

**THE HUMAN WHOLE BLOOD/IL-1
IN VITRO PYROGEN TEST
(CRYO WB/IL-1)**

Use of cryopreserved human whole blood

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1 Rationale for the Proposed Test Method

1.1 Introduction

1.1.1. Describe the historical background for the proposed test method, from original concept to present. This should include the rationale for its development, an overview of prior development and validation activities, and, if applicable, the extent to which the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards.

Pyrogens, a chemically heterogeneous group of hyperthermia- or fever-inducing compounds, derive from bacteria, viruses, fungi. Subjects react to such microbial products during an immune response by producing endogenous pyrogens such as prostaglandins and the pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) (Dinarello, 1999). Depending on the type and amount of pyrogen challenge and the sensitivity of an individual, even life-threatening shock-like conditions can be provoked. To assure quality and safety of any pharmaceutical product for parenteral application in humans, pyrogen testing is therefore imperative.

Depending on the medicinal product, one of two animal-based pyrogen tests is currently prescribed by the respective Pharmacopoeias, i.e. the rabbit pyrogen test and the bacterial endotoxin test (BET). For the rabbit pyrogen test, sterile test substances are injected intravenously to rabbits and any rise in body temperature is assessed. This *in vivo* test detects various pyrogens but not alone the fact that large numbers of animals are required to identify a few batches of pyrogen-containing samples argues against its use. In the past two decades, the declared intention to refine, reduce and replace animal testing, has lowered rabbit pyrogen testing by 80% by allowing to use the BET as an *in vitro* alternative pyrogen test for certain medicinal products (Cooper et al, 1971).

Bacterial endotoxin, comprising largely lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria that stimulates monocytes/macrophages via interaction with CD14 and toll-like receptor 4 (TLR4) (Beutler and Rietschel, 2003), is the pyrogen of major concern to the pharmaceutical industry due to its ubiquitous sources, its stability and its high pyrogenicity. With the BET, endotoxin is detected by its capacity to coagulate the amoebocyte lysate from the haemolymph of the American horseshoe crab, *Limulus polyphemus*, a principle recognised some 40 years ago (Levin and Bang, 1964). In the US, *Limulus* crabs are generally released into nature after drawing about 20% of their blood and therefore most of these animals survive. However, the procedure still causes mortality of about 30,000 horseshoe crabs per year, which adds to even more efficient threats of the horseshoe crab population like its use as bait for fisheries, habitat loss and pollution (<http://www.horseshoecrab.org/>).

As with the rabbit test the general problem of translation of the test results to the human fever reaction persists. Moreover, although being highly sensitive, the failure of the BET to detect non-endotoxin pyrogens as well as its susceptibility to interference by e.g. high protein or lipid levels of test substances or by glucans impedes full replacement of the rabbit pyrogen test. Hence, hundreds-of-thousands rabbits per year are still used for pyrogen testing.

A test system that combines the high sensitivity and *in vitro* performance of the BET test with the wide range of pyrogens detectable by the rabbit pyrogen test is therefore required in order to close the current testing gap for pyrogen and to avoid animal-based tests. With this intention and due to improved understanding of the human fever reaction (Dinarelo, 1999), test systems based on *in vitro* activation of human monocytes were developed. First efforts date back about 20 years, when peripheral blood mononuclear cells (PBMC) were used to detect endotoxin by monitoring the release of pyrogenic cytokines (Duff & Atkins, 1982; Dinarelo et al, 1984). Meanwhile, a number of different test systems, using either whole blood, peripheral blood mononuclear cells (PBMCs) or the monocytoïd cell lines MONO MAC 6 (MM6) or THP-1 as a source for human monocytes and various read-outs were established (Poole et al., 1988; Ziegler et al, 1988; Tsuchiya et al, 1980; Hartung & Wendel, 1996; Hartung et al, 2001; Poole et al, 2003). These test systems were validated with the aim of developing a tool for formal inclusion into Pharmacopoeias, an important basis for implementing novel alternative pyrogen tests for product-specific validation.

1.1.2 *Summarize and provide the results of any peer review conducted to date and summarize any ongoing or planned reviews.*

All of the five methods are currently under peer review of the ECVAM Scientific Advisory Committee.

1.1.3 *Clearly indicate any confidential information associated with the test method; however, the inclusion of confidential information is discouraged.*

This document does not contain any confidential information.

1.2 Regulatory rationale and applicability

1.2.1 *Describe the current regulatory testing requirement(s) for which the proposed test method is applicable.*

To assure quality and safety of pharmaceutical products for parenteral application in humans, pyrogen testing is imperative. Depending on the drug, one of two pyrogen tests is currently prescribed by the European Pharmacopoeia, i.e. the rabbit pyrogen test and the bacterial endotoxin test (BET), and other national and international guidelines.

1.2.2 *Describe the intended regulatory use(s) (e.g., screen, substitute, replacement, or adjunct) of the proposed test method and how it will be used to substitute, replace, or complement any existing regulatory testing requirement(s).*

Dependent on the product and the presence of relevant clinical data on unexpected pyrogenicity of clinical lots, the proposed test method may be an alternative method for pyrogen testing, thus substituting the rabbit pyrogen test or the BET. In certain cases, the proposed test method may function as a supplementary test method to assess compliance to the licensing dossier.

In case the proposed test method is an alternative for pyrogenicity testing, a thorough cross-validation between the proposed test method and the original method for the specific medicinal product is warranted. In case the proposed test method is an adjunctive test to screen for (unexpected) pyrogenic lots, alert and alarm limits may be established based on consistency of production lots or (preferably) based on actual clinical data.

1.2.3 *Where applicable, discuss the similarities and differences in the endpoint measured in the proposed test method and the currently used in vivo reference test method and, if appropriate, between the proposed test method and a comparable validated test method with established performance standards.*

The current *in vivo* method (rabbit test), as described in the pharmacopoeia, and the proposed *in vitro* test method each determine very different end-points, though the biochemical origins of the response are similar.

The *in vivo* method more resembles a black box, and determines the total rise in body temperature (fever induction) of the animals subjected to the medicinal product, as a result of pyrogens (if any) present in the product.

The proposed test method CRYO WB/IL-1 is an *in vitro* model for the fever response mechanism. It determines the release of cytokines by monocytoïd cells into the culture medium upon the interaction of pyrogens and specific receptors on the monocytoïd cells. It is these cytokines that trigger the fever response *in vivo*.

Main differences between the *in vivo* and *in vitro* methods are that the latter is quantitative and uses cells of human origin, thus better reflecting the physiological situation.

1.2.4 *Describe how the proposed test method fits into the overall strategy of hazard or safety assessment. If a component of a tiered assessment process, indicate the weight that should be applied relative to other measures.*

The proposed test method CRYO WB/IL-1 may be applied for those medicinal products for which the rabbit test is the only or most reliable method for pyrogenicity testing, since a) the medicinal product is not compatible with the BET or b) the medicinal product contains pyrogens other than Gram-negative endotoxin.

Limit concentrations for pyrogens are established based on consistency lots or actual clinical data or, in the case of endotoxin the endotoxin limit concentration (ELC) as defined for many medicinal products.

1.3 Scientific basis for the proposed test method

1.3.1 *Describe the purpose and mechanistic basis of the proposed test method.*

The proposed *in vitro* method is intended to determine the presence of pyrogens in medicinal products for parenteral use. The proposed test method is an *in vitro* model of the human fever response. It determines the release of cytokines upon the interaction of pyrogens and specific Toll-like receptors on the monocytoïd cells (Beutler and Rietschel, 2003). These cytokines trigger the fever response *in vivo*.

1.3.2 *Describe what is known and not known about the similarities and differences of modes and mechanisms of action in the proposed test method as compared to the species of interest (e.g., humans for human health-related toxicity testing).*

An important feature of the proposed test method is that it is based upon the use of monocytoïd cells of human origin. It therefore by definition resembles more closely the actual response of humans. The two other test methods make use of either crustaceans (BET) or rabbits, both species more or less distinct from the human species. The response of humans, horseshoe crabs and rabbits toward Gram-negative endotoxin has been

studied extensively and the methods appear equivalent for this particular pyrogen (Cooper et al 1971; Greisman and Hornick, 1969). However, there are documented cases of medicinal products and specified pyrogenic substances that yield false-positive or false-negative results in either test method. Since the proposed test method is based on human cells, it may therefore predict more accurately the pyrogenicity of such substances in humans.

1.3.3 Describe the intended range of substances amenable to the proposed test method and/or the limits of the proposed test method according to chemical class or physicochemical factors.

The proposed test method is intended for the assessment of pyrogens in all parenteral medicinal products for human use, chemical or biological and including raw materials, bulk ingredients and excipients. Use of the proposed test method in testing environmental samples or medicinal products is suggested and may be feasible, but substantiating data are as yet limited or absent.

2 Test Method Protocol Components

2.1 Overview of test method.

Provide an overview of how the proposed test method is conducted. If appropriate, this would include the extent to which the protocol for the proposed test method adheres to established performance standards.

A highly detailed protocol describing the proposed test method (*Detailed protocol CRYO WB/IL-1: Human Whole Blood Pyrogen Test in 96-well Plates Using Fresh or Cryopreserved Blood; electronic file name: SOP CRYO WB IL 1*) is attached in Appendix A of this Background Review Document (BRD). It covers three variations to the preparation of the whole blood described under point 7: 7A - fresh blood using 96-well plates, 7B - cryopreserved blood according to the so called "PEI (Paul-Ehrlich-Institute, Langen, Germany) method" and 7C - cryopreserved blood according to the so called "Konstanz method".

The present BRD refers to the variation 7B; whereas the results with variation 7A are included in Section 13 of BRD WB/IL-1 and variation 7C in Section 13 of this BRD.

The CRYO WB/IL-1 test method is a two-part assay for the detection of pyrogenic contamination. The test protocol itself can be divided into the following two parts:

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

Ad 1.

Diluted human whole blood is incubated overnight (10-24 hours) together with saline or RPMI and the sample of interest in sterile and pyrogen-free microtiter plate and aliquots are taken for further examination.

Ad 2.

Samples (aliquots of blood stimulation) are distributed into the wells of a microtiter plate which are coated with monoclonal antibodies specific for IL-1 β .

An enzyme-conjugated polyclonal antibody against IL-1 β is added. During a 90-minute incubation, a sandwich complex consisting of two antibodies and the IL-1 β 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-1 β concentration. Bi-chromatic measurement with a 600-690 nm reference filter is recommended.

The WHO-LPS standard (code 94/580, E.coli O113:H10:K-), was used throughout the validation. This standard is identical to USP Reference Standard Endotoxin (EC6).

There are several possibilities to estimate the pyrogenic contamination of the preparations under test: 1) A quantitative estimation can be achieved by the construction of a dose-response curve for endotoxin standard (e.g. 5.0, 2.5, 1.0, 0.5 and 0.25 EU/ml) versus

optical density (OD) value of the IL-1 β ELISA. The contamination of the preparations is expressed in endotoxin–equivalent units. 2) A qualitative test can be achieved by the inclusion of an endotoxin threshold control (e.g. one fixed dilution of the standard curve) which allows for the classification in positive and negative samples (i.e. pyrogenic and non-pyrogenic samples). 3) A qualitative test can also be achieved by inclusion of an appropriate positive product control.

A detailed description of analysis methods used during the validation of the test-method can be found in section 5 of the current BRD.

2.2 Rational for selected test components

Provide a detailed description and rationale, if appropriate, for the following aspects of the proposed test method:

2.2.1 Materials, equipment, and supplies needed.

The materials, equipment and supplies used for the CRYO WB/IL-1 test method are laboratory items that will be already available in a routine QC laboratory. There is no need for sophisticated or dedicated laboratory equipment throughout the test.

For all steps in the procedure, excluding the ELISA procedure, the materials (e.g. tips, containers, solutions) which will be in close contact with samples and blood cells need to be sterile and pyrogen free. The materials, equipment and supplies are specified in the protocol given in Appendix A. It should be realized that equivalent devices may also be used and it is the user's responsibility to validate the equivalence.

