Monocyte-Activation Test as an Alternative Method for Pyrogen Test〈*G4-13-190*〉

(単球活性化試験による発熱性物質試験法の代替法〈*G4-13-190*〉)

1. Introduction

Pyrogen Test <*4.04*> uses rabbits to test the existence of pyrogens. However, from the perspective of animal welfare the monocyte-activation test (MAT) has been developed as an alternative method. The MAT detects not only endotoxins derived from gram-negative bacteria, but also non-endotoxin contaminants including pathogen-associated molecular patterns (PAMPs) derived from gram-positive, gram-negative bacteria, viruses, and fungi, and both biological and chemical contaminants originating from pharmaceutical manufacturing processes.

This general information describes the key considerations when using the MAT as an alternative method of the pyrogen test.

2. Outline of the MAT

The MAT is a method used to detect or quantify substances that activate human monocytes or monocytic cells, inducing the production of endogenous mediators such as pro-inflammatory cytokines including tumor necrosis factor α (TNF-α), interleukin-1β, and interleukin-6. As these substances are involved in pyrogenic mechanisms, the MAT indirectly detects pyrogens in the samples.

Since non-endotoxin contaminants are diverse and usually difficult to identify in a sample solution, the level of these factors in the sample solution is compared to the reaction response either to Endotoxin RS or to the reference lot of the sample solution, and is expressed in an equivalent endotoxin amount (Endotoxin Unit: EU).

Pharmaceuticals containing fever- or inflammatory-response-inducing non-endotoxin contaminants may show steep dose-response curves compared to that of endotoxin. Therefore, preparations that actually or potentially contain non-endotoxin contaminants should be tested at multiple dilutions, including the minimum dilution. This test is conducted in a manner that avoids contamination by pyrogens.

3. Basic Procedures

A sample solution is incubated with a source of human monocytes or human monocytic cells [e.g., peripheral blood mononuclear cells (PBMC), monocytic cell lines or cell lines that have been shown to be equivalent]. The duration of the incubation should be sufficient to allow appropriate evaluation of the selected measurement indicators. The test results of the selected measurement indicators for the sample solution are compared with those of the measurement indicators for Endotoxin RS or the reference lot of the sample solution.

4. Equipment

Dry-heat all glassware and other heat-resistant equipment using a validated process to remove or inactivate pyrogens. If employing plastic equipment, such as multi-well plates and tips for micropipette, use only that which has been shown to be free of pyrogen contamination and which does not interfere with the test.

5. Cell Sources

5.1. Whole Blood

Use whole blood from a single donor or whole blood derived from multiple donors, which meets the requirements described in sections 5.3., 5.4., 5.5., and, where applicable, in section 6.3.

5.2. PBMC

PBMC are isolated from whole blood from a single donor or derived from multiple donors, which meets the requirements described in sections 5.3., 5.4., 5.5., and, where applicable, in section 6.3.

5.3. Qualification of Blood Donors

Blood donors must meet the requirements relating to health, safety, and ethical concerns.

5.4. Quolification of Cells Derived from Multiple Donors

When whole blood derived from multiple donors is used, confirm that non-endotoxin contaminants can be detected as described in section 6.3.

5.5. Quolification of Cryopreserved Cells

The cell source intended for use in the MAT (human whole blood and blood fractions such as PBMC or monocytic cell lines) may be cryopreserved. Cryopreserved cells derived from multiple donors are prepared before freezing or alternatively by mixing of cryopreserved blood from single donors immediately after thawing, for example. Confirm the dose-response curve to endotoxins according to section 6.1. If the purpose is to detect non-endotoxin contaminants, perform the verification according to section 6.3.

5.6. Monocytic Cell Lines

Monocytic cell lines that meet the requirements described below are appropriate for the detection of endotoxins and non-endotoxin contaminants.

A human monocytic cell line is cultured sufficiently to ensure an adequate supply for the MAT. To optimize the supply method, a cloned cell line can be used.

The cells are controlled under aseptic conditions and regularly checked for the absence of contamination such as mycoplasma. Additionally, the cells must be regularly monitored for the properties such as doubling time, morphology, and function. The functional stability of the cell line is evaluated by tracking these properties according to the number of passages. Criteria for the functional stability should be established. They may include growth properties, maximum signal obtained in the test, background noise, and receptor signaling.

