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附件：「單核球活化試驗法」乙份

裝

主旨：修正「中華藥典第七版之通則」增訂「單核球活化試驗法(Monocyte Activation Test，簡稱MAT)」，並自中華民國一百零二年七月一日生效。

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GENERAL PRECAUTIONS

The test is carried out under aseptic conditions according to current regulations for potentially infective material.

The precautions taken to avoid contamination are such that they do not affect any micro-organisms that are to be revealed in the test. The test is performed under working conditions that are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

GROWTH PROMOTION TEST

Use at least 2 suitable enriched culture media (for example, blood culture media) intended for detection of fungi and aerobic and anaerobic bacteria.

Confirm the sterility of each batch of medium by the incubation of representative containers at 35-37 °C for not less than 7 days.

Each batch of medium is tested by the supplier and/or the user for its growth-promoting capacities by inoculating duplicate test containers of each medium with 10-100 viable micro-organisms of each of the strains listed in Table 2.6.27.-1, and incubating for either 7 days for automated detection or 14 days for visual detection of microbial growth at 35-37 °C. The test media are satisfactory if there is clear evidence of growth in all inoculated media containers within this period.

Table 2.6.27.-1. – *Micro-organisms used for growth promotion*

Aerobic medium	
Staphylococcus aureus	for example, ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518
Bacillus subtilis	for example, ATCC 6633, CIP 52.62, NCIMB 8054
Pseudomonas aeruginosa	for example, ATCC 9027, NCIMB 8626, CIP 82.118
Candida albicans	for example, ATCC 10231, IP 48.72, NCPF 3179
Aspergillus brasiliensis	for example, ATCC 16404, IP 1431.83, IMI 149007
Anaerobic medium	
Clostridium sporogenes	for example, ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437
Bacteroides fragilis	for example, ATCC 25285, CIP 77.16, NCTC 9343

METHOD VALIDATION

Depending on the type of product, its method of preparation, the inoculum volume used and the type of test system, the need for validation in the presence of the type of preparation to be examined must be considered. Unless otherwise justified and authorised, the test system is validated with respect to specificity (absence of false positive results), sensitivity (limit of detection) and reproducibility. During validation, particularly to determine the limit of detection, the test is carried out using the preparation deliberately contaminated to different degrees with the following micro-organisms, chosen for the likelihood of contamination and their growth requirements:

- *Aspergillus brasiliensis*, for example, ATCC 16404, IP 1431.83, IMI 149007;
- *Bacillus subtilis*, for example, ATCC 6633, CIP 52.62, NCIMB 8054;
- *Candida albicans*, for example, ATCC 10231, IP 48.72, NCPF 3179;
- *Clostridium sporogenes*, for example, ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437;
- *Propionibacterium acnes*, for example, ATCC 11827;
- *Pseudomonas aeruginosa*, for example, ATCC 9027, NCIMB 8626, CIP 82.118;
- *Staphylococcus aureus*, for example, ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518;
- *Streptococcus pyogenes*, for example, ATCC 19615, CIP 1042.26, NCIMB 13285;
- *Yersinia enterocolitica*, for example, ATCC 9610, CIP 80.27, NCTC 12982.

It may be necessary to modify the list of micro-organisms depending on the origin of the cells and any micro-organisms previously found or associated with the particular type of cells.

Other approaches to validation may also be used, for example, interlaboratory comparison.

TESTING OF THE PREPARATION TO BE EXAMINED

Sample. A representative sample including cells and/or medium is tested. The sample is added to the culture medium as soon as possible after collection. If it is not added promptly after collection, it is stored at 5 ± 3 °C to avoid phagocytosis of micro-organisms by cells present in certain types of products (for example, neutrophils).

For haematopoietic products, the minimum amount to be used for the test depending on the total volume of the product (V mL) is shown below.

Total product volume (millilitres)	Inoculum volume
$V \geq 10$	1 per cent of total volume
$1 \leq V < 10$	100 µL
$V < 1$	Not applicable

For haematopoietic products that require dilution before freezing, the inoculum volume must be increased by the dilution factor. For other cellular products, suitable minimum amounts are defined in terms of volume or number of doses.

