataset.
3. A comprehensive modelling of the variances leads to an improvement of the curve fitting procedures.
4. A dedicated statistical test to detect the effect of the dose on the optical densities is needed.

[4] A non-linear model with an S-shape curve

2. Four-parameter logistic model to describe PyroDetect dataset

PyroDetect dataset

Figure 5 plots a dataset generated with the PyroDetect System. It exhibits non-linearity without asymmetry: monocytes from whole blood reach a reactivity plateau naturally before reaching the saturation of the absorbance reader. As a consequence, for PyroDetect data, the 4-parameters logistics regression model is the most suitable.

We will use this nonlinear model for the PyroDetect datasets and assume homogeneous variances in the fitting process and significance analysis.

Design of the assay

In order to fit the nonlinear logistic 4 parameters model, one needs to get observed optical density (OD) values before and after the turning point of the S-curve. It is recommended that:

- at least one standard is not far from each asymptote
- at least 2 standards fall within the linear area of the curve, on either side of the inflection point

The total number of standards was chosen in order to get enough measurements to estimate the four parameters of the model (4 standards required and 5 recommended for the calculation of the goodness of fit statistics).

Yet, the actual values for which the turning point appears depend on each assay. As a consequence a wider range composed of a blank and five concentrations was used to design experiments with the PyroDetect System:

0 EU/mL, 0.125 EU/mL, 0.25 EU/mL, 0.5 EU/mL, 1 EU/mL, 2 EU/mL.

The aim is to successfully find the turning point of the curve at each assay.

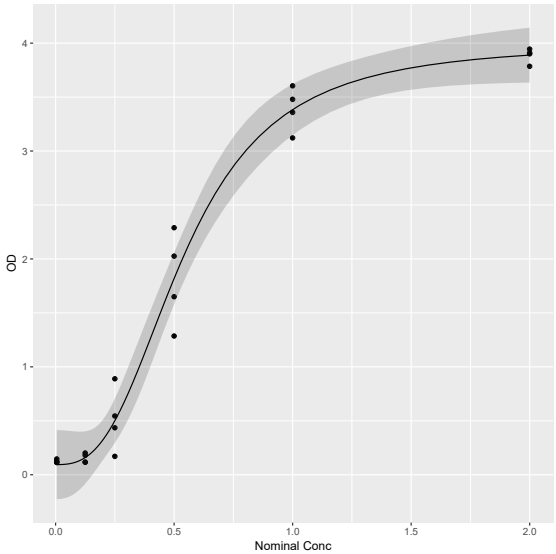

Figure 5: PyroDetect dataset with a 4P logistic fit

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Features of the 4-parameters regression model

Definition

Here is the equation of the log-logistic 4 parameters model:

$$f(x) = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

where a, b, d can be any number and c is a positive number.

Symmetric model

The model is symmetric, which means that if you swap the a and d values and reverse the sign of b, the curve remains unchanged. Hence, one can assume that the slope b is positive without loss of generality or fitting performance. In the following of this white paper, we assume b positive (b>0).

Four parameters

Even though the logistic 4 parameters model seems complicated, it can be easily grasped since its parameters have plain interpretation (remember that b>0), see:

- a is the lower intercept (plateau) at the left of the curve (Y unit: OD)
- b is the unitless slope factor or Hill slope.
- c is the ED_50 (is the concentration that give half-maximal effects), in the same units as X. It also corresponds to the inflexion point (point of greatest slope) and to the point of symmetry.
- d is the upper intercept (plateau) at the right of the curve (Y unit: OD)

The effect of the four parameters on the shape of the curve are plotted on **Figure 6, Figure 7, Figure 8 and Figure 9.**

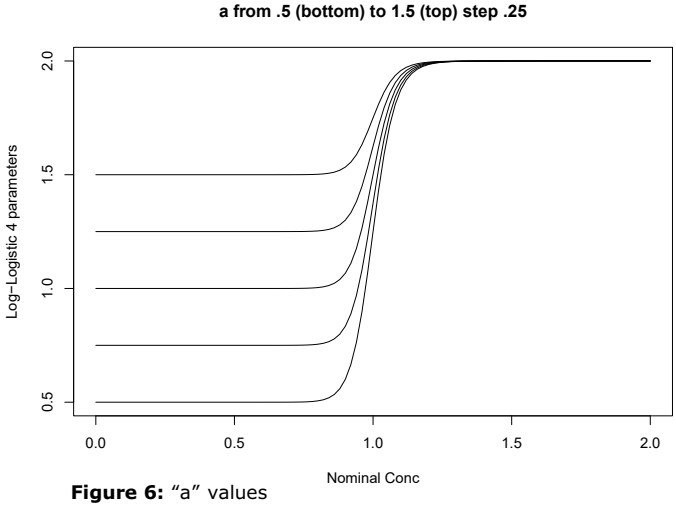


Figure 6: "a" values

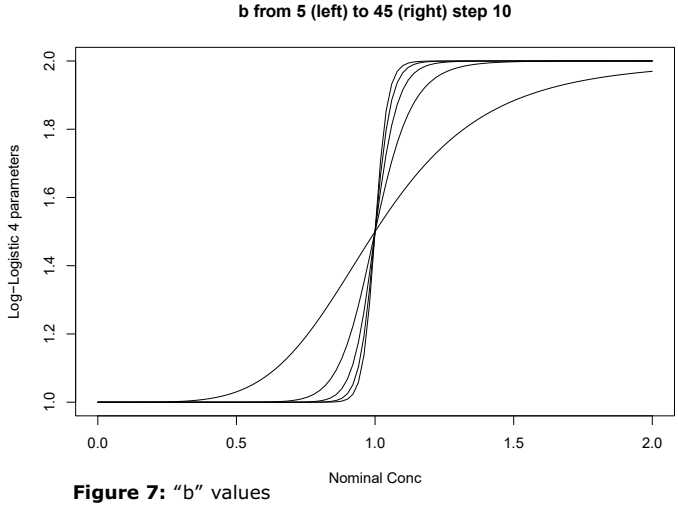


Figure 7: "b" values

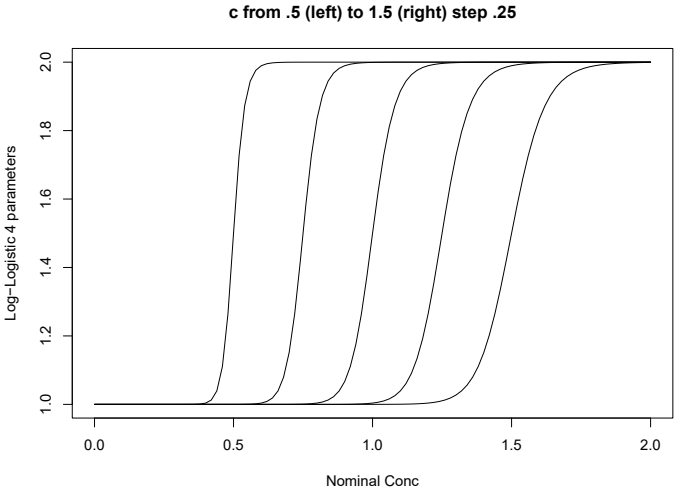


Figure 8: "c" values

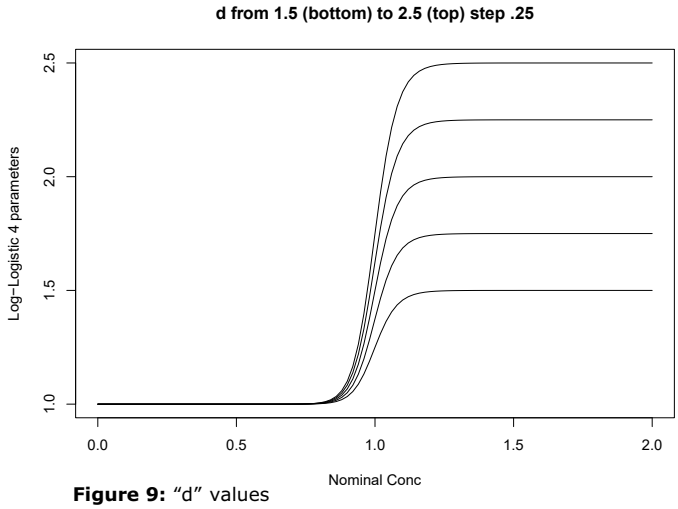


Figure 9: "d" values

Statistical significance testing to fulfill EP requirement

According to the EP, we need to assess the goodness of fit test at a 5% level, then search for a significant dose effect at a 1% level.

