PyroMAT® System
Monocyte Activation Test (MAT)

User Guide for Routine Tests (Method 1 and 2)

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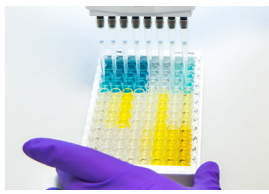
Assay Overview

Step 1: Preparation and incubation with PyroMAT® cells

1	• Prepare endotoxin standard and non-endotoxin pyrogen (NEP) control dilutions • Prepare sample solutions: dilute and spike the samples with the suitable endotoxin standard and NEP control • Load the various solutions into the 96-well cell culture plate • Prepare the PyroMAT® cells and dispense into 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 an IL-6 microplate • Add the IL-6 conjugate to each well • Incubate for 2 hours at room temperature	
4	• Remove the liquid and wash the plate 4 times • Prepare the substrate solution by mixing color reagents A and B, and add the mixture to each well • Incubate for 30 minutes in the dark at room temperature	
5	• Add the stop solution	
6	• Read the plate at 450 nm and 630 nm within 30 minutes after adding the stop solution	

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1. Introduction

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 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.

The MAT has been among the compendial methods for pyrogen detection in the European Pharmacopoeia since 2010 (Chapter 2.6.30). The chapter 2.6.30 has been revised from version 07/2017:20630 to 07/2024:20630 by the European Pharmacopoeia and is since July 2024 the reference method for pyrogen detection.

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 (RPT), 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 several types of cytokines including 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 cryopreserved 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. The IL-6 molecules released into the supernatant by MM6 cells during the incubation phase are transferred onto the ELISA plate and are bound by the immobilized primary antibody. A secondary, enzyme-linked antibody is added to form an IL-6 bound complex. After washing out any unbound molecules, the IL-6 bound complex is detected in a color reaction by 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.

Merck waives all liability for PyroMAT® cells activity and/or test results for PyroMAT® cells that are not used in combination to the PyroMAT® kit.

2. Material provided and storage conditions

To perform the MAT, we recommend using:

- PyroMAT® Cells (Cat. No. Pyr0MATCELLS)
- PyroMAT® Kit (Cat. No. Pyr0MATKIT)
- Reference Standard Endotoxin (Cat. No. 1.44161.0001)

Non-endotoxin pyrogen controls to be chosen among the following available references:

- NEP Control HKSA (Cat. No. MATHKSA)
- NEP Control Flagellin (Cat. No. MATFLAGELLIN)
- NEP Control PAM3CSK4 (Cat. No. MATPAM3CSK4)
- NEP Control FSL-1 (Cat. No. MATFSL1)
- NEP Control RESIQUIMOD R848 (Cat. No. MATRESIQUIM0D)

2.1 PyroMAT® Cells

Part	Qty.	Description	Storage of unopened material
Cells	2 x 1 mL	Cryopreserved human monocytic Mono-Mac-6 cells	-80°C or below* within the expiration date

* Storage in liquid nitrogen is also possible

2.2 PyroMAT® Kit

Part	Qty.	Description	Storage of unopened material	Storage of opened material
Water	125 mL	Pyrogen-free water	2–8 °C	1 month at 2–8 °C, within the expiration date
96-well plate	1 plate	96-wells plate for cell incubation		
RPMI	50 mL	Cell culture medium, with L-glutamine and HEPES		
IL-6 microplate	1 plate	12 strips of 8 wells each, coated with a monoclonal antibody specific for human IL-6		
Assay diluent	11 mL	Buffered protein base with preservatives		
IL-6 conjugate	21 mL	Polyclonal antibody specific for human IL-6 conjugated to horseradish peroxidase with preservatives		
Wash buffer	21 mL	25-fold concentrated solution of buffered surfactant with preservatives*		
Color reagent A	12 mL	Stabilized hydrogen peroxide		
Color reagent B	12 mL	Stabilized chromogen tetramethylbenzidine		
Stop solution	6 mL	2N sulfuric acid		
Plate sealers	4 strips	Adhesive strips		

* Wash buffer may turn yellow over time. This has no impact on the test.

2.3 Reference Standard Endotoxin

Part	Qty.	Description	Storage of unopened material	Storage of reconstituted material
Reference Standard Endotoxin	1 vial	10,000 EU, standard endotoxin, lyophilized	–20 °C	Aliquot at –40 °C or below

Note: 1 vial of RSE is sufficient for ~100 tests or plates

2.4 NEP Control HKSA

Part	Qty.	Description	Storage of unopened material	Storage of reconstituted material
NEP Control HKSA	1 vial	Lyophilized cells of heat-killed *Staphylococcus aureus*.	2–8 °C	Aliquot at –20 °C*

Note: 1 vial is sufficient for ~20 tests or plates
* Refer to the technical data sheet of the product for the shelf life upon resuspension

2.5 NEP Control Flagellin

Part	Qty.	Description	Storage of lyophilized material	Storage of reconstituted material
NEP Control Flagellin	1 vial	Lyophilized Flagellin from *Salmonella typhimurium*.	–20 °C	Aliquot at –20 °C*

Note: 1 vial is sufficient for ~10 tests or plates

*Refer to the technical data sheet of the product for the shelf life upon resuspension

2.6 NEP Control PAM3CSK4

Part	Qty.	Description	Storage of lyophilized material	Storage of reconstituted material
NEP Control PAM3CSK4	1 vial	Lyophilized synthetic triacylated lipopeptide.	2–8 °C	Aliquot at –20 °C*

Note: 1 vial is sufficient for ~20 tests or plates

*Refer to the technical data sheet of the product for the shelf life upon resuspension

2.7 NEP Control FSL-1

Part	Qty.	Description	Storage of lyophilized material	Storage of reconstituted material
NEP Control FSL-1	1 vial	Lyophilized synthetic lipoprotein of *Mycoplasma salivarium*.	2–8 °C	Aliquot at –20°C*

Note: 1 vial is sufficient for ~20 tests or plates

*Refer to the technical data sheet of the product for the shelf life upon resuspension

2.8 NEP Control RESIQUIMOD R848

Part	Qty.	Description	Storage of lyophilized material	Storage of reconstituted material
NEP Control RESIQUIMOD R848	1 vial	Lyophilized imidazoquinoline compound with potent anti-viral activity.	–20 °C	Aliquot at –20 °C*

