Validation of Biomedical Testing Methods

In vitro Pyrogen Test Using Freshly Taken or Cryopreserved PBMC

(SP+PB var. Novartis)

Standard Operating Procedure

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Standard Operating Procedure

In vitro Pyrogen Test Using Freshly Taken or Cryopreserved PBMC (SP+PB var. Novartis)

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1 INTRODUCTION

Parenteral pharmaceutical products must be shown to be free from pyrogenic (fever-inducing) contamination. While a pyrogen may in general be defined as any substance that causes fever, the pyrogens that almost invariably contaminate parenteral pharmaceuticals are bacterial endotoxins (lipopolysaccharides, LPS) from Gram-negative bacteria (Mascoli and Weary, 1979a, 1979b). There are two Pharmacopoeial tests for pyrogenic contamination: the rabbit pyrogen test and the Limulus amoebocyte lysate (LAL) test. The rabbit pyrogen test, which detects LPS and other pyrogens, involves measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined. In contrast, the LAL test detects only LPS: it is described in Pharmacopoeias as the bacterial endotoxins test (BET). The principle of the LAL-test is that LPS causes extracellular coagulation of the blood (haemolymph) of the horseshoe crab, *Limulus polyphemus.* (Levin & Bang, 1964). Although the LAL test is gradually superseding the rabbit pyrogen test, hundreds of thousands of rabbit pyrogen tests are still carried out each year around the world, largely on products which cannot, for one reason or another, be tested in the LAL test. While proving generally reliable, both the rabbit pyrogen test and LAL test have shortcomings. The rabbit pyrogen test uses experimental animals, is costly and is not quantitative. The LAL test gives false negatives with certain products, can overestimate the pyrogen content of other products and does not detect pyrogens other than bacterial endotoxin (LPS), such as Gram-positive exotoxins, viruses and fungi (Dinarello et al., 1984; Poole et al., 1988; Ray et al., 1990; Taktak et al., 1991; Fennrich et al., 1999).
The basis of the rabbit pyrogen test is the in vivo stimulation by exogenous pyrogens (usually LPS) of rabbit peripheral blood monocytes to produce the endogenous pyrogens that cause fever. The endogenous pyrogens are pyrogenic cytokines such as tumour necrosis factorα (TNFα), interleukin-1 (IL-1α and IL-1β, two separate gene products), IL-6 and IL-8 (Dinarello et al., 1999). In view of the shortcomings of the rabbit pyrogen test and the LAL test, in vitro pyrogen tests that utilise the exquisite sensitivity to exogenous pyrogen of monocytes have been proposed. In such tests, products are incubated with human peripheral blood monocytes (or mononuclear cells, PBMC, or leukocytes) and the conditioned media assayed for pyrogenic cytokines (Duff & Atkins, 1982; Dinarello et al., 1984; Poole et al., 1988; Poole, 1989; Hansen and Christensen, 1990; Taktak et al., 1991; Bleeker et al., 1994).

2 PURPOSE

By quantifying the amounts of cytokines released by PBMC stimulated with the USP reference preparation for endotoxin or the international standard (IS) for endotoxin (LPS), various non-endotoxin pyrogens and with medicinal products spiked with endotoxin, it is the objective that an in vitro pyrogen test be developed that will serve as a replacement for the rabbit pyrogen test.

3 SCOPE / LIMITATIONS

The method described below is for the evaluation of in vitro PBMC/cytokine release tests using IL-6 as the cytokine that serves as the readout (measured variable). It is not a 'finalised' test system for the testing of medicinal products. Especially the prediction model has to be developed cautiously.

The method may be applied only to preparations that have been validated with the method, i.e. shown not to interfere by causing inhibition or enhancement of LPS (STD)-induced cytokine production.

4 METHOD OUTLINE

Freshly taken human whole blood is heparinised, diluted with phosphate buffered saline (PBS) and the PBMC's isolated. For storage and shipping purposes PBMCs may be cryopreserved. Freshly taken or thawed PBMCs are then stimulated for 16-24h with the USP reference preparation for endotoxin, the international standard for endotoxin, (LPS) or samples of related materials, e.g. other endotoxins, non-endotoxin pyrogens and medicinal products unspiked and spiked with endotoxin. Following this stimulation, the concentrations of the cytokine in the PBMC-conditioned medium are quantified using a specific ELISA for IL-6 (which is calibrated in terms of the appropriate international standard). The construction of dose-response curves for endotoxin (LPS) versus concentrations of released cytokines permits the estimation of the endotoxic/cytokine-releasing activity contained in the samples.