Goodness of fit

The classical lack-of-fit test is applied (Bates and Watts, 1988[5]). This test compares the dose-response model to a more general analysis of variance (ANOVA) model using an approximate F-test. It assumes that the responses of the individual standard concentrations are approximately normally distributed and this distribution allows us to determine the probability that curve fits having a particular value of lack of fit, or worse, will occur. The fit F-Prob test (Fisher Snedecor) is the probability of goodness of fit. It requires replicates but no weighting.

A significant test result is alarming and means that the model does not fit the data and that it must be changed.

Effect of dose

The dose effect is assessed using three different criteria:

1. Parameter *a* must be lower than *d* (the effect is increasing)
2. The curve must be monotonic: mean OD value for 0.125 EU/mL < mean OD value for 0.250 EU/mL < mean OD value for 0.500 EU/mL < mean OD value for 1.000 EU/mL,
3. The upper intercept (parameter *d*) must be significantly different from 0 (OD) at the 1% level.

The third criteria was chosen among several ones that are sensible for logistic four parameters models: significant dose effect is often tantamount to high *b* values (*b* is significantly > 0), **Figure 7,** or *d* » 0 (*d* is significantly greater than a *null value*), **Figure 9.**

Those significance criteria are based on Student's t-test. A p-value smaller than or equal to the alpha level 0.01=1% is equivalent to a t-value larger than, or equal to, the critical value tcrit(*Df,*1-α)[6,7], with *Df* the degree of freedom of the test (=24-4=20).

For our experimental design (4 replicates for 6 doses) and where a 4P model yields: *tcrit*(20, 0.99)=2.528.

The Statistical Table[7a] for T-Student Critical values at the alpha level 0.01 is used as in some cases masking data in order to remove outliers is changing the degree of freedom:

Statistical Table for T-Student Critical values

df	12	13	14	15	16	17	18	19	20	21	22	23	24
p0.01	2.681	2.650	2.624	2.602	2.583	2.567	2.552	2.539	2.528	2.518	2.508	2.500	2.492

We now detail the test for d=0. Based on the previous data set and using Gen5 software, the d parameter value is 3.983 and its standard error is 0.148 (see **figure 10**).

One can use a student statistic to test if *d* » 0. The Student Ratio is equal to

$$\text{SRatio} = \frac{d_{upper} - 0}{\text{Std error}} = \frac{3.983}{0.148} = 26.912$$

The critical value for a one-sided test of significance at the 1% level is tcrit(20, 0.99)=2.528 and hence the absolute value of the Student Ratio must be equal or greater than 2.528 for the test to be significant at the 1% level. Since 26.912>2.528, the test is significant at the 1% level.

[5] Bates, D. M. and Watts, D. G. (1988) Nonlinear Regression Analysis and Its Applications, New York: Wiley & Sons (pp. 103–104).

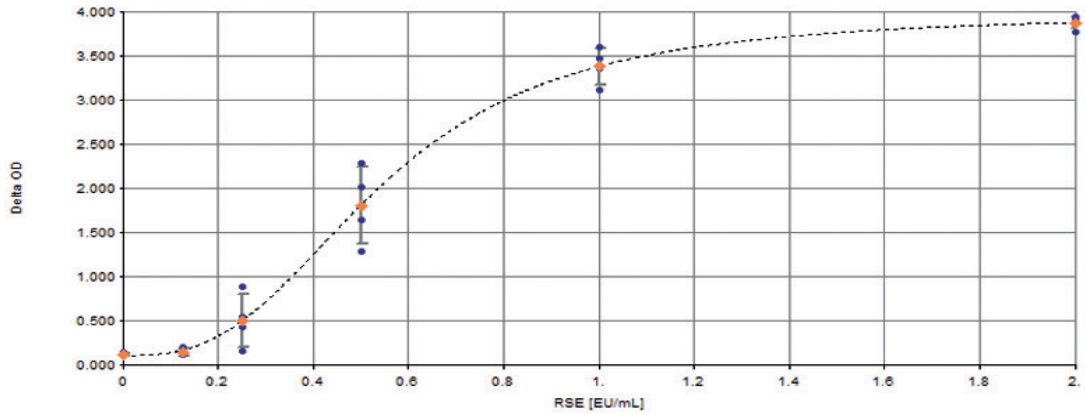
[6] Aide-mémoire pratique des techniques statistiques. Pour ingénieurs et techniciens supérieurs. / CERESTA – Centre d'enseignement et de recherche de statistique appliquée. Paris in Revue de Statistique Appliquée, vol. XXXIV - n° spécial (1986)

[7] Pearson, E. S., und H. O. Hartley: Biometrika Tables for Statisticians, Vol. 1, 2. Aufl. Cambridge University Press, Cambridge 1962.

[7a] Revue de Statistique Appliquée, 1988 - Tables Statistiques - Tables N°3 - Fractiles de la loi de Student

R statistical software used to confirm implementation in Gen5™

The results obtained with the Gen5™ software were confirmed using the R software, a well-known statistical language[8]. This process of verification was carried out by creating scripts and dedicated packages in order to reproduce all the statistical analyses done by Gen5. Both are equal if rounded at 3 decimal places.



Results: Std Curve fitting Details Edit

Curve Name	Curve Formula	Parameter	Value	Std. Error
StdCurve	Y = (A-D)/(1+(X/C)^B) + D	A	0.113	0.085
		B	2.813	0.397
		C	0.545	0.029
		D	3.983	0.148

Figure 10: PyroDetect data. Regression and Values of the 4 parameters generated by Gen5

4P Parameters given by Gen5

Curve Name	Curve Formula	Parameter	Value	Std. Error	SRatio
StdCurve	Y = (A-D)/(1+(X/C)^B) + D	A	0.113	0.085	
		B	2.813	0.397	
		C	0.545	0.029	
		D	3.983	0.148	26.912

4P Parameters given by 'R'

Curve Name	Curve Formula	Parameter	Value	Std. Error	SRatio
StdCurve	Y = (A-D)/(1+(X/C)^B) + D	A	0.112842	0.084915	
		B	2.813270	0.396769	
		C	0.544765	0.029152	
		D	3.983084	0.148003	26.912

4P Parameters given by 'R' rounded at 3 decimals

Curve Name	Curve Formula	Parameter	Value	Std. Error	SRatio
StdCurve	Y = (A-D)/(1+(X/C)^B) + D	A	0.113	0.085	
		B	2.813	0.397	
		C	0.545	0.029	
		D	3.983	0.148	26.912

Error (in %) between parameters given by Gen5 and R

Curve Name	Curve Formula	Parameter	Value	Std. Error	SRatio
StdCurve	Y = (A-D)/(1+(X/C)^B) + D	A	0.000	0.000	
		B	0.000	0.000	
		C	0.000	0.000	
		D	0.000	0.000	0.000

Figure 11: Comparison of the regression generated by Gen5 and R.

[8] R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2018, https://www.R-project.org

3. Five-parameter logistic model to describe PyroMAT™ data

PyroMAT™ data set

Figure 12 plots a dataset generated with PyroMAT™ System.

As seen previously, monocytes from whole human blood react to pyrogens in dose-response manner until reaching a plateau, in the dynamic range of usual absorbance reader (OD between 0 – 4).

Mono-Mac-6 cells, used in the PyroMAT™ System are more sensitive, react strongly to pyrogens and do not reach the plateau in the dynamic range of readers. The observed plateau is artificial and is due to the saturation of the signal. Therefore, the actual dose-response curve shows asymmetry and requires an adequate regression model.

To control asymmetry of the curve, the 4PL model can be extended by adding a fifth parameter *e*. This model is called the five-parameter logistic (5PL) model[9]. Asymmetry means that the curve does not near the lower or higher intercept at the same pace. **Figure 13** displays curve with no asymmetry (bold curve) and two curves with asymmetry values greater than 1 that nears the higher asymptote faster than they near the slower one.

