Validation of Biomedical Testing Methods

***In vitro* pyrogen test using MONOMAC 6 CELLS**

Standard Operating Procedure

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Standard Operating procedure

In vitro pyrogen test using MONOMAC 6 TEST

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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, PBMNC CELLS, 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).

The isolation of monocytes/leukocytes from whole blood is labour–intensive and time–consuming, technically sophisticated, requires expensive reagents and does not guarantee the isolation of cells in a non–activated state. This prompted the evaluation of various cell lines which retain monocytic characteristics, including the capacity to synthesise and secrete pyrogenic cytokines (Taktak et al., 1991). Taktak *et al*. measured LPS–induced IL–1β and IL–6 release from MONO MAC 6 cells and THP–1 cells and concluded that IL–6 release by MONO MAC 6 cells was the most appropriate readout for an *in vitro* pyrogen test ('monocyte test') because immunoreactive IL–6, unlike immunoreactive IL–1 and TNFα, is secreted entirely into the cell–conditioned medium in large quantities, permitting its complete estimation. The test was applied to three batches of therapeutic human serum albumin (HSA) that had caused adverse reactions in recipients. The MONO MAC 6/IL–6 test

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detected pyrogenic contamination in the HSA that had not been detected in the rabbit pyrogen test and the LAL test.

2. PURPOSE

To develop an *in vitro* pyrogen test that will serve as a replacement for the rabbit pyrogen test.

3. SCOPE / LIMITATIONS

The method described below is for the evaluation of an in vitro MONOMAC 6/IL–6 release test. It is not a 'finalised' test system for the testing of medicinal products. The method may be applied only to preparations that have been validated with the method, i.e. shown not to interfere in the test system: see Heading 8.

4. METHOD OUTLINE

MONOMAC 6 cells are stimulated for 16–24h with standard endotoxin (LPS) and preparations under test. Following this stimulation, the concentration of IL–6 in the cell–conditioned medium is quantified using a specific ELISA (which is calibrated in terms of the appropriate international standard). The construction of a dose–response curve for endotoxin standard versus OD-value of IL–6 ELISA permits the estimation of the pyrogenic contamination of the preparations under test. The contamination is measured in endotoxin–equivalent units.

5. DEFINITIONS / ABBREVIATIONS

µg	microgram
µl	microlitre
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
HSA	Human Serum Albumin
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
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
No.	number
nm	nanometre
OD	optical density
PBS	Dulbecco's phosphate buffered saline
PC	Personal Computer
PF	pyrogen–free (items purchased as sterile and pyrogen–free or baked at 250°C for 30–60 min.)
POD	horseradish peroxidase conjugate
R	endotoxin standard
rpm	rounds per minute
RPMI	RPMI 1640 cell culture medium
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

6.1. Cell line

The cell line used is human monocytic cell line MonoMac-6 (obtained from Prof. H.W.L. Ziegler-Heitbrock (Institute for Immunology, University of Munich, Munich, Germany) A Master Cell Bank and a Working Cell Bank was established at the NIBSC. Cells can be obtained from this laboratory.

6.2. Technical equipment

Incubator (37°C ± 2 °C, 5% ± 0.5 % CO_2, humidified)
Inverted microscope
Haematocytometer
Laminar flow clean bench (Class II)
Laboratory centrifuge (refrigerated) suitable for 50 ml tubes
Laboratory centrifuge suitable for 1 ml vials
Water bath (adjustable to 37 °C, 56°C and 70°C)
pH meter
ELISA-reader
Platewasher
Pipettes adjustable to 2-20 µl, 50-200 µl, 20-100 µl or 200-1000 µl (e.g. Gilson)
12-channel or 8-channel pipette
Vortex mixer

6.3. Other materials

All materials must be sterile and pyrogen free.