5 DEFINITIONS / ABBREVIATIONS

Ab	Antibody
BSA	Bovine serum albumin
CO_2	Carbon dioxide
°C	Degrees Celsius (Centigrade)
DMSO	Dimethylsulfoxide
D-R	Dose-response
E. coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
EP	European Pharmacopoeia
EU	Endotoxin units
FDA	Food and Drug Administration (USA)
g	Gram
h	Hour
HIFCS	Heat-inactivated (+56°C for 30 min) foetal calf serum
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
IL	Interleukin
IS	International standard
IU	International unit
l	Litre
KOH	Potassium hydroxide
LAL	Limulus amoebocyte lysate
LPS	Lipopolysaccharide
M	Molar
MAb	Monoclonal antibody
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
µg	Microgram
µl	Microlitre
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$NaHCO_3$	Sodium hydrogen carbonate
NaH_2PO_4	Sodium di-hydrogen phosphate
Na_2HPO_4	di-Sodium hydrogen orthophosphate
nm	Nanometre
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Dulbecco's Phosphate Buffered Saline
PF	Pyrogen-free (items purchased as sterile and pyrogen-free or baked at 250° for 30-60 min.
POD	Horseradish peroxidase conjugate
R	Endotoxin standard
rpm	Rounds per minute
RPMI	RPMI 1640 cell culture medium
RPMI-C	RPMI 1640 cell culture medium + human AB serum at a final concentration of 2% v/v
RSE	Reference Standard Endotoxin
RT	Room temperature
TMB	Tetramethyl benzidine
S	Test sample
STD	Standard

UNK	Unknown
USP	United States Pharmacopoeia
x g	x gravity

6 MATERIALS

Sterile, pyrogen-free Dulbecco's phosphate buffered saline w/o Ca^{++} and Mg^{++} (Life Technologies)
Polyoxyethylene-sorbitan monolaurate (TWEEN 20), cell culture grade, (Sigma, P-2287)
Hydrochloric acid, 0.1M, sterile filtered (Sigma, H-9892)
Sodium hydroxide (reagent grade)
1M H_2SO_4 (Merck)
Mouse monoclonal anti-IL-6 antibody from clone 16
Horseradish peroxydase conjugated sheep polyclonal anti-IL-6 antibody
3,3',5,5'-Tetramethyl benzidine (e.g. Fluka Cat. No. 87748)
Acetone (reagent grade)
Ethanol (reagent grade)
Phenole (e.g. Merck Cat. No. 100206)
Potassium hydroxide (reagent grade)
Sodium dihydrogen phosphate (e.g. Merck Cat. No. 106346)
Disodium hydrogen phosphate (e.g. Merck Cat. No. 106580)
Tris (hydroxymethyl) aminomethane (e.g. Fluka Cat. No. 93352)
Kathon MW/WT, Christ Chemie AG, Reinach, Switzerland
Albumin from bovine serum (e.g. Fluka Cat. No. 05480)
Citric acid monohydrate e.g. Fluka Cat. No. 27490)
Human AB serum (Sigma)
Trypan blue stain (Sigma)
USP Reference Standard Endotoxin [EC6 lot G], identical to the WHO international standard for bacterial endotoxin (LPS, vial code 94/580)
Fragmin (Dalteparin, 10000 IU/ml, Pharmacia)
RPMI 1640 medium (Life Technologies™, Paisley, Scotland)
L-Glutamine 200mM (Life Technologies™, Paisley, Scotland)
Penicillin/Streptomycin solution (Seromed Cat. No. A2213)
Lymphoprep (Nycomed, Oslo, Norway)
Dimethylsulfoxide, LAL tested, to be free of detectable endotoxins.
Nunc-Immuno 96-well plate MaxiSorp (F96, Life Technologies™, Paisley, Scotland)
Falcon Microtest tissue culture plate, 96-well (353072, Beckton Dickinson Labware)
Falcon serological pipettes (5ml,10ml, 25ml, Beckton Dickinson Labware)
Centrifuge tubes (Falcon 2070 Blue Max™)
Polypropylene conical tubes (Falcon 2069 Blue Max™)
Eppendorf Biopur Tips 100ul & 1000ul (Eppendorf-Netheler-Hinz-Gmbh,Germany)
0.22 μm sterile filters (MilliPak 60, Millipore)
Eppendorf® volumetric pipettes
Pyrogen-free reservoir liner, 12-well (PMP-380-507L, Fisher, UK)

All other consumables are purchased as sterile and pyrogen-free and other reagents are pro analysis grade.

