PYROLAB® Monocyte Activation Test (MAT) Kit

REF : KBPL9110

Ver 1.0

RUO

Enzyme Immunoassay for the Quantitative Determination of pyrogens in pharmaceutical product

RUO	**For Research Use Only**	**REF**	**Catalog Number**
	**Store At**	**LOT**	**Batch Code**
	**Manufactured By**		**Biological Risk**
	**Expiry Date**		**Consult Operating Instructions**

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REF KBPL9110 Σ 96 tests

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Introduction:

The Monocyte Activation Test (MAT) is an in vitro assay designed to detect pyrogenic substances in pharmaceutical, biological products and medical apparatus by measuring the immune response of human monocytes.
Monocytes, as key components of the human innate immune system, recognize pyrogens through Toll-like receptors (TLRs) and respond by releasing proinflammatory cytokines, such as interleukin-6 (IL-6). The concentration of IL-6 secreted into the culture supernatant reflects the degree of pyrogenic stimulation.
The PYROLAB® MAT Kit utilizes cryopreserved human monocytic cells (MAT cells) and a human IL-6 ELISA assay to quantitatively determine the presence of pyrogens in test samples.
This method enables detection of both endotoxin and non-endotoxin pyrogens (NEPs), providing a physiologically relevant alternative to traditional rabbit pyrogen test (PRT).
Prior to routine testing, the assay should be validated for each sample to exclude potential interference and confirm assay sensitivity for both endotoxin and non-endotoxin pyrogens.

Materials Provided:

1. Anti-Human IL-6 Microtitre coated plate – 12 x 8 wells
2. Microtitre plate (96 wells) – 1 plate
3. Culture Medium – 2 x 25 ml
4. Water for BET – 8 ml
5. Anti-Human IL-6:Biotin Conjugate (200X) – 60 ul
6. Streptavidin HRP (100X) – 140 ul
7. Diluent – 25 ml
8. Wash Buffer (10X) – 2 x 25 ml
9. TMB substrate – 12 ml
10. Stop solution – 6 ml
11. Plate Sealer – 3 nos.
12. Culture Medium Supplement – 1.5 ml
13. Reference Standard Endotoxin (RSE) – 1 vial
14. MAT cells – 2 vials
15. Instruction manual

Materials to be provided by the End-User:

1. Desktop low-speed centrifuge (for 96-well plates)
2. Water bath
3. CO2 incubator
4. Biological safety cabinet (or ultra clean workbench)
5. Large volume electronic dispenser
6. Multi-channel micropipettes
7. Vortex mixer
8. Microplate shaker
9. Automated microplate washer (optional)
10. Cryo-freezer (-150 °C, for cell storage)
11. Microplate reader with an absorbance wavelength of 450 nm and 595 nm/600 nm/620 nm

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.

Reagent Preparation (all reagents should be diluted immediately prior to use):

DAY-1

Preparation of RSE Solutions:

1. Preparation of 30 EU/ml RSE solution:

Dilute Reference Standard Endotoxin with suitable volume of Water for BET according to concentration labeled on the bottle, vortex for 15 minutes to mix well, and further dilute with Water for BET to a final concentration of 30 EU/ml.

Prepare the standard curve solutions as shown in Table below:

Standard Concentration	Standard Vial	Dilution Particulars
3.0 EU/ml	Standard No.8	100 ul RSE + 900 ul culture medium
1.0 EU/ml	Standard No.7	400 ul Standard No.8 + 800 ul culture medium
0.6 EU/ml	Standard No.6	600 ul Standard No.7 + 400 ul culture medium
0.3 EU/ml	Standard No.5	500 ul Standard No.6 + 500 ul culture medium
0.1 EU/ml	Standard No.4	400 ul Standard No.5 + 800 ul culture medium
0.05 EU/ml	Standard No.3	500 ul Standard No.4 + 500 ul culture medium
0.025 EU/ml	Standard No.2	500 ul Standard No.3 + 500 ul culture medium
0.0125 EU/ml	Standard No.1	500 ul Standard No.2 + 500 ul culture medium
0 EU/ml	Standard No.0	800 ul culture medium

Sample Preparation:

Method Validation
Before performing routine MAT testing on a given sample, method validation must be conducted to rule out potential sample interference and to confirm the assay's sensitivity in detecting endotoxins and non-endotoxin pyrogens (NEPs) in the sample matrix.

Elimination of Interference
Interference from the test sample can often be eliminated by diluting the sample to a certain concentration, referred to as the maximum valid dilution (MVD). The MVD is the maximum allowable dilution of a sample at which the contaminant limit can be determined. The calculation of the MVD is based on the endotoxin reference standard. Greater assay sensitivity permits higher dilution of the test sample, reliably detecting pyrogens while reducing matrix interference.

Determine the MVD using the following expression:

$$\frac{CLC \times C}{Test\ sensitivity}$$

CLC = Contaminant limit concentration
C = Concentration of test solution
The test sensitivity of the PYROLAB® MAT kit is 0.0125 EU/ml.