Note: 1 vial is sufficient for ~20 tests or plates

*Refer to the technical data sheet of the product for the shelf life upon resuspension

3. Additional equipment required

- Incubator (37 °C) with water reservoir to ensure humidified atmosphere
- Water bath (37 °C)
- Centrifuge with buckets for 50 mL centrifuge tubes
- Microplate reader to measure absorbance at 450 nm and 630 nm (reference wavelength): we recommend using a BioTek reader from Agilent to ensure data integrity in combination with Gen5 Software for analysis.
- Cryo-freezer (–150 °C or –80 °C)
- Freezer (–20 °C)
- Refrigerator (2–8 °C)
- Vortex mixer: a multi vortex mixer can be used (eg. Heidolph™ Multi Reax test tube shaker)
- Pipette (10 mL, 25 mL)
- Adjustable pipettes: (10–100 µL, 20–200 µL, 200–1000 µL)
- Suitable sterile, endotoxin-free pipette tips
- Endotoxin-free microcentrifuge tubes
- Endotoxin-free glass tubes with caps
- 50 mL endotoxin-free tubes with caps
- Multichannel pipettes with suitable containers
- Deionized or distilled water
- Chemical sanitary gloves
- 500 mL glass bottle

4. Warnings and precautions

Not suitable for *in vitro* diagnostic use.
Please refer to the MSDS on our website prior to use.

Caution:

- The test must be performed by well-trained and authorized laboratory personnel.
- All reagents should be handled in accordance with Good Laboratory Practice using appropriate precautions.
- Do not use reagents after the expiration date printed on the label.
- Do not use reagents with any evidence of turbidity or microbial contamination.
- Vortex steps are recommended at maximum speed but should not exceed 1400 rpm.
- IL-6 is detectable in saliva. Take precautionary measures to prevent contamination of the kit reagents while running the assay.
- The color reagent B may cause skin, eye and respiratory irritation. Avoid breathing fumes. Please refer to the MSDS.
- The stop solution contains sulfuric acid. Wear eye, hand, face and clothing protection when using this material. Please refer to the MSDS.
- HKSA is fatal if swallowed. Take precautionary measures and refer to the MSDS.

Limitation of Liability

Merck waives all liability for PyroMAT® cells activity and/or test results for PyroMAT® cells that are not used in combination to the PyroMAT® kit.
Notwithstanding our attempts to observe the rules specified in national and international guidelines, we cannot guarantee the proper calculations and subsequent interpretations. Please refer to the relevant chapters in the European Pharmacopoeia (EP chapter 2.6.30) as well as other documents relevant for your specific purpose when utilizing this test.

5. Preparation of the Reference Standard Endotoxin (RSE) and Non-Endotoxin Pyrogens (NEP) control

5.1 Resuspension and storage of the RSE

Endotoxin standard from European Pharmacopeia contains 10,000 International Units per vial. Endotoxin activates the TLR4 receptor of monocytic cells.

Reconstitution of the vial:

- Add 5 mL of pyrogen-free water to the vial.
- Reconstitute by mixing intermittently for 30 min, using a vortex mixer at maximum speed.
- The resulting solution of 2,000 EU per mL is used as a stock solution for preparing serial dilutions.

Immediately after reconstitution:

- Divide stock solution as 50 µL aliquots into endotoxin-free microcentrifuge tubes, and store at –40 °C or below.

5.2 Resuspension and storage of HKSA

Heat-killed *Staphylococcus aureus* is a crude bacterial whole cell extract, representative of real contamination from Gram-positive bacteria. HKSA activates the TLR2 receptor of monocytic cells.

Reconstitution of the vial:

- Add 1 mL of pyrogen-free water to the vial.
- Reconstitute by mixing for 1 minute or until complete homogenization.
- The resulting solution is concentrated at 1000X and is used as a stock solution for preparing serial dilutions with 15 seconds of vortexing time between each dilution.

Immediately after reconstitution:

Divide stock solution as 50 µL aliquots into endotoxin-free microcentrifuge tubes, and store at –20 °C.

5.3 Resuspension and storage of Flagellin

Flagellin is a preparation of Flagellin from *Salmonella typhimurium*. Flagellin activates the TLR5 receptor of monocytic cells.

Reconstitution of the vial:

- Add 500 µL of pyrogen-free water to the vial.
- Reconstitute by mixing for 1 minute or until complete homogenization.
- The resulting solution is concentrated at 1000X and is used as a stock solution for preparing serial dilutions with 15 seconds of vortexing time between each dilution.

Immediately after reconstitution:

- Divide stock solution as 50 µL aliquots into endotoxin-free microcentrifuge tubes, and store at –20 °C.

5.4 Resuspension and storage of PAM3CSK4

PAM3CSK4 is a synthetic triacylated lipopeptide. PAM3CSK4 mimics the acylated amino terminus of bacterial Lipopeptides (LPs). The bacterial Lipopeptides are a family of pro-inflammatory cell wall components found un both Gram-positive and Gram-negative bacteria. PAM3CSK4 activates TLR2/TLR1 receptors of monocytic cells.

Reconstitution of the vial:

- Add 1 mL of pyrogen-free water to the vial.
- Reconstitute by mixing for 1 minute or until complete homogenization.
- The resulting solution called 1000X is used as a stock solution for preparing serial dilutions with 15 seconds of vortexing time between each dilution.

Immediately after reconstitution:

- Divide stock solution as 50 µL aliquots into endotoxin-free microcentrifuge tubes, and store at –20 °C.

5.5 Resuspension and storage of FSL-1

FSL-1 is a synthetic lipoprotein of *Mycoplasma salivarium*. FSL-1 contains a structure like in mycoplasma lipoprotein which plays a crucial role in the initial recognition of microbial lipoprotein by the host innate immune system. FSL-1 activates TLR2/TLR6 receptor of monocytic cells.

Reconstitution of the vial:

- Add 1 mL of pyrogen-free water to the vial.
- Reconstitute by mixing for 1 minute or until complete homogenization.
- The resulting solution called 500 000X is used as a stock solution for preparing serial dilutions with 15 seconds of vortexing time between each dilution.

Immediately after reconstitution:

- Divide stock solution as 50 µL aliquots into endotoxin-free microcentrifuge tubes, and store at –20°C.

