Validation of Biomedical Testing Methods

Human Whole Blood Pyrogen Test

Standard Operating Procedure for the Validation Phase

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JOINT RESEARCH CENTRE
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Human Whole Blood Pyrogen Test

Standard Operating Procedure

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1. INTRODUCTION

The whole blood pyrogen test (in vitro pyrogen test IPT) is a two-part assay for the detection of pyrogenic contamination. It involves incubation of the sample with human blood, followed by an enzyme immunoassay for the measurement of IL-1b.

A pyrogen is a substance that causes fever. Bacterial contaminations, which contain exogenous pyrogens, can be deadly. This problem is of great significance for drug safety.

Also, medical devices and biologically produced substances obtained from bacteria and other microorganisms may cause release of endogenous pyrogens (e.g., IL-1b).

Exogenous pyrogens include metabolic substances and cell-wall components of microorganisms. These substances are present during the "normal" course of an infectious disease. Infections by gram-negative and gram-positive bacteria are equal in frequency. Both of these bacterial types can activate the release of endogenous pyrogens, which cause fever through the thermoregulatory center in the brain. Although these reactions can occur during the "normal" course of an infectious disease, a deadly shock syndrome can occur in the worst case.

Due to these risks, product safety legislation demands rigorous quality checks for pyrogenic contamination of drugs and devices intended for parenteral use. For example, testing in rabbits for medical end products is required in Germany. Products in development and a few end products are allowed to be controlled by the Limulus assay. The first pyrogen assay, based on human whole blood stimulation by pyrogens, was developed by Hartung et al. (3,4).

2. PURPOSE

This assay simulates *in vitro* the normal human reaction to exogenous pyrogens. A few drops of human blood are mixed with the sample, and exogenous pyrogens in the sample are recognized by immunocompetent cells in the human blood.
These cells release IL-1b, which is measured by an integrated ELISA system.

3. SCOPE / LIMITATIONS

Limit of detection is ≤ 0,25 EEU/ml, not suitable for test samples interfering with blood cytokine release.

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4. METHOD OUTLINE

The procedure has two parts:

1. Incubation of the sample with (diluted) human blood
2. An enzyme immunoassay for the measurement of IL-1b.

Ad 1) Blood incubation

Diluted human whole blood is incubated for 10-24 hours together with saline and the sample in pyrogen-free reaction tubes and the supernatant is taken off for further examination.

Ad 2) Capture of Endogenous Pyrogens (ELISA procedure)

Samples (supernatants of blood stimulation) are distributed into the wells of a microplate which are coated with monoclonal antibodies specific for IL-1b.

An enzyme-conjugated polyclonal antibody against IL-1b is added. During a 90-minute incubation, a sandwich complex consisting of two antibodies and the IL-1b is formed. Unbound material is removed by a wash step.

A chromogenic substrate (3,3´,5,5´ -tetramethylbenzidine, TMB) reactive with the enzyme label is added. Color development is terminated by adding a stop solution after 30 minutes. The resulting color, read at 450 nm, is directly related to the IL-1b concentration. Bi-chromatic measurement with a 600-690 nm reference filter is recommended.

5. DEFINITIONS / ABBREVIATIONS

The following abbreviations are used in this work-book.

Ab	antibody
°C	degrees Celsius (Centigrade)
EC	endotoxin control
EEU	endotoxin equivalent unit
ELISA	Enzyme-Linked ImmunoSorbent Assay
EU	endotoxin unit of the international standard
h	hour
H_2SO_4	sulphuric acid
IL	interleukin
LPS	lipopolysaccharide (exogenous pyrogen from Gram-negative bacteria)
LTA	lipoteichoic acid (exogenous pyrogen from Gram-positive bacteria)
µl	microlitre
mg	milligram
ml	millilitre
min	minute
MVD	maximum valid dilution
NaCl	sodium chloride, 0,9%
nm	nanometre
NPC	negative product control
PPC	positive product control
OD	optical density
rpm	rounds per minute
RT	room temperature
TMB	3,3´,5,5´-Tetramethylbenzidine
WDB	wash/dilution buffer
x g	x gravity

6. MATERIALS

6.1. Materials required and not provided

The components listed below are recommended, but equivalent devices may also be used: it is the users responsibility to validate the equivalence.
For all steps excluding the ELISA procedure sterile and pyrogen-free materials have to be used (e.g. tips, containers, solutions).