Analysis. Samples are inoculated into containers of culture medium as soon as possible after collection and incubated at 35-37 °C for not less than 7 or 14 days, depending on the detection system used. A suitable proportion of the inoculum is added to the medium to be incubated in aerobic conditions and the remainder of the inoculum to the medium to be incubated in anaerobic conditions.

OBSERVATION AND INTERPRETATION OF RESULTS

Examine media, visually or with automated systems at least daily, and at the end of the observation period for evidence of microbial growth. If no growth is observed during or at the end of the observation period, the product is 'culture negative' at the limit of detection. If growth is observed in a valid test, the product is 'culture positive'; the contaminant is identified to a suitable taxonomic level (genus, species) and an antibiogram is established.

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2.6.30. MONOCYTE-ACTIVATION TEST

1. INTRODUCTION

The monocyte activation test (MAT) is used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators: such as pro-inflammatory cytokines, for example tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). These cytokines have a role in fever pathogenesis. Consequently, the MAT will detect the presence of pyrogens in the test sample. The MAT is suitable, after a product-specific validation, as a replacement for the rabbit pyrogen test.

Pharmaceutical products that contain non-endotoxin pyrogenic or pro-inflammatory contaminants often show very steep dose-response curves in comparison with endotoxin dose-response curves. Frequently the greatest response to such

contaminated products is obtained with undiluted solutions of the preparations being examined or small dilutions of the preparations being examined. For this reason preparations that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.

The following 3 methods are described in the present chapter.

Method A. Quantitative test

Method B. Semi-quantitative test

Method C. Reference lot comparison test

The test is carried out in a manner that avoids pyrogen contamination.

2. DEFINITIONS

The maximum valid dilution (MVD) is the maximum allowable dilution of a sample at which the contaminant limit can be determined. Determine the MVD using the following expression:

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

CLC = contaminant limit concentration;

C = concentration of test solution;

LOD = limit of detection.

The acceptance criterion for a pass/fail decision is the contaminant limit concentration (CLC), which is expressed in endotoxin equivalents per milligram or millilitre, or in units of biological activity of the preparation being examined.

The CLC is calculated using the following expression:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass;

M = maximum recommended bolus dose of product per kilogram of body mass.

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.

Where an endotoxin limit concentration (ELC) has been specified for a product, the CLC is the same as the ELC, unless otherwise prescribed. In this case, the concentration of test solution is expressed in mg/mL if the endotoxin limit is specified by mass (IU/mg), in Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit), in mL/mL if the endotoxin limit is specified by volume (IU/mL).

Endotoxin equivalents are values for the contaminant concentration read off the standard endotoxin dose-response curve (Method A) or estimated by comparison with responses to standard endotoxin solutions (Method B). The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

The cut-off value is calculated using the following expression:

$$\bar{x} + 3s$$

$\bar{x}$ = mean of the 4 replicates for the responses to the blank (R_0);

s = standard deviation of the 4 replicates of the responses to the blank (R_0).

The cut-off value is expressed in units appropriate to the read-out.

The limit of detection (LOD) is determined using the endotoxin standard curve. The LOD is the concentration of endotoxin corresponding to the cut-off value. For the purpose of the test, the LOD is expressed as endotoxin equivalents per millilitre.

3. GENERAL PROCEDURE

A solution of the preparation being examined is incubated with a source of human monocytes or human monocytic cells, e.g. from human heparinised peripheral blood that is preferably not more than 4 h old, or a monocyte-containing fraction of that blood, such as human peripheral blood mononuclear cells (PBMC) isolated, e.g. by density-gradient centrifugation, or a human monocytic cell line. Human heparinised peripheral blood is usually diluted with culture medium or saline e.g. to 2-50 per cent *V/V* (final concentration). PBMC or monocytic cell lines, in culture medium and with either the donor's own plasma or AB serum, are typically used at a final cell density of 0.1-1.0 × 10^6 cells per well, tube or other receptacle. For monocytic cell lines, heat-inactivated foetal bovine serum may be substituted for AB serum. The cell culture is carried out at 37 ± 1 °C, in an atmosphere appropriate for the culture medium, e.g. 5 per cent CO_2 in humidified air. The duration of the culture is sufficient to allow accumulation of the chosen read-out. The responses of the chosen read-out, e.g. a pro-inflammatory or pyrogenic cytokine, to a solution of the preparation being examined are compared with responses to standard endotoxin or to a reference lot of the preparation being examined. The chosen read-out method is calibrated using the appropriate standard.

