Human Whole Blood/Interleukin (IL)-1 Beta In Vitro Pyrogen Test (WBT)
DB-ALM Protocol n° 133

**Objective and Applications**

**LEVEL OF ASSESSMENT**: Hazard identification

**PURPOSE OF TESTING**: Safety

**Context of Use:**
Regulatory purpose: the test method shall be used for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of pyrogenic contaminations (ESAC, 2006), in materials which are usually evaluated and characterised by rabbit pyrogen tests as indicated by the 2.6.30 Monocyte-activation test of the European Pharmacopoeia (EDQM, 2009) and US Food and Drug Administration (FDA, 2009).

**Applicability Domain:**

*Pharmaceuticals*: parenteral medications (aqueous liquid samples).

The test method has the capacity of detecting pyrogenicity produced by a wide range of potential pyrogens, such as lipoteichoic acid, lipopeptides and peptidoglycan from Gram-positive bacteria, exotoxins, enterotoxins, viruses and fungal components. However, for detection of non-endotoxin monocyte-activating contaminants, the method should be specifically validated as determined by the 2.6.30 Monocyte-activation test of the European Pharmacopoeia (EDQM, 2009) and US Food and Drug Administration (FDA, 2009).

**Rationale**

Pyrogenic contaminations can activate the release of endogenous mediators, such as pro-inflammatory cytokines that have a role in fever pathogenesis (EDQM, 2009).

The Human Whole Blood/Interleukin (IL)-1 beta In Vitro Pyrogen Test (WBT) was developed by Hartung et al. (1995, 2001) as an alternative to the rabbit pyrogen and the *Limulus* amoebocyte lysate tests. In this method, human immune cells are used to detect exogenous pyrogens by measuring the release of IL-1 beta.

No isolation procedures, preactivation or cell culture of the immune cells are required. The source of monocytes is human whole blood where immune cells and serum components are present in their natural composition (Daneshian et al., 2009).

**Experimental Description**

**Endpoint and Endpoint Measurement:**

INTERLEUKIN(IL)-1 BETA RELEASE: release of IL-1 beta by human whole blood monocytes in response to exposure to pyrogenic stimulation measured by enzyme-linked immunosorbent assay (ELISA).

**Endpoint Value:**

EU: Endotoxin units (relative)

**Experimental System(s):**

HUMAN WHOLE BLOOD: human whole blood containing monocytes
Basic procedure
Whole fresh blood is incubated for 10-24 h at 37°C with the test samples, endotoxin-spiked test samples, endotoxin controls and negative controls. The blood monocytes produce pro-inflammatory cytokines in response to any pyrogen present in a concentration-dependent manner and the quantity of produced IL-1 beta is measured by enzyme-linked immunosorbent assay (ELISA).

The ELISA plate is coated with the primary antibody. The IL-1 beta in the sample is sandwiched between the primary coat antibody and a secondary horseradish peroxidase-labeled detection antibody. The unbound material is removed by washing. The peroxidase metabolises the substrate 3,3',5,5'-tetramethylbenzidine (TMB), giving a soluble blue reaction product. The reaction is stopped with hydrochloric or sulphuric acid, forming a yellow reaction product, and the optical density (OD) is measured at 450 nm against a reference wavelength of 600 to 690 nm. No IL-1 beta reference material is required to quantify IL-1 beta protein as the ELISA OD readout is compared with that of the endotoxin controls (Daneshian et al., 2009).

Data Analysis/Prediction Model
During the “Human(e) Pyrogen Test” project (2000-2003), the following prediction model has been used. Test samples are considered to be positive for pyrogenic contaminations if the test sample-induced IL-1 beta release is higher than the positive control sample (containing 0.5 Endotoxin Units (EU)/ml)-induced IL-1 beta release. The endotoxin control at a concentration of 0.5 EU/ml corresponds to 50 pg/ml of the WHO international reference standard from E.coli O113:H10 and is the threshold endotoxin concentration that causes fever in 50% of animals of the most sensitive rabbit strains (Daneshian et al., 2009).

In the 2.6.30 Monocyte-activation test of the European Pharmacopoeia, a test substance is considered pyrogenic when the endotoxin concentration of the test substance (found from endotoxin calibration curve) exceeds the Endotoxin Limit Concentration (ELC) for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (EDQM, 2009).