6.1.1 Materials for Blood Incubation

Equipment
· Incubator or thermoblock (37°C ± 1°C)
· Multipette or adjustable 100 to 1000 µl pipetters
· Centrifuge (recommended)
· Vortex mixer

Consumables
· Heparinized tubes for blood sampling (e.g. Sarstedt S-MONOVETTE 7.5 ml, 15 IU/ml Li-Heparin)
· Sarstedt multifly needle set, pyrogenfree, for S-Monovette
· 1.5 ml closable, pyrogen-free reaction tubes
· Reservoir for saline
· 12 ml (PS)or 15 ml (PP) tubes from greiner bio-one for dilution of substances
· Sterile and pyrogen-free tips 100 µl and 1000 µl **or**
· Combitips for multipette, 10 ml and 2,5 ml for pipetting saline and blood

6.1.2 Materials for ELISA procedure

Equipment

· Multichannel pipettor

· Microplate mixer

· Microplate washer

· Microplate reader capable of readings at 450 nm (optional reference filter in the range of 600-690 nm)

· A software package for facilitating data generation, analysis, reporting, and quality control

Consumables

· Graduated cylinder and plastic storage container for Buffered Wash Solution

· Tip-Tubs for reagent aspiration with Multichannel pipettor

6.2. Materials Supplied in ELISA kit

Components supplied in that kit are *not* interchangeable with other lots of the same components.

IL-1b Ab-coated Microplate: One 96-well polystyrene microplate, packaged in a zip-lock foil bag, with desiccant. The plate consists of twelve strips mounted in a frame. Each strip includes eight anti-IL-1b Ab-coated wells. Additionally, individual wells can be separated from the strip to enable the complete use of all the wells of a kit. Well positions are indexed by a system of letters and numbers (A through H, 1

through 12) embossed on the left and top edges of the frame. Store refrigerated: stable at 2-8°C until the expiration date marked on the label.

Enzyme-Labeled Antibody : One amber vial containing 16 ml of liquid reagent, ready-to-use. The reagent contains horseradish peroxidase-labeled, affinity-purified, polyclonal (rabbit) anti-IL-1b antibodies, with preservative. Store refrigerated: stable at 2-8°C for 30 days after opening, or until the expiration date marked on the label. *Do not freeze.*

Endotoxin Control: One vial of an endotoxin control. The control is supplied lyophilized. Store refrigerated: stable at 2-8°C until the expiration date marked on the label. At least 30 minutes before use, reconstitute control vial with saline. Prepare serial dilutions in saline (see 7. Methods). Mix by vortexing. After preparation, the stock solution can be stored (see 7. Methods).

Saline: Three glass vials, each containing pyrogen-free saline. This is intended for the dilution of donor blood samples and for reconstitution of the Endotoxin Control. Store refrigerated: stable at 2-8°C until the expiration date marked on the label. Use immediately after opening and discard unused volumes.

TMB/Substrate Solution: Two amber vials, each containing 11 ml of a buffered reagent, ready-to-use. The reagent contains a hydrogen peroxide substrate and 3,3´,5,5´-tetramethylbenzidine (TMB). Store refrigerated and protected from light: stable at 2-8°C until the expiration date marked on the label. *Do not freeze*.

Buffered Wash Solution Concentrate: One vial containing 75 ml of a concentrated (10X) buffered saline solution, with surfactants and preservative. Using a transfer container, dilute the contents of the vial with **675 ml** distilled or deionized water for a total volume of **750 ml**.

Store refrigerated: stable at 2-8°C for 7 days after preparation, or until the expiration date marked on the label.

Stop Solution: One vial containing an acidic solution, for terminating the color reaction. The reagent is supplied ready-to-use. Handle with care, using safety gloves

and eye protection. Store refrigerated: stable at 2-8°C for 8 weeks after opening, or until the expiration date marked on the label.

7. METHODS

7.1. Blood Incubation

Blood Collection

Collect blood by venipuncture into heparinized tubes. The blood collection system must be pyrogen-free. The procedure calls for 100 µl of heparinized whole blood per reaction tube. The blood can be stored in the collection tube at room temperature (15-28°C) for 4 hours. Incubation of the sample should be started within this time.

Note:

1. Blood donors should show no evidence of disease or need of medication during the last two weeks.
2. Each assay should include the Endotoxin Controls and the saline control in quadruplicate.
3. Use disposable tip pipets to avoid contamination of reagents and samples.
4. During ELISA procedure, the wells should be washed carefully.
5. The test samples should be done in quadruplicate.
6. The contents of the wells must be decanted or aspirated completely before pipetting wash solution.
7. Deviations from the procedure (incubation time/temperature) may cause erroneous results. The ELISA procedure should be run without interruption. Diluted samples should be tested within an hour.