4. APPARATUS

Depyrogenate all glassware and other heat-stable apparatus in a hot-air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipetters, use apparatus shown to be free of detectable pyrogens and which do not interfere with the test.

5. CELL SOURCES AND QUALIFICATION

5-1. *WHOLE BLOOD*

Whole blood is obtained from single donors or from pooled whole blood which are qualified according to the requirements described under section 5-3 and section 5-4, respectively.

5-2. *PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)*

PBMC are isolated from blood obtained from single donors or from pooled whole blood which are qualified according to the requirements described under section 5-3 and section 5-4, respectively.

5-3. *QUALIFICATION OF BLOOD DONORS*

Blood donors are to satisfy the following qualification criteria, together with other requirements in force that relate to consent, health and safety and ethical considerations. Blood donors are to describe themselves as being in good health, as not to be suffering from any bacterial or viral infections and to have been free from the symptoms of any such infection for a period of at least 1 week prior to the donation of blood. Blood donors are not to have taken non-steroidal anti-inflammatory drugs during the 48 h prior to donating blood and steroidal anti-inflammatory drugs during the 7 days prior to donating blood. Individuals who have been prescribed immunosuppressant or other drugs known to influence the production of the chosen readout are not to serve as blood donors. Blood donation are to be tested for infection markers according to national requirements for transfusion medicine.

5-4. *QUALIFICATION OF CELLS POOLED FROM A NUMBER OF DONORS*

Pools (of whole blood or blood fractions, e.g. PBMC), must consist of donations from a minimum of 4 individual donors but preferably 8 or more donors, where practicable, taking from each donation an approximately equal volume of blood, or cells from an approximately equal volume of blood. For the qualification of pooled cells proceed as follows: within 4 hours of collection of blood, generate dose-response curves from the pool using standard endotoxin with at least 4 geometrically diluted

endotoxin concentrations, e.g. in the range of 0.01 IU/mL to 4 IU/mL. The dose-response curves are to meet the 2 criteria for the standard curve described under section 6-1.

5-5. *QUALIFICATION OF CRYO-PRESERVED CELLS*

The cell source intended for use in a MAT, e.g. human whole blood, blood fractions, such as PBMC or monocytic cell lines, may be cryo-preserved. Pools of cryo-preserved cells are obtained by pooling before freezing, or by pooling single cryo-preserved donations immediately after thawing. Pools must consist of donations from a minimum of 4 individual donors but preferably 8 or more donors where practicable, taking from each donation an approximately equal volume of blood, or cells from an approximately equal volume of blood. Qualification of cryo-preserved blood or cells is performed immediately after thawing (and pooling if necessary): dose-response curves for cryo-preserved blood or cells are to comply with the 2 criteria for the standard curve as described under section 6-1.

5-6. *MONOCYTIC CONTINUOUS CELL LINES*

A human monocytic cell line is continuously cultured in order to warrant a sufficient supply for the MAT. To optimise the method, clones derived from the cell line can be used.

Cells must be maintained under aseptic conditions and regularly tested for the presence of mycoplasma contamination. Additionally, cells must be regularly checked for identity (e.g. doubling time, morphology, and function) and stability. The functional stability of a cell line is assessed by monitoring its performance in relation to the number of passages during routine testing. Criteria for functional stability are to be established and may include growth criteria, maximum signal obtained in the test, background noise and receptor expression. The receptor expression may be tested with specific ligands e.g. lipopolysaccharide (LPS) for toll-like receptor 4 (TLR4), lipoteichoic acid (LTA) for toll-like receptor 2 (TLR2), synthetic bacterial lipoprotein for TLR2-TLR1 or synthetic bacterial lipoprotein for TLR2-TLR6.

