Validation of Biomedical Testing Methods

In vitro pyrogen test using MONOMAC 6 CELLS

Standard Operating Procedure

Only the responsible of the GLP/QA Unit is allowed to make copies of this document. Extra examples can be obtained at the GLP/QA Unit. Quality Documents are only valid if they are signed by the responsible of the GLP/QA Unit in and provided with a blue copy number	Copy number

Sop-MM6v08

JOINT RESEARCH CENTRE
EUROPEAN COMMISSION
Institute for Health and Consumer Protection
ECVAM

Standard Operating procedure

In vitro pyrogen test using MONOMAC 6 TEST

Drafted by:	Name	Stephen Poole
	Date	26-09-01
	Signature	

Reviewed by:	Name	Ria Nibbeling
	Date	28-02-02
	Signature	

Reviewed by:	Name	Ria Nibbeling
	Date	23-04-02
	Signature	

Reviewed by:	Name	Esther van Tienhoven
	Date	01-10-02
	Signature	

*Owner/Trainer: Signature: Date:

PAGE OF CHANGES

Date of change/ Date of draft:	Version–number:	Changed page(s):	Summary of the change(s):	Changed by/Sign.:
280202	2		Overall revision	RN
140402	3	12	Pre-incubation of cells	RN
230402	4	15	Chapter 8	RN
011002	5		Overall revision	EvT
09/10/2002	6	14	Table 1 update	EvT
09/10/2002	6	15	Preperation of Negative and Positive Product Controls updated	EvT
29/10/2002	7	15	Sample preparation [at] 0.5 ml of the respective undiluted drug will be spiked with 23.3 µl corresponding blinded spike solution (see table 2). Then dilute the drug to its MVD according to Table 3.	
29/10/2002	7	16	*Modified Table 2. Sample preparation*	
			Modified Table 3. Dilution of the substances	
30/10/2002	8	14	*Modified Table 3. Dilution of the substances*	

TABLE OF CONTENTS Page No.

THIS SOP WAS AMENDED FOR THE VALIDATION PHASE ONLY. IT DOES THEREFORE ONLY REPLACE THE PREVIOUS VERSION FOR THIS SERIES OF EXPERIMENTS.

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 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.

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
mvd	maximum valid dilution
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)
(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.

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 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.

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 extensively to make 1.5 ml of a 200 IU/ml solution of LPS = Solution S. Then prepare as indicated in Table 1.

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	*Not for culture*	
C	500 µl of Solution B = 2 IU	500 µl	2 IU/ml	*0.4 IU/ml*	R5
D	500 µl of Solution C = 1 IU	500 µl	1 IU/ml	*0.2 IU/ml*	R4
E	500 µl of Solution D = 0.5 IU	500 µl	0.5 IU/ml	*0.1 IU/ml*	R3
F	500 µl of Solution E = 0.25 IU	500 µl	0.25 IU/ml	*0.05 IU/ml*	R2
G	500 µl of Solution F = 0.125 IU	500 µl	0.125 IU/ml	*0.025 IU/ml*	R1
H	None	1 ml	0 IU/ml	*0 IU/ml*	R0

Thoroughly 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)

Preparation of samples for test [at]

Sample preparation [at]
0.5 ml of the respective undiluted drug will be spiked with 23.3 µl corresponding blinded spike solution (see table 2). Then dilute the drug to its MVD according to Table 3.

Preparation of the Negative Product Control (NPC) [at]
Dilute each testproduct to its MVD to a total volume of 0.5 ml. Spike with 25 µl of pyrogen free saline (see table 2).

Preparation of the Positive Product Control (PPC) [at]
The Positive Product Control (PPC) is used for the prediction model of the validation study as demarcation value to discriminate between positive and negative testproducts.
Dilute each testproduct to its MVD to a total volume of 0.5 ml. Spike with 25 µl of the unblinded PPC-LPS spike solution (1:21 dilution of the spike, final concentration 50 pg/ml) (see table 2).

Preparation of the Positive assay Control (PC) [at]
Take 0.5 ml of saline. Spike with 25 µl of the unblinded PPC-LPS spike solution (1:21 dilution of the spike, final concentration 50 pg/ml)

Preparation of the Negative assay Control (NC) [at]
Take 0.5 ml of saline.