Materials for part 1: Blood Incubation with cryopreserved blood

Equipment

- Incubator (37°C + 5%CO₂)
- Multipette or adjustable 20 to 100 μ l pipetters
- Multicannel pipettor 8 or 12 channels)
- Vortex mixer
- Laminar flow bench (recommended)

Consumables

- Non-pyrogenic 96-well polystyrene tissue culture microtiter plate (Falcon, 353072)
- Sterile and pyrogen-free tips 20 and 100 μ l.
- Combitips for multipette, 1.0 ml and 0.5 ml
- Reservoir for RPMI and saline
- Non-pyrogenic test tubes, preferably 12 or 15 ml centrifuge tubes (Greiner)

Materials for part 2: 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 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
- Non-sterile pipette tips
- Non-sterile deionized water
- The IL-1 β -ELISA kit (commercially obtained), containing:
 - IL-1 antibody coated micoplates.
 - Enzyme labeled antibody. Horseradish peroxidase-labeled, affinity-purified, polyclonal(rabbit) anti-IL-1 β antibodies.
 - Endotoxin control.
 - Saline
 - TMB/Substrate solution
 - Buffered Wash Solution Concentrate (saline solution, with surfactants and preservative)
 - Stop Solution (acidic solution)

2.2.2 Dose-selection procedures, including the need for any dose range-finding studies or acute toxicity data prior to conducting a study, if applicable.

For every kind of test compound the interference with human blood and the IL-1 β ELISA kit is determined. For this purpose, a preliminary “dose finding” test is conducted to establish a suitable (interference free) dilution for every new test compound. For the validation study (as described in section 4 of this BRD), the tested products were diluted according to their known ELC, which was usually far beyond interfering concentrations. The ELCs of the tested products or drugs were calculated according to the European Pharmacopoeia.

2.2.3 Endpoint(s) measured.

The proposed test method is an *in vitro* model of the fever response mechanism. It determines the release of interleukin-1 β (IL-1 β) by monocytoïd cells present in human blood. IL-1 β is released into the culture medium upon the interaction of pyrogens and specific receptors on the monocytoïd cells. The measured endpoint IL-1 β is one of the cytokines that trigger the fever response *in vivo*.

2.2.4 Duration of exposure.

The cryopreserved human whole blood is exposed to possible pyrogenic components in samples overnight (10-24 hours) in an incubator at 37°C + 5% CO₂. The monocytoïd cells produce endogenous pyrogens if triggered by pyrogens. Before transferring the stimulation aliquots onto the IL-1 β ELISA plate, the contents of the wells are thoroughly mixed.

2.2.5 Known limits of use.

The CRYO WB/IL-1 method described in the method protocol is not a finalized test system for the testing of all medicinal products. The method may be applied only to preparations that have been validated with this method, i.e. shown not to interfere with the blood and the IL-1 β readout system at a specified dilution of the preparation. A paragraph describing the interference testing is included in the protocol (see Appendix

A). However, at this moment there are no medicinal products known that cannot be tested with the method.

2.2.6 Nature of the response assessed.

The proposed test method is an *in vitro* model of the fever response mechanism. Upon the interaction of exogenous pyrogens and specific receptors on the monocytoïd cells endogenous pyrogens (e.g. interleukins, TNF- α and prostaglandins) are produced. In the body the fever response is triggered by these endogenous pyrogens. Immunoreactive IL-1 β , the measured endpoint for the current method, is one of these endogenous pyrogens.

2.2.7 Appropriate vehicle, positive, and negative controls and the basis for their selection.

Throughout the development and validation phase the test compounds are diluted in 0.9% (w/v) clinical saline. This 0.9% clinical saline is considered an appropriate vehicle as no interference with active substances of a drug is to be expected.

In addition the test includes several controls.

A negative control: 0.9% clinical saline (sodium chloride)

A positive control: WHO-LPS 94/580, 0.5 EU/ml in clinical saline.

A negative product control: clean, released batch for each drug.

A positive product control: test item spike with WHO-LPS (code 94/580) at 0.5 EU/ml

The positive and negative controls are the same in every assay and are needed to establish the sensitivity of the test system. In addition, a product-based set of controls is used to reveal product-related interference.

2.2.8 Acceptable range of vehicle, positive and negative control responses and the basis for the acceptable ranges.

A CRYO WB/IL-1 assay is considered acceptable for further analysis if the mean OD value of the positive control (0.5 EU/ml) exhibits an OD that is greater than 1.6 times the mean OD over the negative control (0.9% clinical saline). The mean OD of the PPC is at 1.6 times the mean OD of the NPC or greater. The mean OD of the PPC has to be in the 50-200% range of the mean OD of the 0.5 EU/ml endotoxin control. The mean OD of the negative saline control is at 0.1 OD or lower. Moreover the response to different concentrations of the positive control should show a dose response relationship. To be able to quantify the responses to the positive control this should be well within the maximum response that can be measured with the test system.

As regards the substances to be tested, for products with an established ELC, specified in EU/ml, the product is diluted to its maximum valid dilution (MVD). The negative product control should be negative at the MVD. The response to the positive product control should be between 50% and 200% of the response to the positive control, indicating a possible pyrogenicity can be detected using these conditions.

2.2.9 Nature of the data to be collected and the methods used for data collection.

The raw data collected are the read-outs (absorbance) of the IL-1 β ELISA, measured by an automated laboratory ELISA-plate reader. The wavelength is dependent on the chromogenic substrate applied, but when using TMB, the ELISA-plate is read at a

wavelength of 450 nm. Bi-chromatic measurement with a reference wavelength of 600-690 nm is recommended.

2.2.10 Type of media in which data are stored.

Data are stored in electronic files (windows98 compatible software) and as hard copy.

2.2.11 Measures of variability.

As part of the development of the WB/IL-1 test method (using fresh blood, see BRD WB/IL-1) the intralaboratory repeatability was assessed by independent and identical replicated measurement of the different concentrations of WHO-LPS. Furthermore, the limit of detection and its dependence from known but uncontrollable variables such as operator and blood donor were investigated. These variables and the inherent variation of biological systems make up to the total variation of the method. However, this part of the development was performed with fresh whole blood samples in reaction tubes. This part of the analysis of the variability was not repeated for the CRYO WB/IL-1 test method in 96-wells microtiter plates. However, while tested drugs spiked with LPS it is shown that the variability is comparable (one blinded experiment performed in three laboratories).

2.2.12 Statistical or nonstatistical methods used to analyze the resulting data, including methods to analyze for a dose-response relationship. Justify and describe the method(s) employed.

All experiments are run with four replicates of the test compound with cryopreserved blood from one batch on one plate. A standard curve in quadruplicate, using the International Standard for Endotoxin (calibrated in EU) is included. The assay should fulfill the minimum assay suitability requirements as detailed in the SOP. This includes the following criteria: The mean OD of the 0.5 EU/ml endotoxin control is at 1.6 times the mean OD of the negative saline control or greater. The mean OD of the PPC is at 1.6 times the mean OD of the NPC or greater. The mean OD of the PPC has to be in the 50-200% range of the mean OD of the 0.5 EU/ml endotoxin control.

The further analysis of the data was performed according to the procedure described in section 5.3 of the current CRYO WB/IL-1 test method.

2.2.13 Decision criteria and the basis for the prediction model used to classify a test chemical (e.g., positive, negative, or equivocal), as appropriate.

A prediction model (PM) was developed in order to classify substances as “pyrogenic for humans” or “non-pyrogenic for humans”. To be able to define a dichotome result in the alternative pyrogen test, a threshold pyrogen value of 0.5 EU/ml was chosen. This threshold value was based on historical data with rabbits (described in section 4.1). The suitability of the PM was assessed by testing substances which were artificially contaminated with endotoxin (substances are described in section 3.2 and 3.3).

The statistical approach, including quality criteria, is detailed in section 5.3

2.2.14 Information and data that will be included in the study report and availability of standard forms for data collection and submission.

Raw data were collected using a standard form. These were submitted to the quality department of ECVAM.

2.3 Basis for selection of this test method

Explain the basis for selection of the test method system. If an animal model is being used, this should include the rationale for selecting the species, strain or stock, sex, acceptable age range, diet, and other applicable parameters.

In view of the shortcomings of the rabbit pyrogen test and the BET test, *in vitro* pyrogen tests that utilize the exquisite sensitivity to exogenous pyrogen of monocytoïd cells have been proposed. In such tests, products are incubated with human cell and the conditioned media assayed for pyrogenic cytokines (Duff & Atkins, 1982; Dinarello et al., 1984; Poole et al., 1988; Poole et al, 1989; Hansen and Christensen, 1990; Taktak et al., 1991; Bleeker et al., 1994).

The human whole blood assay was developed as a real *in vitro* alternative to the rabbit pyrogen test. The basic idea was to mimic the fever reaction in humans. In general, the detection of exogenous pyrogens (e.g. endotoxin) by blood cells causes them to release endogenous pyrogens like IL-1, IL-6 and TNF α . These cytokines affect the thermal regulation centre in the brain and increase the body temperature by changing its set point. In the past, several test methods have been developed that use the sensitivity of human peripheral blood monocytes to exogenous pyrogens. In an attempt to increase the sensitivity of these tests the monocytes/leukocytes were isolated from whole blood. In addition, various cell lines, which retain monocytoïd characteristics, including the capacity to synthesize and secrete pyrogenic cytokines, have been studied.

However, the isolation of monocytes/leukocytes from whole blood as well as the maintenance of a cell-line is labour-intensive and time-consuming, technically sophisticated and requires expensive reagents. It is clear that using whole blood implies considerably simplified handling and that costs are limited. In an early stage of development of the assay, interleukin-1 α was most promising as the endogenous pyrogen used as the readout. In addition, a standardised version of the test in form of an interleukin-1 α kit is commercially available.

Fresh whole blood samples must be used with 4 hours after collection, which put logistic constraints on the WB/IL-1 assay. The method is limited by the availability of freshly drawn blood, putative safety concerns in the case of infected donors and interindividual donor differences. To overcome these limitations a method was developed and optimized to produce batches of cryopreserved blood that can be used directly after thawing without any washing steps. Applying cryopreserved blood (stored at -80°C or liquid nitrogen) considerably improves the flexibility of the WB/IL-1 test method.

2.4 Proprietary components

If the test method employs proprietary components, describe what procedures are used to ensure their integrity (in terms of reliability and accuracy) from "lot-to-lot" and over time. Also describe procedures that the user may employ to verify the integrity of the proprietary components.

T. Hartung and A. Wendel are named as inventors in 436518000 (USPTO) 04/28/05 - Test procedure with biological system - Preparations containing deep-frozen blood are

used for test procedures for determining blood response.

It is stated in the method protocol that components supplied in the IL-1 α - ELISA kit are not interchangeable with other lots of the same components. Including the appropriate positive and negative controls in each run ensures the reliability and accuracy of the CRYO WB/IL-1 test method. As a positive control a specified amount of the Endotoxin Standard is used. Minimum assay suitability requirements are set and are described in the SOP (also summarized in section 2.2.12 of this BRD).

2.5 Replicates

Describe the basis for the number of replicate and repeat experiments; provide the rationale if experiments are not replicated or repeated.

All experiments are run with four replicates of the test compound on one plate. Outliers are rejected only after checking according to the Grubbs test ($p > 0.05$). Four replicates is considered the minimal amount for the Grubbs test.

During the development phase of the WB/IL-1 test method, the intralaboratory reproducibility as well as the interlaboratory reproducibility of the WB/IL-1 test method was established by applying repeated experiments (described in WB/IL-1 BRD). The test method reliability (repeatability /reproducibility) was shown to be satisfactory while using freshly drawn blood. As only the fresh blood was replaced by cryopreserved blood it was considered feasible to accept this result for the CRYO WB/IL-1 as well. Therefore the accuracy of the CRYO WB/IL-1 while testing pharmaceutical substances (detailed in table 3.3.1) was established by performing single experiment in three participating laboratories (described in section 6 and 7). It is shown that accuracy of the CRYO WB/IL-1 method applying cryopreserved blood is indeed comparable with the WB/IL-1 applying freshly drawn blood. Appendix D includes data on the intralaboratory reproducibility of CRYO WB/IL-1 and KN CRYO WB/IL-1 (Section 13) during their development phase.

2.6 Modifications applied after validation

Discuss the basis for any modifications to the proposed test method protocol that were made based on results from validation studies.

