Validation of Biomedical Testing Methods

Human Whole Blood Pyrogen Test

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

Identity

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

Version number: _____
Applicable from: _____
Expired at: _____

Drafted by:	*Name* *Date* *Signature*	*Thomas Hartung,* *28/02/02*	*M. D. Ph.D.*
Reviewed by:	*Name* *Date* *Signature*		
Approved by:	*Name* *Date* *Signature*		
Issued by:	*Name* *Date* *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.:
01.04.01				
29.06.01		10		
20.09.01		2, 4, 5, 6, 8, 10, 11, 12, 13, 14 16		
14.12.01		2, 6, 8, 9, 10, 11		
23.02.02		2, 5, 6,7, 8, 9, 10,11		
28.2.02		2, 6, 7, 10, 12		

3

TABLE OF CONTENTS Page no.

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,

Institute for Health and Consumer Protection

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 (see 8: Data analysis and associated errors).

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. It is then centrifuged 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

Institute for Health and Consumer Protection

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
ESS	Endotoxin Stabilizing Solution
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)
l	litre
µg	microgram
µl	microlitre
mg	milligram
ml	millilitre
min	minute
MAb	monoclonal antibody
NaCl	sodium chloride, 0,9%
nm	nanometre
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

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 user's 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

A. Tube method

Equipment
· Incubator or thermoblock (37°C ± 1°C)
· Multipette
· Centrifuge (recommended)
· Vortex mixer

Consumables
· Sterile and pyrogen-free tips 100 µl and 1000 µl
· 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
· Combitips for multipette, 10 ml and 2,5 ml for pipetting saline and blood
· Non-pyrogenic borosilicate test tubes or other qualified materials that can be used for the preparation of standards and for the dilution of samples.

B. Microtiter plate method

Equipment
·Incubator or thermoblock (37°C)
·Multipette or adjustable 20 to 100 µl pipetters
·Vortex mixer

Consumables
·Sterile and pyrogen-free tips 20 µl and 100 µl or
·Combitips for multipette, 2,5 and 1,0 ml
·Heparinized tubes for blood sampling(e.g. Sarstedt S-MONOVETTE 7.5 ml, 15 IU/ml Li-Heparin)
7

Institute for Health and Consumer Protection

·Sarstedt multifly needle set, pyrogen-free, for S-Monovette
·Non-pyrogenic tissue culture microtiter plate
·Reservoir for saline
·Non-pxrogenic borosilicate test tubes or other qualified materials that can be used for preparing standards and diluting samples

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

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. Mix thoroughly before use. *Do not freeze.*
Endotoxin Stabilizing Solution: for reconstitution and dilution of the endotoxin control.
Endotoxin Control: One vial of an endotoxin control in a buffer matrix, with preservative. 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 ESS (see 7. Methods).
8

Institute for Health and Consumer Protection

Mix by vortexing. After preparation, the stock solution can be stored for up to 1 week hours at 4 °C.
LTA control: One vial of LTA control. The control is supplied lyophilized. Store refrigerated at 2-8°C until the expiration date. Before use, reconstitute the lyophilisate with 1 ml saline. Mix for at least a minute by vortexing. After preparation, the solution can be stored for up to 4 weeks at 4°C.
Saline: Three glass vials, each containing pyrogen-free saline. This is intended for the dilution of donor blood samples. 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 freez*e.
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 30 days after preparation, or until the expiration date marked on the label.
For longer storage aliquot and freeze: stable at -20°C for 6 months.
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.
Adhesive Microplate Covers: Two clear plastic adhesive covers. Remove backing and place over the top of the microplate during incubation to avoid evaporation.

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 assay. 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.
9

2 Each assay should include the Endotoxin Controls in duplicate (EC 0.5 in triplicate) and the saline control in triplicate.
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 triplicate.
· The contents of the wells must be decanted or aspirated completely before pipetting wash solution.
· The wells should be covered during the incubation to avoid evaporation.
6 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.

Endotoxin dilution

NOTE:
Quantitative IPT assays may use endotoxin concentrations of 5.0, 2.5, 1.0, 0.5 and 0.25 EU/ml + saline control in triplicate.

Qualitative IPT assays (threshold assays) should use the 0.5 EU/ml + saline control in triplicate.

Dissolve the contents of the vial with ESS according to directions stated in the Certificate of Analysis, yielding a stock solution = solution S

Solution	amount added to ESS	Volume of ESS	Resulting solution for use in blood incubation
Stock (5IU/ml)	500 µl	500 µl	Endotoxin Control (2,5 EEU/ml)
Endotoxin Control (2,5 EEU/ml)	400 µl	600µl	Endotoxin Control (1,0 EEU/ml)
Endotoxin Control (1,0 EEU/ml)	500 µl	500 µl	Endotoxin Control (0.5 EEU/ml)
Endotoxin Control (0.5 EEU/ml)	500 µl	500 µl	Endotoxin Control (0.25 EEU/ml)

LTA dilution

Reconstitute the vial with 1 ml saline. Mix by vortexing for 3 minutes.