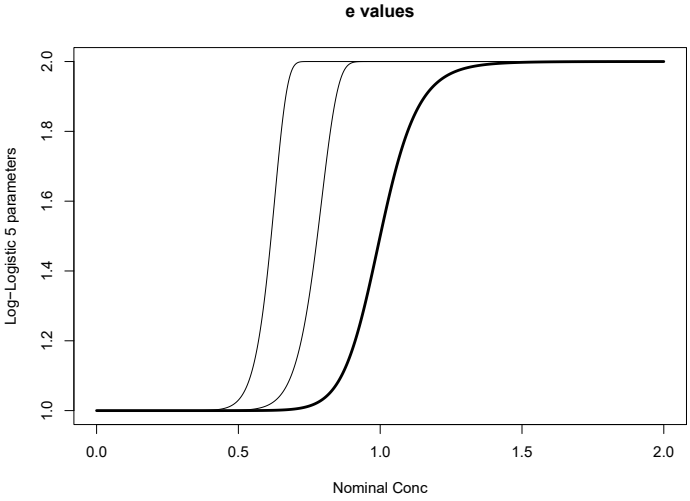


Figure 13: e values (from left to right 1000; 30; 1)

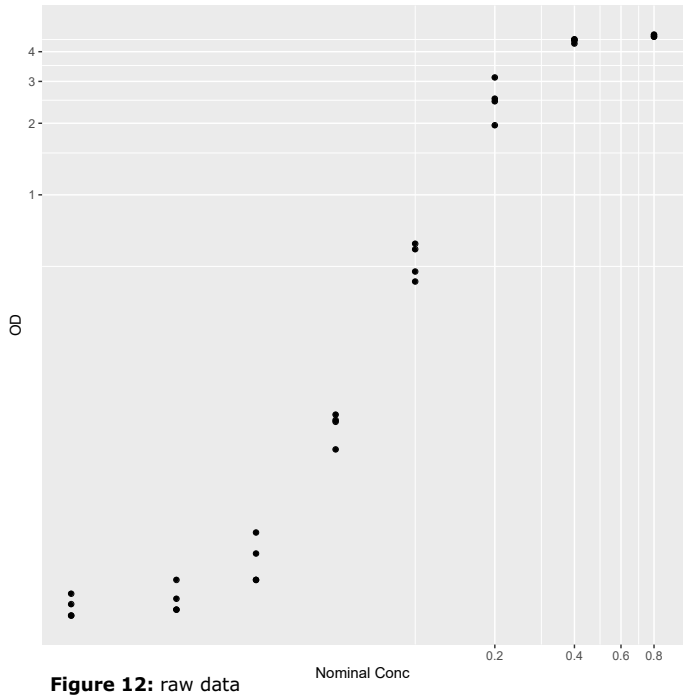


Figure 12: raw data

As shown on the **figure 14,** the 5-parameter logistic model fits PyroMAT™ data.

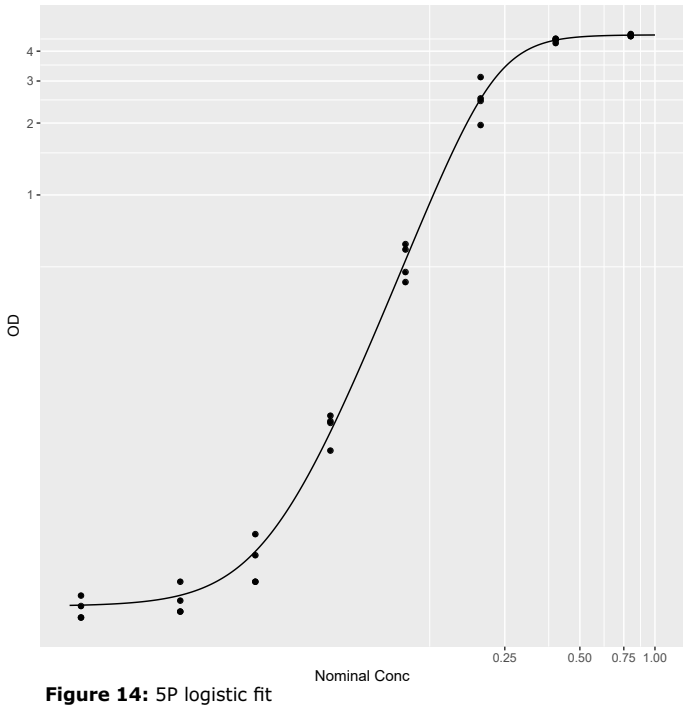


Figure 14: 5P logistic fit

[9] In The five-parameter logistic: A characterization / P.G. Gottschalk, J.R. Dunn / Anal. Biochem. 343 (2005) 54–65, the authors have compared the 5PL to the 4PL in many thousands of dose–response curves from a wide variety of immunoassay and bioassay technologies. With the extra flexibility afforded by its asymmetry parameter, the 5PL model is able to virtually eliminate the lack-of-fit error that occurs when the 4PL is fitted to asymmetric dose–response data.

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Design of the assay

In order to fit the nonlinear logistic 5-parameter model, one needs to get observed optical densities (OD) values before and after the turning point of the S-curve. It is recommended that:

- at least one standard is not far from each asymptote
- at least 3 standards fall within the linear area of the curve, one at the inflection point and one on either side of it.

The design of experiment for the PyroMAT™ System satisfies these criteria.

Yet, the actual values for which the turning point appears depend on each assay and as a consequence a wider range of seven concentrations was used to design experiments in order to fit the 5P model with the PyroMAT™ system:

0.0125 EU/mL, 0.025 EU/mL, 0.05 EU/mL, 0.1 EU/mL, 0.2 EU/mL, 0.4 EU/mL, 0.8 EU/mL.

The aim is to successfully find the turning point of the curve at each assay.

One can spot that additional low concentrations values were selected in order to improve the fit in the region used for back fitting and deriving concentrations of unknown samples.

The dynamic range of usual absorbance readers starts from 0 up to 4 OD. Hence, we will need specific care to deal with values over that threshold.

Features of the 5-parameters regression model

Definition

Here is the equation of the logistic 5 parameters model:

$$f(x) = \frac{a - d}{1 + \left(\left(\frac{x}{c}\right)^b\right)^e} + d$$

Asymmetric model

The 5P logistic model is asymmetric, see Figure 13, unlike the 4P logistic model, introduced in Section 2. Five parameters are required because it is the fewest number of parameters that any general asymmetric sigmoidal function can possess.

Five parameters

Without hindering the fitting flexibility of the 5P model, we assume in the following of this white paper that b is positive (b>0). Its parameters have plain interpretation:

- *a* is the lower intercept (plateau) at the left of the curve (Y unit: OD).
- *b* is unitless and sets the overall length of the function's transition region between asymptotes. It controls alone the rate of approach to the *a* asymptote and jointly with *e* controls the approach to the *d* asymptote.
- *c* sets the location of the transition region, in the same units as X.
- *d* is the upper intercept (plateau) at the right of the curve (Y unit: OD).
- *e* is the degree of asymmetry. It controls, with *b*, the rate of approach to the upper intercept *d*.

The *a* and *d* parameters have the same interpretation as for the four parameters logistic model. Yet, in a five parameters logistic model, other analogies with the four parameter logistic model are misleading.

- The *b* parameter of the model is no longer proportional to the slope at the inflexion point. Moreover, the slope depends more on the *e* parameter than on the *b* one.
- The location of the curve ED_{50} point does not correspond anymore to the *c* parameter: it can be larger or smaller than *c*, depending on the values of *b* and *e*.
- The inflection point of the 5PL curve is not the same as the ED_{50}. It can be larger or smaller than *c* depending on the values of *b* and *e*.

Weighing of the 5-parameters model: Mean-Variance relationship

Unfortunately, it is a common feature of dose-response experiments to show that the variance of the response (i.e. the optical density values) of each point (i.e. RSE concentration) depends on the mean level of this response.

This phenomenon can easily be seen on the PyroMAT™ data set presented in **Figure 15:** the spread of the values increases then decreases with the values of concentrations. The highest value of standard deviation is about 250 times bigger than the smallest.

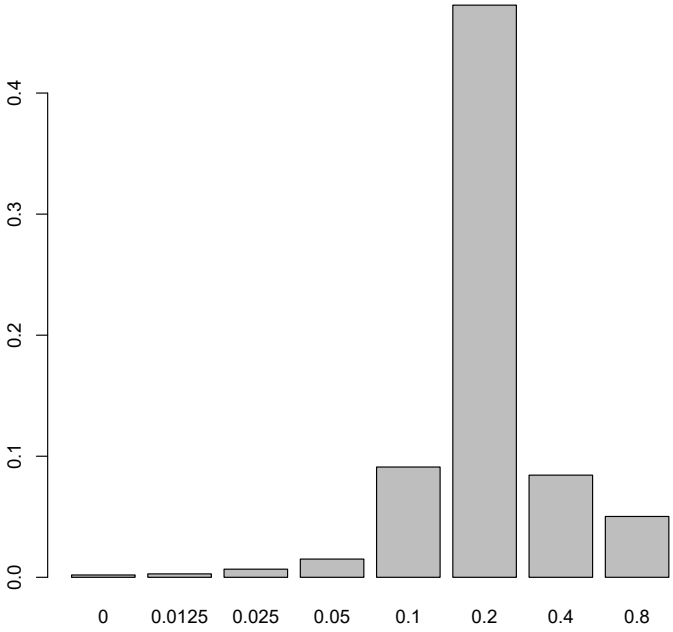


Figure 15: PyroMAT™ dataset - standard deviations (Y axes) in function of RSE concentrations (X axis)

As a consequence, each point of the curve is not determined with the same accuracy. This is taken into account by weighting the curve fit with the variance of the responses.