Tissue culture flasks, 25 cm^2, 75 cm^2 and 150 cm^2 (e.g. Costar)
Centrifuge tubes, 15 ml and 50 ml (e.g.Greiner or Falcon)
pyrogen free tips (e.g. Greiner)
96-wells tissue culture plates with lid (e.g.Costar)
polystyrene tubes (e.g.Greiner or Falcon)
plate sealers, non toxic (e.g.Dynatech Laboratories)
cryotubes, 2 ml (e.g. Nunc)
serological pipettes (5ml,10ml, 25ml, e.g. Beckton Dickinson Labware)
0.22 µm sterile filters (MilliPak 60, Millipore)

6.4 Chemicals and culture media

Sterile, pyrogen–free phosphate buffered saline (e.g. Life Technologies)
Hydrochloric acid, 0.1M, sterile filtered (Sigma, H–9892)
Pyrogen free sodiumchloride 0.9% (i.e. saline)(e.g. NPBI)
Water for injections (e.g. NPBI)
Trypan blue stain (e.g Sigma)

RPMI 1640 (e.g. Lifetechnologies, Gibco BRL, code 31870–025)
Hepes (e.g. 1M, Gibco)
Insulin (Sigma, code 1–4011)
L–Glutamine (e.g. 200 mM , Gibco)
Oxaloacetic acid (e.g. Sigma)
Sodium pyruvate (e.g. 100 mM, Gibco)
MEM non–essential amino acid solution (e.g. Gibco)
Fetal Bovine Serum (FBS) (e.g. Myoclone Super Plus FBS, Gibco, code 16000-036).
Note: The endotoxin content must be < 0.1 ng/ml, checked by LAL, after heating for 30 min. at 70°C.
Dimethyl sulphoxide (e.g. Merck)
Penicillin/Streptomycin, (10,000 IU/ml penicillin, 10 mg/ml streptomycin (e.g. Gibco, code 1514-0114))
International Standard for Endotoxin, 10,000 IU per vial (NIBSC, code 94/580)
International Standard for IL-6, 1µg/ 100000 IU per ampoule (NIBSC, code 89/548)

(HSA, 1% (a dilution in sterile PF saline of clinical grade HSA, 4.5%))
(Human serum AB (e.g. Sigma))

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

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7. METHODS

7.1. STEPS PRIOR TO CELL–CULTURE

Steps marked (^{at}) are carried out in a Class 2 laminar flow sterile cabinet, using aseptic technique and reagents and consumables that are sterile and pyrogen–free.

Preparation of aliquots of the LPS (endotoxin) standard (STD)^{at}

Make aliquots of the LPS STD: take a vial of the current IS for endotoxin (vial code 94/580, 10000 IU = EU/vial, infinite shelf life when stored at –20°C or below), and reconstitute the contents of the vial with 5 ml pyrogen free water and vortex for 30 min. This gives a 2,000 IU/ml stock solution of LPS. The stock solution may be kept at 2–8°C for up to 14 days. Alternatively, aliquot LPS standard into labelled cryotubes, freeze them upright and store them at –20°C or below (shelf life = 12 months) The following primary standards (Reference Standard Endotoxins) are identical to the IS and may be substituted for it: EC6 (USP), Lot G (FDA), BRP3 (EP). Alternatively, a working standard (control standard endotoxin) calibrated against one of these primary standards may be substituted.

Preparation of aliquots of the IL–6 standard^{at}

To make aliquots of the IL–6 standard, take a vial of the IS for IL–6 (ampoule code 89/548, 1 μg/100000 IU/ampoule) and reconstitute with 1 ml of PBS + 1% BSA (or HSA – not critical). This gives a 1 μg/ml stock solution of IL–6†.
(†: concentrations of IL–6 are expressed in pg/ml rather than IU/ml to avoid confusion with IU/ml of LPS)

Aliquot the stock solution into labelled cryo–tubes (of 2 ml capacity), freeze them upright and store them in this frozen state at –20°C or below (shelf life = six months).

A working standard, previously calibrated against the IS may be substituted for the IS. Each new batch of working standard is to be calibrated against the IS.

Preparation of medium^{at}

Prepare and store the RPMI as described by the supplier. Adjust the pH and sterile filter (0.22 μm) the medium if required.
Test a sample of the medium (from one bottle) for LPS contamination in an LAL test according to the current SOP's for LAL testing. Use the batch of medium only if the level of contamination is not greater than 0.06 IU/ml.