The proposed test method protocol has not been modified during or after validation.

2.7 Differences with similar test methods

If applicable, discuss any differences between the protocol for the proposed test method and that for a comparable validated test method with established performance standards.
Not applicable.

3 Substances Used for Validation

3.1 Selection of substances used

Describe the rationale for the chemicals or products selected for use in the validation process. Include information on the suitability of the substances selected for testing, indicating any chemicals that were found to be unsuitable.

Selected test items were medicinal products available on the market. Released clinical batches were considered clean, i.e. containing no detectable pyrogens. To test the specificity, sensitivity and the interlaboratory reproducibility of the proposed test method, the products were spiked with pyrogen. For the present studies endotoxin (LPS) was selected as the model pyrogen, since it is well defined, standardized and readily available.

For the sensitivity and specificity the test items were assessed at their MVD. The MVD is the quotient of the ELC and the detection limit. The European Pharmacopoeia prescribes for various types of parenterals the amount of endotoxin that is maximally allowed in a medicinal product, i.e. the ELC, taking into consideration the dose, the route of administration and the dosing regimen of the product.

The aim of the study was to discriminate between negative and positive samples. Based on the selected pyrogen threshold value (see 4.1) and the PM applied, positive samples were defined as containing 0.5 EU/ml or more. Hence, to determine the MVD, the value of 0.5 EU/ml was defined as the detection limit.

Test items were assessed as such (negative product control), spiked with endotoxin at 0.5 IU/ml (positive product control) and after spiking with endotoxin at 5 levels (blinded samples). In addition a negative control (saline) and positive control (0.5 EU/ml in saline) were included to establish assay validity.

For interlaboratory reproducibility, the test items were tested, at a predefined dilution above the MVD, independently in 3 different laboratories, 3 times each. Based on the selected pyrogen threshold value (see 4.1) and the PM applied, positive samples were defined as containing 0.5 EU/ml or more. The test items were tested after spiking with endotoxin at four levels. For no other reasons but practical ones, i.e. availability of test materials, different test items were selected for this part of the validation study.

It was determined earlier whether candidate test items interfered with the outcome of the proposed test method. Interference was considered when the response of endotoxin in the diluted test item was below 50% or above 200% of the response of endotoxin in saline (spike-recovery). It was shown that none of the test items interfered with the assay at the selected dilutions (data not shown).

3.2 Number of substances

Discuss the rationale for the number of substances that were tested.

A total of 13 test items were selected for the validation study (see 3.3): 10 test items for determining sensitivity and specificity (table 3.3.1), 3 different test items for determining interlaboratory reproducibility (table 3.3.2). Test items and their spikes were

appropriately blinded by ECVAM before distribution to the participating testing facilities.

For sensitivity and specificity, each test item was tested after spiking at its individual MVD. Hence they each came with their own specific set of 5 endotoxin spike solutions: 0.0, 0.25, 0.5, 0.5 and 1.0 EU/ml. Simple logistics limited the amount of test items for this part of the validation study to 10. Since test items were assessed with 5 different endotoxin levels at 3 independent test facilities, this yielded a total of 150 data points, biometrically considered to be sufficient for further analysis.

For reproducibility each test item was spiked at 4 different levels (0.0, 0.0, 0.5 and 1.0 EU/ml) and tested at specified dilutions, 3 times at 3 laboratories.

3.3 Description of substances used

Table 3.3.1: Test items (parenteral drugs) used for determining sensitivity and specificity

Drug	code	Source	Agent	Indication	MVD (-fold)
Glucose 5% (w/v)	GL	Eifel	Glucose	nutrition	70
Ethanol 13% (w/w)	ET	B.Braun	Ethanol	diluent	35
MCP®	ME	Hexal	Metoclopramid	antiemetic	350
Orasthin®	OR	Aventis	Oxytocin	initiation of delivery	700
Binotal®	BI	Aventis	Ampicillin	antibiotic	140
Fenistil®	FE	Novartis	Dimetindenmaleat	antiallergic	175
Sostril®	SO	GlaxoSmithKline	Ranitidine	antiacidic	140
Beloc®	BE	Astra Zeneca	Metoprolol tartrate	heart dysfunction	140
Drug A*	LO	-	0.9% NaCl	-	35
Drug B*	MO	-	0.9% NaCl	-	70

*Drugs A and B were included as saline controls using notional ELCs.

Negative control: 0.9% clinical stock saline solution.

Positive control: WHO-LPS 94/580 (*E. coli* 0113:H10:K-), 0.5 EU/ml in clinical stock saline.

Table 3.3.2: Test items (parenteral drugs) used for determining reproducibility.

Drug	Source	Agent	Indication
Gelafundin®	Braun melsungen	Gelatin	Transfusion
Jonosteril®	Fresenius	Electrolytes	Infusion
Haemate®	Aventis	Factor VIII	Hemophilia

Negative control: 0.9% clinical stock saline solution.

Positive control: WHO-LPS 94/580 (*E. coli* 0113:H10:K-), 0.5 EU/ml in clinical stock saline.

3.4 Sample coding procedure

Describe the coding procedures used in the validation studies.

All test items are registered medicinal products and were obtained from a pharmaceutical supplier. Test items and endotoxin spiking samples were prepared, blinded where appropriate and coded under GLP. The blinding was performed by the quality assurance of ECVAM (Ispra, Italy). The Steinbein-Center InPut (University of Konstanz, Germany) was responsible for the preparation of samples and spikes and the subsequent shipment to each of the appropriate test facilities participating in the study.

For the sensitivity and specificity part of this study, test items and their respective spikes (5 per test item) were all blinded. For reproducibility testing, only the spikes (4) were blinded, the test items were not.

3.5 Recommended reference chemicals

For proposed test methods that are mechanistically and functionally similar to a validated test method with established performance standards, discuss the extent to which the recommended reference chemicals were tested in the proposed test method. In situations where a listed reference chemical was unavailable, the criteria used to select a replacement chemical should be described. To the extent possible, when compared to the original reference chemical, the replacement chemical should be from the same chemical/product class and produce similar effects in the in vivo reference test method. In addition, if applicable, the replacement chemical should have been tested in the mechanistically and functionally similar validated test method. If applicable, the rationale for adding additional chemicals and the adequacy of data from the in vivo reference test method or the species of interest should be provided.

The reference pyrogen material used was the international endotoxin standard WHO-LPS 94/580 (*E. coli* 0113:H10:K-). Where appropriate, the material was diluted in clinical saline solution (0.9%(w/v) sodium chloride). The saline was also used as negative control (blank).

4 *In vivo* Reference Data on Accuracy

4.1 Test protocol *in vivo* reference test method.

Provide a clear description of the protocol(s) used to generate data from the in vivo reference test method. If a specific guideline has been followed, it should be provided. Any deviations should be indicated, including the rationale for the deviation.

For ethical reasons, no rabbit pyrogen tests were performed for this study. However, Dr. U. Lüderitz-Püchel, Paul-Ehrlich Institute, Germany, kindly provided historical data, accumulated over several years, from 171 rabbits (Chinchilla Bastards). The respective pharmacopoeiae do not prescribe a rabbit strain for the *in vivo* pyrogen test, but Chinchilla rabbits are reported as a relatively sensitive strain for pyrogen testing.

The rabbits were injected with endotoxin and their rise in body temperature over the next 180 minutes was recorded (figure 4.1.1). From these data it was established that 50% of the rabbits got fever when treated with endotoxin at 5 EU/kg (Hoffmann et al, 2005a). Fever in rabbits is defined as a rise in body temperature over 0.55°C. On the basis of these historical animal data and corrected for the maximal volume allowed in rabbits, i.e. 10 ml per animal, a pyrogen threshold value of 0.5 EU/ml was defined for the PM in the proposed test method.

4.2 Accuracy

Provide the in vivo reference test method data used to assess the accuracy of the proposed test method. Individual human and/or animal reference test data, if available, should be provided. Provide the source of the reference data, including the literature citation for published data, or the laboratory study director and year generated for unpublished data.

As mentioned, no animal studies were done for ethical reasons. However, a theoretical assumption on specificity and sensitivity can be made (Hoffmann et al, 2005a). Taking the prevalence of the different final concentrations of LPS in the 5 spikes into account (1.0 EU/ml: 20%; 0.5 EU/ml: 40%; 0.25 EU/ml: 20% and 0.0 EU/ml: 20%) and calculating probabilities of misclassification using the chosen study design and defined threshold of pyrogenicity, i.e. 0.5 EU/ml, the theoretical sensitivity of the rabbit pyrogen test is 57.9% and the theoretical specificity of the rabbit pyrogen test is 88.3%.

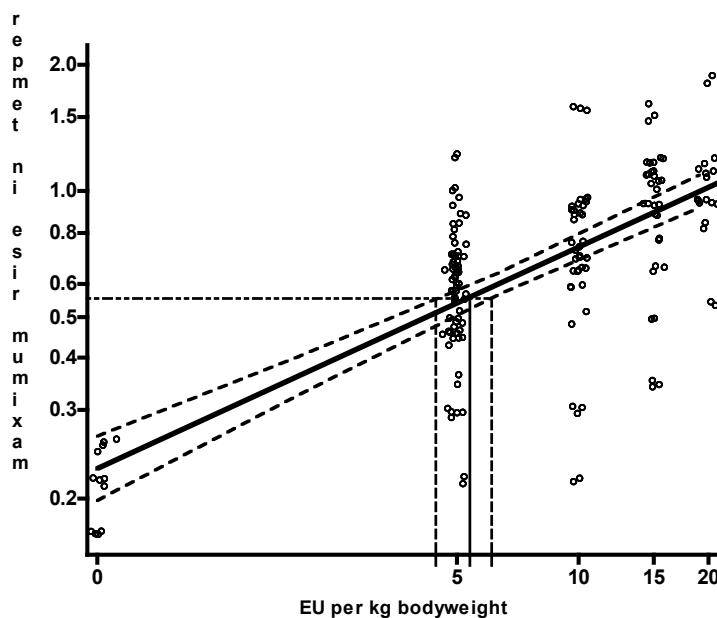


Figure 4.1.1 Dose-temperature of standard endotoxin applied to Chinchilla Bastards (n=171). Rabbits were treated with 1 ml saline containing 0, 5, 10, 15 and 20 EU of *E. coli* LPS (WHO-LPS 94/580 (*E. coli* O113:H10:K)) and their body temperature was measured over 180 min. Linear regression analysis was performed after logarithmic transformation of the data. Data are shown as dots to which a jitter-effect was applied in order to be able to distinguish congruent data. The full line depicts the linear regression whereas the dashed lines represent the 95%-confidence bounds. Furthermore, a horizontal line for a 0.55°C raise of temperature is added which is often defined as the rabbit threshold for fever. At the interception point of this line and the regression line 50% of the rabbits are to be expected to develop fever.

4.3 Original records

If not included in the submission, indicate if original records are available for the in vivo reference test method data.

The recognition of pyrogenic substances as bacterial by-products and the identification of a variety of pyrogenic agents enabled the development of a proper test to demonstrate non-pyrogenicity of the pharmaceutical product. As early as the 1920s, studies were done to select the most appropriate animal model. Results indicated that most mammals had a pyrogenic response, but only a few, including rabbits, dogs, cats, monkeys and horses showed a response similar to that in humans. For practical reasons, other species but rabbits and dogs were considered not practical. In 1942, Co Tui & Schrifft described that rabbits are less thermo-stable as compared to dogs. Hence, rabbits are more suited for the purpose of testing for the absence of pyrogens, since a negative result is more significant.

4.4 Quality of data

Indicate the quality of the in vivo reference test method data, including the extent of GLP compliance and any use of coded chemicals.

Documented procedures were employed that were GLP-concordant. These were quality assured by quality assurance officers from ECVAM.

4.5 Toxicology

Discuss the availability and use of relevant toxicity information from the species of interest (e.g., human studies and reported toxicity from accidental or occupational exposure for human health-related toxicity testing).

Over time, a number of studies were done to correlate the rabbit test to pyrogenic reactions in humans. A conclusive study by Greisman and Hornick, published in 1969, who compared three purified endotoxin preparations (*Salmonella typhosa*, *E. Coli* and *Pseudomonas*) in New Zealand rabbits and in male volunteers, showed that the induction of a threshold pyrogenic response, on a weight basis, was similar to rabbit and man. At higher doses, rabbits respond less severe as compared to man.