Note: *Since test sensitivity is an actual point on the standard curve, it is to be confirmed (>cut-off *value) in every test to confirm the MVD calculation.*

*Cut–off: The cut-off value may be calculated using the following expression:

$$\mathbf{x + 3\,s}$$

x = mean of the responses obtained for the 4 blank replicates.
s = standard deviation of the responses obtained for the 4 blank replicates.

Interference Test Design:
The preparation of test solutions for the interference study was designed in accordance with the requirements of the European Pharmacopoeia and the Chinese Pharmacopeia. The test setup is summarized in Table below, which lists the preparation of interference test solutions.

No.	Solution	Replicates	Note
1.	Sample, solution / A*	4	A< MVD
2.	Sample, solution / 2A	4	2A< MVD
3.	Sample, solution / 4A	4	4A< MVD
4.	Sample, solution / A spiked with a Middle concentration from the endotoxin standard curve (Spiking RSE)	4	NA
5.	Sample, solution / 2A spiked with a Middle concentration from the endotoxin standard curve (Spiking RSE)	4	NA
6.	Sample, solution / 4A spiked with a Middle concentration from the endotoxin standard curve (Spiking RSE	4	NA

Designation of Test Solution (A): The solution of the preparation being examined designated as corresponds to the dilution level used in the interference test. This is defined as the highest concentration or lowest dilution of the sample at which the endotoxin recovery remains within 50%-200% of the nominal value.

Note: *Vortex each endotoxin standard solution for at least 1 minute before preparing the next dilution. Each condition should be tested in four replicates. The dilution factor between adjacent concentrations should not exceed 10-fold.*

Preparation of Monocyte Suspension:

1. Pre-warm the Culture medium at 37°C in water bath. Allow the Culture medium supplement to equilibrate to room temperature before use.
2. Transport MAT cells from liquid nitrogen tank on dry ice.
   Note: *When taking out MAT cells from liquid nitrogen, place the vials horizontally to allow liquid nitrogen to evaporate within the tube. This prevents cracking due to internal pressure buildup.*
3. Quickly thaw the cryovial of MAT cells in a 37°C water bath. Once completely thawed, remove the cryovial, disinfect the outer surface with 75% ethanol, wipe dry, and perform all subsequent operations in a biosafety cabinet.
4. Add 17 ml Culture medium and 305 ul Culture medium supplement to a 50 ml sterile, endotoxin-free centrifuge tube. Gently transfer the cells from the vial to the tube. Mix the contents by gentle pipetting or briefly vortex 3 times to avoid cell clumping.
5. Transfer the MAT cell suspension into a 50 ml sterile, endotoxin-free reservoir.

DAY-2

1. Before use, allow the entire ELISA kit to equilibrate at room temperature (25 ± 3°C) for 20 minutes. Return all components to 2-8℃ immediately after use.
2. Select the required number of Anti-hIL-6 Microtiter Strips according to the experimental design. Return unused strips to the foil bag with desiccant and store at 2-8℃ for up to 90 days.
3. 1×Wash Buffer: Dilute 1 volume of 10×Wash Buffer Concentrate with 9 volumes of ultra-pure water. For example, add 25 ml Wash Buffer Concentrate (10×) to 225 ml of ultra-pure water to prepare 250 ml of 1×Wash Buffer. Prepare fresh and mix well before use.
4. 1×Anti- Human IL-6 Biotinylated Conjugate: Dilute the 200×Anti- Human IL-6: Biotinylated Conjugate with Diluent in a sterile centrifuge tube to prepare the 1×Anti-Human IL-6:Biotinylated Conjugate. Mix gently and prepare it freshly before use.
5. 1×Streptavidin-HRP: Dilute the 100×Streptavidin-HRP with Diluent in a sterile centrifuge tube. Mix gently and prepare it freshly before use.

Assay Procedure:

DAY-1:

1. Add 100 ul of standard/sample per well.
2. Add 150 ul of **Monocyte Suspension** using a multichannel pipette.
3. Incubate the plate at 37°C, 5% CO_2, for 24 hours.

Use the recommended plate layout:

Method 1: semi-quantitative test

96 well plate	1	2	3	4	5	6	7	8	9	10	11	12
A	NCS				Sample 1-①				Sample 2-①			
B	ST7				Sample 1-②				Sample 2-②			
C	ST6				Sample 1-③				Sample 2-③			
D	ST5				Sample 1-④				Sample 2-④			
E	ST4				Sample 1-⑤				Sample 2-⑤			
F	ST3				Sample 1-⑥				Sample 2-⑥			
G	ST2				Sample 1-①+NEP (optional)				Sample 2-①+NEP (optional)			
H	ST1				NEP (optional)				NA			