6. PREPARATORY TESTING

To ensure both the precision and validity of the test, preparatory tests are conducted, to assure that the criteria for the standard curve are satisfied, that the solution does not interfere with the test, that the test detects endotoxins and non-endotoxins contaminants and that the solution does not interfere in the detection system.

6-1. *ASSURANCE OF CRITERIA FOR THE STANDARD CURVE*

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.

The basal release of the chosen read-out (blank) in the absence of added standard endotoxin is to be optimised to be as low as possible.

There are 2 acceptance criteria for the standard curve:

- the regression of responses (appropriately transformed if necessary) on log dose shall be statistically significant ($p < 0.01$);
- the regression of responses on log dose must not deviate significantly from linearity ($p > 0.05$). If analysis for a 4-parameter logistic curve is performed, then the observed curve must not deviate significantly from the theoretical curve as calculated by using the usual statistical methods (see chapter *5.3. Statistical analysis*).

6-2. *TEST FOR INTERFERING FACTORS*

To assure the validity of the test, preparatory tests are conducted to assure that the test solution does not interfere with the test. Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test. Using an appropriate diluent, dilute the preparation to be examined in geometric steps, with all dilutions not exceeding the MVD. Make the same dilutions of the preparation to be examined and add endotoxin at a concentration equal to or near the middle of the standard curve (Method A) or equal to twice the LOD (Method B), or use a diluent containing added endotoxin at a concentration equal to or near the middle of the standard curve (Method A) or equal to twice the LOD (Method B). Test these dilution series in parallel in the same experiment. Use the standard curve to calculate the concentration of endotoxin-equivalents in each solution. Calculate the mean recovery of the added endotoxin by subtracting the mean concentration of endotoxin equivalents in the solution (if any) from that in the solution containing the added endotoxin. The test solution is considered free of interfering factors if, under the conditions of the test, the measured endotoxin equivalents in the test solution to which endotoxin is added is within 50-200 per cent of the added concentration, after subtraction of any endotoxin equivalents detected in the solution without added endotoxin. When this criterion is not met, Method C is to be preferred over Methods A and B.

In Method C, a solution of the preparation being examined is tested at 3 dilutions: the highest concentration (lowest dilution) that stimulates the greatest release of the chosen read-out and the 2-fold dilutions immediately below and above the chosen dilution. Since the concentration that stimulates the greatest release of the chosen read-out may be donor-dependent as well as batch-dependent, the product-specific validation is to be performed in at least 3 independent tests, each using cells from different donors. The highest concentration (lowest dilution) that stimulates the greatest release of the chosen read-out in the majority of donors, and the 2-fold dilutions immediately below and above that dilution are deemed to be validated for further testing. If undiluted test solution stimulates the greatest release of the chosen read-out, subsequent testing is to be performed using undiluted test solution and also test solution diluted in the ratios 1:2 and 1:4 before its addition to the PBMC. The 3 dilutions to be used in subsequent testing are not to exceed the MVD; the dilution factors for these 3 solutions are designated f_1, f_2 and f_3. Following the product-specific validation, the test is routinely performed with cells from 4 individual donors or a single pool or with cells from 1 passage of a human monocytic cell line.

6-3. *METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS*

The preparatory testing is also to show that the chosen test system detects, in addition to bacterial endotoxins, non-endotoxin pro-inflammatory or pyrogenic contaminants. This can be achieved using historic batches that have been found to be contaminated with non-endotoxin contaminants that caused positive responses in the rabbit pyrogens test or adverse drug reactions in man. Where such batches are not available, the preparatory testing is to include validation of the test system using specific ligands for toll-like receptors, e.g. peptidoglycans, lipoteichoic acids or synthetic bacterial lipoproteins.