Modifications of the Method
The original method was developed for the detection of pyrogens in aqueous liquid samples such as parenteral medications (Hartung et al., 1995, 2001). The adaptations of this protocol allows to assess lipiddic, toxic or immunomodulatory substances, to detect low-grade contaminations in large volume parenterals, to assess the pyrogenicity of solid materials, e.g. medical devices, and to evaluate airborne pyrogenic burden (Daneshian et al., 2009).

It is recommended by Daneshian et al. (2009) to dilute whole blood in clinical-grade saline (150 mM) to a final dilution of 1:12, which results in a high release of cytokines using a minimal amount of blood. The cytokine inhibitor alpha-1-antitrypsin is no longer active at this dilution (Pott et al., 2009), therefore the cytokine release is higher and thus more robustly detectable in diluted samples (Daneshian et al., 2009).

Discussion
The rabbit pyrogen test has been traditionally used to evaluate pyrogenic contamination of parenterals (Welch et al., 1943; McClosky et al., 1943). However, the essential limitations of this test are that animal handling could give false-positive or negative results (Grant, 1950), the sensitivity to pyrogens in humans differ from that in rabbits, and a range of modern pharmaceutical products (e.g., biologicals, cancer drugs and immunomodulatory drugs) could not be tested due to interference effects (Hartung et al., 2001).

Another test to assess the pyrogenicity – the Limulus amoebocyte lysate test, where the blood of horseshoe crab Limulus polyphemus is used, could detect only lipopolysaccharides but not their different potencies from different bacterial species observed in humans (Fennrich et al., 1999; Dehus et al., 2006). It also could give false-positive results in the presence of glucans and a number of herbal preparations (Hartung et al., 2001).

In comparison with these tests, the human whole blood IL-1 beta in vitro pyrogen test does not
require components of animal origin. It also can detect non-lipopolysaccharide pyrogens. This test requires no cell preparation steps or cell culture facilities and is standardised and easy to perform (Daneshian et al., 2009).

**Status**

**Participation in Validation Studies:**
The herewith presented Standard Operating Procedure of the Human Whole Blood IL-1 Beta In Vitro Pyrogen Test has been developed as an outcome of the “Human(e) Pyrogen Test” project (2000-2003), sponsored by the 5th Framework Programme of the European Commission. The overall aim of this project was to develop, evaluate and validate a method based on the human fever reaction to replace the rabbit pyrogen and the Limulus amoebocyte lysate tests.

In 2006, ESAC endorsed the scientific validity of the Human Whole Blood IL-1 beta In Vitro Pyrogen Test for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of pyrogenic contaminations in materials usually evaluated and characterized by rabbit pyrogen test (ESAC, 2006).

**Regulatory Acceptance:**
In 2009, the 2.6.30 Monocyte-activation test that covers the Human Whole Blood IL-1 beta In Vitro Pyrogen Test, has been included in the European Pharmacopoeia as a replacement for the rabbit pyrogen test (EDQM, 2009).

**Proprietary and/or Confidentiality Issues**
None

**Abbreviations and Definitions**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ECVAM</td>
<td>European Centre for the Validation of Alternative Methods</td>
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<td>EDQM</td>
<td>European Pharmacopoeia</td>
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<td>ELC</td>
<td>Endotoxin Limit Concentration</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ESAC</td>
<td>ECVAM Scientific Advisory Committee</td>
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<td>EU</td>
<td>Endotoxin Units</td>
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<td>FDA</td>
<td>US Food and Drug Administration</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MVD</td>
<td>Maximum Valid Dilution</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PFS</td>
<td>pyrogen-free saline</td>
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<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine (TMB)</td>
</tr>
<tr>
<td>WBT</td>
<td>the Human Whole Blood/Interleukin (IL)-1 Beta In Vitro Pyrogen Test</td>
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*Last update: 7 June 2011*
PROCEDURE DETAILS, 7 June 2011

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DB-ALM Protocol n° 133

Note: This protocol represents the standard operating procedure compliant with the 2.6.30 Monocyte-activation test of the European Pharmacopoeia.

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Health and Safety Issues

General Precautions

All procedures that use human blood-derived materials should follow national/international procedures for handling blood potentially contaminated with pathogens.