'Completion' of maintenance culture medium (RPMI–M)[at]

RPMI 1640 medium	500 ml
Heat–inactivated (+56°C for 30 min) foetal calf serum (HIFCS)	50 ml
L–Glutamine, 2 mM	
MEM non–essential amino acid, 0.1 mM	
Bovine insulin, 0.23 IU/ml	
Oxaloacetic acid, 1 mM	
Sodium pyruvate, 1 mM	
Hepes, 20 mM	

'Completion' of assay medium (RPMI–C)[at]

RPMI –1640 medium	500 ml
Heat–inactivated (+56°C for 30 min) foetal calf serum (HIFCS)*	10 ml
L–Glutamine, 2 mM	
MEM non–essential amino acid, 0.1 mM	
Bovine insulin, 0.23 IU/ml	
Oxaloacetic acid, 1 mM	
Sodium pyruvate, 1 mM	
Hepes, 20 mM	

After completion, RPMI-M and RPMI-C can be stored at +4°C during three weeks.

('low–endotoxin' heat–inactivated foetal calf serum is not a crucial reagent and may be substituted by human serum AB. Also penicillin and streptomycin may be added to the RPMI-M to reduce the risk of cultures becoming contaminated)*

7.2 CELL CULTURE PROCEDURES

Starting up a cell culture [at]

Take an ampoule of frozen cells out of the liquid nitrogen container and put the ampoule on ice to thaw the cells gradually. Continue with the procedure as soon as the cells are thawed. Clean the outer surface of the ampoule with ethanol 70%. Transfer the cells to a 50 ml centrifuge tube and add 10 ml medium (+4°C). Centrifuge at 100 x g for 5 min (at +4°C). Decant the supernatant carefully and resuspend the cells in 10 ml RPMI–M (+4°C). Centrifuge at 100 x g for 5 min at (+4°C). Decant the supernatant carefully and resuspend the cells in 2 ml RPMI–M. Add 8 ml RPMI–M to a 25 cm^2 tissue culture flask and transfer the 2 ml cell suspension to the flask. Check the quality of the cells visually, using a microscope. The cells should not clump together.
Incubate the cells in a CO_2–incubator (37°C, 5% CO_2, high humidity)
(*Note: As an alternative, the cells may be thawed at 37°C and washed at RT*)

Propagation of the cells [at]

Take the culture flask from the CO_2–incubator. Estimate the number of viable cells using Trypan blue exclusion: viable cells exclude Trypan blue. Take an aliquot of 100 µl of the culture flask and add 850 µl RPMI and 50 µl 0.4% w/v Trypan blue solution. Count the number of viable cells in this solution using a haematocytometer. This

procedure is described in detail in the Sigma (Biochemical and Reagents For Life Science Research) Catalogue 2000/2001, pages 1848–9.
Centrifuge the cell suspension 100 x g for 8 min at RT. Pour off the supernatant phase, resuspend the cell pellet in 4 ml RPMI-M using a serological pipette (gently aspirate and expel several times, avoid bubbles, do not vortex).

Add a part of the cell–suspension to a flask and add new medium to the cells until a final concentration of 2 x 10^5 cells/ml. The total volume depends on the size of the culture flask (For a 25, 75, 150 and 175 cm^2 flasks use 10–15 ml, 25–30 ml, 45–55 ml and about 60 ml, respectively).
It is also valid to skip the centrifugation of the cells: Remove a part of the cell-suspension from the flask and add new medium to the cells (final concentration of 2 x 10^5 cells/ml) In general the cell suspension should be diluted between 1 in 4 up to 1 in 6.
Check the quality of the cells visually, using a microscope. The cells should not cluster. Incubate the cells in a CO_2–incubator (37°C, 5% CO_2, high humidity)

In general this procedure should be performed twice a week.

Preparation of a cell bank [at]

Centrifuge the cell culture at 100 x g for 8 min at RT (or +4°C). Decant the supernatant carefully and resuspend the cells in FBS at RT (or +4°C). Adjust the cell concentration to ≥ 4 x 10^6 cells/ml. Put the cell suspension on ice for 10 minutes.
Add dropwise an equal volume of a cold solution of FBS + 10% DMSO to the cell suspension (final cell concentration is ≥ 2 x 10^6 cells/ml with 5% DMSO).
Transfer the cell suspension to sterile and pyrogen free cryotubes (1 ml/tube) and close the tubes firmly.
Put the tubes in a well insulated polystyrene box and store the box at –70 °C or below for about 48 h. Finally transfer the tubes to a liquid nitrogen container.