5.6 Resuspension and storage of RESIQUIMOD R848

RESIQUIMOD is an imidazoquinoline compound with potent anti-viral activity. RESIQUIMOD activates the intracellular TLR7/TLR8 receptor of monocytic cells.

Reconstitution of the vial:

- Add 1 mL of pyrogen-free water to the vial.
- Reconstitute by mixing for 1 minute or until complete homogenization.
- The resulting solution called 100X is used as a stock solution for preparing serial dilutions with 15 seconds of vortexing time between each dilution.

Immediately after reconstitution:

- Divide stock solution as 50 µL aliquots into endotoxin-free microcentrifuge tubes, and store at –20 °C.

6. Planning test execution

6.1 Product Specific Validation

The European Pharmacopoeia chapter 2.6.30. Monocyte-activation test has been revised from version 07/2017:20630 to 07/2024:20630, the protocols used for testing and the user guide are compliant with the current regulatory text version.

Before routine testing of a pharmaceutical product with MAT, a product specific validation (PSV) must be performed to ensure that the product will not interfere with the assay, namely that the endotoxins and non-endotoxin pyrogens in the product will be detected using the method chosen.

Interferences can be eliminated 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 Test sensitivity of the system. The more sensitive the system is, the more the product can be diluted to remove interferences.

According to EP the Test sensitivity is defined as the lowest endotoxin reference standard concentration on the standard curve whose response exceeds the cut-off value. When using the PyroMAT® Kit a Test sensitivity of 0,05 EU/mL or lower is expected. To allow consistent and stringent calculation of the MVD, a Test sensitivity of 0,05 EU/mL will be retained and use for MVD calculation.

The Test sensitivity of the PyroMAT® system is 0.05 EU/mL.

Therefore, the MVD can be calculated as below:

$$MVD = \frac{CLC \times C}{\text{Test sensitivity}}$$

CLC = contaminant limit concentration
C = concentration of test solution
Test sensitivity = 0.05 EU/mL

The product specific validation must be performed for Method 1 and permits determination of the valid product dilutions, not exceeding the MVD, that will be tested.

If the interference cannot be removed by dilution of the product within the MVD range or a specific sample preparation, Method 2 is preferred over Method 1.

6.2 Routine Testing

Based on the results from the product specific validation, the suitable method is selected for routine testing:

Method 1: semi-quantitative test

Method 2: reference lot comparison test

The following section describes the experimental protocols for routine testing with methods 1 and 2.

7. Step 1: Preparation and incubation with PyroMAT® cells

Step 1 of the assay should be performed in a laminar flow hood to prevent any bacterial contamination.
The reagents used for this step should be equilibrated at room temperature before use (Water and 96-well plates).

Before starting step 1, prewarm the RPMI at 37 °C, using a water bath for example.

7.1 Method 1: semi-quantitative test

European Pharmacopeia 07/2024:20630:

"Method 1 involves a comparison of the preparation being examined with a standard endotoxin dose-response curve. To pass the test, the contaminant concentration of the preparation to be examined is to be lower than the contaminant limit concentration (CLC)."

Using the plate layout recommended, Method 1 includes a non-endotoxin pyrogen (NEP) control and allows for testing 2 samples per plate.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK = 0 EU/mL				Sample 1, dilution 1				Sample 2, dilution 1			
B	0.0125 EU/mL				Sample 1, dilution 1 + spike RSE				Sample 2, dilution 1 + spike RSE			
C	0.025 EU/mL				Sample 1, dilution 2				Sample 2, dilution 2			
D	0.05 EU/mL				Sample 1, dilution 2 + spike RSE				Sample 2, dilution 2 + spike RSE			
E	0.1 EU/mL				Sample 1, dilution 3				Sample 2, dilution 3			
F	0.2 EU/mL				Sample 1, dilution 3 + spike RSE				Sample 2, dilution 3 + spike RSE			
G	0.4 EU/mL				Sample 1, dilution 1 + spike NEP				Sample 2, dilution 1 + spike NEP			
H	0.8 EU/mL				NEP in water				-			

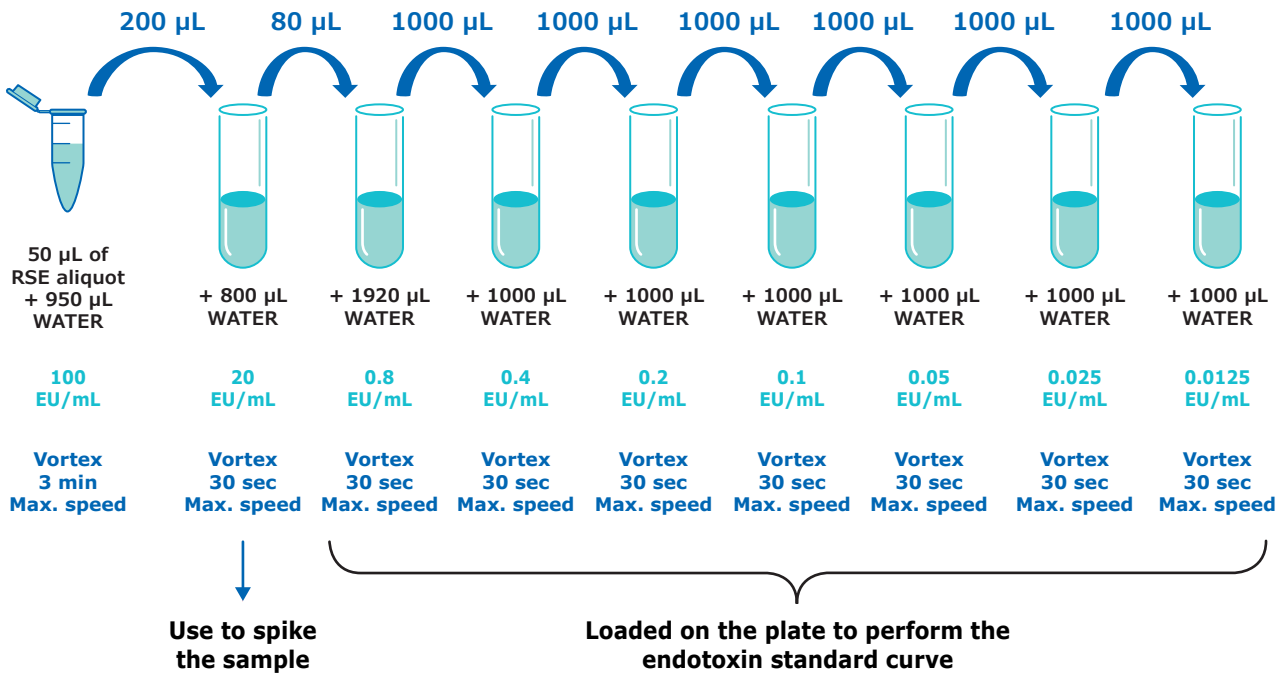
RSE Standard Curve

Preparation of the reference standard endotoxin (RSE) curve

The standard endotoxin solutions are prepared from the RSE stock solution at 2,000 EU/mL. Seven (7) endotoxin concentrations (0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 EU/mL) are prepared to generate the standard curve.