6-4. *INTERFERENCE IN THE DETECTION SYSTEM*

Once the optimum dilution of the solution of the preparation being examined for further testing has been identified, this dilution is tested for interference in the detection system (e.g. ELISA) for the chosen read-out. The agreement between a dilution series of the standard for the chosen read-out, in the presence and absence of the test preparation, is to be within ± 20 per cent.

7. METHODS

7-1. *METHOD A: QUANTITATIVE TEST*

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 to pass the test.

7-1-1. **Test procedure**

Using the validated test method, prepare the solutions shown in Table 2.6.30.-1 and culture 4 replicates of each solution with

cells from each of 4 individual donors or a single pool or with cells from 1 passage of a human monocytic cell line.

Table 2.6.30.-1

Solution	Solution	Added endotoxin (IU/mL)	Number of replicates
A	Test solution/*f*	None	4
B	Test solution/2 × *f*	None	4
C	Test solution/4 × *f*	None	4
D	Test solution/*f*	Middle dose from endotoxin standard curve (R_3)	4
R_0	Pyrogen-free saline or test diluent	None (negative control)	4
R_1-R_4	Pyrogen-free saline or test diluent	4 concentrations of standard endotoxin	4 of each concentration

Solution A = Solution of the preparation being examined at the dilution, here designated *f*, at which the test for interfering factors was carried out, i.e. the highest concentration (lowest dilution) for which the endotoxin recovery is within 50-200 per cent.

Solution B = 2-fold dilution of solution A, not exceeding the MVD.

Solution C = 2-fold dilution of solution B, not exceeding the MVD.

Solution D = solution A spiked with standard endotoxin: the middle dose from endotoxin standard curve (R_3).

Solution R_0 = negative control.

Solutions R_1-R_4 = solutions of standard endotoxin at the concentrations used in the test for interfering factors.

7-1-2. Calculation and interpretation

All data to be included in the data analysis are to relate to cells for which the 2 criteria for the standard curve are satisfied. The endotoxin equivalents recovery calculated from the endotoxin equivalents concentration found in solution D after subtracting the endotoxin equivalents concentration found in solution A, is within the range of 50-200 per cent. For each different cell source, e.g. individual donation, donor pool, or cell line, use the endotoxin standard curve R_1-R_4 to calculate the concentration of endotoxin equivalents in each of the replicates of solutions A, B and C. The preparation being examined complies with the requirements of the test for a given cell source if the mean concentrations of endotoxin equivalents measured in the replicates of solutions A, B and C, after correction for dilution and concentration, are all less than the CLC specified for the preparation being examined.

7-1-3. Pass/fail criteria of the preparation

When cells from individual donors are used, the preparation being examined is required to comply with the test with the cells from each of 4 different donors. If the preparation being examined passes the test with cells from 3 of the 4 donors (1 donor excluded for failing to comply with test performance criteria or showing a positive reaction), the test is continued with cells from a further 4 donors, none of whom provided cells for the 1st test, and the preparation being examined is required to pass the test with cells from 7 of the 8 different donors (i.e. a maximum of 1 positive reaction in 8 donors is allowed). When the source of monocytes consists of cells pooled from a number of individual donors, the preparation being examined is required to pass the test with 1 pool of cells. Where a human monocytic cell line is used for the test, the preparation being examined is required to pass the test with 1 passage of the cell line.

7-2. *METHOD B. SEMI-QUANTITATIVE TEST*

Method B involves a comparison of the preparation being examined with standard endotoxin. The contaminant concentration of the test preparation it to be less than the CLC to pass the test. Solution A must be chosen for the release decision, unless otherwise justified and authorised.

7-2-1. Test procedure

Using the validated test method, prepare the solutions shown in Table 2.6.30.-2 and culture 4 replicates of each solution with cells from each of 4 individual donors or a single pool or with cells from 1 passage of a human monocytic cell line.