7.3 ONSET OF THE TEST

Pre-incubation of cells for a test [at]

Centrifuge 30–50 ml of cell suspension at 100 x g for 8 min at room temperature. Pour off the supernatant phase, and resuspend the cells in approximately 2 ml of RPMI–C using a serological pipette (gently aspirate and expel several times, avoid bubbles, do not vortex). Count the cells and dilute the needed amount of viable cells in RPMI-C until a final concentration of 4x10^5 cells/ml. The total volume depends on the size of the culture flask and the number of cells needed for the test. (Generally 2x10^7 cells pre-incubated in 50 ml RPMI-C in a 150 cm^2 flask is enough for one 96-well assay plate). Incubate the cells during approximately 24 hours in a CO_2–incubator (37°C, 5% CO_2, high humidity)

Preparation of cells for a test [at]

Centrifuge 30–50 ml of cell suspension at 100 x g for 8 min at room temperature. Pour off the supernatant phase, and resuspend the cells in approximately 2 ml of RPMI–C using a serological pipette (gently aspirate and expel several times, avoid

bubbles, do not vortex). Count the cells and dilute the viable cells with RPMI–C to a volume that gives a concentration of 2.5 x 10^6 viable cells/ml. (Each 96–well assay plate requires about 10 ml of 2.5 x 10^6 viable cells/ml.) Prepare the solution of cells just prior to addition to the culture plate.

Preparation of samples for test[at]

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.

Equilibration of reagents for the test

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

Preparation of the LPS standard curve [at]

Prepare the LPS standard curve by making serial dilutions in saline of an aliquot of the stock solution of the current IS.

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

Add 1.35 ml saline to an aliquot (300 IU=EU in 150 µl) of the LPS standard and vortex to make 1.5 ml of a 200 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 IU	900 µl	20 IU/ml	*Not for culture*
B	200 µl of Solution A = 4 IU	800 µl	4 IU/ml	*0.8 IU/ml*
C	500 µl of Solution B = 2 IU	500 µl	2 IU/ml	*0.4 IU/ml*
D	500 µl of Solution C = 1 IU	500 µl	1 IU/ml	*0.2 IU/ml*
E	500 µl of Solution D = 0.5 IU	500 µl	0.5 IU/ml	*0.1 IU/ml*
F	500 µl of Solution E = 0.25 IU	500 µl	0.25 IU/ml	*0.05 IU/ml*
G	None	1 ml	0 IU/ml	*0 IU/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)

Testprocedure

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.)

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

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

Gently swirl the solution of MONOMAC 6 cells to reduce settling of the cells and to distribute the cells more evenly throughout the RPMI–C solution immediately before aliquots of cells are taken. Do not vortex.

Add 100 µl of MONOMAC–6 cells to the wells of columns 1 – 10 as in Template 1, see below.

Using a pipette with a tip of wide diameter, the cells are added by row in the following sequence: A, E, B, F, C, G, D, H (see template 1, below). A repeating pipette 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.

Remark: It is also valid to test the sample in a total culture volume of 200 µl. Take 40 µl of sample dilution, 110 µl medium and 50 ul of a cell solution (cell concentration 4 x 10^6 viable cells/ml)

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: MONOMAC 6 CELLS culture plate (example)

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

Key:
S1 – S14 = test samples #1 – #14
R0 – R5 = Reference Standard Endotoxin, R0 = 0 IU/ml, R1 = 0.25 IU/ml, R2 = 0.5 IU/ml, R3 = 1 IU/ml, R4 = 2 IU/ml and R5 = 4 IU/ml (The final concentrations are: 0.05, 0.1, 0.2, 0,4 and 0.8 IU/ml).

Harvesting and storage of supernatants.

At the end of the cell culture incubation the supernatant is harvested as follows:
Transfer from each well ca. 140 µl (or more if possible) of the supernatant above the MONOMAC 6 cells to the corresponding well a new 96-wells cell culture plate. Do not disturb the cells. The use of an eight channel pipette will facilitate the transfer. Change the pipette tips between each column transfer.
Cover the plate with the supernatants with a seal. Store the plate at –70 °C (or below) until required or proceed with the detection of IL6. When the plates are stored at –20 °C, determination of IL6 should be performed within two weeks.

7.4 DETECTION OF IL–6 IN THE SUPERNATANT MEDIUM BY ELISA

Immunoreactive IL–6 in aliquots of the cell culture fluid (cell–conditioned medium) is quantified using a validated ELISA, in which the IS for IL–6 (89/548) or an IL–6 standard calibrated against the IS is used as the assay calibrant.
Two validated IL-6 ELISA's are described in the ANNEX. (The NOVARTIS ELISA for IL–6 and the Human IL–6 ELISA kit (CLB, Amsterdam, The Netherlands, code M1916) Other validated ELISAs may be substituted.