10

Whole Blood Stimulation-test tube method

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.

With some substances, interference with the ELISA may occur. Therefore, it might be necessary to test the samples in different dilutions.

Step 1: add **1000 µl** saline into each reaction tube.

Step 2: add **100 µl** of each sample into the prepared reaction tubes or **100 µl** of the Endotoxin Control in duplicate (EC 0.5 in triplicate) and the negative control (saline) in triplicate.

Step 3: add **100 µl** of donor blood, mixed by gentle inversion, into each reaction tube.

Step 4: Close the tubes and invert them once or twice before starting the incubation.

Step 5: Incubate the closed reaction tubes in an incubator or a heating block overnight (10-24 hours) at 37°C ± 1°C.

Step 6: 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.

Interference testing (PPC)

For each new sample, to determine whether it requires dilution prior to assay, perform the following experiment in triplicate.
The experiment checks for interference between the sample and the whole blood, and is needed only when the interference status of the sample has not yet been established. First assay 100 µl of the sample, undiluted, in combination with saline, Endotoxin Control (0.5 EEU) and whole blood, as follows

Step 1: add **900 µl** of saline into each tube

Step 2: add **100 µl** of (diluted) sample

Step 3: add **100 µl** of 1,0 EU/ml Endotoxin control
11

Step 4: add **100 µl** of donor blood, mixed by gentle inversion

Continue with Step 4 of Whole Blood Stimulation- test tube method procedure.

Whole blood stimulation- microtiter plate method

Step 1: Using a non-pyrogenic tissue culture treated microtiter plate, draw up an incubation plan designating the layout of endotoxin controls (i.g. 4x3), negative saline controls (3x), Gram-positive control (3x) and your samples (3x) in your assay (corresponding to template)

Step 2: Pipet 200 µl negative saline control into each of the reaction wells that will be used for the standards (endotoxin and Gram-positive control) and samples.

Step 3: Add 20 µl of endotoxin controls, Gram-positive control, negative saline control or samples into their respective reaction wells according to the prepared incubation plan.

Step 4: Add 20 µl of whole blood to all reaction wells.

Step 5: Cover with the dedicated plastic plate cover and mix thoroughly on a microtiter plate mixer.

Step 6: Transfer the mixed microtiter plate to a 37°C incubator for an overnight incubation (10 to 24 hours).

Step 7: Following the overnight incubation, remove plate from incubator place onto a plate mixer. Mix until all bloods cells have been re-suspended.

Step 8: The re-suspended blood mixtures may be ELISA tested for IL-1ß immediately or stored frozen at –20°C for testing at a later time (at least 150 µl).

Interference testing (PPC)

For each new sample, to determine whether it requires dilution prior to assay, perform the following experiment in triplicate.
The experiment checks for interference between the sample and the whole blood and is needed only when the interference status of the sample has not yet been established. First assay 20 µl of the sample, undiluted, in combination with saline, Endtoxin Control (1,0 EU/ml) and whole blood, as follows

12

Institute for Health and Consumer Protection

Step 1: Pipet **180 µl** of saline into the wells used for interference testing

Step 2: Pipet **20 µl** of (diluted) sample into each well

Step 3: Pipet **20 µl** of 1,0 EU/ml Endotoxin Control into the wells

Step 4: Pipet **20 µl** of donor blood into the wells

Continue with Step 5 of the Whole Blood Stimulation-microtiter plate method procedure.

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) and the LTA control are used. P1, P2, etc. are the stimulation supernatants of the test probes.

2 Sample distribution: see Microplate Template below (quantitative assay)

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC 5,0	EC 5.0	EC 5.0	P02	P04	P07	P10	P12	P15	P18	P20	P23
B	EC 2,5	EC 2.5	EC 2.5	P02	P05	P07	P10	P13	P15	P18	P21	P23
C	EC 1.0	EC 1.0	EC 1.0	P02	P05	P08	P10	P13	P16	P18	P21	P24
D	EC 0.5	EC 0.5	EC 0.5	P03	P05	P08	P11	P13	P16	P19	P21	P24
E	EC 0.25	EC 0,25	EC 0.25	P03	P06	P08	P11	P14	P16	P19	P22	P24
F	saline	saline	saline	P03	P06	P09	P11	P14	P17	P19	P22	P25
G	LTA	LTA	LTA	P04	P06	P09	P12	P14	P17	P20	P22	P25
H	P 01	P 01	P01	P04	P07	P09	P12	P15	P17	P20	P23	P25

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

3 Pipet **100 µl** of supernatants of Endotoxin Controls, LTA control, those of the

13

negative (saline) control and of the samples into the wells prepared.
Use a disposable-tip micropipet for the samples, changing the tip between samples, to avoid contaminations.
5 Cover the plate and mix for **90 minutes** on a microplate mixer.
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; this will greatly enhance precision. A technical Data Sheet describing the procedure in detail is available on request.
Before adding the TMB/Substrate solution, tap the plate face down on adsorbant paper to shake off all residual droplets, 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

8. DATA ANALYSIS AND ASSOCIATED ERRORS

The Endotoxin Controls and a negative control (saline) should routinely be assayed in each run.
The assay should be considered acceptable only if the following criteria are met:

.