Table 2.6.30.-2

Solution	Solution	Added endotoxin (IU/mL)	Number of replicates
A	Test solution/*f*	None	4
B	Test solution/f_1	None	4
C	Test solution/f_2	None	4
D	Test solution/*f*	Standard endotoxin at 2 × LOD for the test system	4
E	Test solution/f_1	Standard endotoxin at 2 × LOD for the test system	4
F	Test solution/f_2	Standard endotoxin at 2 × LOD for the test system	4
R_0	Pyrogen-free saline or test diluent	None (negative control)	4
R_1	Pyrogen-free saline or test diluent	Standard endotoxin at 0.5 × LOD for the test system	4
R_2	Pyrogen-free saline or test diluent	Standard endotoxin at 1 × LOD for the test system	4
R_3	Pyrogen-free saline or test diluent	Standard endotoxin at 2 × LOD for the test system	4
R_4	Pyrogen-free saline or test diluent	Standard endotoxin at 4 × LOD for the test system	4

Solution A = solution of the preparation being examined at the dilution, here designated *f*, at which the test for interfering factors was completed.

Solution B = solution of the preparation being examined at a dilution, here designated f_1, not exceeding the MVD, chosen after a review of data from the product-specific validation, e.g. 1:2 × MVD (i.e. a 2-fold dilution above the MVD).

Solution C = solution of the preparation being examined at a dilution, here designated f_2, not exceeding the MVD, chosen after a review of data from the product-specific validation, e.g. MVD.

Solution D = solution A spiked with standard endotoxin at 2 × LOD for the test system (as determined in preparatory testing).

Solution E = solution B spiked with standard endotoxin at 2 × LOD for the test system.

Solution F = solution C spiked with standard endotoxin at 2 × LOD for the test system.

Solution R_0 = negative control.

Solution R_1 = standard endotoxin at 0.5 × LOD for the test system.

Solution R_2 = standard endotoxin at 1 × LOD for the test system.

Solution R_3 = standard endotoxin at 2 × LOD for the test system.

Solution R_4 = standard endotoxin at 4 × LOD for the test system.

7-2-2. **Calculation and interpretation**

All data to be included in the data analysis are to relate to cells for which mean responses to solutions R_0-R_4 increase progressively. The mean response to R_0 may be equal to the mean response to R_1. For each different cell source, e.g. individual donation, donor pool, or cell line, the mean response to solution R_2 is to be greater than a positive cut-off value. Data below this cut-off value are considered negative. If the mean response to R_1 or R_2 exceeds the cut-off value, the response to the solution chosen for the pass/fail decision must be negative (= pass). For each negative solution of the test preparation (A, B and C), the mean response to the corresponding spiked solution (D, E or F respectively) is compared with the mean response to R_3 to determine the percentage spike recovery. The contaminant concentration of the preparation being examined is less than the CLC for a given cell source if the solution of the test preparation designated for the pass/fail-decision and the dilutions below all give negative results and the endotoxin spike recovery is within the range of 50-200 per cent.

7-2-3. **Pass/fail criteria of the preparation**

The criteria are the same as for method A (see 7-1-3).

7-3. *METHOD C: REFERENCE LOT COMPARISON TEST*

Method C involves a comparison of the preparation being examined with a validated reference lot of that preparation. The reference lot is selected according to criteria that have been justified and authorised. The test is intended to be performed in cases where a preparation being examined shows marked interference but cannot be diluted within the MVD to overcome the interference because it contains or is believed to contain non-endotoxin contaminants. Responses to non-endotoxin contaminants may dilute out more rapidly than responses to endotoxin, which makes it necessary to perform the test at a range of dilutions that include minimum dilution. The test procedure is described below and includes an example for the comparison of a test lot with the reference lot.

7-3-1. **Test procedure**

Using the validated test method, prepare the solutions shown in Table 2.6.30.-3 and culture 4 replicates of each solution with cells from each of 4 individual donors or a single pool or with cells from 1 passage of a human monocytic cell line.

Table 2.6.30.-3

Solution	Solution/dilution factor	Number of replicates
A	Solution of reference lot/f_1	4
B	Solution of reference lot/f_2	4
C	Solution of reference lot/f_3	4
D	Solution of test preparation/f_1	4
E	Solution of test preparation/f_2	4
F	Solution of test preparation/f_3	4
G	Positive control (standard endotoxin)	4
R_0	Diluent (negative control)	4

Solutions A, B and C are solutions of the reference lot diluted by the dilution factors, f_1, f_2 and f_3, determined in the test for interfering factors.