8. DATA ANALYSIS

All experiments are run with four replicates of the test compound on one plate. A standard curve using the International Standard for Endotoxin (calibrated in IU) is included, ranging from 0.25 IU/ml up to 4 IU/ml. A test is valid if the 0.5 IU/ml is significant elevated over the background (defined by mean +2SD (n-1)). The endotoxin value of each replicate is calculated from the endotoxin calibration curve of the IS for endotoxin, applying the 4-parameter logistic model and expressed as endotoxin-equivalents/ml (EU/ml). Outliers are rejected only after checking according to the Dixon's test ($p \geq 0.05$). Subsequently, the mean endotoxin value of all replicates (usually quadruplicates) of a test compound is calculated and multiplied by the dilution factor (if applicable). The mean endotoxin concentration is compared with the endotoxin limit concentration (ELC) for the test compound. Where the ELC is not specified for a product, it is calculated as described below in the prediction model.

Alternative analyses are permitted provided these are consistent with the relevant ICH guidelines.

Test validation and interference testing

To assure the precision or validity of the test method, preparatory tests are conducted to assure that the criteria for the standard curve are valid and that the test solution does not interfere in the test. The test method is validated and a test for interfering factors repeated whenever there is any change in either the test method or the test preparation that is likely to influence the result of the test.

Interference test cell culture

Spike an aliquot of the test solution of the preparation being examined with an endotoxin concentration at or near the middle of the endotoxin standard curve. Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the test solution (if any) from that containing the added endotoxin. Calculate the spike recovery for each dilution in percent, taking the theoretical value (spike concentration e.g. 1 EU/ml) as a 100%. The test solution is considered free of interfering factors if the measured concentration of the endotoxin added to the test solution is within 50–200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin. If the test solution interferes in the test, i.e. does not give 50–200% endotoxin spike recovery, the test for interfering factors is repeated with the test solution diluted as far as is necessary to achieve 50–200% endotoxin spike recovery. The lowest dilution (highest concentration) of the product that yields an endotoxin spike recovery of 50-200% is determined. This should not exceed the maximum valid dilution (MVD) for the product. The MVD is the maximum allowable dilution of a preparation at which the endotoxin limit concentration (ELC) can be determined (MVD = ELC/limit of detection of the test).

Interference test readout system

An unknown test compound should be tested for possible interference with the IL6-ELISA itself. For this purpose, a IL6 concentration at or near the middle of the IL6 standard curve is mixed with the various dilutions of the test compound in RPMI-C

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(and as a control with RPMI-C alone) and subsequently tested in the IL-6 ELISA. The test compound dilutions should be comparable to those used in the cell culture test.

Prediction model

For every kind of test compound the interference in the test cell culture and the test readout system should be determined. The test compound should be tested in the lowest dilution showing no interference (endotoxin spike recovery of 50-200%) and not exceeding the maximum valid dilution for the product. The test should be performed according to the SOP and the endotoxin concentration of the test compound should be calculated as described above. The test compound is considered pyrogenic when the endotoxin concentration of the test preparation exceeds the endotoxin limit concentration (ELC) for the preparation.

Where an ELC is not specified, it is calculated as follows:
The sensitivity of rabbits to endotoxin is 5 IU/kg. So, for a product injected (i.e. tested) at 1 ml/kg, the detection limit is 5 IU endotoxin/ml/kg, giving an ELC of 5 IU endotoxin/ml, whereas for a product injected at 10 ml/kg, the detection limit is 5 IU endotoxin/10 ml/kg = 0.5 IU endotoxin/ml/kg, giving an ELC of 0.5 IU endotoxin/ml.

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 laboratory safety procedures.

Cultures of human material should be treated as biologically hazardous waste and disposed of according to the laboratory safety procedures.

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.

ELISA Substrate: TMB ***(Suspected mutagen, wear gloves when handling).*** Store and use in accordance with manufacturer's instructions.

11. REFERENCES

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11. ANNEX

NOVARTIS IL–6 ELISA:

Materials, reagents.

