Validation of Biomedical Testing Methods

In vitro test for pyrogen/endotoxin using human whole blood

22 07 02

Standard Operating Procedure

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Standard Operating procedure
In vitro *pyrogen test using human whole blood*

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TABLE OF CONTENTS ***Page No.***

1 INTRODUCTION ***5***
2 PURPOSE ***5***
3 SCOPE /LIMITATIONS ***5***
4 METHOD OUTLINE ***5***
5 DEFINITIONS ABBREVIATIONS ***5***
6 MATERIALS ***5***
7 METHODS ***5***
7.1. STEPS PRIOR TO BLOOD–CULTURE 5
7.1.1. Preparation of aliquots of the LPS (endotoxin) standard (STD)[at] 5
7.1.2. Preparation of aliquots of the IL–6 standard[at] 5
7.1.3. Coating of IL–6 ELISA plates 5
7.1.4. Preparation of samples for testing[at] 5
7.1.5. Collection of human blood 5
7.1.6. Storage of blood 5
7.1.7. Equilibration of reagents for cell culture 5
7.1.8. Preparation of the LPS standard curve[at] 5
7.2. BLOOD CULTURE[at] 5
8 DATAANALYSIS ***5***
9 HEALTH SAFETY AND ENVIRONMENT ***5***
10 ANNEX ***26***
11 REFERENCES ***5***

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, or leukocytes) and the conditioned media assayed for pyrogenic cytokines (Duff & Atkins, 1982; Dinarello et al., 1984; Poole et al., 1988, 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. Human whole blood produces cytokines in response to pyrogen/LPS (Desch et al., 1989; Finch–Arietta and Cochran, 1991; Hartung and Wendel, 1996) and *in vitro* pyrogen tests using human whole blood are being evaluated in a number of laboratories, particularly in Germany (Hartung and Wendel, 1996; Fennrich et al., 1999) and South Africa (Pool et al., 1998). The major differences between these whole blood *in vitro* pyrogen tests are the cytokine chosen as the readout (measured variable), the duration of incubation of the product (or LPS standard) with the blood, and the inclusion/omission of interferonγ as co–stimulus (priming agent). The preferred readout is IL–1β (Hartung and Wendel, 1996; Fennrich et al., 1999) or IL–6 (Pool et al., 1998), although TNFα also would appear to be suitable (Desch et al., 1989; Finch–Arietta and Cochran, 1991; Hartung and Wendel, 1996). The duration of incubation is determined to some extent by convenience but also by the choice of readout. A short

(4h) incubation of (diluted) blood with a very large dose of LPS (10 μg/ml, i.e. some 100000 IU/ml) stimulated the production of IL–1β (and TNFα) whereas a longer incubation (24h) of (diluted) blood was required for much smaller doses of LPS (1 pg/ml and above) to stimulate the production of IL–1β (and TNFα, Hartung and Wendel, 1996). A lengthy incubation (18 h), in the presence of interferonγ, was also favoured when IL–6 was the readout (Pool et al., 1998).

Our previous work with monocytes/monocytic cells pointed to the merits of IL–6 as the readout because IL–6, unlike IL–1β and TNFα, is secreted entirely into the cell–conditioned medium, in large quantities, permitting its complete estimation (Poole et al., 1989; Taktak et al., 1991).

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 whole blood/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 ANNEX.

This SOP is optimised for the detection of bacterial endotoxins. An alternative SOP, SOP.WBT.NEP.NIBSC.030702, is optimised for the detection of endotoxin and non-endotoxin pyrogens. SOP.WBT.NEP.NIBSC.030702 is to be used to test products suspected of contamination with non-endotoxin pyrogens.

4 METHOD OUTLINE

Freshly taken human whole blood is heparinised, diluted with saline and 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 concentration of released IL–6 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)
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
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
PBMNC	peripheral blood mononuclear cells
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
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 (Life Technologies)
HSA, 1% (a dilution in sterile PF saline of clinical grade HSA, 4.5%)
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 peroxidase 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)
Phenol (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 standards for bacterial endotoxin (LPS, vial code 94/580)
Fragmin (Dalteparin, 10000 IU/ml, Pharmacia)
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
IL–6 from human lymphocytes (Boehringer Mannheim, Cat. No. 1299972)

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

Buffers for the NOVARTIS IL–6 ELISA:

Coating Buffer

Dissolve
5.0 g of sodium dihydrogen phosphate and
2.9 g of disodium hydrogen phosphate
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	12.1 g
Dissolve in distilled water	400 ml
Add Kathon MW/WT	0.1 ml

Use 4 M HCl to adjust the pH to 7.5.