4.6 Background on assay performance

Discuss what is known or not known about the accuracy and reliability of the in vivo reference test method.

As mentioned, animal studies were not performed for ethical reasons. However, a theoretical assumption on specificity and sensitivity can be made (Hoffmann et al, 2005a). Taking the prevalence of the different final concentrations of LPS in the 5 spikes into account (1.0 EU/ml: 20%; 0.5 EU/ml: 40%; 0.25 EU/ml: 20% and 0.0 EU/ml: 20%) and calculating probabilities of misclassification using the chosen study design and defined threshold of pyrogenicity, i.e. 0.5 EU/ml, the theoretical sensitivity of the rabbit pyrogen test is 57.9% and the theoretical specificity of the rabbit pyrogen test is 88.3%.

5 Test Method Data and Results

5.1 Test method protocol

Describe the proposed test method protocol used to generate each submitted set of data. Any differences from the proposed test method protocol should be described, and a rationale or explanation for the difference provided. Any protocol modifications made during the development process and their impact should be clearly stated for each data set.

The detailed protocol used during the catch-up validation of the CRYO WB/IL-1 test is provided in Appendix A of this BRD. It covers the precise step-by-step description of the test method and lists the necessary reagents and laboratory procedures for generating data. For the sake of clarity the protocol contains a detailed description of spiking with WHO-LPS and the dilution of the samples applied during the catch-up validation. A summary of the sample preparation is presented in table 5.1.2. The analysis described in section 5.3 of this BRD was applied to the data produced during catch-up validation.

The CRYO WB/IL-1 method was transferred from the developing laboratory (DL) to two other laboratories (denoted as naive laboratory 1 [NL1] and naive laboratory 2 [NL2]). All laboratories had to meet the validity criteria (minimum assay suitability requirements) as laid down in the SOP before the studies with medicinal substances were conducted.

The **interlaboratory reproducibility** was assessed by testing 3 different medicinal substances, Gelafundin, Jonosteril and Haemate (described in table 3.3.2, section 3.3.). Test items and their spikes were appropriately blinded. Test items were tested, at a predefined dilution above the MVD, independently in 3 laboratories. Test items were tested after spiking with WHO-LPS at four different levels, the spikes were blinded and coded by QA ECVAM. In addition a negative control (saline) and positive control (0.5 EU/ml) in saline were included to establish assay validity.

Although this part of the study was designed for assessment of interlaboratory reproducibility, a preliminary estimate of the accuracy could be derived from the data. Applying the PM to the results and evaluating the concordance in a two-by-two contingency table assessed accuracy.

To assess **accuracy** of the proposed test method 10 substances (listed in table 3.3.1), were spiked with five different concentrations of the WHO-LPS (one of which is negative). To permit the application of the chosen PM, each drug was diluted to its individual MVD, which has been calculated using the ELC to that drug (listed in table 3.3.1.). Each substance had their own specific set of endotoxin spike solution, ensuring that after spiking the undiluted drug, it contained 0.0; 0.25; 0.5; 0.5 and 1.0 EU/ml respectively when tested at its MVD. A detailed description of the sample preparation was supplied to the three independent laboratories (shown in table 5.1.2). To put more weight to the result of this part of the validation, the spikes were blinded and coded by QA ECVAM. The raw data of the CRYO WB/IL-1 assay are shown in paragraph 5.2. Accuracy was assessed by applying the PM to the results and evaluating the concordance in a two by two table. Intralaboratory reproducibility was (successfully) shown in

previous experiments with fresh whole blood samples and it is assumed that the variability was not affected by the change to cryopreserved blood assayed in 96-wells plates (see also Appendix D). Interlaboratory reproducibility was also assessed for the CRYO WB/IL-1 test method.

Table 5.1.2: Sample preparation for the testing of 10 substances spiked with 5 different concentrations of WHO-LPS.

<i>unblinded</i>			<i>blinded</i>				
dilution of drug up to MVD ↓			spiking of undiluted drug: 0.5 ml each				
diluted drug	NPC	PPC	+ 25 µl of Spike 1	+ 25 µl of Spike 2	+ 25 µl of Spike 3	+ 25 µl of Spike 4	+ 25 µl of Spike 5
0.5 ml	+ 25 µl saline	+ 25 µl PPC-LPS-spike *					
		(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

5.2 Accuracy and reliability

Provide all data obtained to evaluate the accuracy and reliability of the proposed test method. This should include copies of original data from individual animals and/or individual samples, as well as derived data. The laboratory's summary judgment regarding the outcome of each test should be provided. The submission should include data (and explanations) from all studies, whether successful or not.

See figures 5.2.1 and 5.2.2 for the prevalidation data. The data of the validation are presented as tabulated results in section 5.4.

Figure. 5.2.1 Prevalidation data for the CRYO WB/IL-1 test method (Cryopreserved blood prepared by the PEI method). Three different drugs were spiked (blinded) with WHO-LPS at 0.0, 0.0, 0.5 and 1.0 IU/ml, respectively. Experiment was run independently at three different laboratories. Treatment and controls are abbreviated (J = Jonosteril; G = Gelafundin ; H = Heamate. C- = negative control (saline); C+ = positive control (0.5 IU/ml in saline).

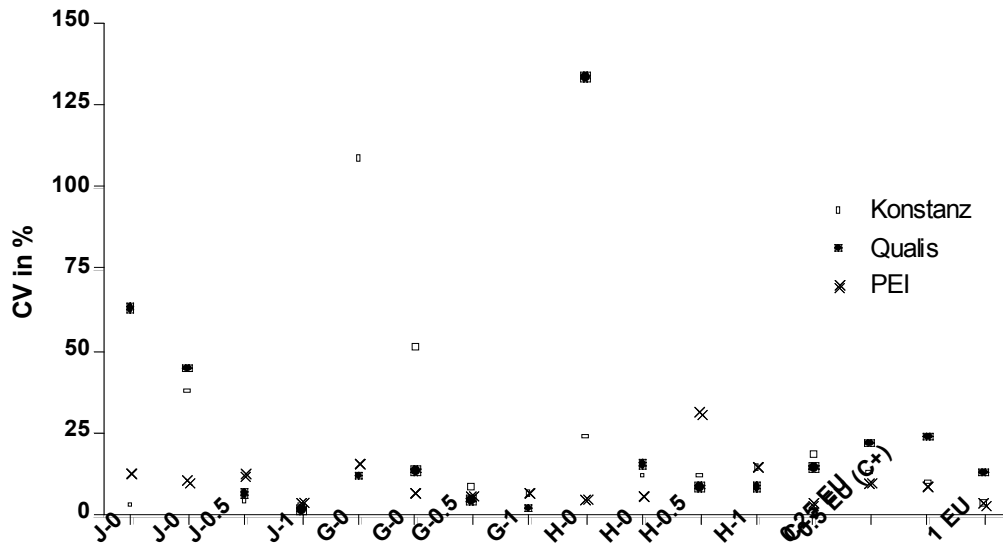


Figure. 5.2.2: Coefficient of variation (CV) of the prevalidation data (readout of IL-1 □ ELISA) of CRYO WB/IL-1 test method. CV of the three different drugs spiked (with WHO-LPS at 0.0, 0.0, 0.5 and 1.0 IU/ml, respectively). Experiment was run independently at three different laboratories (Konstanz, Qualis and PEI). Treatment and controls are abbreviated (J = Jonesteril; G = Gelafundin ; H = Heamate. C- = negative control (saline); C+ = positive control (0.5 IU/ml in saline)).

5.3 Statistics

Describe the statistical approach used to evaluate the data resulting from studies conducted with the proposed test method.

A generally applicable analytical procedure was employed. This procedure includes a universal PM as well as quality criteria. First, a two-step procedure consisting of a variance-criterion and an outlier-test was applied. For this, the Dixon's test (Barnett and Lewis, 1984), which is USP approved, was chosen with the significance level of $\alpha=0.01$ and applied to identify and eliminate aberrant data.

Next, the negative and the respective positive control are compared to ensure a suitable limit of detection. For this, a one-sided t-test with a significance level of $\alpha=0.01$ is applied to the ln-transformed data to ensure that the response to the positive control is significantly larger than that of the respective negative control.

Finally, the samples are classified as either negative or positive by the outcome of a one-sided version of the t-test, which is based on the assigned pyrogen threshold value. The final results will be given in 2 x 2 contingency tables (table 5.3.1). These tables allow for estimation of accuracy (sensitivity and specificity) and reproducibility of the proposed test method.

Table 5.3.1: 2x2 contingency table.

		<i>pre-defined class</i> (“truth”)		Σ
		<i>1</i>	<i>0</i>	
<i>Classification</i> <i>by test system</i> <i>and PM</i>	<i>1</i>	<i>a</i>	<i>b</i>	<i>a+b = n₁</i>
	<i>0</i>	<i>c</i>	<i>d</i>	<i>c+d = n₀</i>
Σ		<i>a+c = n_{1.}</i>	<i>b+d = n_{0.}</i>	<i>n</i>

Accuracy:

The most important statistical tool to determine accuracy (specificity and sensitivity) is the PM (Hothorn, 1995). In general, it is a statistical model, which classifies a given drug by an objective diagnostic or deciding rule. The objective of a dichotomous result requires a clear cut PM, which assigns a drug in one of the two classes “pyrogenic for humans” and “non-pyrogenic for humans”. Since a threshold pyrogen value will be used, a one-sided test is appropriate for the task. Because the data are normalised by a ln-transformation, a t-test is chosen. Although the variances over the range of concentration converge by the transformation, the assumptions of equal variances do generally not hold true, because it depends on additional covariates. Therefore, the one sided Welch-t-test (Snedecor and Cochran, 1989) is applied. Due to the safety aspect of the basic problem, the hypotheses of the test are

$$H_0 : \mu_{S_{i,j}} > \mu_{S_+} \quad \text{vs} \quad H_1 : \mu_{S_{i,j}} < \mu_{S_+},$$

where μ_{\dots} denotes the parameter of location of the respective ln-transformed distribution. This approach controls the probability of false positive outcomes directly by means of its significance level α , which is chosen as 0.01, because it assumes hazard, respectively pyrogenicity, of the tested drug in H_0 , and assures safety, i.e. non-pyrogenicity. The test statistic is

$$T_{S_{i,j}} = \frac{\bar{x}_{S_+} - \bar{x}_{S_{i,j}}}{\sqrt{\frac{s_{S_+}^2}{n_{S_+}} + \frac{s_{S_{i,j}}^2}{n_{S_{i,j}}}}}.$$

The PM is built by means of the outcome of the test. Let 0 denote safety and 1 denote hazard. The classification of S_{i-j} is then determined by

$$S_{ij} = 0, \text{ if } T_{S_{i,j}} > t_{0.99; n_{S_+} + n_{S_{i,j}} - 2},$$

$$S_{ij} = 1, \text{ else,}$$

where $t_{0.99; n_{S_+} + n_{S_{ij}} - 2}$ the 0.99-quantile of the t-distribution with $n_{S_+} + n_{S_{ij}} - 2$ degrees of freedom. The number of replicates for every control and sample, i.e. $n_{...}$, was harmonised to be four. Due to the possibility of removing one observation by the outlier test, the number of replicates could be reduced to three. The classification of a version of a drug is regarded as an independent decision. Therefore, the niveau α is local.

Finally, the classifications of the drugs will be summarised in 2x2 contingency table (table 3). From these tables, estimates of the sensitivity (S_E), i.e. the probability of correctly classified positive drugs and specificity (S_P), i.e. the probability of correctly classified negative drugs, will be obtained by the respective proportions. Where

$$S_E = a / (a + c) * 100\%$$

and

$$S_P = d / (b + d) * 100\%.$$

Furthermore, these estimates will be accompanied by confidence intervals, which will be calculated by the Pearson-Clopper method [15]. For example, let \hat{p}_{SE} denote the proportion, namely the sensitivity, under investigation. Then the confidence interval to a niveau α is calculated as

$$\left[P_{SE}^L = \frac{aF_{2a; 2(n_1 - a + 1); \frac{\alpha}{2}}}{n_1 - a + 1 + aF_{2a; 2(n_1 - a + 1); \frac{\alpha}{2}}}; P_{SE}^U = \frac{(a + 1)F_{2(a + 1); 2(n_1 - a); 1 - \frac{\alpha}{2}}}{n_1 - a + (a + 1)F_{2(a + 1); 2(n_1 - a); 1 - \frac{\alpha}{2}}} \right],$$

where F denotes the respective quantile of the F-distribution and n_1 is the sample size of the positive drugs and a the number of correctly classified drugs.