6.1 Buffers and Reagents for Novartis IL-6 ELISA:

Coating Buffer

Dissolve
5.0 g of sodium dihydrogen phosphate, (e.g. Merck Art. No. 106346), and
2.9 g of disodium hydrogen phosphate, (e.g.Merck Art. No. 106580),
in 400 ml of distilled water.

Use 1 N NaOH to adjust the pH to 7.5, and make up to 500 ml with distilled water.

Remains stable for 6 months at 2 -8°C.

Blocking Buffer

Tris(hydroxymethyl)aminomethane, (e.g.Fluka Art. No. 93352)	12.1 g
Dissolve in distilled water	400 ml
Kathon MW/WT, Christ Chemie AG, Switzerland	0.1 ml

Use 4 M HCl to adjust the pH to 7.5.

Albumin from bovine serum, (e.g.Fluka Art. No. 05480)	5.0 g
Add distilled water to make up to 500 ml.	

Remains stable for 6 months at 2 - 8°C.

Stopping Solution

Distilled water	500 ml
H_2SO_4	26.6 ml

Wash Solution

Demineralised water	2000 ml
Tween-20	1 ml

Interleukin-6 Standard

Use IL-6 from human lymphocytes (e.g. Boehringer Mannheim, Cat. No. 1299972). 1 vial contains 200 000 units of natural human IL-6.
Dilute the contents of a vial (1 ml) of human IL-6 with 4 ml of RPMI-C, and freeze aliquots of 100 µl at about – 80 °C.
Before the first use of a new batch, this IL-6 used as the assay calibrant has to be calibrated against the IS for IL-6 (89/548).

Mouse Anti-Human IL-6 Monoclonal Antibody

(*Reagent 1)*
derived from clone 16.

Detection Antibody (POD)

(Reagent 2)
Sheep anti-human IL-6 antibody, horseradish peroxidase conjugated. Remains stable for at least 6 months at 2-8°C.

Human IL-6 Standard Solution

(Reagent 3)
Thaw 1 frozen aliquot of interleukin-6 standard (4000 units in 100 µl) and dilute with 900 µl of RPMI-C.
Add 100 µl of this dilution to 900 µl of RPMI-C = 400 units/ml (standard initial concentration, equivalent to 4000 pg/ml).
Prepare the solution shortly before use; do not store.

Dilution Buffer

(*Reagent 4)*
Prepare the dilution buffer as follows:

Tris(hydroxymethyl)aminomethane, (e.g.Fluka Art. No. 93352)	2.1 g
Distilled water	400 ml
Kathon MW/WT, Christ AG, Switzerland	0.1 ml
Phenol, (e.g.Merck Art. No. 100206)	0.5 g
Heat-inactivated (30 minutes/56 °C) fetal bovine serum	25 ml

Mix to dissolve the substances, then adjust the pH to 7.5 with 4 M HCl. Make up to 500 ml with distilled water.

Remains stable for at least 6 months at 2 - 8 °C.
In the absence of the stabilizers Kathon and phenol the stability is only 1 day.

TMB Solution

(Reagent 5)

Prepare the TMB solution as follows:

3,3',5,5'Tetramethylbenzidine (e.g.Fluka Art. No. 87748)	240 mg
Reagent-grade acetone	5 ml

Dissolve, then add

Reagent-grade ethanol	45 ml
Perhydrol (30 % H_2O_2), (e.g.Merck Art. No. 107209)	0.300 ml

Remains stable for at least 6 months at 15 - 25 °C when sealed and protected from light.