Nunc-immuno MaxiSorp F96)
Mouse monoclonal anti–IL–6 antibody from clone 16 (Novartis)
Horseradish peroxidase conjugated sheep polyclonal anti–IL–6 antibody (Novartis)
3,3',5,5'–Tetramethyl benzidine (e.g. Fluka Cat. No. 87748)
Acetone (reagent grade)
Ethanol (reagent grade)
Phenol (e.g. Merck Cat. No. 100206)
Potassium hydroxide (reagent grade)
Sodium hydroxide (reagent grade)
Hydrochloric acid (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)
Sulphuric acid (2 M H_2SO_4, reagent grade)

Preparation of buffers

Coating Buffer
Dissolve 5.0 g sodium dihydrogen phosphate and 2.9 g disodium hydrogen phosphate in 400 ml 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
Dissolve 12.1 g Tris (hydroxymethyl)aminomethane in 400 ml distilled water.
Add 0.1 ml Kathon MW/WT. Use 4 M HCl to adjust the pH to 7.5.
Add 5.0 g BSA. Add distilled water to make up to 500 ml.
Remains stable for 6 months at 2–8°C.

Stopping Solution
Add 26.6 ml H_2SO_4 to 500 ml distilled water.

Wash Solution
Add 1 ml Tween –20 to 2000 ml of demineralised water.

Dillution Buffer
Dissolve 2.1 g Tris(hydroxymethyl)aminomethane in 400ml distilled water.
Add 0.1 ml Kathon MW/WT, 0.5 g phenol and 25 ml heat–inactivated (30 minutes at +56°C) foetal bovine serum.

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 stabilisers Kathon and phenol the stability is only 1 day.

TMB Solution #
Dissolve 240 mg TMB in 5 ml acetone. Add 45 ml ethanol and 0.3 ml Perhydrol (30 % H_2O_2).
Remains stable for at least 6 months at 15–25°C when sealed and protected from light.

Substrate Buffer#
Dissolve 6.3 g citric acid monohydrate in 800 ml distilled water.
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.

(# TMB solution and substrate buffer may be replaced by a TMB ready-to-use substrate system (e.g. Sigma, T8665)).

Coating of IL–6 ELISA plates

For the NOVARTIS IL–6 ELISA, 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 h.

Aspirate and discard the coating solution. Wash the coated plate 3 times with demineralised water and tap out onto absorbent material, e.g. paper towel. Pipette 200 µl of blocking buffer into each well to block the residual protein–binding capacity of the coated plates. Seal the microtiter plates with adhesive film and store in a humidified atmosphere at 2–8°C (shelf life: two months).

DETECTION OF IL-6 IN THE CELL CONDITIONED MEDIUM BY ELISA

Equilibration of reagents

Bring an aliquot of the IL–6 standard and other assay reagents to room temperature before proceeding.

Preparation of IL–6 standard curve

About 30 min before the end of the tissue culture, prepare the IL–6 standard curve by making serial dilutions, in RPMI–C, of an aliquot of the stock solution of the current IS (or working STD for IL–6 calibrated against the IS).

When using the IS for IL–6, label nine tubes H – P. Add the specified volumes of RPMI–C to the tubes – see table 2 below.

Add 180 µl RPMI–C to an aliquot (20 ng in 20 µl) of the IL–6 standard and vortex to make 200 µl of a 100 ng/ml solution of IL–6 = Solution G.

Table 2. Preparation of the IL–6 standard curve

Tube	IL–6 added	RPMI–C	[IL–6] in tube	→ *[IL–6] in well*
H	100 µl of Solution G = 10 ng	900 µl	10 ng/ml	*Not for ELISA*
I	800 µl of Solution H = 8 ng	1.2 ml	4 ng/ml	*4000 pg/ml*
J	1 ml of Solution I = 4 ng	1 ml	2 ng/ml	*2000 pg/ml*
K	1 ml of Solution J = 2 ng	1 ml	1 ng/ml	*1000 pg/ml*
L	1 ml of Solution K = 1ng	1 ml	500 pg/ml	*500 pg/ml*
M	1 ml of Solution L = 500 pg	1 ml	250 pg/ml	*250 pg/ml*
N	1 ml of Solution M = 250 pg	1 ml	125 pg/ml	*125 pg/ml*
O	1 ml of solution N = 125 pg	1 ml	62.5 pg/ml	*62.5 pg/ml*
P	None	2 ml	0 pg/ml	*0 pg/ml*

Vortex each of Solutions H – P 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 H – P and an IL–6 standard calibrated against the IS for IL–6 may be substituted for the IS.)