To fulfill this requirement, a specific variance analysis has been performed on a large pool of historical data.

The **figure 16** shows a pool of observed data from RSE standard curves generated with PyroMAT™ System:

- Variance increases with the Delta OD signal, on the OD range from 0 to 4
- Variance starts to decrease for the highest values of OD, greater than 4, due to the saturation of the reader
- There are less values of observed variances for OD between 2 and 4.

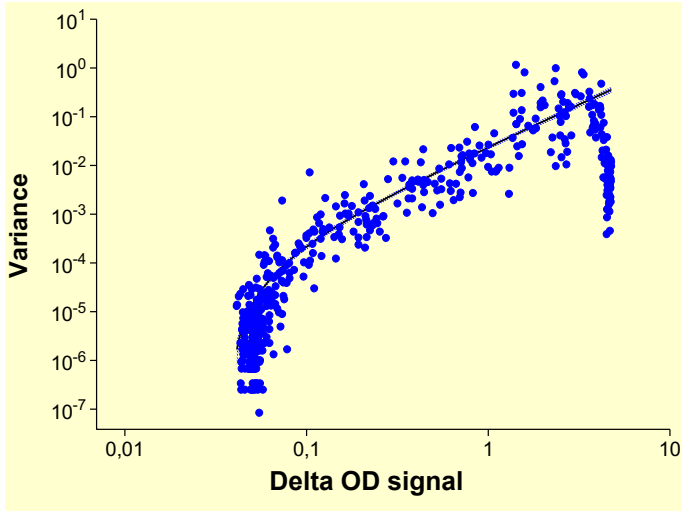


Figure 16: Observed variance versus Delta OD values

In order to determine the relationship between the mean OD and the variance of the OD, on the whole OD range, the following approach has been used:

- **Using a variance function model (see below) on the reduced OD range from 0 to 4.** In particular, this model allows to find accurate variance values for OD values in the 2 to 4 range.

To model the mean-variance relationship, we approximated the variance of the standards by the standard 3-parameter variance function[10a] of the response:

$$\sigma^2(Y) = (\beta_1 + \beta_2 * Y)^J$$

where σ^2 denotes variance, Y denotes the mean and β_1, β_2 and J are the parameters. This function is always monotone over the range of the mean values; either an increasing function of the mean over the entire range, or a decreasing function of the mean.

[10a] According to regression theory, the weights should be set equal to the inverse variance of the responses at that concentration: weighting the squared errors in this way causes the fitting procedure to adjust the curve to be tighter around those standard responses with the smallest variance (error).

Accurately estimating the true variance function from a single assay would require an impractical number of replicates. As a consequence, a large pool of historical assay data was collected during the development of the PyroMAT™ System and then used to compute this variance function, once and for all, using a dedicated software, Variance function program 14[10,11].

- **using empirical variances for the OD values greater than 4.**

As the variance modelling is an increasing function, it was not fitting well the mean-variance relationship for OD values greater than 4, for which the variance decreases due to the saturation of the reader. As a consequence, empiric values from a large pool of historical assay data were considered.

This mixed approach allows to determine the parameters to weigh the 5-parameters regression model.

This regression model is robust to various cells reactivity profiles that could be observed even if not reaching the plateau at the RSE concentration of 0.4 EU/mL.

Statistical significance testing to fulfill EP requirement

According to the EP, the endotoxin standard curve has to fulfill several acceptance criteria:

- the goodness of fit test at a 5% level, then
- a significant dose effect at a 1% level.

Goodness of fit: a χ^2 test

Description of the χ^2 goodness of fit test.

Nonlinear regression minimizes the sum of the squared residuals (i.e. vertical distances between the data point and the curve). The value of that sum-of-squares (SS) depends on the number of data points collected and the units used to express the response. Hence, we need some reference values to carry out relevant interpretation.

The χ^2 (chi-square) statistic aims to compare the observed dispersion of the points (scatter) around the curve with the expected (based on theory or a large number of observations) amount of experimental scatter.

From a numerical point of view, this is done by computing the chi-square statistics:

$$\chi^2 = \sum_{i=1}^{n} \left(\frac{y_i - y_{model}}{\sigma_i} \right)^2$$

The standard deviation values must be computed from lots of data to yield accurate estimates (Subsection on Mean-Variance relationship).

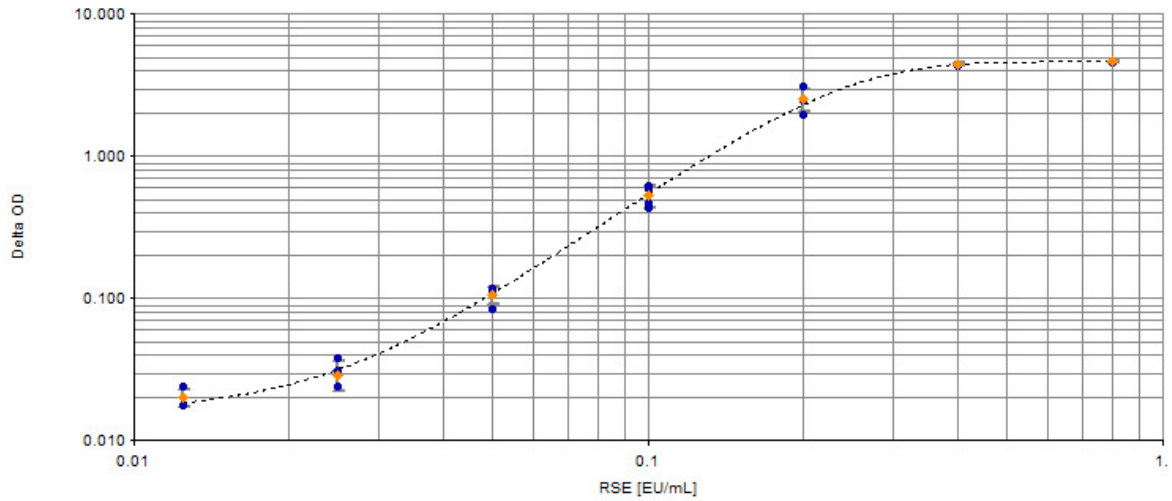
[10] Sadler WA, Smith MH. A reliable method of estimating the variance function in immunoassay. Comp Stat & Data Anal, 3:227-239 (1986). Sadler WA, Smith MH. A computer program for estimating imprecision characteristics of immunoassays. Comput Biomed Res, 23:105-114 (1990) Sadler WA. A new Win32 computer program for estimating immunoassay variance functions. Computer Meth & Prog in Biomed, 67:195-199 (2002) Sadler WA. An upgraded Win32 computer program for estimating immunoassay variance functions. Clin Chem Lab Med, 39(Suppl):S133 (2001)

[11] Available from the website of the Australasian Association of Clinical Biochemists at the http address https://www.aacb.asn.au/professionaldevelopment/useful-tools/variance-function-program-version-140.

Use of the χ^2 goodness of fit test.

If the model is correctly specified, the errors (Y_i-Y_{model}) are Gaussian and the σ_i values are accurately known, then the χ^2 statistics is distributed as a χ^2 distribution with a number of degrees of freedom, which equals the number of data points minus the number of parameters. A *p*-value can be computed accordingly and the fit of the model can be rejected if that p-value is lower than a given level of risk. A α = 5% risk level is recommended by the EP.

With the PyroMAT™ data set (**figure 12**), the following results are obtained with Gen5™:



Results: Std Curve fitting Details Edit

Curve Name	Curve Formula	Parameter	Value	Std. Error	Fit Chi2 Prob
StdCurve	Y = (A-D)/(1+(X/C)^B)^E + D	A	0.016	0.004	0.878
		B	2.572	0.081	
		C	0.441	0.151	
		D	4.653	0.028	
		E	5.528	3.841	

Figure 17: PyroMAT™ data. Regression and Values of the 4 parameters generated by Gen5

The Fit-Chi2-Prob value of the test is equal to 0.878 which is greater than 0.05=5%. Hence the test is not significant at the 5% level and the model does not missfit the data. The goodness of fit criteria is valid for this endotoxin standard curve.

Note: the main difference between the χ^2 and the Fit F-Prob test, is that the variance of the errors must be estimated using the sample for the Fit F-Prob test whereas it is estimated using accurate previous knowledge of the process (a large amount of sample is required) for the χ^2 test, see Subsection on Mean-Variance relationship.

Effect of dose

Why a global test of effect?

