Monocyte Activation Test Kit

User Guide for routine testing
(Method 1 and 2)

MAT RESEARCH

Monocyte activation test Manual

Quick-start protocol

Step 1: Sample and control preparation followed by incubation with MATR cells

1. Prepare and pre-warm cell culture medium
2. Prepare the samples
3. Prepare reference endotoxin standard
4. Prepare the endotoxin and non-endotoxin pyrogen controls
5. Prepare the MATR cells
6. Incubate for 20 ± 2 hours

Step 2: Detection of pyrogens with IL-6 ELISA

7. Prepare IL-6 ELISA plate
8. Transfer the supernatants collected from the MATR plate onto the IL-6 ELISA plate
9. Incubate for 2 hours on a plate shaker at room temperature
10. Remove liquids and wash plate 4 times
11. Add detection antibody mix to each well
12. Incubate for 1 hour on a plate shaker at room temperature
13. Remove liquids and wash plate 4 times
14. Add Streptavidin-HRP mix to each well
15. Incubate for 1 hour on a plate shaker at room temperature
16. Remove liquids and wash plate 4 times
17. Add TMB substrate solution to each well
18. Incubate for 10 minutes at room temperature
19. Read plate at 450 nm with a plate reader

Step 3: Data analysis with appropriate software

20 You can log into our software here: https://www.matresearch.com/resources/

Contents

6. Definitions and abbreviations

1. Kit contents and storage conditions

1.1. MATR Cells

Item	Contents	Unit	Storage temp.
MATR cells	Cryopreserved peripheral blood mononuclear cells	1x	short term -80°C long term -150°C or below

1.2. MATR Kit

Item	Contents	Unit	Storage temp.
Sealers	ELISA plate sealers	3x unit	Room temperature
Cell culture medium	RPMI	45 mL	+4°C
Culture medium supplement	Fetal Bovine Serum	5 mL	-20°C
Microplate: 96 well, Cell Culture	Packed plate (sterile)	1x unit	Room temperature
Broad range IL-6 ELISA kit	Reagents and pre-coated plates	1x unit	+4°C
IL-6 standard	Recombinant IL-6 protein	1x unit	-20°C
LAL water	Endotoxin free water	1x	+4°C

1.3. Reference endotoxin standard and non-endotoxin controls

Item	Contents	Unit	Storage temp.
Reference standard endotoxin	LPS EDQM (200 EU/mL)	50 µL	-20°C
NEP control	PAM3CSK4	50 µL	-20°C
NEP control	Flagellin B.S.	50 µL	-20°C
NEP control	a variety of NEP controls are available upon request		

2. Introduction

2.1. Background information

Pharmaceutical products can contain pyrogens that originate from bacteria, viruses, fungi and environmental factors, or from the production process itself. Pyrogen contamination is considered a serious health hazard that can induce fever-like symptoms, which in severe cases may result in septic shock and death. Detection of pyrogens (either endotoxin or non- endotoxin) is currently performed by Rabbit Pyrogen Test (RPT) which is inaccurate and promotes animal cruelty. MAT Research provides the monocyte activation test (MAT) to aid our customers in more sensitive and animal-friendly pyrogen testing.

2.2. Principle and procedure

The monocyte activation test (MAT) has been qualified and validated as a method to detect and quantify endotoxin and non-endotoxin pyrogenic contaminations, which activate human monocytes to release endogenous mediators. MAT Research uses cryopreserved human Peripheral Blood Mononuclear Cells (PBMC) as a source of monocytes to detect pyrogens with the highest sensitivity. The test is performed by an overnight co-culture of freshly thawed cryopreserved PBMCs together with a test substance and measuring IL-6 production as the readout for pyrogen-induced monocyte activation. Lipopolysaccharide (LPS) from Gram-negative bacteria, is used as a standard for endotoxin pyrogens, while PAM3CKS4 and Flagellin B.S. are used as non-endotoxin controls.

3. Methods and preparatory testing

Note: For more support with choosing a method and performing the preparatory tests for your product, please contact our helpdesk.