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

The OD of the PPC satisfies the requirement stated in the Interference testing for products.

Interference testing for products

A. Quantitative IPT assay:

There is no interference if

0.5 x the median $EC_{1,0}$ < median interference test < 2x median $EC_{1,0}$
14

If the median assay result (in terms of OD) falls outside the 50 to 200% range of the median of the incubation of the 1,0 EU/ml control in the absence of sample, repeat the experiment using 100 µl of *diluted* sample until it yields an OD reading inside the range. (Dilute the sample with saline, e.g. 1:10, 1:100, etc.). Samples exhibiting interference should be assayed at the lowest dilution not causing interference.

B. Threshold IPT assay:

There is no interference if

0.5 x the median EC $_{0,5}$ median interference test < 2x median EC $_{0,5}$

Interpretation

A. Quantitative IPT assay
???????????

B. Threshold IPT assay (rabbit equivalent test)

The results are given as *positive* or *negative* (non-pyrogenic). A sample is considered positive if the mean OD of the sample is equal or greater than the mean OD of the 0.5 EU/ml standard.

9. PREDICTION MODEL

Rabbits are likely to develop fever if tested with 10ml/kg of the sample if

$$OD_{Sample} > OD_{mean\ (0.5\ EEU)}$$

10. 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.
This kit contains components of human origin which, when tested by FDA-approved
15

methods, were found non-reactive for hepatitis B surface antigen and for HIV antibody. No known tests can guarantee, however, that products derived from human blood will not be infectious. Handle, therefore, as if capable of transmitting infectious agents.
.

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

16

Institute for Health and Consumer Protection

11. 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 EEU)	Endotoxin Control (1 EEU)	Endotoxin Control (2 EEU)	LTA control	Test sample	Donor blood	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.
3	Endotoxin Control (0.5 EEU)	1000	100	-	-		-	100		
2	Endotoxin Control (1 EEU)	1000	-	100	-		-	100		
2	Endotoxin Control (2 EEU)	1000	-	-	100		-	100		
2	LTA control	1000				100		100		
3	Blank (0)	1100						100		
3	Interference test,	900	100	-	-		100 (diluted)	100		
3	Test samples 1, 2, 3	1000	-	-	-		100	100		

3

Part 2: Procedure (all values in µl)

Well	Supernatants from Stimulation	Enzyme-labeled Antibody		Substrate		Stop solution	
D2, E2, F2 (Blank)	100	150	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
EC: see template schedule	100	150		200		50	
G1, G2 (LTA control)	100	150		200		50	
Samples: see template schedule	100	150		200		50	

4

12. REFERENCES

1 Hartung T., Wendel A. : Detection of pyrogens using human whole blood. In Vitro Toxicology; 9(4): 353-59.
2 Fennrich S., Fischer M., Hartung T., Lexa P., Montag-Lessing T., Sonntag H.-G., Weigand M. und Wendel A. : Detection of endotoxins and other pyrogens using human whole blood . Dev Biol Stand.Basel , 1999, 101:131-39.
3 Hartung T., Aaberge I., Berthold S., Carlin G., Charton E., Coecke S., Fennrich S., Fischer M., Gommer M., Halder M., Haslov K., Montag-Lessing T., Poole S., Schechtman L., Wendel A. und Werner-Felmayer, G. : ECVAM workshop on novel pyrogen tests based on the human fever reaction. ATLA 2001, 29:99-123.
4 Morath S., Geyer A., Hartung T. : Structure-function relationship of cytokine induction by lipoteichoic acid from Staphylococcus aureus. J. Exp. Med., 2001, 193:393-397.
5 Bonenberger J., Diekmann W., Fennrich S., Fischer M., Friedrich A., Hansper M., Hartung T., Jahnke M., Löwer J., Montag-Lessing T., Petri E., Sonntag H.-G., Weigand M., Wendel A. und Zucker B. : Pyrogentestung mit Vollblut-Zusammenfassung eines Status-Workshops am Paul-Ehrlich-Institut, Langen, am 22.11.99. Bundesgesundheitsbl-Gesundheitsforsch-Gesundheitsschutz 2000, 43:525-533.
6 Petri E., van de Ploeg A., Habermaier B. and Fennrich S.: Improved detection of pyrogenic substances on polymer surfaces with an ex vivo human whole-blood assay in comparison to the Limulus amoebocyte lysate test. In: Balls M., van Zeller A.-M. and Halder M.: Progress in the reduction, refinement and replacement of animal experimentation. Elsevier 2000, 339-345.
7 Fennrich S., Wendel A. and Hartung T.: New applications of the human whole blood pyrogen assay (PyroCheck). ALTEX 1999, 16:146-149.