Store Solutions H – P at 2– 8°C until required.

Addition of samples

Immediately prior to adding standards and samples, empty the blocking buffer from the ELISA plate(s) and expel any remaining fluid by tapping the inverted plate onto absorbent material, e.g. paper towel.
Make the supernatant more homogeneous by aspirating and expelling three times before transferring the test- amount of supernatant from each of the wells of columns 1 – 10 of the tissue culture plate into the corresponding wells on the ELISA plate – see Template 1, above and Template 2, below. (The wells in columns 11 and 12 are for the cytokine standard curve – see below). The use of an eight channel pipette will facilitate the mixing and transfer. Change the pipette tips between each column transfer.

Addition of standards

Add 50 µl of IL–6 standards to the wells in columns 11 and 12, as shown in Template 2, below. Start at the lowest concentration to permit using the same tip for additions of all the standards.

Template 2: ELISA plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	R0 0	R1 0.25	R2 0.5	R3 1	R4 2	R5 4	S3	S4	0	0
B	S1	S2	R0 0	R1 0.25	R2 0.5	R3 1	R4 2	R5 4	S3	S4	62.5	62.5
C	S1	S2	R0 0	R1 0.25	R2 0.5	R3 1	R4 2	R5 4	S3	S4	125	125
D	S1	S2	R0 0	R1 0.25	R2 0.5	R3 1	R4 2	R5 4	S3	S4	250	250
E	S5	S6	S7	S8	S9	S10	S1 1	S1 2	S1 3	S1 4	500	500
F	S5	S6	S7	S8	S9	S10	S1 1	S1 2	S1 3	S1 4	1000	1000
G	S5	S6	S7	S8	S9	S10	S1 1	S1 2	S1 3	S1 4	2000	2000
H	S5	S6	S7	S8	S9	S10	S1 1	S1 2	S1 3	S1 4	4000	4000

Key:
S1 – S14 = test samples #1 – #14
R0 – R5 = Reference Standard Endotoxin, R0 = 0 IU/ml, R1 = 0.25 IU/ml, R2 = 0.5 IU/ml, R3 = 1 IU/ml, R4 = 2 IU/ml and R5 = 4 IU/ml (The final concentrations are: 0.05, 0.1, 0.2, 0,4 and 0.8 IU/ml).
Values 1 – 4000 in columns 11 and 12 are concentrations in pg/ml of the IS for IL–6 (ampoule code 94/580, 1 μg/ampoule, 1 pg = 0.1 IU).

Addition of (2nd) antibody–HRP conjugate (POD)

Add 200 µl of detection antibody POD (horseradish peroxidase conjugated to sheep anti–IL–6 antibodies: stable for at least 6 months at 2–8°C) pre–diluted with dilution buffer (usually 1/200 to 1/500, as determined in optimisation experiments) to each well, seal the plates with adhesive film, and allow to stand for 2–3 hours at 20–25°C. (100 ml of diluted POD is sufficient for 4 ELISA plates.)

After incubation, wash plate three times with about 250 µl per well wash solution and then three times with demineralised water. Empty plate and expel any remaining fluid by tapping the inverted plate onto absorbent material, e.g. paper towel.

Addition of substrate solution and reading of optical densities

Prepare the substrate solution shortly before use. Transfer 90ml of substrate buffer to a plastic bottle, add 4.5 ml of TMB solution and mix.

Pipette 200µl 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 an ELISA plate reader using a 540–590nm corrective filter. Subtract the values of the measurement with the corrective filter from values measured with the 450 nm filter.

IL-6 ELISA kit (CLB, Amsterdam, The Netherlands, code M1916)

The reagents provided in the ELISA kit used are:

- Coating antibody, 100-fold concentrated
- Blocking reagent, 50-fold concentrated
- IL-6 standard, 4500 pg/ml (calibrated against the WHO International Standard)
- Biotinylated IL-6 antibody, 100-fold concentrated
- Streptavidin-HRP conjugate, 10,000-fold concentrated
- Dilution buffer, 5-fold concentrated
- Microtiter plates + lid (Nunc-Immuno Maxisorp F96)
- Plate seals

Additional materials needed are:
Carbonatebuffer (pH 9.6)
Ethanol 96%
Tween 20
Phosphate buffered saline
Sulphuric acid (2 M H_2SO_4, reagent grade)
3,3, 5,5 Tetramethylbenzidine
Peroxide (30 % H_2O_2)
sulphuric acid (2M)
sodium-acetate (1.1 M, pH 5.5)
Instead of the last four items, a TMB ready-to-use substrate system can be applied (e.g. Sigma, T-8665)

Preparation of reagent solutions used in the IL-6 ELISA
The following solutions are prepared prior to each test:

Washing buffer
Add 50 µl Tween 20 (or 500 µl 10% Tween 20) to 1000 ml PBS.