3.1. Methods

There are two main methods of the MAT.

- Method 1: the semi-quantitative test.
- Method 2: the reference lot comparison test.

Method 1 is a comparative method that measures the product of interest to a reference endotoxin dose-response curve. To pass the test, the contaminant concentration in the sample is to be lower than the contaminant limit concentration (CLC, explained below).

Method 2 involves a comparison of the product of interest to a validated reference lot of that product, which has been found to be safe and efficacious throughout its clinical use. This method is intended to be performed when the product of interest shows significant interference and cannot be diluted further within the Maximum Valid Dilution (MVD, explained below) to overcome such interference, or because it is suspected to contain non-endotoxin contaminants.

3.2. Testing for interfering factors in the MAT

For each novel product that is examined, preparatory tests have to be performed to validate that the content of the sample does not interfere with the MAT, thereby limiting its capacity to accurately determine pyrogenic contamination. Testing for interfering factors in the MAT is essential to ensure the validity of the results.

To test for interference, create two (2-fold) dilution series of the samples in RPMI without medium supplement, ensuring not to exceed the Maximum Valid Dilution (MVD) of the sample.

The MVD is calculated as follows:

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

CLC:	**contaminant limit concentration**
C:	**concentration of test solution**
Test sensitivity:	**mean of blank + 3*sd**

Following this, spike one of the dilution series with endotoxin, and determine the endotoxin recovery. The dilution factor *f*0 is the highest concentration (lowest dilution) of the product for which the endotoxin recovery is within 50-200%.

3.3. Testing for interference in the IL-6 detection system

Following testing for interfering factors in the MAT, the previously determined product dilution *f*0 is tested for interference in the IL-6 detection system. To this end, a series of IL-6 protein dilutions is prepared and measured by ELISA in presence or absence of the product at *f*0. The absolute difference in measured OD value between IL-6 in absence or presence of the product sample, should be below 20%. If the difference exceeds the 20% limit, the product at dilution *f*0 interferes with the IL-6 detection system and *f*0 has to be redetermined, if possible, until there is no interference in the IL-6 detection system.

If *f*0 shows no interference in the MAT and the IL-6 detection system, it can be used as the highest concentration (lowest dilution) of the product for Method 1 of the MAT.

3.4. Recommended plate layouts

Recommended plate layout for Method 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	R1	R1	R1	R1	A1	A1	A1	A1	A2	A2	A2	A2
B	R2	R2	R2	R2	B1	B1	B1	B1	B2	B2	B2	B2
C	R3	R3	R3	R3	C1	C1	C1	C1	C2	C2	C2	C2
D	R4	R4	R4	R4	AS1	AS1	AS1	AS1	AS2	AS2	AS2	AS2
E	R5	R5	R5	R5	BS1	BS1	BS1	BS1	BS2	BS2	BS2	BS2
F	R6	R6	R6	R6	CS1	CS1	CS1	CS1	CS2	CS2	CS2	CS2
G	R7	R7	R7	R7	AS1 Flag.	AS1 Flag.	AS1 Flag.	AS1 Flag.	AS2 Flag.	AS2 Flag.	AS2 Flag.	AS2 Flag.
H	R8 blank	R8 blank	R8 blank	R8 blank	AS1 PAM.	AS1 PAM.	AS1 PAM.	AS1 PAM.	AS2 PAM.	AS2 PAM.	AS2 PAM.	AS2 PAM.

R1-7	Serial dilutions of the reference endotoxin standard
R8 blank	negative control
A1-2	Sample dilution at 1 x *f*0
AS1-2	Sample dilution at 1 x *f*0 + spiked with endotoxin
B1-2	Sample dilution at 0.5 x *f*0
BS1-2	Sample dilution at 0.5 x *f*0 + spiked with endotoxin
C1-2	Sample dilution at 0.25 x *f*0
CS1-2	Sample dilution at 0.25 x *f*0 + spiked with endotoxin
AS1-2 Flag.	Sample dilution at 1 x *f*0 + spiked with Flagellin B.S.
AS1-2 PAM.	Sample dilution at 1 x *f*0 + spiked with PAM3CSK4