Table 2. Sample preparation

unblinded			*blinded*				
dilution of drug up to **MVD**			Spiking of **undiluted** drug: 0.5 ml each				
diluted drug	**NPC**	**PPC**					
0.5 ml	+ 25 µl saline	+ 25 µl PPC-LPS-spike *	+ 23.3 µl of Spike 1	+ 23.3 µl of Spike 2	+ 23.3 µl of Spike 3	+ 23.3 µl of Spike 4	+ 23.3 µl of Spike 5
		(final conc. = 50 pg/ml)	**dilution** to **MVD**				
	test	test	test	test	test	test	test

* *PPC-LPS-spike contains 1050 pg/ml = 21fold 50 pg/ml*
NPC = Negative Product Control, PPC = Positive Product Control,
MVD = Maximal Valid Dilution

Table 3. Dilution of the substances

	Substance	*MVD (ELC/0.5 EU/ml)*	*_l of substance*	*_l of saline*
1	Glucose 5%	70	40	2760
2	EtOH 13%	35	80	2720

3	MCP	350	8	2792
4	Orasthin	700	4	2796
5	Binotal	140	20	2780
6	Fenistil	175	16	2784
7	Sostril	140	20	2780
8	Beloc	140	20	2780
9	Drug A	35	80	2720
10	Drug B	70	40	2760

Each substance has to be vortexed for about 5 seconds immediately before use.

Test procedure

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 samples to wells as in Template 1, see below.
Add 50 µl of the PPC to wells as in Template 1, see below.
Add 50 µl of the NPC to wells as in Template 1, see below.
Add 50 µl of the PC to wells as in Template 1, see below.
Add 50 µl of the NC to wells as in Template 1, see below.

Add 100 µl of RPMI–C to the wells of columns 1 – 12 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 – 12 as in Template 1, see below.

Template 1: MONOMAC 6 CELLS culture plate (example)

	1	2	3	4	5	6	7	8	9	10	11	12
A	D1-NPC	D1-PPC	R0	R1	R2	R3	R4	R5	D2-2	D2-4	NC	PC
B	D1-NPC	D1-PPC	R0	R1	R2	R3	R4	R5	D2-2	D2-4	NC	PC
C	D1-NPC	D1-PPC	R0	R1	R2	R3	R4	R5	D2-2	D2-4	NC	PC
D	D1-NPC	D1-PPC	R0	R1	R2	R3	R4	R5	D2-2	D2-4	NC	PC

E	D2-NPC	D2-PPC	D1-1	D1-2	D1-3	D1-4	D1-5	D2-1	D2-3	D2-5	void	void
F	D2-NPC	D2-PPC	D1-1	D1-2	D1-3	D1-4	D1-5	D2-1	D2-3	D2-5	void	void
G	D2-NPC	D2-PPC	D1-1	D1-2	D1-3	D1-4	D1-5	D2-1	D2-3	D2-5	void	void
H	D2-NPC	D2-PPC	D1-1	D1-2	D1-3	D1-4	D1-5	D2-1	D2-3	D2-5	void	void

D = testproduct (drug)
NPC= negative product control
PPC= positive product control
*R0 – R5 = Reference Standard Endotoxin, R0 = 0 IU/ml, R1 = 0.125 IU/ml, R2 = 0.25 IU/ml, R3 = 0.5 IU/ml, R4 = 1 IU/ml and R5 = 2 IU/ml (The final concentrations are: 0.*05, 0.1, 0.2, 0,4 and 0.8 IU/ml).
NC= negative control
PC= positive control
Using a pipette with a tip of wide diameter. 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.

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.
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 in quadruplicate is included, using the International Standard for Endotoxin (calibrated in IU), ranging *as appropriate* from 0.25 IU/ml up to 4 IU/ml *or 0.125 IU/ml up to 2 IU/ml*.
The endotoxin value of each replicate is calculated from the endotoxin calibration curve of the IS for endotoxin plotted against the final OD at 450 nm, applying the 4-parameter logistic model and expressed as endotoxin-equivalents/ml (EU/ml).

8.1 Assay acceptance criteria

The mean OD at 450 nm is calculated within a quadruplicate ($\times 4$). One datapoint per quadruplicate may be designated as an outlier, as determined using Dixon's test (P>0.05), and removed from the data set used for calculation.
The positive control (final concentration of the spike when diluted in saline) should be between ± 20% of the theoretical value (expected concentration).
The OD at 450 nm of the positive product control (final concentration of the spike diluted in product) should be over 50% of the positive control and below the 200% of the positive control (50–200% endotoxin spike recovery). The OD at 450 nm of the positive controle should be higher then the limit of quantification (LOQ).
The LOQ is defined as the mean OD at 450 nm of the R0 (Negative Cell Control) + 10xSD mean OD at 450 nm of the R0.
The OD at 450 nm of the negative control (blank) should be less then 0.200.
In the LPS standard curve the mean OD at 450 nm of the Rn should be less then the mean OD at 450 nm of the Rn+1. A minimum of 4 data points is needed for a valid reference curve.