Substrate Buffer

(Reagent 6)

Reagent-grade citric acid monohydrate (e.g.Fluka Art. No. 27490)	6.3 g
Distilled water	800 ml

Mix to dissolve, then adjust the pH to 4.1 by adding 4 M KOH.
Make up to 1000 ml with distilled water and add 0.2 ml of Kathon MW/WT

Remains stable for about 6 months at 15 - 25 °C.
In the absence of the Kathon the stability is only 1 day.

Culture Medium (RPMI-C)

The preparation of the medium has to be carried out in a class 2 laminar flow sterile cabinet, using aseptic technique and reagents and consumables that are sterile and pyrogen-free.

RPMI 1640 medium	500 ml
Human serum AB	5 ml
L-Glutamine, 200 mM	5 ml
Penicillin/Streptomycin solution	10 ml

7 METHODS

Steps marked (*) are carried out in a class 2 laminar flow sterile cabinet, using aseptic technique and reagents and consumables that are sterile and pyrogen-free.

7.1 Coating of IL-6 ELISA plates

Dilute the coating anti-IL-6 antibody (Clone 16) with coating buffer to 2.5 µg/ml and swirl to mix, e.g. 1 mg of antibody in 400 ml of coating buffer. Add 200 µl to each well of a 96-well plate (Nunc-Immuno MaxiSorp F96)
Stack the microtitre plates and allow to stand in the dark at 15 -25°C.for 16-24 hours.

Aspirate and discard the coating solution. Wash the coated plate 3 times with demineralised water, and tap out onto cellulose.
Pipette 200 µl of blocking buffer into each of the wells to block the residual protein-binding capacity of the coated plates.

Seal the plates with adhesive film, and store in a humidified atmosphere at 2 - 8 °C (shelf life: two months).

7.2 Preparation of samples for assay (*)

Samples are tested at a dilution of 1 in 5, i.e. 50 µl of sample in a total culture volume of 250 µl. To test samples at dilutions greater than 1 in 5, pre-dilute samples before addition to the assay plate, e.g. to test a sample at a dilution of 1 in 10, pre-dilute the sample 1 in 2 with saline and add 50 µl of this diluted sample to the assay plate.

7.3 Collection of human blood

Qualification of blood donors: Blood donors are to describe themselves as being in good health, not suffering from any bacterial or viral infections (including colds and influenza). Blood donors are not to be taking drugs known to influence the production of cytokines. Also, See Section 8. (below) for the criteria for the rejection of data as having come from a test utilising blood from a donor not in good health.

***Procedure(*)*:** Dilute 1 ampoule of Fragmin (10000 IU in 1 ml) with 1 ml pyrogen-free distilled water. Draw back the piston of a 30 ml syringe by about 3 mm, then inject, using a syringe calibrated in µl, 50 µl of the diluted Fragmin via the luer lock adapter into the space between the syringe wall and the piston. Then attach a 19 mm, 21 gauge butterfly system to the 30 ml syringe. Alternatively a 40mm 21 gauge needle may be used.

Using this prepared (heparinised) syringe, draw 30 ml blood from the median cubital or cephalic vein of the left or right arm of a single donor. Mix the blood with the anticoagulant by tilting the syringe several times.

Afterwards remove the butterfly or needle and transfer 15 ml of the blood into each of two 50 ml sterile, pyrogen-free centrifuge tubes.

7.4 Isolation of PBMCs(*)

PBMCs are isolated using Lymphoprep. The procedure is a modification of the manufacturers' instructions that may permit cleaner separation of the PBMC. Start the procedure not later than 2 hours after blood withdrawal.

Procedure: Add 15 ml PBS to each 15 ml of heparinised whole blood to make 2 tubes of 30 ml of diluted blood. Break off the end of a 10 ml pipette to remove the cotton and then place the pipette carefully (point down) in the tube containing the diluted blood. Add 20 ml Lymphoprep to each of the two tubes, via the inserted pipette, to form a lower layer. Centrifuge at 340 x g for 45 min at room temperature (18 – 25 °C), with the centrifuge brake set to off/zero. After centrifugation, the PBMC form a white band at about the 25 ml graduation of the tube. Carefully draw off the uppermost 9ml of each of the supernatants from the same donor and pipette to a new 50 ml tube if it is intended to freeze the PBMCs, otherwise discard. Aspirate and discard the remaining supernatants from above the PBMCs. Take up the PBMCs with a 10 ml pipette and transfer to a new 50 ml centrifuge tube.