Recommended plate layout for Method 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	blank	blank	blank								
B	LPS control	LPS control	LPS control	LPS control								
C	AR1	AR1	AR1	AR1	AR2	AR2	AR2	AR2	AR3	AR3	AR3	AR3
D	BR1	BR1	BR1	BR1	BR2	BR2	BR2	BR2	BR3	BR3	BR3	BR3
E	CR1	CR1	CR1	CR1	CR2	CR2	CR2	CR2	CR3	CR3	CR3	CR3
F	AS1	AS1	AS1	AS1	AS2	AS2	AS2	AS2	AS3	AS3	AS3	AS3
G	BS1	BS1	BS1	BS1	BS2	BS2	BS2	BS2	BS3	BS3	BS3	BS3
H	CS1	CS1	CS1	CS1	CS2	CS2	CS2	CS2	CS3	CS3	CS3	CS3

Blank	negative control
LPS control	positive control
AR1-3	Reference batch at sample dilution *f*0
BR1-3	Reference batch at sample dilution 0.5 x *f*0
CR1-3	Reference batch at sample dilution 0.25 x *f*0
AS1-3	Test batch at sample dilution 1x *f*0
BS1-3	Test batch at sample dilution 0.5 x *f*0
CS1-3	Test batch at sample dilution 0.25 x *f*0

4. Equipment and Reagents

4.1. Equipment

- Biological safety cabinet
- Humidified incubator (37°C, 5% CO2)
- Water bath 37°C
- Vortex mixer
- Single/multi-channel adjustable volume pipettes suitable for 5 to 1000 µL

4.2. Materials

- Pyrogen-free 50 mL tubes
- Pyrogen-free pipette tips
- Sterile 96-well flat-bottom microtiter plates (included in kit)
- Non-sterile 96-well flat-bottom microtiter plates (included in kit)
- Pyrogen-free Polystyrene disposable pipettes
- Sterile liquid reservoirs
- Flasks, beakers and liquid containers necessary for preparation of reagents
- Ethanol (70%)
- Round bottom polystyrene tube

4.3. IL-6 ELISA kit

- IL-6 concentration in the harvested supernatant is determined using an broad range (10-3160 pg/ml) IL-6 ELISA (included in kit)

4.4. Software

- SoftMax Pro 7.0 or our web-based tool http://www.matresearch.com/analysis

NOTE *For support on data analysis, please contact our helpdesk at info@matresearch.com*

4.5. Important Notes

- Always perform the assay in a biological safety cabinet
- Use sterile/pyrogen-free equipment/consumables
- Regularly clean surfaces with 70% ethanol
- Wear protective gloves and lab coat
- Immediately proceed during, as well as following dilution and mixing of samples: do not let the mixtures sit

5. Protocols

The protocol must be performed using sterile and pyrogen-free reagents and materials, in a sterile working environment.

5.1. Preparing the cell culture medium

The cell culture medium is composed of RPMI with medium supplement. This cell culture medium is used to wash and resuspend the MATR cells. The following steps describe how the cell culture medium is prepared.

1. Thaw the medium supplement bottle.
2. Transfer 3 mL of the culture medium supplement to 12 mL RPMI and mix gently to create the cell culture medium solution. Warm the cell culture medium to 37°C in a water bath before use.

NOTE I *Mix gently, avoid creating bubbles.*

NOTE II *Warm the cell culture medium to 37°C in a waterbath before use.*

5.2. Preparing the product samples

NOTE I *Preparation of the product samples is done using RPMI <u>without</u> medium supplement.*

1. Add 4 replicates of 50 µL RPMI to the plate for each sample dilution both for pure samples and spiked samples, with the exception of the highest concentration (which is left empty).