As stated in the 4P model section, if a dose-response experiment is modelled using a four parameters logistic model, different criteria can be used to detect and evaluate the significance of a dose effect, either based on the *b* parameter or *d* parameter (upper asymptote).

Yet, it is not convenient or even misleading to use those criteria with a five parameters logistic model and the design of experiments set up for the PyroMAT™ System because:

- the *b* parameter of the model is no longer proportional to the slope at the inflexion point. Moreover, the slope depends more on the *e* parameter than on the *b* one. Hence, testing if the *b* parameter is equal to 0 is not testing for a 0 slope.
- the uncertainty on the *d* parameter is increased because of:
  - the presence of the asymmetry parameter *e*
  - the choice of the concentrations that favors lower values with OD before the inflection point
  - even if the uncertainty on the *d* parameter was low, it is most often estimated using OD values lying beyond the dynamic range of the reader.

On the contrary, the no effect test assesses, using the whole dose-response curve, whether there is a dose effect or not and does not focus only on some of the parameters.

In addition to the no effect test, additional criteria were implemented to assess the effect of dose and ensure a strictly monotonous increase of the curve:

Value of the parameter *a* should be below the value of the parameter *d*

Value of each standard should increase progressively: STD (0.05 EU/mL) < STD (0.1 EU/mL) < STD (0.2 EU/mL) < STD (0.4 EU/mL)

Description of the no effect test

A significance test[12] is provided for the comparison of the dose-response model considered and the simple linear regression model with slope 0 (a horizontal regression line corresponding to no dose effect).

Hypotheses tested by the no effect test

The aim of the no effect test is to decide between two hypotheses, named null hypothesis H_0 and alternative hypothesis H_1, one of the two being the more likely to be true. Those two hypotheses are mutually exclusive (they cannot hold simultaneously):

- the H_0 hypothesis is that there is no dose effect at all,
- the H_1 hypothesis is that there is a dose effect.

The model used to describe the H_0 hypothesis is a model with a constant OD value whatever the concentration value, which is tantamount to no concentration effect on the OD. That constant value is the best one possible[13] and is equal to the mean of all observed OD values.

The model used to describe the H_1 hypothesis is a model with dose-response relationship modelled by a five parameters logistic curve.

The test aims to compare those two models by evaluating whether there is a significant improvement when fitting a five parameters logistic model instead of the simpler constant one. The EP recommends a α = 1% level of significance for that no effect test. By having a test with a small value of α, we feel that we have actually "proved" something when we reject the null hypothesis.

A p-value smaller than or equal to α = 0.01 = 1% -*p*-value<0.01- is equivalent to a Chi-square test value larger than, or equal to, the critical value *chisqcrit* (*Df*,1-α)[14,15], with *Df* the degrees of freedom of the test.

With our experimental design and 5P model yields to: chisqcrit(*Df*,1-α) = 13.277.

As a result, if the test is significant at the α=1%, then we reject the null hypothesis H_0 and decide that there is a significant dose effect, the risk of wrong decision being limited to 1%.

With the PyroMAT™ dataset (**figure 12**), the following results are obtained with Gen5:

Chi-square test	Df
176.426	4.000

Since the Chi-square test is equal to 176.426 which is larger than 13.277, the test is significant at the 1% level: there is a significant dose effect.

[12] From a statistical point of view, this no effect test is a likelihood ratio test and is based on a chi-square distribution whose number of degrees of freedom is 4 -the number of free parameters 5 minus 1-. For more statistical details on the no effect test, see appendix 1.

[13] For the least squares criterion

[14] *Aide-mémoire pratique des techniques statistiques. Pour ingénieurs et techniciens supérieurs.* / CERESTA – Centre d'enseignement et de recherche de statistique appliquée. Paris in Revue de Statistique Appliquée, vol. XXXIV - n° spécial (1986)

[15] Pearson, E. S., und H. O. Hartley: *Biometrika Tables for Statisticians,* Vol. 1, 2. Aufl. Cambridge University Press, Cambridge 1962.

4. Handling of outliers

When performing a MAT test, it happens that the endotoxin standard curve does not fulfill all the defined acceptance criteria. Therefore, a deeper analysis of the raw data is required and the handling of outlier can be a solution. Outliers can have several effects, not only on the validity of the standard curve but also on the test conclusion on pyrogenicity of the sample.

What is an outlier?

The MAT test requires to perform 4 replicates of each condition (e.g. endotoxin standard, samples, positive controls...). An outlier is a measurement that strongly deviates from the other replicates (extreme value).

The MAT test is based on a biological reaction of living cells. Therefore, it is expected that the test may demonstrate some variability and a broader distribution of the measured values than chemical reactions.

As a manually performed method, the test can also be strongly affected by handling issues such as pipetting errors or carry over contaminations from well to well.

What is the effect of an outlier ?

When computing a mean value, the smaller the sample size, the more influence an outlier will exert.

Depending on its location on the plate, outliers can have several impacts on the assay:

- Outlier in the blank can lead to invalid Limit of Detection
- Outlier in the standard curve can lead to a poorer curve fit or invalid criteria
- Outlier in the sample spiked with RSE can lead to invalid spike recovery
- Outlier in the sample could lead to inaccurate conclusions on pyrogenicity of the samples.

In a word, using or not these extreme values can change the result of the statistical analysis. As it may lead to wrong conclusion or results, outliers should be examined carefully.

Dealing with outliers

As a consequence, one should use dedicated statistical methods to deal with such a measurement and, from a data integrity point of view, cannot bluntly suppress it from the data. In order to temper the influence that such a single outlying measurement may have on the dose-response modelling or test conclusion, three solutions are commonly used:

- significance testing,
- median-based statistics,
- robust statistics.

In this white paper we focus on the first approach. The Gen5 software allows users to mask any value and it is the way to exclude any detected outlier from further analysis. To justify the presence of an outlier, 2 statistical tests can be used: Grubb’s test and Dixon’s test.

Grubbs' test

Grubbs' test is recommended by ISO. It computes the ratio of the deviation between the suspect value and the mean value by the standard deviation of the sample. Only one outlier can be detected by this test: the value the farther from the mean value of the sample.

Grubbs' test aims to select between two competing hypotheses, the null hypothesis H_0 and the alternative hypothesis H_1:

H_0: Keep the tested value
H_1: Reject the tested value

For this, the T statistic is calculated:

$$T = \frac{|\text{suspect value-sample mean value}|}{\text{sample standard deviation}}$$

where both the sample mean value and the sample standard deviation are computed using the complete sample (hence including the suspect value). This test assumes that we are dealing with an approximately normal population[16].

Grubbs recommends to use a smaller risk level than the one used to perform the forecoming statistical analysis of the data (typically the 1% level if the statistical analysis will be performed at the 5% level). Hence, in our case we will use the 1% level.

There are three types of critical values –see **figure 18**– for the T statistic of the Grubbs' test.

1. Testing a suspect value among the large values of the sample (T_n case).
2. Testing a suspect value among the small values of the sample (T_1 case).
3. Testing a suspect value among both the large and the small values of the sample (T case). It is generally the case, if we do not know beforehand (before carrying out the experiment) if we will be looking for a high valued or a low valued outlier.

Number of observation *n*	Upper 0.1% significance level	Upper 0.5% significance level	Upper 1% significance level	Upper 2.5% significance level	Upper 5% significance level	Upper 10% significance level
4	1.499	**1.496**	**1.492**	1.481	1.463	1.425

Figure 18: Table of critical value of T (and one-sided tests T_1 or T_n)[17]

For small sample sizes, the outlying value must highly differ from the other values to be detected by Grubbs' test at a common risk level, namely the 1% level in our case. One of the major issue of Grubbs' test is masking effects due to more than one outlier being in the sample.