7.5 Washing PBMCs(*)

To the isolated PBMCs add sufficient PBS to give a total volume of 50 ml and centrifuge at 340 x g for 15 min.

Aspirate the supernatant phase, and resuspend the sediment with 10 ml of PBS using a serological pipette (aspirate and expel several times, do not vortex). Make up to a total volume to 50 ml with PBS and centrifuge at 340 x g for 10 min. Aspirate the supernatant phase and resuspend the sediment with 15 ml of RPMI-C. Pool the resuspended sediments for each donor into one tube per donor and distribute the cells with a volume of RPMI-C equal to the initial volume of blood from which the PBMC were isolated. This suspension of PBMC in RPMI-C is used in the cell-culture.
The cells are to be cultured with endotoxin or samples within 4 hours of blood withdrawal.

7.6 Procedure for cryopreserving and thawing PBMCs(*)

Add 2 ml of endotoxin-free DMSO to 18 ml of separated supernatant from each donor to produce the cryo-protective solution and cool to 2 to 8°C. Proceed as under point 7.5 of "Washing PBMCs" but centrifuge only once. Discard the supernatant and resuspend the cell pellet with 6 ml of the chilled, homologous cryoprotective solution. Pool the cell suspensions from the same donor and make aliquots of 1.0 ml in screw cap cryotubes. One 1.0 ml aliquot contains the PBMC of 5 ml whole blood. Slowly cool down the aliquoted cell suspensions to –80°C using a styrofoam box to provide thermal insulation. For prolonged storage transfer the tubes after 72 hours to liquid nitrogen (-196°C).

Take two tubes (2 x 1.0 ml) of cryopreserved cells from one donor from the liquid nitrogen and submerge the tubes (but not their caps) immediately in a water bath at a temperature of 37°C. After thawing, pool the cell suspension in a 50ml centrifuge tube and add culture medium to give a total volume of 40 ml. Take care not to contaminate the contents with the water from the water bath. Centrifuge for 10 minutes at 340 x g, pour off the supernatant phase and resuspend the sedimented cells in 10 ml of culture medium (RPMI-C). If desired, a viability test can be performed. (In our experience the viability is above 95%.)

7.7 Equilibration of reagents for cell culture

Bring a vial of the LPS standard, the samples for assay and a bottle of RPMI-C to room temperature.

7.8 Preparation of the LPS standard curve (*)

Add 5 ml of LAL reagent water to the lyophilised contents of one vial of the current USP Reference Standard Endotoxin or the IS to produce a stock solution of 2000 EU(IU)/ml. Vortex for at least 30 min. The stock solution remains stable for 14 days if stored at 2 – 8°C.
Prepare the LPS standard curve by making serial dilutions in saline of the stock solution of endotoxin:

Label seven tubes, A – G. Add the volumes of saline to the tubes specified in table 1, below.

Take 200µl of endotoxin stock solution and add 1.8 ml of saline and vortex to make 2.0 ml of a 200 EU (IU)/ml solution of LPS = Solution S.

Table 1. Preparation of the LPS standard curve:

Tube	LPS added to tube	Saline	[LPS] in tube	→ *[LPS] in well*
A	100 µl of Solution S = 20 EU	900 µl	20 EU/ml	*Not for culture*
B	100 µl of Solution A = 2 EU	1900 µl	1 EU/ml	*0.2 EU/ml*
C	500 µl of Solution B = 0.5 EU	500 µl	0.5 EU/ml	*0.1 EU/ml*
D	500 µl of Solution C = 0.25 EU	500 µl	0.25 EU/ml	*0.05 EU/ml*
E	500 µl of Solution D = 0.125 EU	500 µl	0.125 EU/ml	*0.025 EU/ml*
F	500 µl of Solution E = 0.063 EU	500 µl	0.063 EU/ml	*0.0125 EU/ml*
G	None	1 ml	0 EU/ml	*0 EU/ml*

Vortex each of Solutions A - G after its preparation and then use each solution for the preparation of the subsequent dilution.
(Multiples of the above volumes may be used to generate larger volumes of Solutions A – G and an LPS standard calibrated against the USP RSE or the IS for endotoxin may be used.)