Storage of the substances

- please keep all substances at 4°C

Spiking of the substances

Part 1)

5 blinded spikes have been sent out by PEI

They are bearing a code for

a) the respective drug
b) the test method, in this case WBT-KN
c) a random blinding number

- please pipet 500 µl of the respective substance into an Eppendorf tube
- vortex the respective vial with the blinded spike for about 5 seconds
- add 25 µl of the spike to the substance and vortex for another 5 seconds
- perform the dilutions according to the instructions below

Dilution of the substances

- for dilution, please use either 12 ml or 15 ml tubes from greiner bio-one
- each substance has to be vortexed for about 5 seconds immediately before performing Step 3 of the Whole Blood Stimulation.

Substance 1: Glucose 5%

Maximum valid dilution =1:75; add 40 µl of substance to 2960 µl of saline

Substance 2: EtOH 13%

Maximum valid dilution = 1:37.5 ; add 80 µl of substance to 2920 µl of saline

Substance 3: MCP

Maximum valid dilution: 1:375; add 8 µl of substance to 2992 µl of saline

Substance 4: Orasthin

Maximum valid dilution: 1:750: add 4 µl of substance to 2996 µl of saline

Substance 5: Binotal

Maximum valid dilution: 1:150; add 20 µl of substance to 2980 µl of saline

Substance 6: Fenistil

Maximum valid dilution: 1:187.5; add 16 µl of substance to 2984 µl of saline

Substance 7: Sostril

Maximum valid dilution: 1:150; add 20 µl of substance to 2980 µl of saline

Substance 8: Beloc

Maximum valid dilution: 1:150; add 20 µl of substance to 2980 µl of saline

Substance 9: Drug A

Maximum valid dilution: 1:37.5; add 80 µl of substance to 2920 µl of saline

Substance 10: Drug B

Maximum valid dilution: 1:75; add 40 µl of subsubstance to 2960 µl of saline

Part 2)

(unblinded)

- Positive Product Control (PPC)

dilute the respective substance according to the instructions above

vortex for about 5 seconds

pipet 500 µl of the diluted substance into an Eppendorf tube

add 25 µl of the unblinded PPC-LPS spike handed out by PEI

- Negative Product Control (NPC)

dilute the respective substance according to the instructions above

vortex for about 5 seconds

pipet 500 µl of the diluted substance into an Eppendorf tube

add 25 µl of saline

Endotoxin dilution for the Dose-Response Curve

IPT assays must include the 0.5 EU/ml + saline control in quadruplicate.

Dissolve the contents of the vial containing O113 provided by NIBSC with 5 ml of saline yielding a stock solution of 2000 EU/ml.

EC = Endotoxin Control, for use in the assay.

Solution	amount added to saline	Volume of saline	Resulting solution
Stock (2000 EU/ml)	100 µl	900 µl	200 EU/ml
200 EU/ml	100 µl	900µl	20 EU/ml
20 EU/ml	100 µl	900 µl	2 EU/ml
2 EU/ml	500 µl	500 µl	1 EU/ml (EC)
1 EU/ml	500 µl	500 µl	0,5 EU/ml (EC)

The stock solution of the Endotoxin Standard may be aliquoted (e.g. 100 µl aliquots) and kept at –20 °C for up to 6 months.

Whole Blood Stimulation

Perform incubation of blood samples in 1.5 ml pyrogen-free reaction tubes. Preferably, use a laminar-flow bench**.** All consumables and solutions have to be sterile and pyrogen-free.

Step 1: Draw up an incubation plan according to the template below

Step 2: Add **1000 µl** saline into each reaction tube.

Step 3: Add **100 µl** of Endotoxin Controls and negative saline control or samples in quadruplicate into the respective reaction tubes according to the prepared incubation plan.

Step 4: Add **100 µl** of donor blood, mixed by gentle inversion, into each reaction tube.

Step 5: Close the tubes and invert them once or twice before starting the incubation.

Step 6: Incubate the closed reaction tubes in an incubator or a heating block overnight (10-24 hours) at 37°C ± 1°C.

Step 7: Mix the incubation tubes thoroughly by inverting the tubes. Incubations are to be centrifuged for 2 minutes at 10.000 g and the clear supernatant is used for the ELISA procedure. Take aliquots of ≥ **150 µl.**

The supernatants can be tested immediately by the ELISA System or may be stored at -20°C for testing at a later time.

Freeze additional aliquots.

7.2. ELISA Procedure

All components must be at room temperature (15-28°C) before use. Do *not* thaw frozen specimens by heating them in a waterbath. The ELISA is carried out at room temperature.