For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes. If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

Materials and Preparations
Cell or Test System

Human whole blood containing monocytes

Equipment

Fixed Equipment
- Adjustable 20 to 100 µl pipetters
- Centrifuge (recommended)
- Incubator or thermoblock (37°C ± 1°C)
- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 600-690 nm)
- Microplate washer
- Multichannel pipetter (8- or 12-channel)
- Software package for facilitating ELISA data generation, analysis, reporting, and quality control
- Vortex mixer

Consumables
- Combitips; repeating pipetter (1.0 and 2.5 ml)
- Flat-bottom tissue culture plates, 96-well (e.g. TC-plate, Greiner Bio-one, cat. No. 655 180)
- Pyrogen-free borosilicate test tubes (e.g., KairoSafe, cat. No. 1959000)
- Pyrogen-free reaction tubes, 1.5 ml sealable (e.g., Eppendorf, cat. No. 0030 102.002)
- Pyrogen-free reservoirs (e.g., 50 ml Falcon, Greiner Bio-one, cat. No. 210261)
- Sterile and pyrogen-free pipette tips (e.g., Biosphere Tips blue, Sarstedt., cat. Nos. 70.760.202 and 70.762.200)
- Sterile needle set (e.g., Multifly Set 21G tubing 8", Starstedt, cat. No. 85.1638.035)
- Sterile, endotoxin-free, heparinised tubes for blood sampling (e.g., Starstedt S-Monovette 7.5 ml, LH, Sarstedt, cat. No. 01.1608.001)

Media, Reagents, Sera, others

An ELISA that measures IL-1 \( \beta \) release is used. The IL-1 \( \beta \) ELISA should be calibrated using an IL-1 \( \beta \) international reference standard.

ELISA kit components:

<table>
<thead>
<tr>
<th>Item</th>
<th>Description/use</th>
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<tbody>
<tr>
<td>ELISA plates coated with anti-human IL-1 ( \beta ) capture antibody; monoclonal or polyclonal</td>
<td>96-well polystyrene microplates, consisting of twelve strips mounted in a frame. Each strip includes eight anti-human IL-1 ( \beta ) Ab-coated wells.</td>
</tr>
<tr>
<td>Enzyme-labeled detection antibody</td>
<td>A vial containing 16 ml of liquid reagent, ready-to-use. The reagent contains horseradish peroxidase-labeled, affinity-purifies, polyclonal (rabbit) anti-IL-1 ( \beta ) antibodies, with preservative.</td>
</tr>
<tr>
<td>Pyrogen-free saline</td>
<td>Glass vials, containing pyrogen-free saline. This is intended for the dilution of donor blood samples and for reconstitution of the Endotoxin control.</td>
</tr>
<tr>
<td>TMB/Substrate solution</td>
<td>Vials, each containing 11 ml of a buffered reagent, ready-to-use. The reagent contains a hydrogen peroxide substrate and 3,3',5,5'-tetramethylbenzidine (TMB) (store refrigerated and protected from light, stable at 2-8°C, do not freeze).</td>
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Buffered Wash Solution

A vial, containing 75 ml of a concentrated (10x) buffered saline solution, with surfactants and preservative. Using a transfer container, dilute the contents of the vial with 675 ml distilled or deionised water for a total volume of 750 ml. Store refrigerated: stable at 2-8°C for 30 days after preparation. For longer storage aliquot and freeze: stable at -20°C for 6 months.

Stop Solution

One vial containing an acidic solution, for terminating the color reaction. The reagent is supplied ready-to use. Store refrigerated: stable at 2-8°C for 8 weeks after opening.

Endotoxin control: the international World Health Organisation (WHO) reference standard from *E.coli* O113:H10 (e.g., National Institute for Biological Standards and Control, cat.no. 94/580), or an LPS that has been calibrated against the WHO reference standard (e.g., CSE, Charles River Laboratories International, cat. No. E110).

Preparations

*Media and Endpoint Assay Solutions*

See MEDIA, REAGENTS, SERA, OTHERS sector. All test substances, endotoxin and endotoxin-spiked solutions should be stored as specified in the manufacturer’s instructions.