7.9 Cell culture (*)

Add 100 µl of RPMI-C to wells of columns 1 – 10 as in template 1, see below.

Add 50 µl of the test samples S1 – S14 to wells as in template 1, see below.

Add 50 µl of LPS standards to wells as in template 1, below.

Solution G into wells A3 – D3 (STD R0)
Solution F into wells A4 – D4 (STD R1)
Solution E into wells A5 – D5 (STD R2)
Solution D into wells A6 – D6 (STD R3)
Solution C into wells A7 – D7 (STD R4)
Solution B into wells A8 – D8 (STD R5)

(The above order of addition permits the same tip to be used for additions of all the standards.)

Gently swirl the solution of PBMC to reduce settling of the cells and to distribute the PBMC more evenly throughout the RPMI-C solution immediately before aliquots of PBMC are taken. Do not vortex.

Add 100 µl of PBMC to the 96-well plate (see template 1, below). Add the PBMC by row in the following sequence: A, E, B, F, C, G, D, H if a repeating pipette is used. Alternatively a multipipette may be used for these additions provided that the aliquots are added briskly to minimise the settling of cells.

Gently swirl the resulting cultures to mix the contents of the wells without cross-contaminating wells.
Incubate the cultures without vibration (to allow the cells to settle) at 37°C for 16 - 24h in an atmosphere of 5% CO_2 in humidified air.

Template 1: PBMC culture plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	R0 0	R1 0.063	R2 0.125	R3 0.25	R4 0.5	R5 1	S3	S4	void	void
B	S1	S2	R0 0	R1 0.063	R2 0.125	R3 0.25	R4 0.5	R5 1	S3	S4	void	void
C	S1	S2	R0 0	R1 0.063	R2 0.125	R3 0.25	R4 0.5	R5 1	S3	S4	void	void
D	S1	S2	R0 0	R1 0.063	R2 0.125	R3 0.25	R4 0.5	R5 1	S3	S4	void	void
E	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	void	void
F	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	void	void
G	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	void	void
H	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	void	void

Key:
S1 – S14 = test samples #1 - #14
R0 – R5 = Reference standard for endotoxin , R0 = 0 EU/ml, R1 = 0.063 EU/ml, R2 = 0.125 EU/ml, R3 = 0.25 EU/ml, R4 = 0.5 EU/ml and R5 = 1 EU/ml (*The final concentrations are: 0.0125, 0.025, 0.05, 0,1 and 0,2UE/ml).*

7.10 Detection of IL-6 in the supernatant medium by ELISA

Immunoreactive IL-6 in aliquots of the tissue culture fluid is quantified using a validated ELISA, in which the IL-6 standard used as the assay calibrant is calibrated against the IS for IL-6 (89/548).

7.10.1 Preparation of IL-6 standard curve

Prepare the IL-6 standard dilutions as follows:
Fill each of 7 polystyrene tubes (12ml) with 500 µl of RPMI-C.
Add 900 µl of RPMI-C to one frozen aliquot (100µl) of IL-6 standard (=Reagent 3)
Dilute this concentration (Solution I) by transferring 500 µl to tube J (1 in 2). Further dilute by transferring 500 µl from this tube to the next, 500 µl from that tube to the following one, and so on, ending with the tube marked with O. Use the RPMI-C in tube P as a blank (see table 2).

Table 2. Preparation of the IL-6 standard curve from an aliquot of the IL-6 standard.

Sol.	IL-6 added	RPMI-C	IL-6 in tube
I	100 µl of frozen sol.= 4000 pg	900 µl	4000 pg/ml
J	500 µl of Solution I = 2000 pg	500 µl	2000 pg/ml
K	500 µl of Solution J = 1000 pg	500 µl	1000 pg/ml
L	500 µl of Solution K = 500 pg	500 µl	500 pg/ml
M	500 µl of Solution L = 250 pg	500 µl	250 pg/ml
N	500 µl of Solution M = 125 pg	500 µl	125 pg/ml
O	500 µl of Solution N = 62.5 pg	500 µl	62.5 pg/ml
P	None	500 µl	0 pg/ml

Vortex each of Solutions I – O after its preparation and then use each solution for the preparation of the subsequent dilution.
(*Multiples of the above volumes may be used to generate larger volumes of Solutions I – P).*

7.10.2 Addition of standards and samples

Volumes are sufficient for 4 microtiter plates.
Dilute detection antibody (Reagent 2) with dilution buffer (Reagent 4) (e.g. 1 in 500 [200 µl of Reagent 2 + 100 ml of Reagent 4]), and mix without causing foam to form Test each batch of detection antibody in separate experiments to determine the optimum dilution.