Thaw a 50 µL-aliquot of RSE and vortex at maximum speed over 1 min.

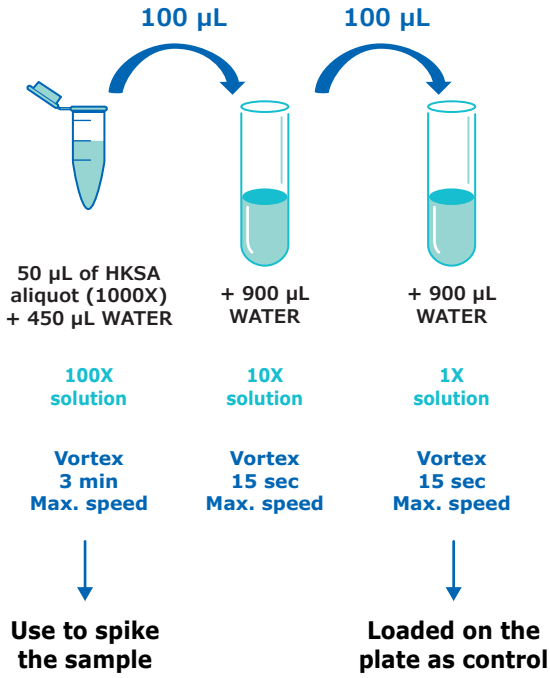
Perform serial dilutions with pyrogen-free water, using endotoxin-free glass tubes, as described below. Make sure to vortex all the dilutions before use.



Preparation of heat-killed *Staphylococcus aureus* (HKSA) solutions

Heat-killed *Staphylococcus aureus* has been included in each experiment as a positive control, to assess the detection of non-endotoxin pyrogens by the system.

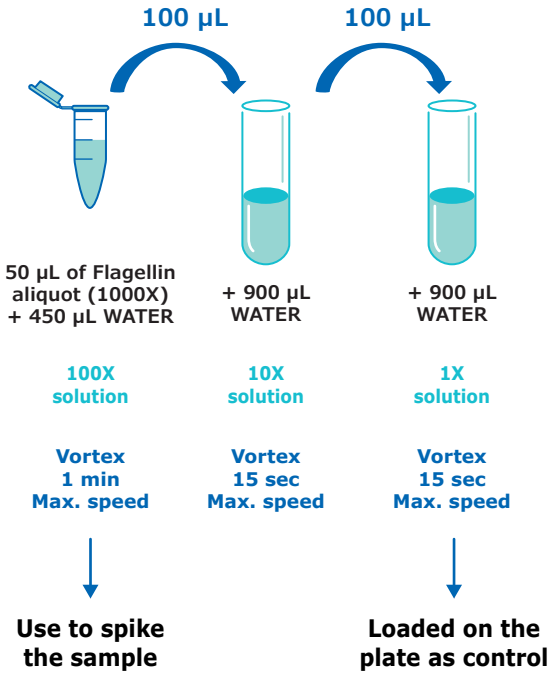
Thaw a 50 µL-aliquot of HKSA at 1000X and vortex at maximum speed over 1 min. Perform serial dilutions with pyrogen-free water, using endotoxin-free glass tubes, as described in the figure below. Make sure to vortex all the dilutions before use.



Preparation of Flagellin solutions

Flagellin can be included in each experiment as a positive control, to assess the detection of non-endotoxin pyrogens by the system.

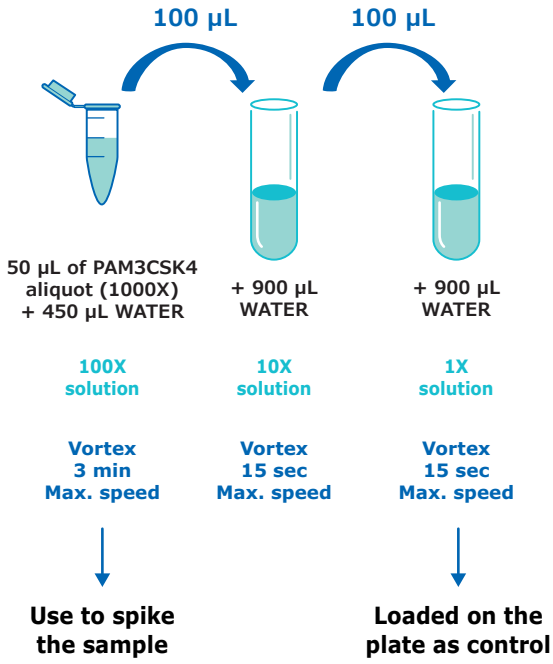
Thaw a 50 µL-aliquot of Flagellin at 1000X. Perform serial dilutions with pyrogen-free water, using endotoxin-free glass tubes, as described in the figure below. Make sure to vortex all the dilutions before use.



Preparation of PAM3CSK4 solutions

PAM3CSK4 can be included in each experiment as a positive control, to assess the detection of non-endotoxin pyrogens by the system.