By contaminating the drugs artificially and by defining a threshold value, which is assumed to be appropriate, the class of a drug is determined beforehand. The versions of drugs, which are effectively contaminated, but below the threshold dose, are considered to be negative, respectively safe, because their contamination is not crucial for humans in terms of ELC.

Reproducibility:

The analysis of the interlaboratory reproducibility was assessed from the three identical and independent runs conducted in each of 3 laboratories. The comparison of the three runs was carried out blindly such that the testing facility did not know the true classification of the sample, either pyrogenic or non-pyrogenic. By this procedure only the randomness and reproducibility of the methods was assessed and not systematic errors, which may have arisen from other sources, e.g. logistics or preparation of the samples. This means that if a sample was (mis)classified in all three runs the result is reproducible regardless of the (mis)classification of the sample. Therefore, a measure of similarity, i.e. complete simple matching with equal weights, was preferred to the coefficient of correlation for 2x2 contingency tables.

The study was designed as follows: each laboratory had to conduct three independent runs with the same 12 samples (3 test items with 4 blinded spikes each) and two controls, i.e. saline as a negative control (C-) and a 0.5 EU/ml LPS-spike in saline as a positive controls (C+). The samples were derived from the three substances Gelafundine, Haemate and Jonosteril. Per run, each substance was blindly spiked twice with saline, once with 0.5 EU/ml LPS and once with 1 EU/ml LPS, which resulted in a balanced design with regard to positive and negative samples, i.e. samples expected to be pyrogenic and non-pyrogenic, respectively.

The three independent runs per testing facility provide the information on which the assessment of the intralaboratory reproducibility is based. The combined results of the three runs per testing facility were used to determine interlaboratory reproducibility. The correlation of the prediction (in terms of the Bravais-Pearson coefficient of correlation) between all runs is calculated, independent of whether that classification is true or false. A BP-correlation of 1 is calculated, if two runs gave exactly the same predictions for the twelve substances. If one run gives adverse classifications for all substances than the other, the correlation is -1 . As these calculations do not need information of the true status of a sample, they were carried out blinded.

5.4 Tabulated results

Provide a summary, in graphic or tabular form, of the results.

See tables 5.4.1 and 5.4.2.

Table 5.4.1: Results of prevalidation, testing of 3 spiked substances by the three involved laboratories. Classifications after applying the PM (compare to fig. 5.2.1)
“0”denotes “non-pyrogenic”; “1” denotes “pyrogenic”.

drug	Code	spike	laboratory		
		in EU	Konstanz	Qualis	PEI
Jonosteril	J - 0 (1)	0	0	0	0
	J - 0 (2)	0	0	0	0
	J - 0.5	0.5	1	1	1
	J - 1	1	1	1	1
Gelafundin	G - 0 (1)	0	0	0	0
	G - 0 (2)	0	0	0	0
	G - 0.5	0.5	1	1	1
	G - 1	1	1	1	1
Haemate	H - 0 (1)	0	0	0	0
	H - 0 (2)	0	0	0	0
	H - 0.5	0.5	1	1	0
	H - 1	1	1	1	1

Table 5.4.2: Results of the validation study of 10 drugs, spiked with WHO-LPS at 0.0, 0.25, 0.5, 0.5 and 1.0 IU/ml, respectively and tested in 3 different laboratories. Samples and spikes were blinded. Classifications after applying the PM (compare to fig. 5.2.7).

drug (code)	spike EU/ml	“truth”	results		
			PEI	Qua	Nov
Beloc (BE)	0.00	0	0	0	CV
	0.25	0	0	1	CV
	0.50	1	1	1	1
	0.50	1	1	1	1
	1.00	1	1	1	1
Binotal (BI)	0.00	0	0	0	0
	0.25	0	0	1	1
	0.50	1	0	1	1
	0.50	1	1	1	1
	1.00	1	1	1	1
Ethanol 13% (ET)	0.00	0	0	0	nq
	0.25	0	CV	1	nq
	0.50	1	1	1	nq
	0.50	1	1	1	nq
	1.00	1	1	1	nq
Fenistil (FE)	0.00	0	0	0	0
	0.25	0	0	1	1
	0.50	1	1	1	CV
	0.50	1	1	1	1
	1.00	1	1	1	1
Glucose 5% (GL)	0.00	0	0	0	0
	0.25	0	CV	0	0
	0.50	1	1	1	1
	0.50	1	1	1	1
	1.00	1	1	1	1
"Drug A" 0.9% NaCl (LO)	0.00	0	CV	0	nq
	0.25	0	0	0	nq
	0.50	1	1	1	nq
	0.50	1	1	1	nq
	1.00	1	1	1	nq
MCP (ME)	0.00	0	0	0	0
	0.25	0	CV	1	CV
	0.50	1	1	1	1
	0.50	1	1	1	1
	1.00	1	1	1	1
"Drug B" 0.9% NaCl (MO)	0.00	0	0	0	nq
	0.25	0	0	0	nq
	0.50	1	1	1	nq

drug (code)	spike EU/ml	“truth”	results		
			PEI	Qua	Nov
	0.50	1	1	1	nq
	1.00	1	1	1	nq
Orasthin (OR)	0.00	0	0	0	0
/Syntocinon	0.25	0	0	CV	CV
	0.50	1	1	1	1
	0.50	1	1	1	1
	1.00	1	1	1	1
Sostril (SO)	0.00	0	0	0	nq
	0.25	0	0	1	nq
	0.50	1	0	1	nq
	0.50	1	1	1	nq
	1.00	1	1	1	nq

0 = considered/classified negative

1 = considered/classified positive

Grey shading indicates that for these drugs the PPCs did not qualify so that the PC was used in the PM. CV = sample showed a variability resulting in exclusion, i.e. CV > 45% and no significant outlier present.

nq = not qualified according to quality criteria, i.e. failure of PPCs and PCs

False classifications are in bold type.

5.5 Coding of data

For each set of data, indicate whether coded chemicals were tested, whether experiments were conducted without knowledge of the chemicals being tested, and the extent to which experiments followed GLP guidelines.

Blinding of drugs and/or spikes is indicated with the data.

5.6 Circumstances

Indicate the “lot-to-lot” consistency of the test substances, the time frame of the various studies, and the laboratory in which the study or studies were conducted. A coded designation for each laboratory is acceptable.

In each part of the study, all samples are derived from one (clinical) lot.

5.7 Other data available

Indicate the availability of any data not submitted for external audit, if requested.

All relevant data were submitted with the present BRD.

6 Test Method Accuracy

6.1 Accuracy

Describe the accuracy (e.g., concordance, sensitivity, specificity, positive and negative predictivity, false positive and negative rates) of the proposed test method compared with the reference test method. Explain how discordant results in the same or multiple laboratories from the proposed test were considered when calculating accuracy.

Test method accuracy was assessed in two large scale experiments performed with the drugs outlined in table 3.3.1 and table 3.3.2 in section 3 respectively. As described before, a prevalidation test was conducted with 3 different drugs and in the final validation experiment ten drugs were tested in the three participating laboratories. From the first experiment a preliminary estimate of sensitivity and specificity can be calculated, whereas the second is regarded as the established accuracy for the CRYO WB/IL-1 assay.

6.1.1 Preliminary estimate of the accuracy of the CRYO WB/IL-1 test. In the prevalidation phase of the study the developing laboratories (DLs) determined for each drug (outlined in table 3.3.2, section 3.3) the smallest dilution within the MVD that showed no interference or an acceptable degree of interference with the spike recovery. In general the lowest dilution of the sample allowing for a 50-200% spike recovery was chosen. In addition, the positive control (PC) set at 0.5 EU/ml saline was used as the classification threshold. The laboratory procedure as described in the method protocol was maintained throughout the study. Although it was realized there were some drawbacks to the concept for interference testing and applying the PC as a threshold, this small scale study allows for a preliminary estimate of the accuracy of CRYO WB/IL-1 method. It has to be noted that this part of the study was designed to provide an estimate of the interlaboratory reproducibility. Therefore it will also be discussed in detail in section 7 (Test Method Reliability).

According to the PM applied during an early phase of the study the outcome (positive/negative) is related to the positive control (PC=0.5 EU/ml). If absorbance of sample > absorbance of PC, then the sample is classified as positive. If absorbance of sample < PC, then the sample is classified as negative. While performing the experiments during this phase it was realized that there is a flaw between interference testing and the PM. After the interference testing, the lowest dilution of the sample allowing for a 50-200% spike recovery was chosen. It is obvious that even with a flawless repeatability of the assay; a spike recovery between 50%-100% would be classified as negative according to the preliminary PM. In addition, due to unforeseen problems with the preparations of the spike, the recovery of the spikes was far below 100%. (This is outside the scope of the study and will not be discussed). As a consequence of the employed preliminary setup of the study the sensitivity will be underestimated, and the specificity will be overestimated.

In short, three test substances were spiked with WHO-LPS at four levels (0, 0, 0.5 and 1.0 EU/ml respectively), which resulted in 12 samples with a balanced design with regard to positive and negative samples (i.e. samples expected to be pyrogenic and non-

pyrogenic respectively. These 12 samples were tested in three laboratories. In total there were 36 classifications in 3 laboratories. Results are described in detail in section 7. A 2x2 contingency table was constructed (table 6.1.1), from which the estimates of sensitivity and specificity can easily be derived.

Table 6.1.1: 2x2 contingency table. The prediction model applied to a preliminary validation study.

		True status of samples		Total
		+	-	
PM	+	17	0	16
	-	1	18	20
Total		18	18	36

The specifications of specificity and sensitivity described in section 5.3 were applied to these results. The specificity (Sp) of the CRYO WB/IL-1 assay is 100% ($18/(18+0)*100\%$). The sensitivity (Se) calculated for this data set is 94.4% ($17/(17+1)*100\%$). As outlined previously the specificity is overestimated and the sensitivity is underestimated as a result of the design of this part of the study.

6.1.2 Test method accuracy of the proposed CRYO WB/IL-1 method. To assess accuracy of the proposed method, 10 substances (listed in table 3.1.1, section 3) were spiked with five different concentrations of the WHO-LPS (one of which is negative). Thus, in total, 50 samples have been tested in each laboratory.

To permit the application of the chosen PM, each drug was diluted to its individual MVD, which has been calculated using the ELC to that drug (listed in section 3). Lesser dilutions were tested by the DL, and showed no interference. Therefore interference was not expected at the individual MVD. Each substance had their own specific set of endotoxin spike solution, ensuring that after spiking the undiluted drug, it contained 0.0; 0.25; 0.5; 0.5 and 1.0 EU/ml respectively when tested at its MVD. A detailed description of the sample preparation was supplied to the three independent laboratories (shown in table 5.1.1 for convenience). To put more weight to this part of the validation, the spikes were blinded and coded by QA ECVAM. Accuracy was assessed by applying the PM to the results (summarized in table 5.4.2) and evaluating the concordance in this section in a two by two contingency table (table 6.1.2 and 6.1.3). As described above 10 substances, spiked with 5 different WHO-LPS concentrations were tested in three laboratories and consequently a maximum of 150 data were available for analysis.

Of the 150 available data for the CRYO WB/IL-1 method, eleven sets of 4 replicates showed a high variability resulting in exclusion, i.e. $CV > 45\%$ and no significant outliers present. Therefore 139 data in total could be used to estimate the specificity and sensitivity of the CRYO WB/IL-1 method. The results are shown separately for each participating laboratory (table 6.1.2) as well as combined for all these laboratories (table 6.1.2).

The specificity that can be estimated from the available results for DL, NL1 and NL2 is 68.4%, 75% and 100% respectively. The estimated sensitivity of the CRYO WB/IL-1 assay was excellent of all three participating laboratories: 93.3%, 100% and 100% respectively (calculated from results in table 6.1.2).