Dilution buffer
The ELISA kit contains one bottle with 5-fold concentrated dilution buffer. Calculate the quantity of dilution buffer required and prepare a working-strength dilution by diluting the concentrated buffer 1 in 5 in distilled water.

Substrate
Dissolve 6 mg TMB in 1 ml 96% ethanol. Add 1.2 ml sodium-acetate (1.1 M, pH 5.5) and 0.2 ml TMB solution to 10.8 ml of distilled water. Add 2.4 µl H_2O_2 directly prior to use. (alternatively: an equivalent TMB substrate system can be applied)

Performing the ELISA
Centrifuge all vials (except the blocking reagent) before use (1 min. at 3000 g). Preferably an ELISA-plate shaker is used during the incubations (except during the coating of the plate))

Coating of the plate

Dilute 120 µl of the coating antibody with 12 ml of carbonate buffer (= 1/100). Add 100 µl of this dilution to each well of the microtiterplate and incubate overnight at RT.

Wash the plate four times using washing buffer (platewasher).
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Blocking of the plate
Dilute 500 µl blocking reagent with 25 ml PBS (= 1/50). Add 200 µl of this dilution to each well of the microtiterplate and incubate during 1 hour at RT. In the meantime bring the storage plate with the supernatant to RT.
Wash the plate four times using washing buffer.

Preparation of the IL6 Standard (provided with the IL6 ELISA kit)
Dilute in polystyrene tubes.

Tube	IL–6 added	dilution buffer	[IL–6] in tube	→ [IL–6] in well
1	50 µl IL6 Standard (4500pg/ml)	450 µl	450 pg/ml	450 pg/ml
2	200 µl of solution 1 (450 pg/ml)	400 µl	150 pg/ml	150 pg/ml
3	200 µl of solution 2 (150 pg/ml)	400 µl	50 pg/ml	50 pg/ml
4	200 µl of solution 3 (50 pg/ml)	400 µl	16.7 pg/ml	16.7 pg/ml
5	200 µl of solution 4 (16.7 pg/ml)	400 µl	5.6 pg/ml	5.6 pg/ml
6	200 µl of solution 5 (5.6 pg/ml)	400 µl	1.9 pg/ml	1.9 pg/ml
7	200 µl of solution 6 (1.9 pg/ml)	400 µl	0.6 pg/ml	0.6 pg/ml
8	None	400 µl	0 pg/ml	0 pg/ml

Addition of the samples (supernatants)
See: -template 2 ELISA-plate (example)

Homogenise the supernatant in the storage plate before transferring the test-amount of supernatant from the storage plate to the ELISA. Add 100 µl of each dilution of the IL-6 standard (IL6) to the assigned wells of column 11 and 12. Add 80 µl dilution buffer and 20 µl of the sample (sample = S) to the assigned wells of the microtiterplate. As a controle for the performance of the ELISA, one of the sample can be replaced with dilution buffer. Incubate for 1 hour at RT. (Plate shaker)

Wash the plate four times using washing buffer.

Conjugate 1: Biotinylated IL-6 antibody
Add 120 µl biotinylated IL-6 antibody to 12 ml dilution buffer (= 1/100).
Add 100 µl of the conjugate dilution to each well and incubate for 1 hour at RT. (Plate shaker)

Wash the plate four times using washing buffer.

Conjugate 2: Streptavidine-Peroxidase
Add 3 µl streptavidine-HRP conjugate to 30 ml dilution buffer (= 1/10,000).
Add 100 µl of the conjugate dilution to each well and incubate for half an hour at RT. (Plate shaker)

Wash the plate four times using washing buffer.

Substrate solution
Add 100 µl of substrate solution to each well and incubate 15 minutes at RT

Stop solution
Add 100 µl of sulphuric acid (2M) to each well. Measure the absorbance at 450 nm with an ELISA plate reader.