Albumin from bovine serum	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

Dilution Buffer

Prepare the dilution buffer as follows:

Tris(hydroxymethyl)aminomethane	2.1 g
Distilled water	400 ml
Kathon MW/WT,	0.1 ml
Phenol	0.5 g
Heat–inactivated (30 minutes at +56°C) foetal 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

Prepare the TMB solution as follows:

3,3',5,5'Tetramethylbenzidine	240 mg
Reagent–grade acetone	5 ml

Dissolve, then add

Reagent–grade ethanol	45 ml
Perhydrol (30 % H_2O_2)	0.3 ml

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

Substrate Buffer

Reagent–grade citric acid monohydrate	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.

7 METHODS

7.1. STEPS PRIOR TO BLOOD–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.

7.1.1. 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), open the vial, reconstitute the contents of the vial with 5 ml PFDW 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 or frozen in aliquots immediately after reconstitution. 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.

To prepare aliquots of the IS for endotoxin:

Aliquot 150 µl of LPS standard into labelled cryotubes (of 2 ml capacity), freeze them upright and store them in this frozen state at –20°C or below (shelf life = 12 months). Label the cryotubes with the following information:

Endotoxin STD 94/580
300 IU in 150 µl
date of reconstitution
initials of the operator

7.1.2. 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), open the vial 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 20 µl of 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). Label the cryotubes with the following information:

IL–6 STD 89/548
20 ng in 20 µl
date of reconstitution
initials of the operator

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

7.1.3. 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).

7.1.4. Preparation of samples for testing[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.

7.1.5. 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), and to have been free from the symptoms of any such infection for a period of one week prior to the donation of blood. Blood donors are not to be taking non–steroidal anti–inflammatory drugs, immunosuppressants, glucocorticoids or any other drugs known to influence the production of cytokines. Also, See Section 8. (below) for the criteria for the rejection of data. Take blood donations from at least three donors because the preparation being examined is required to pass the test with blood donations from at least three different donors.

***Procedure*:** Using a 30 ml syringe and a 40 mm, 21 gauge hypodermic needle, draw 30 ml blood from the median cubital or cephalic vein of the left or right arm of a single donor.

Immediately remove the hypodermic needle and transfer the blood into a 50 ml sterile, pyrogen–free centrifuge tube (e.g. Falcon 2070 Blue Max) containing 10 IU in 10 μl heparin (Fragmin, 1000 IU/ml, prepared by diluting Fragmin, 10000 IU/ml, Pharmacia, 1/10 with saline) for each ml of blood to be collected, e.g. for a blood sample of 30 ml the tube will contain 300 IU/ml heparin, giving 10 IU heparin/ml of blood.

Screw the lid of the tube on tightly and invert slowly five times to ensure thorough mixing of the blood with the heparin. Do not vortex.

(N.B. The blood collection procedure is non-critical. Pyrogen-free heparin may be substituted for Fragmin and a proprietary blood collection device/system may be substituted provided that it is of a type shown to be pyrogen-free.)

7.1.6. Storage of blood

Store the tube upright at room temperature and stimulate with LPS within 4 h of its collection.

7.1.7. Equilibration of reagents for cell culture

Bring an aliquot of the LPS standard, the samples for testing and a bottle of saline to room temperature.

7.1.8. 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 and an LPS standard calibrated against the USP Reference Standard Endotoxin or the IS for endotoxin may be used.)

7.2. BLOOD CULTURE[at]

7.2.1. Add 50 μl of saline the wells of columns 1 – 10 as in Template 1, see below.

7.2.2. Gently mix the blood donation, using the Procedure below, immediately before aliquots of the blood are taken. Do not vortex the blood.

Procedure: Immediately after gently swirling the blood, pour a sample of about 5 ml into a smaller tube, e.g. a screw–top bottle of 7 ml capacity. Screw the lid on the tube and invert twice immediately before aliquots of the blood are taken. Replenish the blood in the smaller tube, as required, with further samples of blood, each taken immediately after gentle swirling of the blood donation.