NOTE II *For plate layouts, see chapter 3.*

2. Prepare the samples by creating a 5x concentrated pre-dilution of your product in RPMI (note that a 2x pre-dilution equals a 10x end-dilution) in polystyrene tubes. Mix by pipetting up and down for at least 20x (avoid creating bubbles).
3. Add 4 replicates of 100 µL of the 5x pre-dilution for each sample and corresponding spike control (please refer to the plate layout in chapter 3)

to the plate. Mix by pipetting up and down with a P100/200 set to 50 µL for at least 20x (avoid creating bubbles).

NOTE III *Spiking of samples with endotoxin is performed in step 5.3.*

4. Serial dilute the samples by moving 50 µL from sample dilution 1 to 2 in the plate. Mix by pipetting up and down with a P100/200 set to 50 µL for at least 20x (avoid creating bubbles).
5. Dilute the samples by moving 50 µL from sample dilution 2 to 3 in the plate. Mix by pipetting up and down with a P100/200 set to 50 µL for at least 20x (avoid creating bubbles).
6. Aspirate and trash 50 µL of sample dilution 3 from the plate.
7. Repeat for all spiked and unspiked samples.
8. Add 100 µL of the RPMI to the pure samples (without spike) from the lowest to the highest sample dilution using a multichannel, being careful not to touch the sample with the tip (do not mix!).

5.3. Preparing the endotoxin standard (LPS)

EDQM Lipopolysaccharide E.Coli, calibrated in International Endotoxin Units, is used as the reference standard endotoxin (RSE) for the assay.

1. Add 3300 µL RPMI without supplements (tube R1) to a round bottom polystyrene tube.
2. Add 1000 µL of RPMI without supplements into 7 round bottom polystyrene tubes (tube R2 - R8).
3. Thaw the LPS vial.
4. Add 950 µL LAL-reagent water (LRW) to the LPS vial to make a solution of 10 EU/mL LPS.

IMPORTANT *Vortex the solution for at least 3 minutes at high speed, immediately proceed to the next step*

5. Transfer 300 µL of the LPS stock solution to the R1 tube to prepare a 0,83 EU/ mL LPS solution (do not vortex).
6. Mix R1 by pipetting up and down using a p1000 pipet set to 1000 µL for at least 20 times (do not vortex), immediately proceed to the next step.

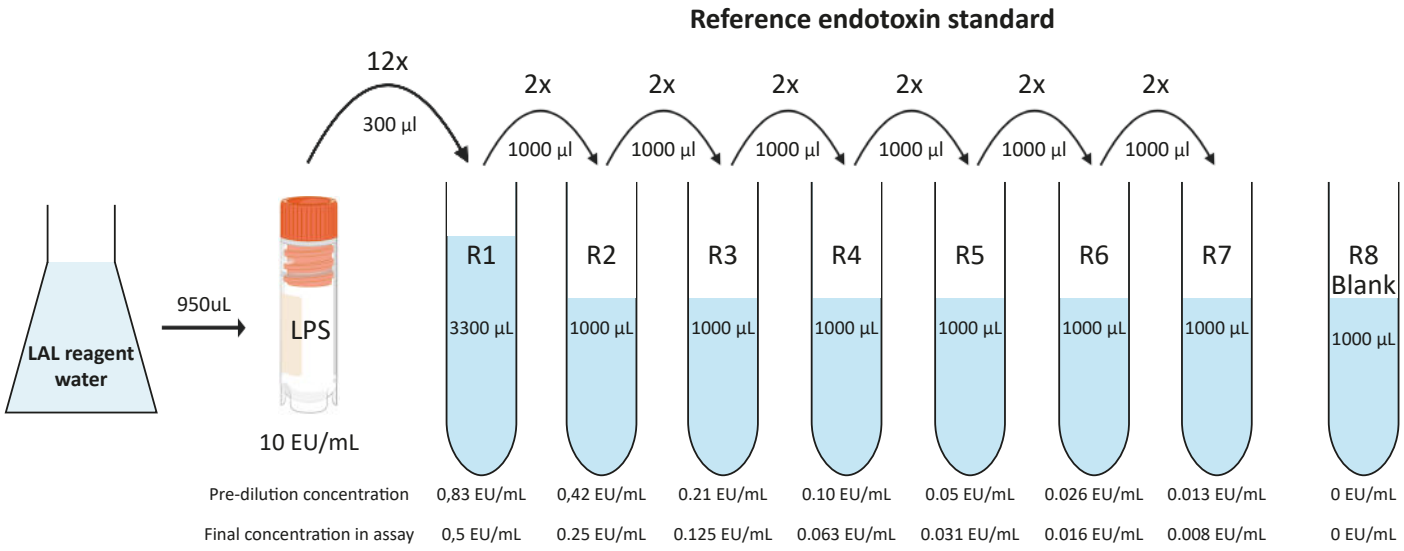
7. Transfer 1000 µL of R1 to R2 and mix by pipetting up and down for at least 20 times (do not vortex), immediately proceed to the next step.
8. Transfer 4 replicates of 150 µL of each serial diluted (RSE) endotoxin standard R1 - R8 (0.5 - 0.0 EU/mL) to the 96-well culture plate. Ensure mixing of each dilution before transferring to the plate.