Solutions D, E and F are solutions of the preparation being examined diluted by the dilution factors, f_1, f_2 and f_3, determined for the reference lot in the test for interfering factors.

Solution G is the positive test control for the viability of the cells and is a standard endotoxin concentration that gives a clear positive response.

Solution R_0 is the diluent used to dilute the preparation being examined and serves as the test blank.

7-3-2. **Calculation and interpretation**

All data to be included in the data analysis are to relate to cells for which solution G and at least one of solutions A, B and C give a response that is greater than the basal release of the read-out (Solution R_0). For each different cell source, e.g. individual donation, donor pool, or cell line, use the standard curve for the read-out (a calibration curve in duplicate with a blank and at least 4 geometrically diluted concentrations of the standard for the chosen read-out) and calculate the mean responses of the replicates of solutions A-F. Sum the mean responses to solutions A, B and C and sum the mean responses to solutions D, E and F. Divide the sum of the mean responses to solutions D, E and F by the sum of the mean responses to solutions A, B and C. The preparation being examined complies with the test for a given cell source if the resulting value complies with a defined acceptance criterion not exceeding 2.5.

7-3-3. **Pass/fail criteria of the preparation**

The criteria are the same as for method A (see 7-1-3).

To quantify more closely the level of contamination, Methods A, B and C may be performed using other dilutions of the solution of the preparation being examined not exceeding the MVD.

The following section is published for information only.

Guidance notes

1. INTRODUCTION

The monocyte activation test (MAT) is primarily intended to be used as an alternative method to the rabbit pyrogen test. The MAT detects pyrogenic and pro-inflammatory contaminants, including endotoxins from gram-negative bacteria and 'non-endotoxin' contaminants, including pathogen-associated molecular patterns (PAMPs), derived from gram-positive and gram-negative bacteria, viruses and fungi, and product-related and process-related biological or chemical entities.

Since non-endotoxin contaminants are a physico-chemically diverse class of molecules, and usually the nature of the contaminant in a preparation being examined is unknown, the level of contamination is expressed either in endotoxin-equivalent units, derived by comparison with responses to standard endotoxin, or by comparison with a reference lot of the test preparation.

In the MAT, responses to standard endotoxin usually dilute out over approximatively 1 log and responses to products contaminated with non-endotoxin contaminants (alone or in combination with endotoxins) often show very steep dose-response curves, usually over only 1 or 2 dilution steps when tested for their capability to stimulate monocytes. Frequently, the largest response to such contaminated products is obtained with undiluted solutions of preparations being examined or small dilutions of the preparations being examined. For this reason test solutions of preparations being examined that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.

2. METHODS

2-1. *INFORMATION REGARDING THE CHOICE OF METHODS*

Methods A, B and C, are not normally applied where a test preparation has the intrinsic activity of stimulating the release of the chosen read-out or where the test preparation is contaminated with the chosen read-out. In both cases, this fact is to be addressed by modifying and validating the chosen method accordingly. The product-specific validation of the chosen method would be expected to identify the frequency

of non-responders to a particular product/contaminant(s) combination and to identify steps to address this, e.g. screening of donors, increasing the number of donors per test, and setting pass/fail criteria of appropriate stringency to maximise the likelihood of detecting contaminated batches. Methods A and B are appropriate when responses to dilutions of a preparation being examined are broadly parallel to responses to dilutions of standard endotoxin. Method B is a semi-quantitative test that can also be applied when responses to dilutions of a test preparation are not parallel to responses to dilutions of standard endotoxin.

Method C, the reference lot comparison test, was developed to address extreme donor variability in responses to certain product/contaminant(s) combinations. In this regard, it should be noted that, while monocytes from most donors respond in a broadly similar manner to bacterial endotoxin, responses of monocytes from different donors to non-endotoxin contaminants can differ markedly, so that it is possible to identify non-responders to certain product/contaminant(s) combinations.