Just before using shake out the blocking buffer from the antibody-coated microtitre plates, place the plates on cellulose with the openings facing down, and tap.
At the end of the tissue culture incubation, transfer 50 µl of supernatant from each of the wells of columns 1 – 10 of the tissue culture plate to the corresponding wells on each of the cytokine ELISA plates – see template 1, above and template 2, below. A multichannel pipette may be used. Ensure that the well contents are mixed by aspirating and expelling 50 µl three times before transferring the liquid. (The wells in columns 11 and 12 are for the IL-6 standard curve – see, below).

Transfer 50 µl of each of the dilutions of the IL-6 standard, and of the blank, into 2 wells each (standard concentrations from 4000 - 62.5 pg/ml).
Add 200 µl of dilute detection antibody (e.g. 1 in 500) to each of the wells, seal the microtitre plates with adhesive film, and allow to stand for 2 - 3 hours at 20 - 25 °C. Use template 2 below.

Template 2: ELISA plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	R0	R1	R2	R3	R4	R5	S3	S4	0	0
B	S1	S2	R0	R1	R2	R3	R4	R5	S3	S4	62.5	62.5
C	S1	S2	R0	R1	R2	R3	R4	R5	S3	S4	125	125
D	S1	S2	R0	R1	R2	R3	R4	R5	S3	S4	250	250
E	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	500	500
F	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	1000	1000
G	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	2000	2000
H	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	4000	4000

Key:
S1 – S14 and R0 – R5 are as defined for Template 1 (see above).
Values in columns 11 and 12 are concentrations in pg/ml for IL-6.

7.10.3 Addition of substrate solution and measuring

Prepare the substrate solution shortly before use. Transfer 90 ml of substrate buffer (Reagent 6) to a plastic bottle, add 4.5 ml of TMB solution (Reagent 5), and mix.
Wash the microtitre plates by immersing them 3 times in wash solution, then rinse 3 times with demineralised water. Place the microtitre plates face down on cellulose and tap.
Pipette 200 µl of substrate solution into each well. After 10 - 15 minutes, stop the enzyme reaction by adding 50 µl/well of stopping solution. Wipe the back of the microtitre plates with a clean tissue, then measure the absorbance at 450 nm in a ELISA plate reader using a 540 nm to 590 nm corrective filter. Subtract the values of the measurement with the corrective filter from values measured with the 450 nm filter.

8 DATA ANALYSIS, PREDICTION MODEL AND RELATED ERRORS

8.1 Acceptance Criteria

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

The ELISA is valid if the OD of the blank control is below 0.15 and the mathematical function (quadratic model) of the IL-6 standard curve produces an r >0.95.

The reactions (in terms of OD) on the endotoxin concentrations give a sigmoidal ascending dose response.

Blood donors are considered low responders if their mean OD value for the endotoxin reference standard concentration 1 EU/ml (R5) is below the mean OD value for 1000 pg/ml of IL-6. Low responders should not be included in the assessment.
Blood donors showing a mean OD value for the negative control (R0) above the mean OD value at 500 pg/ml of IL-6 per millilitre are also not included in the assessment (high responder).
If the test sample show an irregular response (e.g. high SD), check the results obtained for the blank and the standard endotoxin concentrations derived from the donor in question. If the latter results are inconsistent with those which would ordinarily be expected, then the donor in question must be excluded from the assessment.