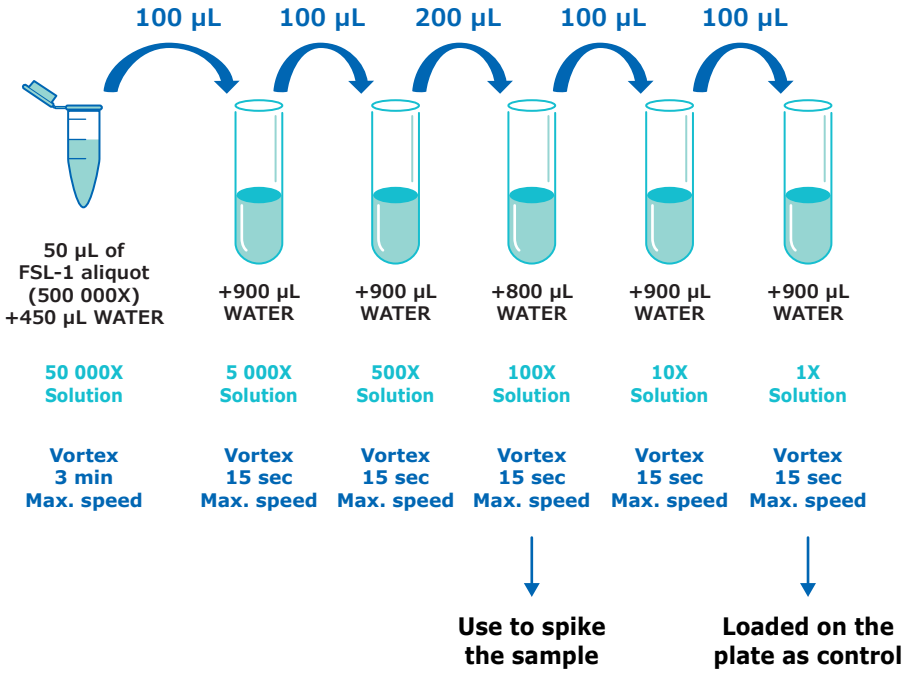
Thaw a 50 µL-aliquot of PAM3CSK4 at 1000X and vortex at maximum speed over 1 min. Perform serial dilutions with pyrogen-free water, using endotoxin-free glass tubes, as described in the figure below. Make sure to vortex all the dilutions before use.



Preparation of FSL-1 solutions

FSL-1 can be included in each experiment as a positive control, to assess the detection of non-endotoxin pyrogens by the system.

Thaw a 50 µL-aliquot of FSL-1 at 500 000X and vortex at maximum speed over 1 min. Perform serial dilutions with pyrogen-free water, using endotoxin-free glass tubes, as described in the figure below. Make sure to vortex all the dilutions before use.

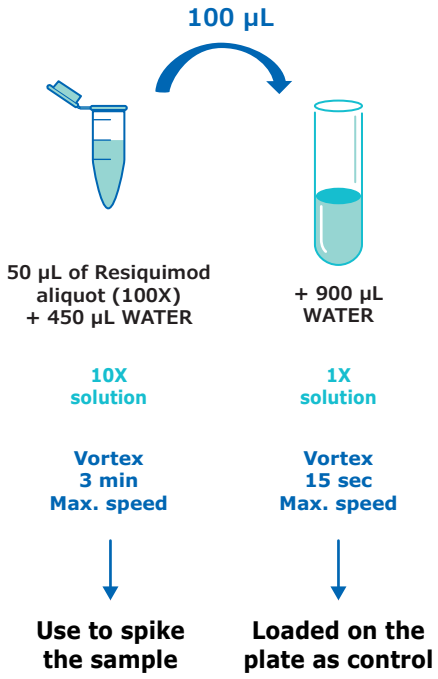


Preparation of RESIQUIMOD R848 solutions

RESIQUIMOD can be included in each experiment as a positive control, to assess the detection of non-endotoxin pyrogens by the system.

Thaw a 50 µL-aliquot of RESIQUIMOD at 100X and vortex at maximum speed over 1 min.

Perform serial dilutions with pyrogen-free water, using endotoxin-free glass tubes, as described in the figure below. Make sure to vortex all the dilutions before use.



Preparation of the sample

Non-spiked sample

The sample is tested at 3 dilution levels: dilution 1 has been previously determined through the product specific validation, with a test for interfering factors. Then, 2-fold serial dilutions are tested (dilutions 2 and 3), not exceeding the MVD. Dilutions are performed in pyrogen-free water using endotoxin-free glass tubes.

Sample spiked with RSE

Each sample dilution is spiked with a known amount of endotoxin to calculate the spike recovery. The spike value is chosen to be near the middle of the endotoxin standard curve (0.2 EU/mL for Method 1). The endotoxin solution at 20 EU/mL is used to spike the sample.

Sample spiked with NEP control

Only dilution 1 of the sample is spiked with a specific amount of non-endotoxin pyrogen. The choice of NEP control should reflect the most likely contaminants of the preparation being examined.

- Sample spiked with HKSA: 10 µL of the 100X solution of HKSA is used to spike the sample.

OR

- Sample spiked with Flagellin: 10 µL of the 100X solution of Flagellin is used to spike the sample.

OR

- Sample spiked with PAM3CSK4: 10 µL of the 100X solution of PAM3CSK4 is used to spike the sample.