ECVAM

7.2.3. Add 50 µl of blood to the wells of columns 1 – 10 as in Template 1, see below.

Using a pipette with a tip of wide diameter the blood is added by row to the wells of columns 1 – 10 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.

Template 1: addition of blood to blood culture plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	First row of ten wells (A1–A10) to which blood is added										Void	Void
B	Third row of ten wells (B1–B10) to which blood is added										Void	Void
C	Fifth row of ten wells (C1-C10) to which blood is added										Void	Void
D	Seventh row of ten wells (D1–D10) to which blood is added										Void	Void
E	Second row of ten wells (E1–E10) to which blood is added										Void	Void
F	Fourth row of ten wells (F1–F10) to which blood is added										Void	Void
G	Sixth row of ten wells (G1–G10) to which blood is added										Void	Void
H	Eighth row of ten wells (H1–H10) to which blood is added										Void	Void

7.2.4. Add 50 µl of LPS standards to wells as in Template 2, 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.)

7.2.5. Add 50 µl of test samples S1 – S14 to wells, as in Template 2, see below.

7.2.6. Add 100 µl of saline to the wells of columns 1 – 10 as in Template 2, see below.

7.2.7. Gently mix the contents of the wells without cross–contaminating wells.

7.2.8. 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 2: addition of standards and samples to the blood culture 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	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	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 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).

7.3. IL–6 ELISA

Immunoreactive IL–6 in aliquots of the tissue culture fluid 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.

The NOVARTIS ELISA for IL–6 is described below. When it is not practicable to use the NOVARTIS ELISA, a different validated ELISA may be substituted, e.g. the MILIENIA IL-6 ELISA kit, CLB IL–6 ELISA kit, R&D IL-6 ELISA kit or an ELISA using matched pairs of anti–IL–6 MAbs purchased from R&D Systems or BioSource.

7.3.1. Equilibration of reagents

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

7.3.2. 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, of an aliquot of the stock solution of the current IS (or working STD for IL–6 calibrated against the IS). The diluent is PBS/1% BSA (or HSA – not critical).
(RPMI–C –see PBMNC SOP – may be substituted for PBS/1% BSA)
When using the IS for IL–6, label nine tubes H – P. Add the specified volumes of PBS/1% BSA to the tubes – see table 2 below.

Add 180 µl PBS/1% BSA 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 from an aliquot of the IS for IL–6

Tube	IL–6 added	PBS/1 % BSA	[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.

7.3.3. 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.

At the end of the tissue culture incubation, carefully transfer 50 µl 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 and ensure that cells are not aspirated by supporting the assay plate such that it slopes down at an angle of about 45° from column 1 to column 12.

7.3.4. Addition of standards

Add 50 µl of IL–6 standards to the wells in columns 11 and 12, as shown in Template 3, below.

Solution P into wells A11 and A12 (0 pg/ml IL–6 STD)
Solution O into wells B11 and B12 (62.5 pg/ml IL–6 STD)
Solution N into wells C11 and C12 (125 pg/ml IL–6 STD)
Solution M into wells D11 and D12 (250 pg/ml IL–6 STD)
Solution L into wells E11 and E12 (500 pg/ml IL–6 STD)
Solution K into wells F11 and F12 (1000 pg/ml IL–6 STD)
Solution J into wells G11 and G12 (2000 pg/ml IL–6 STD)
Solution I into wells H11 and H12 (4000 pg/ml IL–6 STD)

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

Template 3: 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 11	R4 2	R5 4	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 = 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).

7.3.5. 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 h 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.

7.3.6. Addition of substrate solution and reading of optical densities

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

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 microtiter plates with a clean tissue, then measure the absorbance at 450 nm in an ELISA plate reader using a 540–590 nm corrective filter. Subtract the values of the measurement with the corrective filter from values measured with the 450 nm filter.

8 DATA ANALYSIS AND PREDICTION MODELS

Preparatory testing

Preparatory tests are conducted to assure that the criteria for validity and precision for the standard curve are satisfied and that the solution of the preparation being examined (test article) 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 preparation being examined that is likely to influence the result of the test.