Table 6.1.2: 2x2 contingency table. Prediction model applied to the CRYO WB/IL-1 test result of 10 different substances assessed in three different laboratories. Results of each laboratory separately (DL, NL1 and NL2= PEI, Qualis and Novartis respectively).

Results DL		True status of samples		Total
		+	-	
PM	+	28	0	28
	-	2	16	18
Total		30	16	46

Results NL1		True status of samples		Total
		+	-	
PM	+	30	6	36
	-	0	13	13
Total		30	19	49

Results NL2		True status of samples		Total
		+	-	
PM	+	17	2	19
	-	0	6	6
Total		17	8	25

The specificity of the combined results of the three laboratories of the assay is 81.4% ($35/(35+8)*100\%$), 95% confidence interval [0.679-0.920]. The sensitivity equals 97.4% ($75/(75+2)*100\%$), 95% confidence interval [0.907-0.997]. (Summarized in table 6.1.3 and 6.1.4).

Table 6.1.3: 2x2 contingency table. Prediction model applied to the CRYO WB/IL-1 test result of 10 different substances assessed in three different laboratories. Combined results.

		True status of samples		Total
		+	-	
PM	+	75	8	83
	-	2	35	37
Total		77	43	120

Table 6.1.3: Specificity and sensitivity of the CRYO WB/IL-1 assay

	N total	N correctly identified	proportion	95% CI lower limit	95% CI upper limit
Specificity (Sp)	43	35	81.4%	0.679	0.920
Sensitivity (Se)	77	75	97.4%	0.907	0.997

6.2 Concordancy to *in vivo* reference method

Discuss results that are discordant with results from the in vivo reference method.

Not applicable.

6.3 Comparison with reference methods

Discuss the accuracy of the proposed test method compared to data or recognized toxicity from the species of interest (e.g., humans for human health-related toxicity testing), where such data or toxicity classification are available. This is essential when the method is measuring or predicting an endpoint for which there is no preexisting method. In instances where the proposed test method was discordant from the in vivo reference test method, describe the frequency of correct predictions of each test method compared to recognized toxicity information from the species of interest.

Not applicable.

6.4 Strength and limitations

State the strengths and limitations of the proposed test method, including those applicable to specific chemical classes or physical-chemical properties.

It appears the proposed test is applicable to most classes of medicinal products, at least those that are non- or low-toxic to cells *in vitro*. In addition, the test may be employed to assess pyrogenicity of various medical devices, such as (biological) bovine collagen bone implants.

6.5 Data interpretation

Describe the salient issues of data interpretation, including why specific parameters were selected for inclusion.

No issues.

6.6 Comparison to other methods

In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the results obtained with both test methods should be compared with each other and with the in vivo reference test method and/or toxicity information from the species of interest.

Not applicable.

7 Test Method Reliability (Repeatability/Reproducibility)

7.1 Selection of substances

Discuss the selection rationale for the substances used to evaluate the reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) of the proposed test method as well as the extent to which the chosen set of substances represents the range of possible test outcomes.

The rationale for the selection of the substances is described in section 3.3. In short: for the present studies endotoxin (WHO-LPS) was selected as the model pyrogen, since it is well defined biological standard and readily available. Selected test substances were medicinal products available on the market. These batches are released by the manufacturers and comply with the Marketing Authorisation file and European Pharmacopoeia. Therefore these batches are considered to contain no detectable pyrogens. To test the method reliability the medical products were spiked with endotoxin.

7.2 Results

Provide analyses and conclusions reached regarding the repeatability and reproducibility of the proposed test method. Acceptable methods of analyses might include those described in ASTM E691-92 (13) or by coefficient of variation analysis.

Interlaboratory reproducibility.

The interlaboratory reproducibility was assessed in a prevalidation test by testing 3 different medicinal substances, Gelafundin, Jonosteril and Haemate (described in table 3.3.2, section 3.3.). Test substances and their spikes were appropriately blinded. Test substances were tested, at a predefined dilution above the MVD, independently in 3 different laboratories, 3 times each. The three test substances were spiked with WHO-LPS at four levels (0, 0, 0.5 and 1.0 EU/ml respectively), which resulted in 12 samples with a balanced design with regard to positive and negative samples (i.e. samples expected to be pyrogenic and non-pyrogenic respectively). In addition a negative control (saline) and positive control (0.5 EU/ml in saline) were included to establish assay validity. To avoid interference, the DL performed interference testing in terms of the BET, i.e. 50-200% spike recovery, and decided on the dilution of the test substances. Dilutions chosen for Gelafundine, Haemate, Jonosteril were 1:2, 1:20 and 1:2 respectively.

For the CRYO WB/IL-1 test method, the coefficient of variation (CV) of the prevalidation test was assessed. The prevalidation test, three different drugs spiked with WHO-LPS (0.0, 0.0, 0.5 or 1.0 IU/ml), was run independently at three different laboratories (Konstanz, Qualis and PEI). The CVs were calculated for each treatment or control for all laboratories (shown in figure 5.2.2). While the major part of the CVs was smaller than 30%, four samples (Konstanz: both G-0; Qualis: J-0, H-0) showed a CV larger than 45%. In all of these, one replicate was much larger than the others, which gave very low responses (less than 0.02 OD).

Also for the fresh blood WB/IL-1 test, described in a separate BRD, it was concluded that the coefficient of variation for the sets of four replicates in the WB/IL-1 assay is usually below 45%. This is considered acceptable for a biological assay. To harmonize the acceptance criteria between different variations of the WB/IL-1 test, the coefficient of variation was arbitrarily set at $CV < 45\%$ for the CRYO WB/IL-1 test.

The intralaboratory reproducibility of the CRYO WB/IL-1 test method is not assessed in this study (see Appendix D), but is considered to be similar to the WB/IL-1 test method (using fresh blood and conducted in test tubes) and therefore acceptable. As the interlaboratory reproducibility (usually worse than the intralaboratory reproducibility) is indeed shown to be satisfactory for the CRYO WB/IL-1 method presented in this BRD, this assumption was proven to be valid.

The analysis of the interlaboratory reproducibility could be assessed from the identical and independent runs conducted in the three laboratory. The three runs was carried out blindly such that the laboratory did not know the true classification of the sample (either pyrogenic or non-pyrogenic). By this procedure only the randomness and reproducibility of the methods was assessed and not systematic errors, which may have arisen from other sources, e.g. logistics or preparation of the samples. This means that if a sample was misclassified in all three laboratories the result is 100% interlaboratory reproducible (regardless of the misclassification of the sample).

According to the preliminary PM applied during this phase of the study the outcome (positive/negative) was related to the positive control (PC=0.5 EU/ml). If absorbance of sample $>$ absorbance of PC, then sample is classified as being positive. If absorbance of sample $<$ PC, sample is classified as negative (positive/pyrogenic = 1, negative/non-pyrogenic = 0). (NB. During this phase it was realized that there is a flaw between interference testing and the PM. After the interference testing, the lowest dilution of the sample allowing 50-200% spike recovery was chosen. It is obvious that even with a flawless repeatability of the assay, a spike recovery between 50%-100% would be classified as negative according to the preliminary PM.)

The interlaboratory reproducibility of the CRYO WB/IL-1 method was assessed by comparing the results of the preliminary test of the three laboratories. The measure of similarity is the proportion of equally classified samples. These proportions are summarized in table 7.2.3, and indicate that there is a good interlaboratory reproducibility for the CRYO WB/IL-1 test of at least 91.7%.

Table 7.2.3: Interlaboratory reproducibility assessed by interlaboratory correlations. Result of testing 3 substances (with four different spikes) by 3 laboratories.

Laboratories	Interlaboratory reproducibility	Number of equal predictions
DL – NL1	91.7%	11/12
DL – NL2	91.7%	11/12
NL1 – NL 2	91.7%	11/12
Mean	91.7%	
Same in three laboratories	83.3%	10/12

DL = Konstanz; NL1 = Qualis; NL2 = PEI

Also from the result of the large scale study (testing 10 substances spiked with 5 separate spikes), the interlaboratory reproducibility can be estimated (table 7.2.4). None of the laboratories identified all samples correctly. The reproducibility varied from 88.4% to 100% between two laboratories. From the available results it can be concluded that also the reproducibility between all three participating laboratories was satisfactory: All three laboratories found the same result for 21 out of 24 samples (87.5%).

Table 7.2.4: Interlaboratory reproducibility: Assessed by testing of 10 substances, spiked 5 times. One run of 50 samples by three different laboratories.

Laboratories	Interlaboratory Reproducibility	Number of equal predictions
DL - NL1	84.4%	38 / 45
DL - NL2	87.5%	21 / 24
NL1 – NL2	100%	25 / 25
Mean	90.6%	
same result in all laboratories	87.5%	21 / 24

DL = PEI; NL1 = Qualis; NL2 = Novartis.

Conclusion: The results of the prevalidation experiment indicated that the interlaboratory reproducibility was satisfactory. The reproducibility between two laboratories equals 91.7% during prevalidation. This was confirmed in the validation study where the interlaboratory reproducibility ranged from 88.4% to 100%. All three participating laboratories predicted the same in 83.3% and 87.5% of the measurements respectively.

7.3 Historical data

Summarize historical positive and negative control data, including number of experiments, measures of central tendency, and variability.

Not applicable.

7.4 Comparison to other methods

In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the reliability of the two test methods should be compared and any differences discussed.

Not applicable.

8 Test Method Data Quality

8.1 Conformity

State the extent of adherence to national and international GLP guidelines (7-12) for all submitted data, including that for the proposed test method, the in vivo reference test method, and if applicable, a comparable validated test method. Information regarding the use of coded chemicals and coded testing should be included.

The studies were done in accordance to the guidelines for GLP. Written protocols and approved standard operating procedures were followed during the entire course of the study. Deviations were recorded and, where appropriate, approved in amendments. All data are stored and archived. As mentioned, samples were appropriately blinded.

8.2 Audits

Summarize the results of any data quality audits, if conducted.

No audits were conducted.

8.3 Deviations

Discuss the impact of deviations from GLP guidelines or any noncompliance detected in the data quality audits.

Not applicable.

8.4 Raw data

Address the availability of laboratory notebooks or other records for an independent audit. Unpublished data should be supported by laboratory notebooks.

All records are stored and archived by the contributing laboratories and available for inspection.

9 Other Scientific Reports and Reviews

9.1 Summary

Summarize all available and relevant data from other published or unpublished studies conducted using the proposed test method.

Relevant data obtained with the WB/IL-1 using fresh blood (see BRD WB/IL-1) are described in a number of published studies and reports. The most important ones for this BRD are included in the Appendix B as hardcopies and referenced in Section 12, whereas for others only the references are given in section 14. In most of the study reports the WB/IL-1 is named *in vitro* pyrogen test or IPT.

The establishment of the whole blood test as an alternative to the rabbit pyrogen test as well as the comparison to the BET is described below.

Further applications were developed by adaptation to the basic whole blood test e.g. to measuring pyrogenic contaminations of medical devices and measuring the air quality in the working place and references are included in Section 14 in part 2 and 3.

A total of 96 batches of parenteral pharmaceuticals from 21 indication groups were tested using the WB/IL-1 test and compared to data from the rabbit and BET test, if available (Jahnke et al., 2000). For these batches of parenteral drugs it was shown that the results of the three methods correlate well. In one case (an amino acid-containing infusion solution) a pyrogen-containing batch was clearly detected by all three testing systems.

The other parenteral pharmaceuticals remained negative in all assays. It is worth mentioning that all of the products could be tested with the WB/IL-1, in some cases after interfering factors had been excluded. A few drugs (e.g. dopamine) were found to affect the sensitivity of the WB/IL-1 and hence caused interference, but this could be overcome by diluting the drug.

In a preliminary study (Fennrich et al., 1999), the suitability of the WB/IL-1 was tested by determining the LPS retrieval in spiked pharmaceutical samples at the border line concentrations given in the European Pharmacopoeia for endotoxins (ELC), which should be detectable also using the WB/IL-1 test.