The critical values for Grubbs' test with a sample with 4 replicates at the 1% level are:

$$T_{1,crit} = T_{n,crit} = 1.492 \text{ and } T_{crit} = 1.496.$$

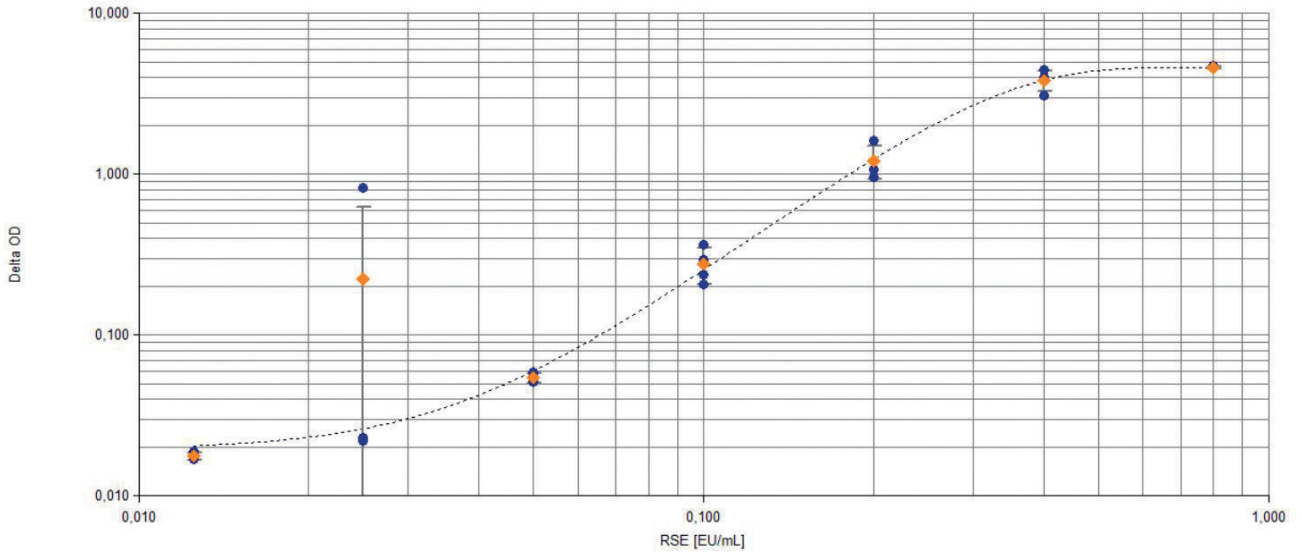
Note that $T_{1,crit}$ and $T_{n,crit}$ are unilateral tests at 1% level, therefore correspond to one-sided tests at 1% level, in the table. T_{crit} is a bilateral test at 1% level, therefore corresponds to a one-sided test at 0.5% level, in the table.

[16] https://www.itl.nist.gov/div898/handbook/eda/section3/eda35h.htm#Normality

[17] Frank E. Grubbs and Glenn Beck, *"Extension of Sample Sizes and Percentage Points for Significance Tests of Outlying Observations"*, Technometrics, 14(4), 847-854 (1972).

Example

Below an example of an endotoxin standard curve obtained with the PyroMAT™ System for which the goodness of fit criteria is INVALID.



Effect of Dose Criteria	Goodness of Fit Criteria	BLK Delta OD Criteria	LOD Criteria
VALID	INVALID	VALID	VALID

Figure 19: Example of pyroMAT data (RSE standard curve) showing a potential outlier and impact on acceptance criteria.

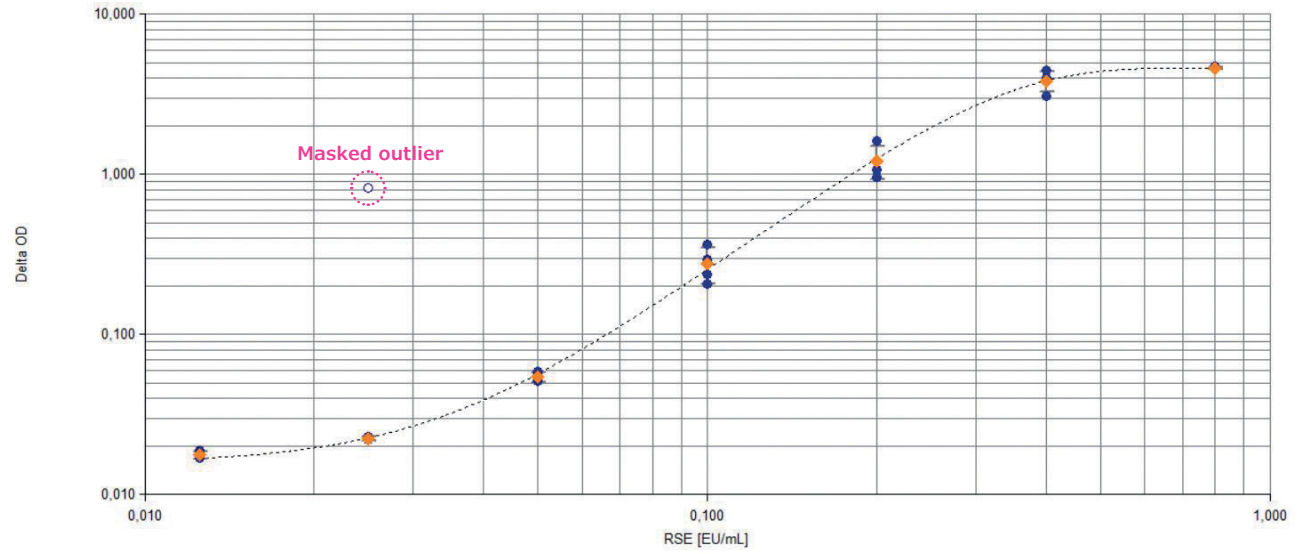
One OD replicates (“0.824”) of the standard at 0.025 EU/mL seems to be suspicious:

Delta OD at 0.025 EU/mL				Mean	Standard Deviation
0.022	0.022	0.824	0.023	0.223	0.401

Applying Grubbs’ test, we get:

$$T = \frac{|0.824 - 0.223|}{0.401} = 1.499$$

The T is higher than the critical T value for $\alpha = 1\%$ (1.496). The test is significant and we can reject the value “0.824” as an outlier, leading to a valid endotoxin standard curve.



Effect of Dose Criteria	Goodness of Fit Criteria	BLK Delta OD Criteria	LOD Criteria
VALID	VALID	VALID	VALID

Figure 20: Example of PyroMAT™ data (RSE standard curve) after masking of an outlier and impact on acceptance criteria.

Dixon's test

The scope of Dixon's test is to detect only one outlier (the same as Grubbs' test), the value the farther from the mean value of the sample. This test assumes that we are dealing with an approximately normal population.

Dixon's test aims to select between two competing hypotheses, the null hypothesis H_0 and the alternative hypothesis H_1:

H_0: Keep the tested value
H_1: Reject the tested value

Dixon's $r_{1,0}$ statistic is the ratio of the difference of the two largest values of the sample by the range (maximum values minus minimum value) of the sample. It aims to detect an outlier among the large values of the sample.

$$r_{1,0} = \frac{\text{largest value-second largest value}}{\text{range (maximum – minimum)}}$$

Dixon's $r'_{1,0}$ statistic is the ratio of the difference of the two smallest values of the sample by the range (maximum values minus minimum value) of the sample. It aims to detect an outlier among the small values of the sample.

$$r'_{1,0} = \frac{\text{second smallest value-smallest value}}{\text{range (maximum – minimum)}}$$

$r_{1,0}$ and $r'_{1,0}$ are combined and define:

$$r = \max(r_{1,0}, r'_{1,0})$$

Dixon's r search for an outlier among both the large and small values of the sample. To find if a Dixon's test is significant at the α level, we use critical values from the table given in **figure 21**. $r_{1,0}$ and $r'_{1,0}$ correspond to a one-sided test and r to a two-sided test.

Number of observation *n*	One sided critical values			Two-sided critical value		
4	***α* = 1%**	*α* = 5%	*α* = 10%	***α* = 1%**	*α* = 5%	*α* = 10%
	0.88940	0.76548	0.67867	**0.92063**	0.82973	0.76550

Figure 21: Critical value for Dixon's outlier test based on MC simulation[18]

The critical values for Dixon's test with a sample with size four at the 1% level are:

$$r_{1,0,crit} = r'_{1,0,crit} = 0.8894 \text{ and } r_{crit} = 0.9206.$$

It is the easiest test to carry out, even easier than Grubbs' test, since it requires very few calculations.

Example

Below an example of 4 replicates of Blank Delta OD obtained with PyroMAT™ System during an assay and for which the LOD criteria is INVALID.

	1	2	3	4
A	BLK	BLK	BLK	BLK
	0,017	0,016	0,116	0,018

LOD Criteria
INVALID

Figure 22: Example of pyroMAT data (blank delta OD) showing a potential outlier and impact on LOD criteria.

The value "0.116" seems to be suspicious and a Dixon's test can be performed to determined if it is a true outlier.

[18] Armin Böhrer, Economic Quality Control, Vol 23 (2008), No. 1, 5 – 13
One-sided and Two-sided Critical Values for Dixon's Outlier Test for Sample Sizes up to n = 30

Applying Dixon's Test, we get:

$r_{1,0} = \frac{0.116 - 0.018}{0.116 - 0.016} = 0.98$	$r'_{1,0} = \frac{0.017 - 0.016}{0.116 - 0.016} = 0.1$	$r = \max(r_{1,0}, r'_{1,0}) = 0.98$

For $\alpha = 1\%$ and $n = 4$, the critical value is equal to 0.8886 for Dixon's $r_{1,0}$ and Dixon's $r'_{1,0}$ and even arger for Dixon's r. As $r = 0.98$ is above the $r_{crit} = 0.9206$, we can reject the value "0.116", which lead to a valid LOD criteria.