NOTE I *For plate layouts, see chapter 3.*

NOTE II *Overview of preparing the endotoxin standard can be observed in figure 1.*

NOTE III *Immediately proceed after diluting and mixing (non-) endotoxin controls, do not let the mixtures sit*

IMPORTANT *Tube R8 is used as blank control (RPMI only).*



5.4. Preparing the non-endotoxin and endotoxin controls

1. To spike the samples with endotoxin, resuspend the endotoxin (RSE) R1 dilution created in 5.3 for at least 20 times using a P1000 pipette set to 1000 µL. Immediately proceed to the next step.
2. Move 150 µL of the resuspended endotoxin R1 dilution into 3850 µL RPMI without supplements in a round bottom polystyrene tube and resuspend at least 20 times using a P1000 pipette set to 1000 µL. Immediately proceed to the next step.
3. Add 100 µL of the endotoxin spike to the plate.
4. Add 100 µL of the RPMI to the pure samples (without spike).

NOTE *Spiked samples should correspond to R4 after transfer to the plate.*

NOTE *PAM3CSK4 and Flagellin B.S. are used as non-endotoxin pyrogen controls.*

NOTE *Preparation of the NEP controls is done using RPMI without medium supplement. PAM3CSK4 and Flagellin B.S. are used as non-endotoxin pyrogen controls.*

5. Thaw the PAM3CSK4 vial (1 µg/mL).
6. Add 950 µL RPMI to make a 50 ng/mL PAM3CSK4 solution and vortex until properly mixed.
7. Dilute 750 µL of the PAM3CSK4 solution in 750 µL RPMI, to obtain a 25 ng/mL solution and vortex until properly mixed.
8. Transfer the diluted PAM3CSK4 to a reagent reservoir and add 4 replicates of 100 µL to the samples in the 96- well culture plate.
9. Thaw the Flagellin B.S vial (1 µg/mL)
10. Add 950 µL RPMI to make a 50 ng/mL Flagellin B.S. solution and mix by resuspension until the solution is properly mixed. Do not vortex!
11. Transfer the diluted Flagellin B.S. to a reagent reservoir and add 4 replicates of 100 µL to the samples in the 96- well culture plate.

5.5. Preparing the MATR cell suspension

The following steps describe the procedure of thawing the MATR-cells. This process is from the moment the cells are removed from the LN_2 storage, through the final step of adding the MATR cells to the plate.

NOTE *Preparation of the MATR cells is done using pre-warmed (37°C) cell culture medium with medium supplement.*

1. Take the MATR cells cryo vial out of the liquid nitrogen tank (or dry ice) and thaw the cells by placing it immediately in a water bath at 37°C, until the last ice crystals are visible.
2. Immediately transfer the thawed MATR cells (±1 mL) from the vial into a sterile and pyrogen-free 50 mL tube.
3. Slowly add 10 mL of pre-warmed (37°C) cell culture medium (1 mL per 5 seconds) and mix by gently swirling the tube while adding the culture medium.
4. Wash the MATR cells in pre-warmed cell culture medium by spinning down the cells at 150 x g for 10 minutes at RT. Discard the supernatant without disturbing the cell pellet.
5. Resuspend the cell pellet in 10.5 mL pre-warmed cell culture medium to create a homogenous cell suspension and move the cells to a reagent reservoir. Add 100 µL of the cell suspension to all wells of 96-well plate (sample dilutions, endotoxin standard, NEP controls, and blanks) using a multichannel pipet.