Human serum albumin belongs to those substances that still are tested in the rabbit pyrogen test. Spreitzer et al (2002) compared the sensitivity of the rabbit test with the WB/IL-1 using 29 defined human albumin samples: plain, spiked with 5 EU/ml and 10 EU/ml respectively. The unspiked samples were negative in both assays. Both the borderline 5 EU/kg and the 10 EU/kg partially led to results with the rabbit test (conducted with 3 rabbits), which would cause further testing with additional animals. In contrast, the WB/IL-1 test detected 100% detection of the 5 EU/ml and 10 EU/ml endotoxin spikes. The WB/IL-1 demonstrated at least the same level of safety for the products as achieved with the rabbit pyrogen test. After further dilution of the 29 spiked albumin samples to endotoxin levels of 0.5 EU/ml, 18 samples were still positive in the WB/IL-1 assay but there were 11 negative results too.

Schindler et al. (2003) directly compared the reactivity of human and rabbit blood *in vitro* towards Gram negative and Gram-positive stimuli using an *in vitro* whole blood test

(endpoint; IL-1) for both species. The reactivity of the two species towards LPS was found to be similar, whereas human blood was more sensitive for LTA (lipoteichoic acid) than rabbit blood. The results suggested that the test with human blood to detect contaminations in e.g. parenteral drugs, might predict the human reaction to real life contamination better than the rabbit pyrogen test.

A Gram-positive standard derived from *B. subtilis* has been developed by the same research group (University of Konstanz) and was reviewed in numerous different articles. This lipoteichoic acid is BET negative which however reacts positive in the WB/IL-1 assay. Identification, isolation and purification of other Gram-positive stimuli are subject of ongoing research.

It is stressed throughout these studies using whole blood that only healthy donors not taking any medication must be used for testing pharmaceuticals. Various drugs such as cortisone suppress interleukin release and would therefore exclude a blood donor from the experiment. An infection can stimulate release, as reflected in an increased baseline value or an abnormally high interleukin response. Therefore, the WB/IL-1 test may only be used if samples have first been shown not to cause interference. The blood group of the human donors does not influence the results of the assay.

9.2 Discussion

Comment on and compare the conclusions published in independent peer-reviewed reports or other independent scientific reviews of the proposed test method. The conclusions of such scientific reports and reviews should be compared to the conclusions reached in this submission. Any ongoing evaluations of the proposed test method should be described.

The validation study summarised in this BRD is the first, which extensively addresses specificity and accuracy using actual medicinal products spiked with endotoxin. Hence, there are no comparing reports in independent peer-reviewed journals available. However, the validation study confirms conclusions of several scientific reports, e.g. several preliminary studies (e.g. Jahnke et al.2000, Fennrich et al., 1999, Spreitzer et al 2002) showed that the WB/IL-1 assay is suitable to test different types of pharmaceuticals. Their findings are confirmed by the current validation study, where 11 different pharmaceuticals were tested. In addition, both studies indicate that (pyrogen free) batches which passed the current batch release scheme and are available on the market, show rarely a false positive reactivity in the WB/IL-1 assay. Jahnke's study was conducted by an experienced laboratory, whereas relatively inexperienced laboratories were also involved in the presented validation study. This may account for the less than 100% specificity in the validation study.

Finally, Charles River Endosafe offers the WB/IL-1 test under the name IPT (*In vitro* Pyrogen Test) worldwide in a highly standardized kit-version. Frequent symposia and workshops with coworkers of Charles River together with the University of Konstanz take place in order to train interested parties and introduce the IPT to users. The introduction and optimization of cryopreserved human whole blood is expected to overcome all final obstacles to standardization.

9.3 Results of similar validated method

In cases where the proposed test method is mechanistically and functionally similar to a validated test method with established performance standards, the results of studies conducted with the validated test method subsequent to the ICCVAM evaluation should be included and any impact on the reliability and accuracy of the proposed test method should be discussed.

As mentioned, *in vitro* methods activating monocytoïd cells for detecting pyrogenic contaminants are being developed over the course of the past two decades. A number of variants have been described, although the underlying principle of each variant remains the same. The test preparation is cultured with monocytoïd cells, either as peripheral blood mononuclear cells (PBMCs), (diluted) whole blood or cells of a monocytoïd cell line such as MONO MAC-6 (MM6). Accuracy and specificity of these test methods are comparable, but in general methods using whole blood, PBMC and the MM6 cell line appear to perform best (Hoffmann et al, 2005b).

Table 9.3.1 summarises the performance of *in vitro* methods presented in the five BRDs and Table 9.3.2 compares the *in vivo* and *in vitro* pyrogen tests regarding their strengths, weaknesses, costs, time, limitations.

However, most studies (as this one) are done with model pyrogens and as yet little experience is available in the field, e.g. as part of the final batch release test-package. Experience and thus confidence in these methods will grow once regulatory authorities approve these methods and more manufacturers start to employ them. Then, on a case by case situation, it should be determined which method is best suited for the actual situation and demonstrates to pick out the appropriate, i.e. pyrogenic batches of the medicinal product.

Table 9.3.1: Summary of the performance of in vitro pyrogen tests based on monocytoïd cells (see Tables 7.2.2; 7.2.4; 6.1.3)

Test	System	Read-out	Intralaboratory reproducibility (%)	Interlaboratory reproducibility (%)	Sensitivity (%)	Specificity (%)
WB/IL-6	whole blood	IL-6	DL: 83.3 NL1: 94.4 NL2: 100	DL-NL1: 85.4 DL-NL2: 85.4 NL1-NL2: 92.0	88.9	96.6
WB/IL-1	whole blood	IL-1 β	DL: 88.9 NL1: 95.8 NL2: 94.4	DL-NL1: 72.9 DL-NL2: 81.6 NL1-NL2: 70.2	72.7	93.2
96-wells WB/IL-1 ¹	whole blood	IL-1 β	-	DL-NL1: 88.1 DL-NL2: 89.7 NL1-NL2: 91.5	98.8	83.6
CRYO WB/IL-1	cryo whole blood	IL-1 β	-	DL-NL1: 91.7 DL-NL2: 91.7 NL1-NL2: 91.7	97.4	81.4
KN CRYO WB/IL-1 ²	cryo whole blood	IL-1 β	-	DL-NL1: 83.3 DL-NL2: 100 NL1-NL2: 83.3	88.9	94.4
PBMC/IL6	PBMC	IL-6	DL: 94.4 NL1: 100 NL2: 94.4	DL-NL1: 84.0 DL-NL2: 86.0 NL1-NL2: 90.0	92.2	95.0
PBMC-CRYO/IL-6 ³	PBMC	IL-6	-	DL-NL1: 96 DL-NL2: 76 NL1-NL2: 80	93.3	76.7
MM6/IL-6	MM6	IL-6	DL: 100 NL1: 94.4 NL2: 94.4	DL-NL1: 90.0 DL-NL2: 89.6 NL1-NL2: 83.3	95.5	89.8

DL = developing laboratory; NL1, NL2 = naive laboratory 1 and 2

1 = data provided in Section 13 of WB/IL-1 BRD

2 = data provided in Section 13 of CRYO WB/IL-1 BRD

3 = data provided in Section 13 of PBMC/IL-6 BRD

Table amended from Hoffmann et al 2005b; results with THP cells not included

Table 9.3.2: Comparison of the in vivo and in vitro pyrogen tests regarding their strengths, weaknesses, costs, time, limitations

	Rabbit pyrogen test	BET / LAL	In vitro pyrogen test
Test materials	Liquids	Clear liquids	Liquids, potentially cell preparations, solid materials
Pyrogens covered	All (possible species differences to humans for non-endotoxin pyrogens)	Endotoxin from Gram-negative bacteria	(probably) all
Limit of detection (LPS)	0,5 EU	0,1 EU (some variants down to 0,01 EU)	0,5 EU (validated PM), some variants down to 0,001 EU
Ethical concerns	Animal experiment	About 10% lethality to bled horseshoe crabs	Some assays: blood donation
Costs*	High (200-600\$/sample)	Low (50-150\$/sample)	Medium (100-350\$/sample)
Time required	27 h	45 min	24-30h**
Materials not testable	Short-lived radiochemicals, anesthetics, sedatives, analgetics, chemotherapeutics, immunomodulators, cytokines, corticosteroids	Most biologicals, glucan-containing preparations (herbal medicinal products, cellulose-filtered products), lipids, microsomes, cellular therapeutics	Not known (some of the materials not testable in rabbits require adaptations)
Others	No positive or negative control included, strain differences, stress affects body temperature	Potency of LPS from different bacterial species in mammals not reflected, false-positive for glucans	Possible donor differences, need to exclude hepatitis/HIV and acute infections / allergies of donors, dedifferentiation of cell lines

* = We consulted the laboratories participating in the validation study and a consultant regarding the costs of the tests. The figures we received vary significantly depending on the facility (e.g. industry, contract laboratory, control authority), frequency of testing, specific test requirements, country, etc.

** = interference testing might increase duration by 24 hours

10 Animal Welfare Considerations (Refinement, Reduction, and Replacement)

10.1 Diminish animal use

Describe how the proposed test method will refine (reduce or eliminate pain or distress), reduce, or replace animal use compared to the reference test method.

Depending on the medicinal product, one of two animal-based pyrogen tests is currently prescribed by the respective Pharmacopoeias, i.e. the rabbit pyrogen test and the bacterial endotoxin test (BET). The rabbit pyrogen test detects various pyrogens but alone the fact that large numbers of animals are required to identify a few batches of pyrogen-containing samples argues against its use. In the past two decades, the declared intention to refine, reduce and replace animal testing, has lowered rabbit pyrogen testing by 80% by allowing to use the BET as an alternative pyrogen test for certain medicinal products. Bacterial endotoxin is the pyrogen of major concern to the pharmaceutical industry due to its ubiquitous sources, its stability and its high pyrogenicity. With the BET, endotoxin is detected by its capacity to coagulate the amoebocyte lysate from the haemolymph of the American horseshoe crab, *Limulus polyphemus*, a principle recognised some 40 years ago (Levin and Bang, 1964). In the US, *Limulus* crabs are generally released into nature after drawing about 20% of their blood and therefore most of these animals survive. However, the procedure still causes mortality of about 30,000 horseshoe crabs per year, which adds to more efficient threats of the horseshoe crab population like its use as bait for fisheries, habitat loss and pollution.

The proposed test method is an alternative for the rabbit test and the BET. By replacing the rabbit test or the BET, the lives of rabbits and horseshoe crabs are spared.

10.2 Continuation of animal use

If the proposed test method requires the use of animals, the following items should be addressed:

10.2.1 *Describe the rationale for the need to use animals and describe why the information provided by the proposed test method requires the use of animals (i.e., cannot be obtained using non-animal methods).*

Not applicable.

10.2.2 *Include a description of the sources used to determine the availability of alternative test methods that might further refine, reduce, or replace animal use for this testing. This should, at a minimum, include the databases searched, the search strategy used, the search date(s), a discussion of the results of the search, and the rationale for not incorporating available alternative methods.*

Not applicable.

10.2.3 *Describe the basis for determining that the number of animals used is appropriate.*

Not applicable.

10.2.4 If the proposed test method involves potential animal pain and distress, discuss the methods and approaches that have been incorporated to minimize and, whenever possible, eliminate the occurrence of such pain and distress.

Not applicable.

11 Practical Considerations

11.1 Transferability

Discuss the following aspects of proposed test method transferability. Include an explanation of how this compares to the transferability of the in vivo reference test method and, if applicable, to a comparable validated test method with established performance standards.

In general, the proposed test method is not unlike other bioassays and immunoassays that are performed routinely in many laboratories.

11.1.1 Discuss the facilities and major fixed equipment needed to conduct a study using the proposed test method.

No extraordinary facilities are required. General laboratory equipment for aseptic operations and analytical instruments for performing immunoassays, e.g. microtiter plate reader and –washer, are sufficient to perform the proposed test method.

11.1.2 Discuss the general availability of other necessary equipment and supplies.

All supplies and reagents are readily available on the market. In contrast, availability of sufficient rabbits of adequate weight and in good health for the *in vivo* reference test is sometimes reported a limitation.

11.2 Training

Discuss the following aspects of proposed test method training. Include an explanation of how this compares to the level of training required to conduct the in vivo reference test method and, if applicable, a comparable validated test method with established performance standards.