OR

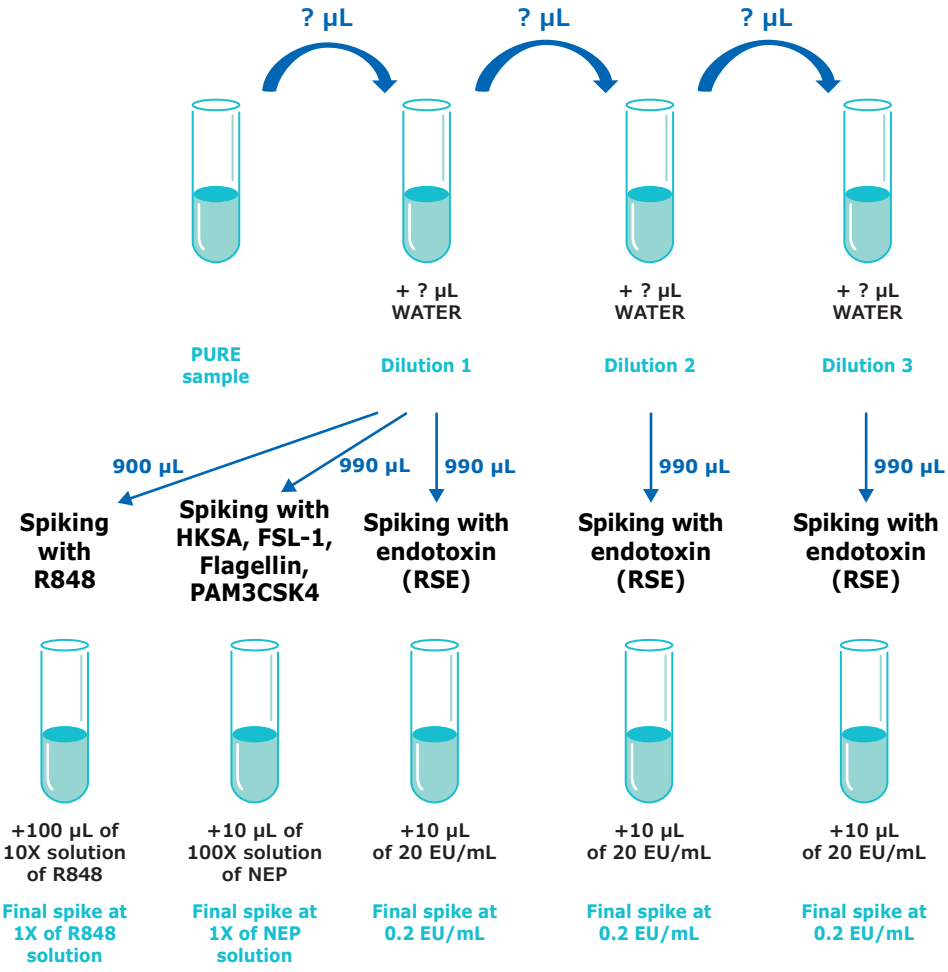
- Sample spiked with FSL-1: 10 µL of the 100X solution of FSL-1 is used to spike the sample.

OR

- Sample spiked with RESIQUIMOD: 100 µL of the 10X solution of RESIQUIMOD is used to spike the sample.

Note: the RESIQUIMOD solution is spiked with a higher volume than the other NEP controls

An example of the sample dilution preparation is shown below:
Make sure to vortex the pure sample, each sample dilution and the RSE solutions maximum 30 seconds at maximum speed. Be careful, for the NEPs it is maximum 15 seconds.



Loading of the cell culture plate with standard and samples

Load the plate according to the plate layout for Method 1. Before loading on the plate, make sure to vortex the pure sample, each sample dilution and the RSE solutions maximum 30 seconds at maximum speed. Be careful, for the NEPs it is maximum 15 seconds.

50 µL/well of:

- Blank: pyrogen-free water
- Endotoxin standard solutions: from 0.0125–0.8 EU/mL
- NEPs: 1X solution
- Spiked and non-spiked sample solutions

4 replicates of each preparation.

Preparation of the PyroMAT® cells

The PyroMAT® cells (Mono-Mac-6 human monocytic cell line) can be stored at –80 °C or lower within the expiration date. The product is delivered in 2 x 1 mL vials, sufficient for setting up one entire cell culture plate. Cryo-protectants (e.g. DMSO) are toxic to cells in culture at room temperature; that's why the cells must be diluted and washed immediately after thawing via a centrifugation step.

Preparation of the cells for one entire 96-well plate:

- From the pre-warmed RPMI bottle (water bath at 37 °C), pipet 20 mL of RPMI into a 50 mL endotoxin-free tube.
- Take 1 vial of PyroMAT® cells from the freezer and immediately thaw it in a water bath at 37 °C for 1 min ±30 sec.
- Add 1 mL of warmed RPMI (20 mL in 50 mL-tube) into the vial, mix by pipetting up and down for 3 times, and transfer back into the rest of RPMI (19 mL).
- Add again 1 mL from the previous mix to wash the vial and transfer back into the 50 mL-tube.
- Repeat the same steps for the thawing of the second vial of PyroMAT® cells.
- Close the 50 mL-tube and centrifuge for 5 min at 1,000 rpm or 200 × g at room temperature.
- After centrifugation, discard the supernatant and resuspend the pellet with the small amount of remaining liquid.
- Add 20 mL of warmed RPMI at 37 °C, and mix by pipetting up and down for 3 times.

Distribution of the cells:

Dispense 200 µL of cells/well using a P1000 pipette to prevent any cell damage. Change tips for each well to avoid cross-contamination. Mix the stock solution of cells regularly to ensure good homogenization

Note:

- One vial of cells is sufficient for half of a plate. When using only one vial, the preparation of cells must be performed in 10 mL of culture medium.
- If the vial is taken from liquid nitrogen, before thawing, quickly open and close it under laminar flow hood to remove gas to avoid any risk of explosion.

Incubation of the cell culture plate

Incubate the cell culture plate for 22 ±2 hours at 37 °C in a humidified atmosphere (i.e. a reservoir with purified water can be placed in the incubator in advance) without CO_2 addition.

In presence of pyrogens, the monocytes will produce cytokines.

7.2 Method 2: reference lot comparison test

European Pharmacopeia 07/2024:20630:

> "Method 2 involves a comparison of the preparation to be examined with a validated reference lot of that preparation, the latter being a lot of the preparation that has been found to be safe and efficacious through clinical studies, or is representative thereof. The type of analysis selected to compare the two is to be justified and validated for each product and is to include test validity criteria."

The reference lot comparison test comprises dilution of the reference lot, sample dilutions as well a positive control and a blank. With the plate layout recommended, Method 2 allows the testing of 3 samples per plate, with one reference lot and one lot being examined for each sample/product.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank (water)											
B	Positive control (endotoxin standard)											
C	Sample 1 — Ref: dilution 1				Sample 2 — Ref: dilution 1				Sample 3 — Ref: dilution 1			
D	Sample 1 — Ref: dilution 2				Sample 2 — Ref: dilution 2				Sample 3 — Ref: dilution 2			
E	Sample 1 — Ref: dilution 3				Sample 2 — Ref: dilution 3				Sample 3 — Ref: dilution 3			
F	Sample 1 — Lot 1: dilution 1				Sample 2 — Lot 1: dilution 1				Sample 3 — Lot 1: dilution 1			
G	Sample 1 — Lot 1: dilution 2				Sample 2 — Lot 1: dilution 2				Sample 3 — Lot 1: dilution 2			
H	Sample 1 — Lot 1: dilution 3				Sample 2 — Lot 1: dilution 3				Sample 3 — Lot 1: dilution 3			

Preparation of the positive control

The positive control is used to assess the viability of the cells and corresponds to a standard endotoxin concentration that gives a clear positive response. The standard endotoxin solution at 0.2 EU/mL is recommended as a positive control.