Validation of the test method

Using the standard endotoxin solution, prepare five endotoxin concentrations (R1–R5) to generate the standard curve (R0 = 0 EU/ml). Perform the test using four replicates of each standard endotoxin solution. The standard curve is to satisfy the criteria for linearity and range described in the *ICH HARMONISED TRIPARTITE GUIDELINE: VALIDATION OF ANALYTICAL PROCEDURES METHODOLOGY* (ICH guideline Q2B Validation of Analytical procedures: methodology, November 1996), together with a restriction on test precision (see below).

Assurance criteria for the standard curve

Reject the data from any test that does not satisfy all four criteria specified below.

(i) The (basal) release of IL–6 in the absence of added LPS, i.e. for the 0 IU/ml dose of LPS, is to be < 200 pg/ml IL–6 (20 IU IL–6/ml).

(The reason for this is as follows. Blood cultures in the absence of added endotoxin release amounts of (immunoreactive) TNFα and IL–1β close to or below the detection limits of the ELISAs used to detect them. In contrast, concentrations of immunoreactive IL–6 are detectable in these cultures. For healthy donors, concentrations of immunoreactive IL–6 are below 200 pg/ml (20 IU IL–6/ml, typically 50 pg/ml or less) but in donors who have recently recovered from minor viral or bacterial infections basal (i.e. unstimulated) concentrations of IL–6 can exceed 200 pg/ml. These findings accord with reports that plasma concentrations of immunoreactive IL–6 (unlike those of TNFα and IL–1β) are likely to be increased in individuals not in good health (Buck et al., 1994; Lin and Huang, 1998; Otto et al., 1999). Consequently, the choice of IL–6 as the readout (measured variable) enables the identification and 'screening out' (albeit retrospectively) of donors who are not in good health, even though they may feel well enough to serve as a blood donor for the test. Based on historic data obtained at NIBSC, a 'cut–off' value of 200 pg/ml IL–6 (20 IU IL–6/ml) for basal immunoreactive IL–6 release is deemed appropriate for the rejection of test data as having come from a test utilising blood from a donor not in good health.)

(ii) Compare the values for the four replicates each of 0 and 0.25 IU/ml endotoxin STD: the three smallest values are to be in the response to 0 IU/ml and the three largest values are to be in the response to 0.25 IU/ml (Wilcoxon rank-sum test, $p<0.05$).

(iii) Compare the values for the four replicates each of the endotoxin STDs 0.25 IU/ml and 0.5 IU/ml: the three smallest values are to be in the response to 0.25 IU/ml and the three largest values are to be in the response to 0.5 IU/ml (Wilcoxon rank-sum test, $p<0.05$).

(iv) Compare the values for the four replicates each of 0 and 0. 5 IU/ml endotoxin STD: the four smallest values are to be in the response to 0 IU/ml and the four largest values are to be in the response to 0.5 IU/ml (Wilcoxon rank-sum test, $p<0.025$).

Interference test

Spike an aliquot of the test solution of the preparation being examined, the test article (1), with an endotoxin concentration at or near the middle dose of the endotoxin standard curve. *[Alternatively, to facilitate comparisons with other test systems, a different spike, e.g. 0.5 IU/ml, may be used, provided that this is within one contiguous (adjacent) dilution of the middle dose 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. The target is 100% spike recovery. If necessary, test doubling dilutions of the test article, not exceeding the MVD (2), to identify the minimum dilution that gives spike recovery as close to 100% as can be achieved. Subsequent testing is to be performed at this dilution, which must give at least 50% spike recovery, or at one further two-fold dilution (3) of the test article, provided that the further dilution is within the MVD.

(1) the test article is to contain the lowest level of contamination with pyrogen/endotoxin that practicably can be achieved for that preparation.

(2) MVD = the maximum valid dilution of a preparation at which the endotoxin limit concentration (ELC) can be determined. MVD = ELC/the second smallest dose in the endotoxin STD curve.

(3) the further dilution is to mitigate variations in LOD, donors/passages, and the balance between interference by the test article and its pyrogenic content.)

(N.B. Testing at minimum dilution of the test article would be expected to increase the probability of detecting non-endotoxin pyrogens since, in general, these dilute out more rapidly than endotoxin. In this regard it should be noted that the interference testing described above, in common with the rest of this SOP, is optimised for the detection of bacterial endotoxins. A number of changes to this SOP would be required to optimise it for the detection of non-endotoxin pyrogens. Consequently a different protocol is used to test products suspected of contamination with non-endotoxin pyrogens.)