	1	2	3	4
A	BLK	BLK	BLK	BLK
	0,017	0,016	*0,116*	0,018

LOD Criteria
VALID

Figure 23: Example of PyroMAT™ data (blank delta OD) with masked outlier and impact on LOD criteria.

5. Conclusion

The Gen5™ protocol developed to support data analysis with PyroDetect System and PyroMAT™ System allows to model the response of monocyte to endotoxin concentrations taking into account the specific features of each method and by using robust regression models that fulfill the EP requirements.

CONTACT US

APPENDIX

The no effect test

Scope of the test

This test assesses a dose effect from a global point of view and not only through one parameter of the model. It is especially useful for the logistic 5-parameter model, since for this model it is difficult to single out one parameter that quantifies the dose effect.

Sample output

Chi-square test	Df	p-value
176.426	4.000	0.000

Output description

Chi-square test
Value of the likelihood ratio test statistic.

Df
It is the number of degrees of freedom for the test = number of independent parameters of the full model minus one (because the reduced model is limited to a single parameter: the intercept). As a result, the Df value is equal to 5-1 = 4 for a five parameters logistic model.

p-value
Value of the likelihood ratio test statistic.

Hypotheses tested

The aim of the test is to decide between the two following hypotheses which one is the more likely to be true on the ground of the observed values.

- H0, there is no dose effect
- H1, there is a dose effect

How is the test statistic derived?

We first need to compute two likelihood values: the likelihood of the reduced model and the likelihood of the full model.

The log likelihood of the reduced model is equal to:

$$llNull = -\frac{1}{2}(28 * (\log(2\pi) + 1 - \log(28) + \log(\text{RSS model is a single intercept" }))),$$

where "RSS" is the residual sum of squares and 28 is the number of values used for the fit (7 doses without the blank with four replicates).

The log likelihood of the 5P logistic model (the full dose-response model) is equal to:

$$ll5P = -\frac{28}{2}(\log(2\pi) + \log(\text{RSS full 5P model }) - \log(28) + 1),$$

where "RSS" is the residual sum of squares and 5 is the number of free parameters of the model.

The likelihood ratio test statistic is equal to:

$$lrt = -2(llNull - ll5P).$$

When is the test significant at the 1% level?

- If the p-value is smaller than, or equal to, the level value 0.01=1%.
- If the observed lrt value (=Chi-square test value in the output) is larger than, or equal to, the 99% quantile (=critical value) of the Chi-square distribution with four degrees of freedom that is equal to 13.277.

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Studies to demonstrate the robustness and sensitivity of the PyroMAT® system for the detection of endotoxin and non-endotoxin pyrogens

Application Notes:

- Detection of **non-endotoxin pyrogens (NEP)** by Monocyte Activation Test (MAT) using the PyroMAT® system
- Quantification of pyrogen in **Hormone** with the PyroMAT® system
- Quantification of pyrogen in **Vaccine** with the PyroMAT® system
- Quantification of pyrogen in **FBS** with the PyroMAT® system
- Quantification of pyrogens in **Albumine** with the PyroMAT® system
- Comparison of **Reference Standard Endotoxins** (RSE)

White Paper:

- Monocyte Activation Test (MAT): **the *in vitro* test for pyrogen detection**
- Monocyte Activation Test: **statistical analysis**

Datasheet:

- **Validation of a cell line-based Monocyte Activation Test** method according to **USP <1225>** Validation of compendial procedures guideline

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Data Sheet

Validation of a cell line-based Monocyte Activation Test method according to USP <1225> Validation of compendial procedures guideline

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Introduction

The Monocyte Activation Test (MAT) was introduced in the European Pharmacopeia (Chapter 2.6.30) in 2010, as a compendial method that can be used to replace the Rabbit Pyrogen Test (RPT). Also, the new version of USP <151> Pyrogen Test, effective since May 2017, indicates the possibility to use a validated and equivalent *in vitro* pyrogen test in place of the *in vivo* RPT. A new cell line-based MAT was developed to detect the full range of pyrogens.

Using the Mono-Mac-6 (MM6) ready-to-use cell line, monocytes are activated if the sample is contaminated with pyrogens (endotoxins and non-endotoxin pyrogens) and produce cytokines including interleukin-6 (IL-6) which is detected in an immunological assay (ELISA).

This method validation study was organized according to EP 2.6.30 MAT, USP <1225> Validation of compendial procedures, and ICH Q2(R1) November 2005, Validation of analytical procedures: text and methodology guidelines.

The method validation characteristics evaluated in the different guidelines were: accuracy, precision, specificity, detection limit, linearity and range. To cover all the aspects of the method performance, ruggedness and robustness were also considered.

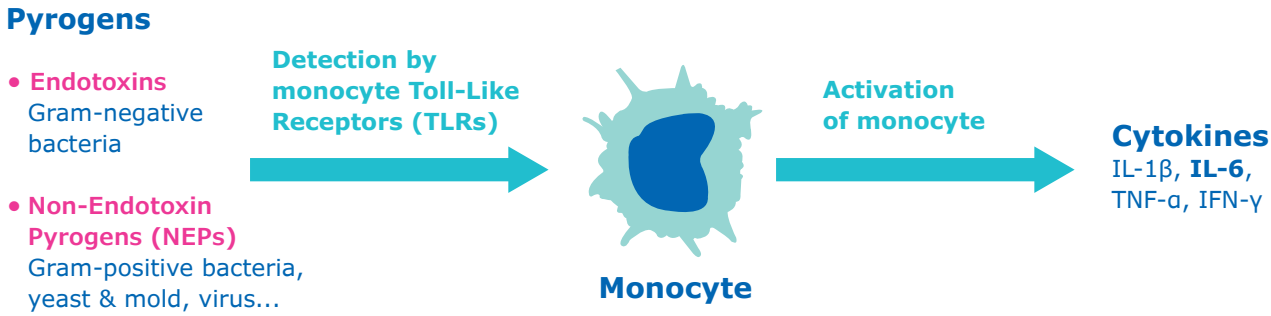


Fig. 1: Monocyte activation test principle Monocyte activation test method detects pyrogenic and proinflammatory contaminants, including endotoxins from Gram-negative bacteria and non-endotoxin contaminants

Methods

Endotoxin standard curves and spiked samples were used for evaluating the monocyte activation test according to the following workflow (see Fig. 2).

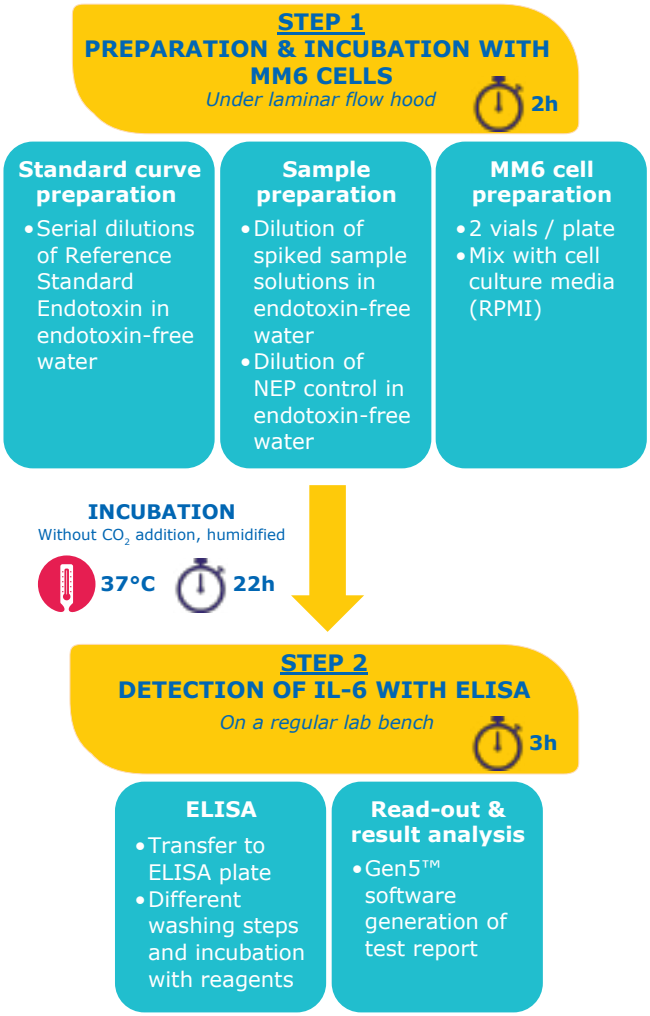


Fig. 2: **Diagram of the test method**
Workflow of the monocyte activation test with the PyroMAT™ system