For the preparation, refer to the Method 1: Preparation of the reference standard endotoxin (RSE) solutions.

Preparation of the sample

For each sample, the reference lot and the lot being examined have to be tested at 3 dilution levels. The dilution factors can be determined in a preliminary testing: for example, the lowest dilution that stimulates the greatest release of IL-6 dilutions immediately below and above the chosen dilution.

Dilutions are performed with pyrogen-free water, using endotoxin-free glass tubes. Make sure to vortex the pure sample and each sample dilution for over 30 seconds at maximum speed before use.

Note: The pyrogen-free water used to dilute the samples is also used for the blank.

Loading of the cell culture plate with standards and samples

Load the plate according to the plate layout for Method 2. Make sure to vortex each solution over 30 seconds at maximum speed before loading onto the plate.

50 µL/well of:

- Blank: pyrogen-free water
- Positive control: endotoxin standard solution at 0.2 EU/mL
- Sample solutions of the reference lot and the lot being examined.

4 replicates of each preparation.

Preparation of the PyroMAT® cells

Refer to Method 1.

Incubation of the cell culture plate

Refer to Method 1.

8. Step 2: Detection of IL-6 with ELISA

Step 2 of the assay is performed on a regular lab bench (no need to perform under a laminar flow hood). The associated reagents should be equilibrated at room temperature before use.

Production of cytokines (interleukin IL-6) is detected with an immunological assay involving specific antibodies and an enzymatic color reaction (ELISA kit). Either a standard or specific version of ELISA can be performed: the standard ELISA is faster and recommended for most of applications. The specific ELISA includes additional washing and incubation steps and can be used in case of interference with complex sample matrices.

Note:

- Vials have been numbered in the order of use for the standard version of ELISA.
- The “assay diluent” reagent is not used with the standard version of the ELISA.
- We recommend numbering each strip of the IL-6 microplate before use.
- Manual or automatic multichannel operation can be used, change tips when necessary, especially for supernatant transfer and addition of the stop solution, to avoid cross-contamination.

This step is identical for Methods 1 and 2.

Cytokine detection by ELISA: standard protocol

- Transfer **100 µL/well of cell supernatant** to the ELISA plate (Item 1 IL-6 Microplate). Take care not to mix the well contents before collecting the supernatant.
- Add **200 µL/well of IL-6 conjugate** (Vial 2) and cover the plate with an adhesive strip.
- Incubate for **2 hours at room temperature**.
- **Prepare the wash buffer** (Vial 3) (20 mL of concentrated wash buffer 25X + 480 mL of purified water).
  Note: If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.
  Note: Wash buffer may turn yellow over time. This has no impact on the test.
- After incubation, remove the adhesive strip and empty the ELISA plate into the chemical waste. Invert the plate and blot it against clean paper towels.
  Note: Complete removal of the liquid at each step is essential to good performance.
- Add **400 µL/well of wash buffer**, empty the ELISA plate, invert the plate and blot it against clean paper towels. Repeat this step 3 more times, for **a total of 4 washing steps**.
- **Prepare the substrate solution:** add the entire content of the **color reagent A** (Vial 4a) bottle to the **color reagent B** (Vial 4b) bottle. Mix by inverting the bottle several times. Protect from light, and use within 15 minutes.
  Note: leave the last wash buffer on the plate while preparing this step; the wells should not remain empty. If only half of the plate is used, equal volumes of color reagents A and B must be mixed together.
- **Add 200 µL/well of substrate solution** and cover the plate with a new adhesive strip.
- **Incubate for 30 minutes at room temperature, protected from light.**
- **Add 50 µL/well of stop solution** (Vial 5) and mix thoroughly by up and down pipetting to ensure color homogenization.
  Note: the color in the wells should change from blue to yellow. If the color in the wells is green, or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing or mix the well content by up and down pipetting.

Reading

The color development is proportional to the amount of IL-6 production initially present in the supernatant. Measure the optical density at 450 nm and 630 nm (reference wavelength) using an absorbance reader. We recommend using a BioTek reader from Agilent to ensure data integrity in combination with Gen5 Software for analysis.

The plate can be read within 30 minutes after the addition of the stop solution.

Cytokine detection by ELISA: specific protocol

- Add 100 µL/well of **assay diluent** to the ELISA plate.
- Transfer **100 µL/well of cell supernatant** to the ELISA plate. Take care not to mix the well contents before collecting the supernatant.
- Cover the plate with an adhesive strip. Incubate for **2 hours at room temperature**.
- **Prepare the wash buffer** (20 mL of concentrated wash buffer 25X + 480 mL of purified water). **Note:** if crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.
- After incubation, remove the adhesive strip, empty the ELISA plate into the chemical waste. Invert the plate and blot it against clean paper towels. **Note:** complete removal of the liquid at each step is essential to good performance.
- Add **400 µL/well of wash buffer**, empty the ELISA plate, invert the plate and blot it against clean paper towels. Repeat this step 3 more times, for **a total of 4 washing steps**.
- Add **200 µL/well of IL-6 conjugate** and cover the plate with a new adhesive strip.
- Incubate for **2 hours at room temperature**.
- **Repeat the 4 washing steps.**
- **Prepare the substrate solution:** add the entire content of the **color reagent A** bottle to the **color reagent B** bottle. Mix by inverting the bottle several times. Protect from light, and use within 15 minutes.
  Note: leave the last wash buffer on the plate while preparing this step; the wells should not remain empty. If only half of the plate is used, equal volumes of color reagents A and B must be mixed together.
- **Add 200 µL/well of substrate solution** and cover the plate with a new adhesive strip.
- **Incubate for 20 minutes at room temperature, protected from light.**
- **Add 50 µL/ well of stop solution** and mix thoroughly by up and down pipetting to ensure color homogenization.

  Note: the color in the wells should change from blue to yellow. If the color in the wells is green, or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing or mix the well contents by up and down pipetting.

Reading

The color development is proportional to the amount of IL-6 production initially present in the supernatant. Measure the optical density at 450 nm and 630 nm (reference wavelength) using an absorbance reader. We recommend using a BioTek reader from Agilent to ensure data integrity in combination with Gen5 Software for analysis.