*Test Compounds*

Liquid test substances should be diluted in pyrogen-free saline (PFS). Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay.

To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD):

\[ \text{MVD} = \frac{\text{ELC} \times \text{PP}}{\lambda} \]

where ELC – endotoxin limit concentration

PP – Product Potency – test sample concentration expressed as mg/ml or ml/ml

\( \lambda \) – assay sensitivity (in EU/ml)

The calculation of the MVD is dependent on the Endotoxin Limit Concentration (ELC) for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period.

For example, for Cyclophosphamide injection, the ELC is 0.17 EU/mg, PP is 20 mg/ml, and the assay sensitivity is 0.065 EU/ml. The calculated MVD would be 1:52. The test substance can be diluted no more than 1:52 prior to testing.

Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

*Positive Control(s)*

Endotoxin controls (0.5 EU/ml) in quadruplicate are used as positive control.

For semi-quantitative assessment endotoxin calibration curve (e.g. 0.25, 0.5, 1.0, 2.5 and 5.0 EU/ml) is used.

For positive control prepare a 2000 EU/ml stock solution of endotoxin using pyrogen-free saline. Aliquots can be stored at -20°C for up to 6 months. Vortex
solutions and all dilutions made from these for at least 60 s directly before use.

Negative Control(s)

Pyrogen-free saline should be included in the test in quadruplicate as negative control.

Method

Test System Procurement

Monocytes from the whole blood are the source of cytokine production in this test. Blood donors have to be in good health, not to be suffering from any bacterial or viral infections and have to be 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 any non-steroidal anti-inflammatory drugs during the 48 h prior to donating blood and any steroidal anti-inflammatory drugs during the 7 days prior to donating blood. Donors could not use immunosuppressant or other drugs known to influence the production of the cytokines. Blood donation has to be tested for infection markers according to national requirements for transfusion medicine (EDQM, 2009).

Blood collection

- Collect blood by venipuncture into heparinised tubes. The blood collection system must be pyrogen-free.
- The blood can be stored in the collection tube at room temperature (15 - 28°C) for 4 hours. Incubation of the sample should be started within this time.
- Prior to use in the assay, an equal volume of whole blood from multiple individual donors (at least 4, recommended 8) should be pooled.

Routine Culture Procedure;

Preparation for the assay

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether a test substance has an effect on cytokine release.

Interference test

- Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an in vitro pyrogen test is performed.
- A fixed concentration of the endotoxin control (i.e., 1.0 EU/ml or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD).
- For non-spiked solutions, 200 µL of pyrogen-free saline (PFS) is added to a well followed by 20 µL of the test substance and 20 µL of whole blood.
- Endotoxin-spiked solutions are prepared by adding 180 µL of PFS to each well followed by 20 µL of the test substance, and 20 µL of whole blood. Then, 20 µL of an endotoxin-spiked solution (1.0 EU/mL) is added to each well.
- The contents of the wells are mixed and incubated as described in TEST MATERIAL EXPOSURE PROCEDURES sector.
- An ELISA is then performed as described in ENDPOINT MEASUREMENT sector.
- The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve.
- The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution.
- The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%.
- If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated.
- The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined.
- The test substance is then diluted in serial two-fold dilutions beginning at this dilution,
not to exceed the MVD, for use in the assay.
• If the data obtained from the interference test suggests the presence of interference at the MVD, then another validated pyrogen test method should be used.

Test Material Exposure Procedures

Calculate the number of samples to be assayed. Include endotoxin controls (e.g., 0.25, 0.5, 1.0, 2.5 and 5.0 EU/ml), a negative saline control, spiked samples (if required) and samples. The endotoxin concentration 0.5 EU/ml must be included to allow qualitative assessment of pyrogenic contamination in the sample. All controls and samples should be tested in quadruplicate. Diluted samples should be tested within an hour.

The whole blood incubation may be carried out in 1.5 ml sealable, pyrogen-free reaction tubes in a total volume of 1200 µl or 96-well flat-bottom tissue culture plates in a total volume of 240 µl.