The performance assessment of the monocyte activation test method was challenged through the following parameters:

- Robustness: incubation time
- Ruggedness: cell lots, culture medium lots, ELISA kit lots, operator
- Accuracy, precision, linearity, range, limit of quantification
- Limit of detection: 0.05 EU/mL
- Specificity: panel of non-endotoxin pyrogens (NEP)

Several concentrations of spiked sample (from 0.05 to 0.6 EU/mL) were used for the robustness test (incubation time), for the ruggedness test (testing different cell lot, culture medium lot and ELISA kit lots) and for the accuracy. Data from accuracy were used for precision, linearity, range and limit of quantification analysis. Cell lot ruggedness was carried out by two different operators. A single concentration of spiked sample at 0.05 EU/mL was used for the limit of detection. Three independent preparations were tested for each spiked sample. All the tests were realized with standard and specific ELISA protocols and handled at 2 different sites (except specificity, only one site). To test specificity, NEPs targeting different surface TLRs were challenged.

Results

Incubation time robustness demonstrates a superposition of the standard curves over the three time-points tested (20, 22 and 24 hours) without variation of endotoxin quantification. Therefore, an incubation time of 22 ± 2 hours is recommended.

Accuracy. Endotoxin recoveries from sample spiked with a known pyrogenic concentration meet acceptance criteria (between 50-200%).

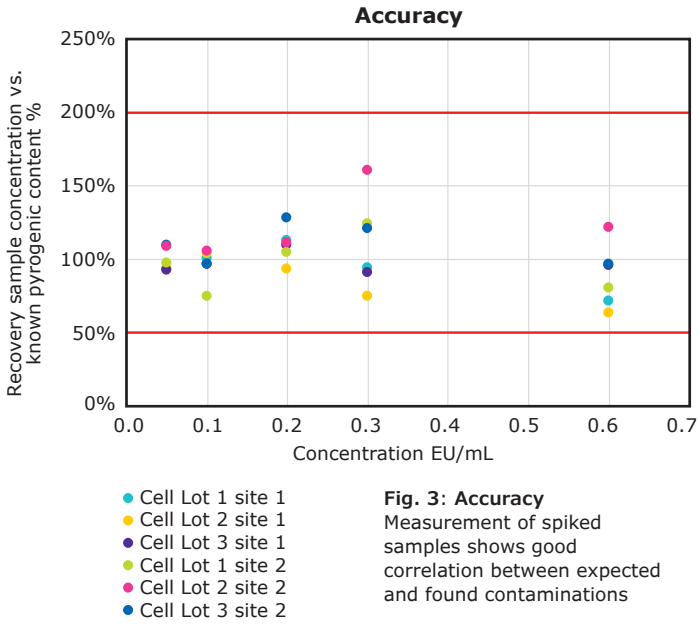


Fig. 3: **Accuracy**
Measurement of spiked samples shows good correlation between expected and found contaminations

Ruggedness test: cell, culture medium and ELISA kit lots and operators. Endotoxin recoveries from sample spiked with a known pyrogenic concentration (not represented here) meet acceptance criteria (between 50-200%). Respective Relative Standard Deviation (RSD) calculation (< 25%) between different variables demonstrates the reproducibility of results for all parameters tested.

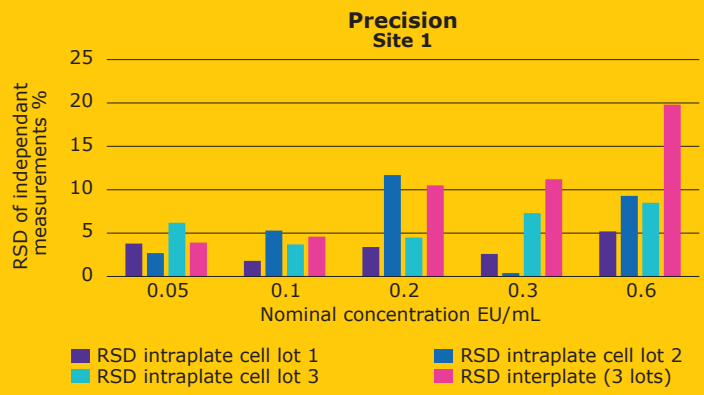
Specificity. Monocytes react to endotoxin and non-endotoxin pyrogens targeting different surface TLRs. Positive signal is demonstrated with lipoteichoic acid (LTA), Heat-Killed *Staphylococcus aureus* (HKSA), peptidoglycan, PAM3CSK4, PAM2CSK4 and flagellin.

RSD (%) for ruggedness of cell lot	Cell lot 1 Operator 1	Cell lot 2 Operator 1	Cell lot 1 Operator 2	Cell lot 2 Operator 2
0.05 EU/mL	3.3	12.3	9.9	6.1
0.1 EU/mL	3.2	1.9	2.4	10.2
0.2 EU/mL	2.2	3.0	11.2	5.8
0.3 EU/mL	8.7	1.2	5.8	7.0

Fig. 4: **Ruggedness of cell lot (site 1)**
Test results with each cell lot and each operator are reproducible (RSD < 25%)

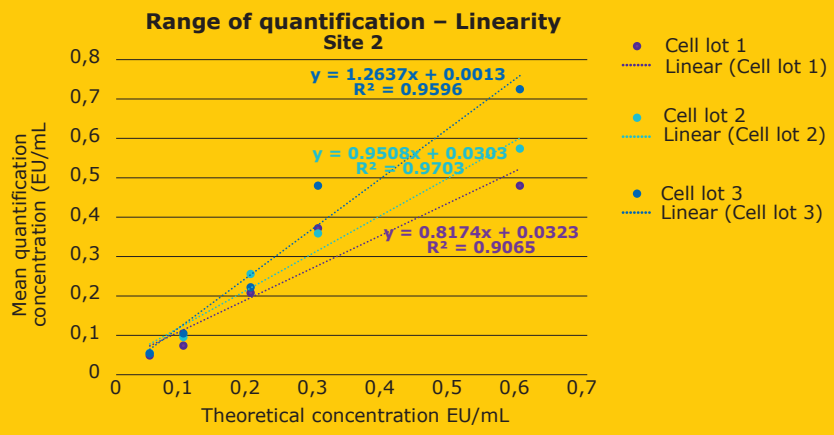
Precision

3 independent measurements of 5 spiked sample concentrations showed a RSD < 25%. The analysis of interplate RSD (< 25%) corresponding to the comparison of the sample measurement from 3 independent monocyte activation tests demonstrates a **reproducibility of test results with precision**.



Range of quantification

The interval between the upper and lower concentrations is derived from accuracy and linearity studies. The specified range of quantification that has been demonstrated with precision, accuracy and linearity is **0.05 to 0.4 EU/mL**.



High sensitivity LOD 0.05 EU/mL

3 independent preparations of Reference Standard Endotoxin at 0.05 EU/mL, with a quantification confirmed by LAL method, were evaluated. At least 23 out of 24 wells for each preparation show a positive signal, confirming the **LOD of 0.05 EU/mL**.

LOD 0.05 EU/mL	Cell lot 1	Cell lot 2	Cell lot 3
Site 1	✔	✔	✔
Site 2	✔	✔	✔

Summary

All the performance assessment was performed in parallel with standard and **specific protocol of ELISA**, standard protocol showing an optimized and reduced time to result (3 hours) and giving reliable and reproducible results with a variety of parameters such as cell lots, operator or site.

The results of this study with the PyroMAT™ system are in accordance with the specification given by USP <1225> Validation of compendial procedures and ICH Q2(R1) November 2005 Validation of analytical procedures: text and methodology guidelines, using the monocyte activation test according to EP 2.6.30 MAT.

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Studies to demonstrate the robustness and sensitivity of the PyroMAT® system for the detection of endotoxin and non-endotoxin pyrogens

Application Notes:

- Detection of **non-endotoxin pyrogens (NEP)** by Monocyte Activation Test (MAT) using the PyroMAT® system
- Quantification of pyrogen in **Hormone** with the PyroMAT® system
- Quantification of pyrogen in **Vaccine** with the PyroMAT® system
- Quantification of pyrogen in **FBS** with the PyroMAT® system
- Quantification of pyrogens in **Albumine** with the PyroMAT® system
- Comparison of **Reference Standard Endotoxins** (RSE)

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