Receptor signaling can be tested with specific ligands (i.e. substances that induce biological responses by binding to specific receptors) for pattern recognition receptors (e.g. Toll-like receptors).

Confirm the dose-response curve to endotoxins according to section 6.1. If the purpose is to detect non-endotoxin contaminants, perform the verification according to section 6.3.

Note that if transcription factors related to the expression of pro-inflammatory cytokines are selected as measurement indicators, the validity of the detection of non-endotoxin contaminants should be demonstrated.

6. Points to Consider in Measurements

To verify the validity of the test, preparatory tests are conducted. The preparatory tests are to confirm that the requirements for the endotoxin standard curve described in section 6.1. are met, that the sample solution does not interfere with the test, that the test detects endotoxins and non-endotoxin contaminants, and that the solution does not interfere with the detection system.

The preparatory test is repeated whenever there is a significant change in the test conditions that could potentially influence the test results.

6.1. Assurance of the Endotoxin Standard Curve

Prepare at least four dilutions of Standard Endotoxin Solution to draw a standard curve and perform the tests with at least n=4 for each of dilutions of Standard Endotoxin Solution.

The measured value of the blank (i.e. no Standard Endotoxin Solution added) is preferably as low as possible.

Assure the validity of the endotoxin standard curve using appropriate statistical methods.

6.2. Verification Concerning Interfering Factors

To ensure the validity of the test, conduct preparatory tests to confirm that the sample does not interfere with the test. Use an appropriate diluent to dilute the sample in serial dilutions, ensuring that no dilutions exceed the maximum valid dilution (i.e. the maximum allowable dilution of the sample where the influence of reaction-interfering factors can be removed by dilution). Using the sample solution and the sample solutions spiked with appropriate concentrations of Endotoxin RS, calculate the concentration of pyrogens that correspond to the concentration of endotoxins in each solution applying the endotoxin standard curve. Calculate the endotoxin concentrations in the solutions spiked with endotoxins by subtracting the average concentration of pyrogens found in the unspiked solutions from the concentrations of pyrogens found in the spiked solutions, then calculate the average recovery of spiked endotoxins. When the average recovery is in the range of 50 – 200%, the test sample is judged to be free of interfering factors.

6.3. Verification Concerning Non-Endotoxin Contaminants

Confirm that non-endotoxin contaminants are detected as well as endotoxins on the chosen test system through preparatory tests. For this confirmation, past batches that were contaminated with non-endotoxin contaminants and judged positive in Pyrogen Test <*4.04*> or determined to be responsible for harmful reactions in humans may be used. When such batches are not available, use a specific ligand for two or more pattern recognition receptors (e.g., Toll-like receptors) to verify the system. When the recovery of non-endotoxin contaminants is in the range of 50 – 200%, the test solution is judged to be free of interfering factors. However, when there is a synergy effect between the sample and the ligand of non-endotoxin contaminants, the recovery rate is acceptable if it is 50% or higher, and acceptable even if it exceeds 200%. The selection of non-endotoxin pyrogens for this verification should reflect the non-endotoxin contaminants likely to be present in the sample solutions. The test system should be confirmed for detecting ligands to at least Toll-like receptor 4 and two other Toll-like receptors.

6.4. Interference in the Detection System

Verify the absence of interference in the detection system (e.g. enzyme-linked immunosorbent assay) for the selected measurement indicators after determining the optimal dilution for the sample solution.

7. Test Methods

There are two methods as described below. These tests are conducted following the preparatory tests.

Method 1: Semi-quantitative test

Method 2: Reference lot comparison test

In Method 1, the sample solution to be tested is compared to the standard curve obtained with Standard Endotoxin Solutions. As with the preparatory tests, prepare an endotoxin standard curve for each test and check for any influence of reaction-interfering factors. In Method 2, the sample solutions to be tested are compared with a verified reference lot (a preparation whose safety and efficacy have been verified through clinical studies, etc.) when the influence of reaction-interfering factors cannot be avoided within the range of the maximum valid dilution, or when it is considered to contain non-endotoxin contaminants. In either method, use a positive control made from Endotoxin RS and an appropriate negative control for each test. For detailed procedures and judgement, follow a validated MAT procedure.

References

1) European Pharmacopoeia Supplement 11.5 (2023) 2.6.30. Monocyte-activation test.
2) European Pharmacopoeia Supplement 11.5 (2023) 2.6.40. Monocyte-activation test for vaccines containing inherently pyrogenic components.