Quantitative estimation of endotoxin equivalents and uncertainty of measurement

Where quantitative estimates of endotoxin-equivalents are required, statistical analysis is carried out in Informatics to provide estimates of endotoxin concentrations for test samples in terms of the endotoxin standard curve using the methods of parallel line assay analysis (see for example, Finney, 1978).

The upper and lower OD response values for the endotoxin standard curve are determined graphically and the OD values for endotoxin standards and test samples are transformed to logit responses and analysed using the in house program WRANL (Gaines Das and Tydeman, 1982). The linearity mean square for the endotoxin standard curve (calculated by the program) provides a measure of the suitability of the graphically determined limits for the curve. The pyrogenic contamination in the solution of the preparation being examined (i.e. test article) is calculated, with confidence intervals, from a standard curve of the IS for endotoxin (which is calibrated in IU) and expressed in endotoxin-equivalents/ml. This value is compared with the endotoxin limit concentration (ELC) for the preparation. Where the ELC is not specified for a product, it is calculated as described in *10. ANNEX*. The preparation being examined complies with the test if the estimated mean endotoxin concentration of the preparation, after correction for dilution and concentration, is less than the endotoxin limit for the preparation. The preparation being examined is required to pass the test with blood donations from at least three different donors.

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

Limit test

This is a simplified test to detect whether or not a solution of the preparation being examined (test article), after correction for dilution and concentration, contains less than 0.5 IU/ml (the threshold dose in the rabbit pyrogen test). This is carried out as follows.

(a) Perform an interference test with an endotoxin spike of 0.5 IU/ml, as described above.

(b) Measure responses to 4 replicates each of 0, 0.25 and 0.5 IU/ml endotoxin and the test article.

(c) Reject the data from any test that does not satisfy all four criteria specified below.

(i) The (basal) release of IL–6 in the absence of added LPS, i.e. for the 0 IU/ml dose of LPS, is to be < 200 pg/ml IL–6 (20 IU IL–6/ml).

(ii) Compare the values for the four replicates each of 0 and 0.25 IU/ml endotoxin STD: the three smallest values are to be in the response to 0 IU/ml and the three largest values are to be in the response to 0.25 IU/ml (Wilcoxon rank-sum test, $p<0.05$).

(iii) Compare the values for the four replicates each of the endotoxin STDs 0.25 IU/ml and 0.5 IU/ml: the three smallest values are to be in the response to 0.25 IU/ml and the three largest values are to be in the response to 0.5 IU/ml (Wilcoxon rank-sum test, $p<0.05$).

(iv) Compare the values for the four replicates each of 0 and 0. 5 IU/ml endotoxin STD: the four smallest values are to be in the response to 0 IU/ml and the four largest values are to be in the response to 0.5 IU/ml (Wilcoxon rank-sum test, $p<0.25$).

(d) Compare the values for the four replicates each of the test article and the LPS STD 0.5 IU/ml: the four smallest values are to be in the response to the test article and the four largest values are to be in the response to 0.5 IU/ml (Wilcoxon rank-sum test, $p<0.25$). The preparation being examined is required to pass the test with blood donations from at least three different donors.

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 NIBSC Safety Compendium.

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

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. ANNEX

LOD. Detection limit. The LOD is to take into account all dilution factors.

LOQ. Quantification limit. The LOQ is to take into account all dilution factors.

[N.B. The calculation of LOD and LOQ is addressed in the ICH HARMONISED TRIPARTITE GUIDELINE: VALIDATION OF ANALYTICAL PROCEDURES METHODOLOGY, ICH guideline Q2B Validation of Analytical procedures: methodology, November 1996. It should be noted that the methods described in the guideline, in common with other methods used to calculate LOD and LOQ, make assumptions about the distribution of data that may not apply.]

Calculation of ELC

ELC = Endotoxin limit concentration for the preparation being examined. 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.

Maximum valid dilution

The MVD is the maximum allowable, i.e. 'valid', dilution of a preparation at which the endotoxin limit concentration (ELC) can be determined. MVD = ELC/the second smallest dose in the endotoxin STD curve.

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