Whole Blood Stimulation – test tube method

• Add 1000 µl saline into each reaction tube.
• Add 100 µl of sample or spiked sample or control (saline or endotoxin) into the prepared reaction tubes.
• Add 100 µl of donor blood, mixed by gentle inversion, into each reaction tube.
• Close the tubes and invert them once or twice before starting the incubation.
• Incubate the closed reaction tubes in an incubator or a heating block overnight (10-24 hours) at 37°C ± 1°C.
• Mix the incubation tubes thoroughly by inverting them. The tubes are to be centrifuged for 2 min at 10,000 g and the clear supernatant is used for the ELISA procedure.
• Take aliquots of ≥ 150 ml.
• The supernatants can be tested immediately by the ELISA System or may be stored at -20°C for testing at a later time.
• Freeze additional aliquots.

Whole Blood Stimulation – microtiter plate method

• Using a non-pyrogenic tissue culture plate, draw up an incubation plan designating the layout of endotoxin controls, negative saline controls and samples in the assay.
• Pipet 200 µl pyrogen-free saline into each of the reaction wells that will be used for the assay.
• Add 20 µl of endotoxin controls, negative saline control or samples into their respective reaction wells according to the prepared incubation plan.
• Add 20 µl of whole blood to all reaction wells.
• Cover with the dedicated plastic plate cover and mix thoroughly on a microtiter plate mixer.
• Transfer the mixed microtiter plate to a 37°C incubator for an overnight incubation (10 to 24 hours).
• Following the overnight incubation, remove plate from incubator place onto a plate mixer. Mix until all blood cells have been re-suspended.
• The re-suspended blood mixtures may be ELISA tested for IL-1 beta immediately or stored frozen at -20°C for testing at a later time (at least 150 µl).

Endpoint Measurement

All components must be at room temperature (15-28°C) before use. The ELISA is carried out at room temperature.

• Add 100 µl Enzyme-Labeled Detection Antibody to every well.
• Pipet 100 µl of supernatants of Endotoxin Controls, those of the negative (saline) control and of the samples into the wells prepared. Use a disposable-tip micropipet for the samples, changing the tip between samples, to avoid contaminations.
• Cover the plate and mix for 90 minutes on a microplate mixer at 350-400 rpm.
• Decant, then wash. For assays using centrifuged blood supernatants, wash each well 4 times with 300 µl Buffered Wash Solution. For assays using resuspended blood, wash 5 to 6 times with 300 µl per well. If this step is performed manually, remove as much moisture as possible during the decanting; this will greatly enhance precision.
• Before adding the TMB/Substrate solution, tap the plate face down on adsorbant paper to shake off all residual droplets, being careful not to dislodge the strips from the frame.
Add 200 µl of TMB/Substrate Solution to every well. Incubate without shaking for 15-30 minutes in the dark at room temperature.

Add 50 µl of Stop Solution to every well. Tapping the plate gently after the addition of Stop Solution will aid mixing and improve precision. The Stop Solution is acidic. Handle carefully, and use safety gloves and eye protection.

Read the OD at 450 nm, within 15 minutes of adding the Stop Solution. Measurement with a reference wavelength of 600-690 nm is recommended.

**Acceptance Criteria**

The assay should be considered acceptable only if the following minimum criteria are met:

The mean optical density of the 0.5 EU/ml endotoxin control exhibits an OD that is greater than 1.6 times the mean optical density of the negative saline control.

**Data Analysis**

Calculate the mean and standard deviation of the OD of the replicate values of all controls and samples. Use the nonparametric Dixon’s test or Grubb’s test for normally distributed samples to detect and exclude possible outliers among the replicates.

**Prediction Model**

The following prediction model was developed during the “Human(e) Pyrogen Test” project (2000-2003):

Rabbits are likely to develop fever if tested with 10ml/kg of the sample if mean OD (sample) > mean OD (endotoxin control 0.5 EU/ml). The endotoxin control at a concentration of 0.5 EU/ml corresponds to 50 pg/ml of the WHO international reference standard from *E.coli* O113:H10 and is the threshold endotoxin concentration that causes fever in 50% of animals of the most sensitive rabbit strains (Daneshian *et al.*, 2009).

In the 2.6.30 Monocyte-activation test of the European Pharmacopoeia, a test substance is considered pyrogenic when the endotoxin concentration of the test substance (found from endotoxin calibration curve) exceeds the Endotoxin Limit Concentration (ELC) for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (EDQM, 2009).

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