Method 2: reference lot comparison test

96 well plate	1	2	3	4	5	6	7	8	9	10	11	12
A	Positive control (standard endotoxin)				NA				NA			
B	NCS				NA				NA			
C	Sample 1-lot 1: ①				Sample 2-lot 1: ①				Sample 3-lot 1: ①			
D	Sample 1-lot 1: ②				Sample 2-lot 1: ②				Sample 3-lot 1: ②			
E	Sample 1-lot 1: ③				Sample 2-lot 1: ③				Sample 3-lot 1: ③			
F	Sample 1-ref:* ①				Sample 2-ref: ①				Sample 3-ref: ①			
G	Sample 1-ref: ②				Sample 2-ref: ②				Sample 3-ref: ②			
H	Sample 1-ref: ③				Sample 2-ref: ③				Sample 3-ref: ③			

DAY-2

1. After 24 hours incubation (Day 1), centrifuge the 96-well MAT cell plate at 2000 × g for 10 minutes.
2. Transfer 50ul of supernatant from each well to the Anti-hIL-6 Microtiter plate with a multi-channel micropipette.
3. Seal the plate with Sealing Film and incubate on a microplate thermoshaker at 600 rpm for 1 hour at room temperature, protected from light.
4. Wash the plate 3 times with 300 ul 1×Wash Buffer per well, allowing 30 seconds soak each time. Try to remove as much liquid as possible from each well and avoid drying before adding the next solution.
5. Add 100 ul of freshly prepared 1×Anti-hIL-6 Biotinylated Conjugate to each well.
6. Seal and incubate with Sealing Film for 45 minutes at room temperature, protected from light.
7. Wash the plate 3 times with 300 ul 1×Wash Buffer per well, allowing 30 seconds soak each time. Try to remove as much liquid as possible from each well and avoid drying before adding the next solution.
8. Add 100 ul of 1×Streptavidin-HRP solution to each well.
9. Seal and incubate with Sealing Film for 30 minutes at room temperature, protected from light.
10. Allow TMB Substrate (PND004) to equilibrate for 20 minutes at room temperature before use.
11. Wash the plate 5 times with 300 ul 1×Wash Buffer per well, soaking 30 seconds each time. Try to remove as much liquid as possible from each well and avoid drying of wells between washes.
12. Add 100 ul of TMB Substrate to each well, and incubate at room temperature for 1-10 minutes, protected from light. The maximum duration should not exceed 20 minutes.
13. Add 50 ul of Stop Solution to each well.
14. Mix for 30 seconds and read the absorbance at 450 nm within 10 minutes

Calculation of Results:

- Based on the absorbance values (OD450 nm) of the endotoxin standards, establish a four-parameter logistic (4-PL) standard curve.
- Calculate the endotoxin concentration of each sample from the fitted curve.
- The recovery rate can be determined as follows:

  R= (Cwith spike RSE -Cwithout spike RSE) / Cwith spike RSE × 100%
- The curve must include at least five standard concentrations (points may be removed from the extremes if necessary). The correlation coefficient R2 should be ≥ 0.980.
- Data analysis can be performed using the microplate reader's built-in software or external tools such as CurveExpert, ELISAcalc, or equivalent.
- For samples with absorbance values above the Calibration standard ST1, determine a suitable dilution factor in a pilot study before retesting. The endotoxin concentration in test samples is calculated by multiplying the measured value by the corresponding dilution factor.
- For spiked samples tested at specific dilution levels, the recovery rate must meet the acceptance criteria defined by the relevant pharmacopoeial or regulatory guidelines.

Pyrogenicity of the Sample:

- For each sample dilution within the range that does not exceed the MVD, the endotoxin spike recovery is calculated based on the measured endotoxin concentration (EU/mL) of the spiked and non-spiked samples.

- The spike recovery is considered valid only when it falls within the range of 50%–200%. Any dilution that does not meet this criterion is deemed invalid and must be further diluted or otherwise treated to eliminate matrix interference. If the interference cannot be eliminated, the test shall not be continued.

- For each validated sample dilution, the OD values are converted to endotoxin concentrations (EU/mL), using the standard curve, multiplied by the corresponding dilution factor, then compared with the CLC to determine whether the sample exhibits pyrogenic activity:

Method 1:

If the value (in EU/mL) of sample is < **CLC→PASS**, indicates that the pyrogen level in the sample is below the CLC.
If the value (in EU/mL) of sample is > **CLC→FAIL**, indicates that the pyrogen level in the sample is above the CLC.

Method 2:

The positive control and at least one dilution of the reference lot should be above the mean OD value of the blank.
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. The OD ratio should not exceed a justified acceptance criterion. This acceptance criterion is to be defined by the end-user.
If the OD ratio is ≤ of the acceptance criterion→**PASS,** indicates that the lot being examined is not pyrogenic compared to the reference lot.
If the OD ratio is > of the acceptance criterion→**FAIL,** indicates that the lot being examined is pyrogenic compared to the reference lot.

Assay Performance:

- Range of the standard curve: 0.0125-1.0 EU/mL (optional, points ≥ 5), 4-PL, R2≥0.980
- QL: 0.025 EU/mL
- DL: 0.0125 EU/mL

Note: *Endotoxin standard curve and the cut-off value need to be calculated for each individual experiment.*

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