8.2 Prediction model

For the purpose of the validation study, a sample is considered positive when the mean OD at 450 nm of the tested product exceeds the mean OD at 450 nm of that of the positive product control.

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

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.

10. REFERENCES

Bleeker, W.K., de Groot, E.M., den Boer, P.J., Biessels, P.T., Aarden, L.A. and Bakker, J.C. (1994) Measurement of interleukin–6 production by monocytes for in vitro safety testing of hemoglobin solutions. Artif Cells Blood Substit Immobil Biotechnol. 22, 835–40.

Dinarello, C.A., Gatti, S. and Bartfai, T. (1999) Fever: links with an ancient receptor. Curr Biol. 9, R147–50.

Dinarello, C.A., O'Connor, J.V., LoPreste, G. and Swift, R.L. (1984). Human leukocyte pyrogen test for detection of pyrogenic material in growth hormone produced by recombinant Escherichia coli. J. Clin. Microbiol. 20, 323–329.

Duff, G.W. and Atkins, E. (1982) The detection of endotoxin by *in vitro* production of endogenous pyrogen: comparison with amebocyte lysate gelation. J Immunol Methods 52, 323–332.

Fennrich, S., Fischer, M., Hartung, T., Lexa, P., Montag–Lessing, T., Sonntag, H.G., Weigandt, M. and Wendel. (1999) A Detection of endotoxins and other pyrogens using human whole blood. Dev Biol Stand. 101, 131–9.

Finney DJ: Statistical Method in Biological Assay, Third Edition. London, Charles Griffin and Company Ltd, 1978.

Gaines Das RE and Tydeman MS. (1982) Iterative weighted regression analysis of logit responses: A computer program for analysis of bioassays and immunoassays. Computer Programs in Biomedicine 15; 13–22.

Hansen, E.W. and Christensen, J.D. (1990) Comparison of cultured human mononuclear cells, Limulus amebocyte lysate and rabbits in the detection of pyrogens. J Clin Pharm Ther. 15, 425–33.

Levin, J. and Bang, F.B. (1964) A description of cellular coagulation in the Limulus. Bull. John Hopkins Hosp. 155, 337–345.

Mascoli, C.C. and Weary, M.E. (1979a) Limulus amebocyte lysate (LAL) test for detecting pyrogens in parenteral injectable products and medical devices: advantages to manufacturers and regulatory officials. J Parenter Drug Assoc. 33, 81–95.

Mascoli, C.C. and Weary, M.E. (1979b) Applications and advantages of the Limulus amebocyte lysate (LAL) pyrogen test for parenteral injectable products. Prog Clin Biol Res. 29, 387–402.

Poole, S., Selkirk, S., Rafferty, B., Meager, A., Thorpe, R. and Gearing, A. (1989) Assay of pyrogenic contamination in pharmaceuticals by cytokine release. Proceedings of the European Workshop on detection and quantification of pyrogen. Pharmeuropa, November 1989, 17–18.

Poole, S., Thorpe, R., Meager, A., Hubbard, A.R. and Gearing, A.J.H. (1988) Detection of pyrogen by cytokine release. Lancet 8577, 130.

Ray, A., Redhead, K., Selkirk, S. and Poole, S. (1990) Variability in LPS composition, antigenicity and reactogenicity of phase variants of Bordetella pertussis. FEMS Microbiology Letters 79, 211–218.

Taktak, Y.S., Selkirk, S., Bristow, A.F., Carpenter, A., Ball, C., Rafferty, B. and Poole, S. (1991) Assay of pyrogens by interleukin–6 release from monocytic cell lines. J Pharm Pharmacol 43, 578–582.

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 assay reagents to room temperature before proceeding.
N.B. No IL-6 standard is used.

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 – 12 of the tissue culture plate into the corresponding wells on the ELISA plate – see Template 1 The use of an eight channel pipette will facilitate the mixing and transfer. Change the pipette tips between each column transfer.

Void wells are filled with Dilution Buffer.

Addition of (2^{nd}) 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
- 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).

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.

Addition of the samples (supernatants)
See: -template 1 above

Homogenise the supernatant in the storage plate before transferring the test-amount of supernatant from the storage plate to the ELISA. Add 80 µl dilution buffer and 20 µl of the sample 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).
Void wells are filled with Dilution Buffer.
N.B. The pate design does not allow the use of the IL-6 standard.

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.