The plate can be read within 30 minutes after the addition of the stop solution.

9. Data analysis

Protocols to support data analysis have been developed on the Gen 5™ Software commercialized by Agilent. The protocols can be downloaded free of charge on the website: **SigmaAldrich.com/pyromat-software**

9.1 Method 1: semi-quantitative test

Method 1 is a semi-quantitative test. Its principle is based on conversion of the OD signal for the sample/product tested to a concentration in EEU/mL (endotoxin equivalents units) using the endotoxin standard curve.

After applying the dilution factor correction, the concentration of the pure sample expressed in EEU/mL is compared to the CLC for the product.

Acceptance criteria for validity of the endotoxin standard curve

Method 1 requires a valid standard curve based on the following criteria:

- **Goodness of fit criteria:** a statistical test that confirms the suitability of the regression model to describe the raw data. The data are modeled with a 5-parameter logistics regression model that requires a minimum of 6 concentrations.
- **Coefficient of regression R squared (R^2):** a statistical test that measures the match between the model and the measured data on a convenient 0 – 1 scale.
- **Test sensitivity criteria:** the test is valid if a Test sensitivity ≤ 0.05 EU/mL is reached.

According to European Pharmacopoeia, current Chapter 2.6.30 the Test sensitivity is defined as the lowest endotoxin reference standard concentration on the standard curve whose response exceeds the cut-off value. The cut-off is expressed in an OD value and corresponds to the mean of blank OD values of the 4 replicates + 3X standard deviation (Blank)

For each plate:

- The data analysis tool shows the lowest mean OD signal of the endotoxin reference standard solution that exceeds the cut-off value.
- The test validity criterion is valid if at least the mean OD signal from the Standard solution at 0,05 EU/mL is above the cut-off value.
- If the Test Sensitivity (TS) criterion is valid, a TS = 0.05 EU/mL is considered for all the calculations even if it could be lower for the test.

Additional criteria are implemented in the protocol to ensure consistency of the Standard Curve:

- **Blank criteria:** the mean of blank OD values is recommended to be below 0.1.
- **Minimum of reactivity:** OD of the 4 replicates of the highest standard (0.8 EU/mL) should be above 3.

(In the case of an overflow signal meaning values exceeding the instrument dynamic range the Minimum of reactivity criterion is valid)

Both criteria are not required by the current European Pharmacopoeia Chapter 2.6.30 and are given as an additional indication for the end-user. When this criterion does not reach expected results, it is recommended to have a deeper look at the raw data to investigate for a root cause. It is up to the end-user to decide regarding the validity of the assay.

Detection of non-endotoxin pyrogens

The NEP tested in water as a positive control is detected if the signal, expressed in EEU/mL using the endotoxin standard curve, is above the Test Sensitivity (TS) = 0.05 EU/mL.

The NEP is detected in the sample if the signal difference between the spiked sample and non-spiked sample, expressed in EEU/mL, is above the TS = 0.05 EU/mL.

Pyrogenicity of the sample

For each sample dilution the endotoxin (RSE) spike recovery is calculated based on the EEU/mL for the spiked and non-spiked samples. The spike recovery is conform if it is within the range of 50%– 200%.

The test is valid if at least one of the sample dilution displays a valid spike recovery and does not exceed the MVD.

For each sample dilution, the OD signal is converted in EEU/mL, corrected with the dilution factor, and compared to the CLC, regardless the spike recovery value

- If value in EEU/mL in the pure sample is **< CLC: CLC_STATUS → OK**, meaning that the pyrogen level in that sample dilution is below the CLC.
- If value in EEU/mL in the pure sample is **> CLC: CLC_STATUS → FAIL**, meaning that the pyrogen level in that sample dilution is above the CLC.

Sample conclusion:

- If the Test is valid and all tested dilution values in EEU/mL in pure sample are **< CLC → sample result is PASS**, meaning that the pyrogen level in the sample is below the CLC.
- If the Test is valid and at least one of the tested dilution values in EEU/mL in pure sample is **> CLC → sample result is FAIL**, meaning that the pyrogen level in the sample is above the CLC.

9.2 Method 2: reference lot comparison test

Method 2 is based on the comparison of the sample OD signal from the 3 dilutions of the reference lot and the lot being examined. The OD ratio should not exceed a justified acceptance criterion. This acceptance criterion is to be defined by the end-user.

Validity of the assay

The positive control and at least one dilution of the reference lot should be above the mean OD value of the blank.

Pyrogenicity of the sample

For each sample, an OD ratio is calculated, corresponding to the sum of the mean response of the 3 dilutions of the lot being examined divided by the sum of the mean response of the 3 dilutions of the reference lot.

- If the OD ratio is **≤ of the acceptance criterion → PASS**, meaning that the lot being examined is not pyrogenic compared to the reference lot.
- If the OD ratio is **> of the acceptance criterion → FAIL**, meaning that the lot being examined is pyrogenic compared to the reference lot.

10. Supplementary Information

Abbreviation	Definition
BLK	Blank
CLC	Contaminant limit concentration
CO_2	Carbon dioxide
ELISA	Enzyme-linked immunosorbent assay
EP	European Pharmacopoeia
EU/mL	Endotoxin units per milliliter
EEU/mL	Endotoxin equivalent units per milliliter
FSL-1	Fibroblast-stimulating lipopeptide -1
G	Gravity
HKSA	Heat-killed Staphylococcus aureus
IL-6	Interleukin-6
MAT	Monocyte activation test
MAX	Maximum
MIN	Minimum
mL	Milliliter

Abbreviation	Definition
MSDS	Material Safety Data Sheet
MVD	Maximum valid dilution
NEP	Non-endotoxin pyrogen
NM	Nanometer
OD	Optical density
PAM3CSK4	Pam3CysSerLys4
PRRs	Pattern Recognition Receptors
QTY	Quantity
R848	Resiquimod
RPM	Revolution per minute
RPT	Rabbit pyrogen test
RSE	Reference standard endotoxin
SD	Standard deviation
Sec	Seconds
TS	Test Sensitivity

11. Standard Warranty

The applicable warranty for the products listed in this publication may be found at **SigmaAldrich.com/terms** (“Conditions of Sale”).

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Frankfurter Strasse 250,
64293 Darmstadt, Germany

To place an order or receive technical assistance

Order/Customer Service:
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