8.2 Interference Test

Interference with the cell system

For each product tested the first time, it is necessary to determine whether it requires dilution prior to assay or not. The following experiment checks for interference between the sample and the PBMC and/or ELISA system.
Perform a dilution series of the product with dilution factor 2 or higher, if necessary. Dilutions should not reach outside the range where the detection limit of the test does not allow to determine the defined **endotoxin limit** of the product. If no endotoxin limit is defined it can easily be estimated by dividing 350EU by the maximum hourly dose. (Example: The maximum hourly dose is 100mg/patient; then the estimated endotoxin limit is 350/100 = 3.5EU/mg)
Split each dilution ("undiluted" may be included) and test it spiked and unspiked in quadruplicates. Spike with an endotoxin concentration from the middle of the endotoxin standard curve (e.g. 0.25 EU/ml). Prepare the spiking solution in RPMI-C instead of saline and prefill the wells for this purpose first with 50µl of RPMI-C and then add 50µl of the spiking solution. The prefill for the unspiked testing of the dilution is 100 µl of RPMI-C per well. Then add 50µl of the product dilution (or undiluted product, resp.) to 4 wells containing the spiking solution and 4 wells containing only RPMI-C.

Proceed according to the testing instructions (see above).
Determine the lowest dilution (highest concentration) of the product that yields an endotoxin spike recovery of 50 to 200%. For that purpose calculate the mean endotoxin values of the unspiked and the spiked product dilutions applying a best fit model on the endotoxin calibration curve. Consider that in this nomenclature "undiluted" means also a dilution. Then subtract the endotoxin value of the unspiked dilution from the endotoxin value of the corresponding spiked dilution. Calculate the spike recovery for each dilution in percent taking the theoretical value (spike concentration e.g. 0.25 EU/ml) as a 100%.
Example: Tested dilutions and spike recovery :"undiluted" = 25%; 1 in 2 = 49%; 1 in 4 = 90% and 1 in 8 = 110%. Then the dilution meeting the requirements is 1 in 4.

8.3 Interference with the ELISA system

Simulate a pyrogen test without cells and without incubation using the chosen test dilution of the product (from testing "interference with the cell system", in the example 1 in 4). Prepare enough replicates to spike with the IL-6 standard curve concentrations (4000 - 62.5 pg/ml and the blank) in duplicate (12 wells). Test the IL-6-spiked solutions directly for interference in the ELISA system together with a unspiked IL-6 standard dilution series. Apply exactly the same methodology as for a real pyrogen test. If interference in the ELISA system appears, the product has to be tested in further dilutions but not exceeding the maximum valid dilution (see above).
The lowest dilution (highest concentration) of the product not interfering with PBMC and the ELISA is considered as the future dilution for routine testing.

8.4 Prediction model

Perform the test with a certain product according to this SOP. Apply a valid test dilution showing no interference with the test systems as written above. Calculate the parameters of the endotoxin standard curve applying a best fit model ($r \geq 0.95$). Reject

outliers only after checking according to Dixon's Test. Test at the level of p = 0.90 or 0.95. Measure the mean OD values of all replicates (at least triplicates). Using the endotoxin standard curve, calculate the pyrogen content of the product in endotoxin equivalents. Multiply the calculated concentration of endotoxin equivalents in the product by the dilution factor (may be 1 in special cases). This value represents the pyrogen content of the sample expressed in endotoxin equivalents for the donor under test. For the product to pass the test, it must comply with its specification (endotoxin limit concentration) when tested with PBMC from three independent donors.

Remark: In order to optimize the prediction model of this test a methodology using a well characterized reference substance (identical in composition to the test substance) is in preparation. With this advanced prediction model interferences in the test due to donor variability will be reduced distinctively.

9 HEALTH SAFETY AND ENVIRONMENT

Human material

Human material should be treated as biologically hazardous and all work using human material is to be carried out according to the procedures specified in the NOVARTIS Safety Guidelines.

Cultures of human material should be treated as biologically hazardous waste and disposed of according to the procedures specified in the NOVARTIS Safety Guidelines.

Bacterial endotoxin is, as its name indicates, a toxic agent and should be handled with care.

Precautions: Cover open cuts before use. Do not get in eyes, on skin, on clothing. Avoid inhaling. Keep container closed.

First Aid: In case of contact, immediately flush eyes or skin with plenty of water for at least 15 minutes. If inhaled, remove to fresh air. If not breathing, give artificial respiration, preferably mouth-to-mouth. If breathing is difficult, give oxygen.

Effects of skin absorption can include fever, headache and hypotension.

Effects of inhalation can include fever, headache and hypotension.

Effects of ingestion - adverse effects are unlikely since ingested endotoxin is rapidly detoxified.

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