   NOTE *Avoid creating bubbles*

6. Place the 96-well plate in the cell incubator for 20 ± 2 hours.

5.6. Analyzing the samples by IL-6 ELISApro

PREPARATION

- Allow the plates and reagents to reach room temperature before starting the ELISA assay (except for the TMB substrate).
- Add 50 mL wash buffer to 950 mL distilled water.
- Briefly centrifuge the micro vials to spin down the solution.

NOTES

- *For accurate assay performance, samples must be diluted at least 2- fold in ELISA diluent.*
- *The use of a plate shaker is recommended during the incubation steps.*
- *For additional help with the ELISApro, please contact our helpdesk.*

1. Wash plate 1 time with 200 µL wash buffer. After the final wash, invert and firmly tap the plate against absorbance paper.

   NOTE *Immediately proceed to the next step. Do not let the wells dry out!*

2. Add 100 µL ELISA diluent to the plate, then add 100 µL of the supernatants from the RSE reference standard, product samples, and NEP controls to the IL-6 ELISA plate.
3. Cover the plate with the adhesive plate cover and incubate for 2 hours at room temperature on a plate shaker.

   NOTE *Prepare the detection antibody mix 15 minutes prior to the next step by adding 12 µL of detection antibody to 12 mL of ELISA diluent. Mix thoroughly before use.*

4. Wash the ELISA plate 4 times with 200 µL wash buffer. After the final wash, invert and firmly tap the plate against absorbance paper.

   NOTE *Immediately proceed to the next step. Do not let the wells dry out!*

5. Add 100 µL of the detection antibody mix into each well, cover the plate with the adhesive cover and incubate for 1 hour at room temperature on a plate shaker.

NOTE *Prepare the Streptavidin-HRP mix 15 minutes prior to next step by adding 12 µL of Streptavidin-HRP with 12 mL of Streptavidin-HRP diluent. Mix thoroughly before use.*

6. Wash the ELISA plate 4 times with 200 µL wash buffer. After the final wash, invert and firmly tap the plate against absorbance paper.

NOTE *Immediately proceed to the next step. Do not let the wells dry out!*

7. Add 100 µL of the Streptavidin-HRP mix into each well, cover the plate with the adhesive cover and incubate for 1 hour at room temperature on a plate shaker.

8. Wash the ELISA plate 4 times with 200 µL wash buffer. After the final wash, invert and firmly tap the plate against absorbance paper.

NOTE *Immediately proceed to the next step. Do not let the wells dry out!*

9. Add 100 µL TMB substrate to each well and incubate for 10 minutes at room temperature.

NOTE *Protect the plate from direct light.*

10. Add 100 µL of stop solution to each well to stop the color development.

11. Measure the optical density using an absorbance plate reader at OD450 within 15 minutes after adding the stop solution. If possible, measure the optical density at OD570/OD630 and subtract the value measured from OD450.

6. Definitions and abbreviations

°C	Degrees Celsius
CLC	Contaminant limit concentration
CO2	Carbon dioxide
DMSO	DiMethyl SulfOxide
E. Coli	Escherichia coli
EDQM	European Directorate for the Quality of Medicines
ELISA	Enzyme Linked Immunosorbent Assay
EU	International endotoxin unit
EP	European Pharmacopoeia
FBS	Fetal Bovine Serum
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MAT	Monocyte Activation Test
MVD	Maximum valid dilution
NEP	Non-endotoxin pyrogen
OD	Optical density
PBMC	Peripheral blood mononuclear cells
RT	Room temperature
STD	Standard

MAT
RESEARCH