2-2. *CALCULATION OF CONTAMINANT LIMIT CONCENTRATION*

The acceptance criterion for a pass/fail decision is the contaminant limit concentration (CLC), which is expressed in endotoxin equivalents per milligram or millilitre or in units of biological activity of the preparation being examined. Where an endotoxin limit concentration (ELC) has been specified for a product, the CLC is the same as the ELC, unless otherwise prescribed. The CLC is expressed in terms of endotoxin equivalents. The CLC is calculated using the following expression:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass;

M = maximum recommended bolus dose of product per kilogram of body mass.

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.

The endotoxin limits depends on the product and its route of administration and is stated in monographs.

Values for K are suggested in Table 2.6.30.-4.

Table 2.6.30.-4

Route of administration	K (IU of endotoxin per kilogram of body mass)
Intravenous	5.0
Intravenous, for radiopharmaceuticals	2.5
Intrathecal	0.2

For other routes, the acceptance criterion for bacterial endotoxins is generally determined on the basis of results obtained during the development of the preparation.

2-3. *INFORMATION REGARDING CRYO-PROTECTANTS*

The influence of cryo-protectants, e.g. dimethyl sulfoxide (DMSO), and their residues in thawed cells, is to be considered: DMSO is toxic to cells in culture and, even when cells have been washed thoroughly, cryo-preservation may have altered cell properties, e.g. cell membrane permeability.

2-4. *INTERFERENCE TESTING*

Where practicable, interference testing is performed on at least 3 different lots of the preparation being examined. Preparations being examined that show marked batch-to-batch variation, that effectively renders each batch unique for the purposes of interference testing, are to be subjected to interference testing within each individual test, i.e. concomitant validation.

Interference testing is preferably performed on batches of the preparation being examined that are free of endotoxins and other pyrogenic/pro-inflammatory contaminants and, where this is not practicable, none of the batches are to be heavily contaminated. If only 1 batch is available the validation has to be performed on that batch in 3 independent tests. Precision parameters for reproducibility, e.g. ± 50 per cent, are to be fulfilled.

3. REPLACEMENT OF THE RABBIT PYROGEN TEST BY THE MONOCYTE ACTIVATION TEST

As noted above, the monocyte activation test (MAT) is primarily intended to be used as an alternative method to the rabbit pyrogen test. Monographs on pharmaceutical products intended for parenteral administration that may contain pyrogenic contaminants require either a test for bacterial endotoxins or a monocyte activation test.

As a general policy:

- in any individual monograph, when a test is required, only 1 test is included, either that for bacterial endotoxins or the MAT. Before including the MAT in a monograph, evidence is required that 1 of the 3 methods described in the MAT chapter can be applied satisfactorily to the product in question;
- the necessary information is sought from manufacturers. Companies are invited to provide any validation data that they have concerning the applicability of the MAT to the substances and formulations of interest. Such data include details of sample preparation and of any procedures necessary to eliminate interfering factors. In addition, any available parallel data for rabbit pyrogen testing that would contribute to an assurance that the replacement of a rabbit pyrogen test by the MAT is appropriate, is to be provided.

4. VALIDATION OF ALTERNATIVE METHODS

Replacement of a rabbit pyrogen test by a MAT, or replacement of a method for detecting pro-inflammatory/pyrogenic contaminants by another method, is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices:

'The test and assays described are the official methods upon which the standards of the European Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the European Pharmacopoeia are alone authoritative.'

The following procedures are suggested for validating a method for the MAT other than the one indicated in the monograph:

- the procedure and the materials and reagents used in the method should be validated as described for the test concerned;
- the presence of interfering factors (and, if needed, the procedure for removing them) should be tested on samples of at least 3 production batches.

MAT should be applied to all new products intended for parenteral administration that have to be tested for the presence of monocyte-activating contaminants according to the requirements of the European Pharmacopoeia.

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2.6.31. MICROBIOLOGICAL EXAMINATION OF HERBAL MEDICINAL PRODUCTS FOR ORAL USE

Total aerobic microbial count (TAMC). Perform the count as described in general chapter *2.6.12.*