1 For control of the ELISA procedure, the stimulation supernatants of the Endotoxin Controls (EC) are used.

2 Sample distribution: see Microplate Template below.

A	NPC (A)	NPC (A)	PPC (A)	PPC (A)	PPC (A)	PPC (A)	1 (B)	1 (B)	1 (B)	1 (B)	2 (C)	2 (C)
B	NPC (A)	NPC (A)	1 (A)	1 (A)	1 (A)	1 (A)	2 (B)	2 (B)	2 (B)	2 (B)	2 (C)	2 (C)
C	EC 1,0	EC 1,0	2 (A)	2 (A)	2 (A)	2 (A)	3 (B)	3 (B)	3 (B)	3 (B)	3 (C)	3 (C)
D	EC 1,0	EC 1,0	3 (A)	3 (A)	3 (A)	3 (A)	4 (B)	4 (B)	4 (B)	4 (B)	3 (C)	3 (C)
E	EC 0,5	EC 0,5	4 (A)	4 (A)	4 (A)	4 (A)	5 (B)	5 (B)	5 (B)	5 (B)	4 (C)	4 (C)
F	EC 0,5	EC 0,5	5 (A)	5 (A)	5 (A)	5 (A)	NPC (C)	NPC (C)	NPC (C)	NPC (C)	4 (C)	4 (C)
G	saline	saline	NPC (B)	NPC (B)	NPC (B)	NPC (B)	PPC (C)	PPC (C)	PPC (C)	PPC (C)	5 (C)	5 (C)
H	saline	saline	PPC	PPC	PPC	PPC	1	1	1	1	5	5

			(B)	(B)	(B)	(B)	(C)	(C)	(C)	(C)	(C)	(C)

A, B, C : e.g. Substances 1, 2, 3

1-5 : blinded spikes 1-5

EC : Endotoxin Control

NPC: negative product control

PPC: positive product control

3 Add **100 µl** Enzyme-Labeled Antibody to every well

4 Pipet **100 µl** of supernatants of Endotoxin Controls, those of the negative saline control and of the samples into the wells prepared.

Use a disposable-tip micropipet for the samples, changing the tip between each sample and control, to avoid contaminations.

5 Mix for **90 minutes** on a microplate mixer at 350-400 rpm.

6 Decant, then wash. For assays using centrifuged blood supernatants, wash each well 4 times with **300 µl** Buffered Wash Solution. For assays using resuspended blood, wash 5 to 6 times with 300 µl per well.

If this step is performed manually, remove as much moisture as possible during the decanting by inverting the washed microplate and tapping out the residual washing buffer on blotting paper or a paper towel, being careful not to dislodge the strips from the frame.

7 Add **200 µl** of TMB/Substrate Solution to every well.

8 Incubate without shaking for **30 minutes** in the dark.

9 Add **50 µl** of Stop Solution to every well.

*Tapping the plate **gently** after the addition of Stop Solution will aid mixing and improve precision. The Stop Solution is acidic.*

Handle carefully, and use safety gloves and eye protection.

10 Read at 450 nm, **within 15 minutes** of adding Stop Solution. Bi-chromatic measurement with a reference wavelength of 600-690 nm is recommended.

MINIMUM ASSAY SUITABILITY REQUIREMENTS

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

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

8. HEALTH SAFETY AND ENVIRONMENT

· For *in vitro* use only.
· Do not use reagents beyond their expiration dates.

Bio-Safety

Human blood has to be considered infectious and handled accordingly.

Stop Solution and TMB/Substrate Solution

Avoid contact with the Stop Solution, which is acidic. Wear gloves and eye protection. If this reagent comes into contact with skin, wash thoroughly with water and seek medical attention, if necessary. The reagent is corrosive; therefore, the instrument employed to dispense it should be thoroughly cleaned after use. The TMB/Substrate Solution contains peroxide. Since peroxides are strong oxidizing agents, avoid all bodily contact with the TMB/Substrate Solution

9. ANNEX (Pipetting scheme for the whole blood assay)

Part 1: Whole blood stimulation (all values in µl)

Tube account	Stimulation sample	saline	Endotoxin Control (0.5 – 1.0 EU/ml)	Test sample	Donor blood		
4	Endotoxin Control (0.5 – 1.0 EU/ml)	1000	100	-	100	Incubate overnight at 37°C	Mix the samples. Centrifuge for 2 minutes at 10000 x g (if necessary). Take 150 µl from the supernatant. Test immediately with the ELISA system or store at -20 °C.
4	Blank (0)	1100			100		
4	Test samples (1-8)	1000	-	100	100		

Part 2: ELISA procedure (all values in µl)

Well	Supernatants from Stimulation	Enzyme-labeled Antibody		Substrate		Stop solution	
G/H 1/2 (Blank)	100	100	Incubate 90 min at RT on a plate mixer at 350-400 rpm. Decant.Wash 4 times with 300 µl Buffered Wash Solution	200	Incubate 30 min at RT	50	Read at 450 nm (600-690 nm reference wave-length recom-mended)
EC: B-F 1/2	100	100		200		50	
Samples: see template schedule	100	100		200		50	

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