11.2.1 Discuss the required level of training and expertise needed for personnel to conduct the proposed test method.

The proposed test method requires personnel trained for general laboratory activities in cell biology and immunochemistry or biochemistry. Techniques they should master are not unlike cell culture (aseptic operations) and immunological techniques (especially ELISA). Such expertise is available in most if not all QC-laboratories.

11.2.2 Indicate any training requirements needed for personnel to demonstrate proficiency and describe any laboratory proficiency criteria that should be met.

Personnel should demonstrate that they master the execution of the test. The candidate should demonstrate to meet all the appropriate assay acceptance criteria and yield accurate results (outcome) using selected test items.

11.3 Cost Considerations

Discuss the cost involved in conducting a study with the proposed test method. Discuss how this compares to the cost of the in vivo reference test method and, if applicable, with that of a comparable validated test method with established performance standards.

Three factors contribute to the cost of the proposed test method: availability of monocytoid cells, cost of the reagents for the immunoassay and, last but not least, personnel.

Since the proposed test method is relatively more labor-intensive, it is estimated that the cost of the proposed test method is more than the BET or the *in vivo* reference test using rabbits. Obviously, a higher throughput of tests (runs/year) such as in a QC-laboratory of a multi-product facility or in a Contract Research Organization will significantly reduce the costs per assay.

However, especially with pharmaceuticals of biological origin, the proposed test method may be cost-effective, since these products all too often are incompatible with the BET and by their nature preclude the re-use of the rabbits.

11.4 Time Considerations

Indicate the amount of time needed to conduct a study using the proposed test method and discuss how this compares with the in vivo reference test method and, if applicable, with that of a comparable validated test method with established performance standards.

Essentially the test stretches two working days. On day one the testing materials are prepared and incubated overnight with the monocytoid cells. On the second day the amount of excreted cytokines is determined by immunoassay. The total time from start to result is approximately 24 hours.

It is thus concluded that the proposed test method will take more time when compared to the alternative tests, either the rabbit test or the BET. It should be noted that rabbits are tested prior to their first use by a sham test.

12 References

List all publications referenced in the submission.

References in bold are included as hardcopies in Appendix B

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13 In vitro Pyrogen Test with Cryopreserved Human Whole Blood according to the Konstanz method (KN CRYO WB/IL-1).

13.1 Rationale

Several laboratories have developed their own method to cryopreserve whole blood. In the main part of this BRD, whole blood cryopreserved according to a procedure developed by PEI is used. Another cryopreserved WB/IL-1 method recently published by Schindler et. al (2004) was also investigated separately, while applying the same study plan. This variant is indicated as the KN (University of Konstanz, Germany) CRYO WB/IL-1 method. The optimisation of the procedure to preserve whole blood is in detail described in the manuscript of Schindler (Schindler et al 2004; Appendix B BRD).

13.2 Test Method Protocol Components

The method follows the original standard protocol, with the obvious exemption of the cryopreservation of the freshly drawn whole blood in the presence of 10% (v/v) DMSO prepared according to the Konstanz method (Schindler et. al, 2004). Details of the test procedure are given in the method protocol under point 7C (Appendix A). The blood has to be stored in the vapour phase of liquid nitrogen. After incubation of the blood (20 µl) with the samples of interest in a 96-wells microtiter plate, the plate is frozen at -20 or -80°C until the contents of the wells are completely frozen. Subsequently the plate is thawed at room temperature or in a water bath (maximum 37°C). The released IL-1 is assessed using the standard IL-1 ELISA.

13.3 Substances Used for Validation

The same 10 parenteral drugs used to determine sensitivity and specificity (see table 3.3.1.) were used for the catch-up validation. Again, each test item was tested after spiking at its individual MVD, thus came with its own specific set of 5 endotoxin spike solutions: 0.0, 0.25, 0.5, 0.5 and 1.0 EU/ml. The test items were assessed with 5 different endotoxin levels at 3 independent test facilities, yielding a total of 150 data points, biometrically considered to be sufficient for further analysis.

The same three drugs (table 3.3.2) as used for the prevalidation of the CRYO WB/IL-1 method were employed. Each drug was tested at an interference free dilution and spiked with 0.0, 0.0, 0.5 and 1.0 EU/ml. The samples were tested at each of the 3 laboratories. The results were used to provide a preliminary estimate of the interlaboratory reproducibility and accuracy.

13.4 Preliminary estimate of the Test Method Accuracy

In short, three test substances were spiked with WHO-LPS at four levels (0, 0, 0.5 and 1.0 EU/ml respectively), which resulted in 12 samples with a balanced design with regard to positive and negative samples (i.e. samples expected to be pyrogenic and non-pyrogenic respectively). These 12 samples were tested in three laboratories (See figure 13.4.1)

Figure 13.4.1: Prevalidation data for KN CRYO WB/IL-1 of the three involved laboratories. The treatments and controls are abbreviated (indicating the endotoxin contamination in EU. (J = Jonosteril; G = Gelafundin; H = Haemate; C- = saline with 0 EU; C+ = positive control)

As Figure 13.4.1 only gives an indication about variability of replicates, the coefficients of variation (CVs) were calculated for each treatment or control for all laboratories (Figure 13.4.2). While the major part of the CVs was smaller than 40%, six samples (mainly from Konstanz) and one standard showed a CV larger than 45%.

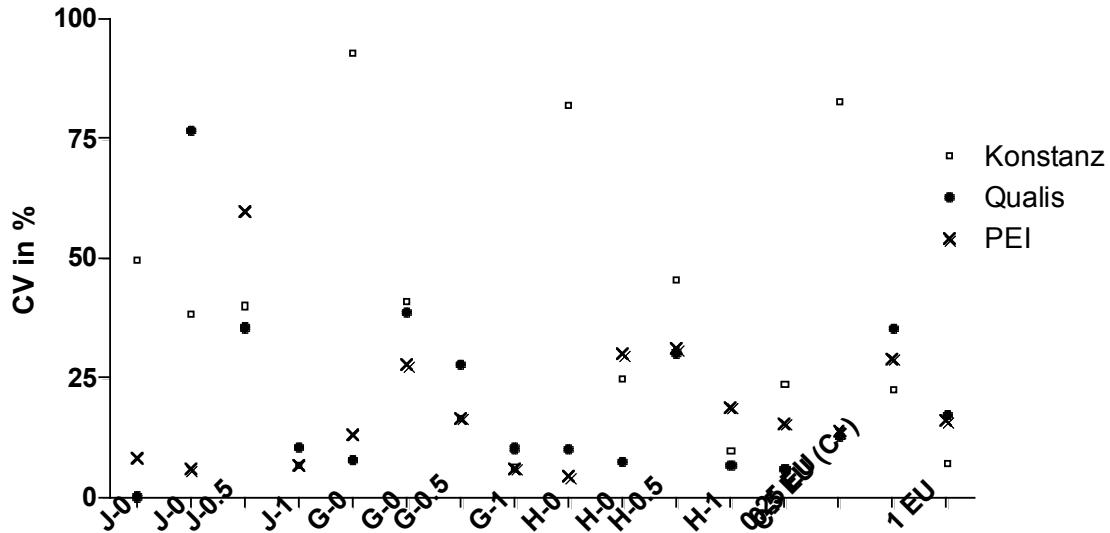


Figure 13.4.2: Coefficients of variation of the prevalidation data from KN WB-CRYO/IL1-B for the three involved laboratories. The treatments and controls are abbreviated indicating the endotoxin contamination in EU. (J = Jonosteril; G = Gelafundin; H = Haemate; C- = saline with 0 EU; C+ = positive control)

Application of the PM to these data resulted in the classifications summarized in Table 13.4.1. Ten out of the twelve spikes were classified in the same way in all laboratories (83.3%). Comparing the laboratories pair wise, showed that 32 of the total of 36 single comparisons, i.e. 88.9% resulted in the same classification.

Assessing in the final step preliminarily the predictive capacity, revealed that one negative samples was classified wrongly (Qualis: J-0) due to one outlying value, and that two times a Haemate 0.5-EU sample (Konstanz and PEI) at the rabbit classification threshold was classified false negative.

These false negative samples had OD-values significantly larger than (PEI) or equivalent to (Konstanz) the respective 0.25 EU-spike of the standard curve.

Table 13.4.1: Classification by the KN CRYO WB/IL-1 of the spikes in the prevalidation in the three involved laboratories

drug	spike in EU	laboratory Konstanz	Qualis	PEI
Jonosteril	0	0	0	0
	0	0	1	0
	0.5	1	1	1
	1	1	1	1
Gelafundin	0	0	0	0
	0	0	0	0
	0.5	1	1	1
	1	1	1	1
Haemate	0	0	0	0
	0	0	0	0
	0.5	0	1	0
	1	1	1	1

Table 13.4.2: Preliminary estimate of interlaboratory reproducibility: Assessed by testing of 3 substances, spiked 4 times. One run of 12 samples by three different laboratories.

Laboratories	Interlaboratory Reproducibility	Number of equal predictions
DL - NL1	83.3%	10 / 12
DL - NL2	100%	12 / 12
NL1 – NL2	83.3%	10 / 12
Mean	88.9%	
same result in all laboratories	83.3%	10 / 12

DL =Konstanz; NL1 = Qualis; NL2 = PEI

A 2x2 contingency table was constructed (table 13.4.3), from which the estimates of sensitivity and specificity can easily be derived.

Table 13.4.3: 2x2 contingency table. The prediction model applied to a preliminary validation study with KN CRYO WB/IL-1. Three different substances were assessed in three different laboratories (derived from table 13.4.1)

	True status of samples		Total	
	+	-		
PM	+	16	1	17
	-	2	17	19
Total		18	18	36

The specifications of specificity and sensitivity described in section 5.3 were applied to these results. The specificity (Sp) of the KN CRYO WB/IL-1 assay is 94.4% ($17/(17+1)*100\%$). The sensitivity (Se) calculated for this data set is 88.9% ($16/(16+2)*100\%$). As outlined previously the specificity is overestimated and the sensitivity is underestimated as a result of the design of this part of the study.

Conclusion: For KN CRYO WB/IL-1, an increased inherent variability and an increased and borderline acceptable limit of detection compared to the WB/IL-1 and the CRYO WB/IL-1 were found. As the variability is still tolerable and because of the interlaboratory reproducibility and the predictive capacity in terms of specificity and sensitivity, the KN CRYO WB/IL-1 method showed sufficiently good results to proceed with the validation of this method.

13.5 Test Method Accuracy

To assess **accuracy** of the proposed test method 10 substances (listed in table 3.3.1), were spiked with five different concentrations of the WHO-LPS (one of which is negative). To permit the application of the chosen PM, each drug was diluted to its individual MVD, which has been calculated using the ELC to that drug (listed in table 3.3.1.) Each substance had their own specific set of endotoxin spike solution, ensuring that after spiking the undiluted drug, it contained 0.0; 0.25; 0.5; 0.5 and 1.0 EU/ml respectively when tested at its MVD. A detailed description of the sample preparation was supplied to the three independent laboratories (as shown in table 5.1.2). To put more weight to the result of this part of the validation, the spikes were blinded and coded by QA ECVAM. Accuracy was assessed by applying the PM to the results and evaluating the concordance in a two by two table.

Table 13.4.1: Results of the validation study of 10 drugs, spiked with WHO-LPS at 0.0, 0.25, 0.5, 0.5 and 1.0 IU/ml, respectively and tested in 3 different laboratories. Samples and spikes were blinded. Classifications after applying the prediction model.

drug (code)	spike EU/ml	"truth"	results		
			PEI	Qualis	Novartis
Beloc (BE)	0.00	0	0	0	0
	0.25	0	0	0	CV
	0.50	1	0	1	1
	0.50	1	1	1	1
	1.00	1	1	1	1
Binotal (BI)	0.00	0	0	0	0
	0.25	0	0	1	CV
	0.50	1	1	1	1
	0.50	1	1	1	1
	1.00	1	1	1	1
Ethanol 13% (ET)	0.00	0	0	0	0
	0.25	0	0	0	CV
	0.50	1	1	1	0
	0.50	1	1	1	0
	1.00	1	1	1	1
Fenistil (FE)	0.00	0	0	0	0
	0.25	0	0	CV	1
	0.50	1	CV	1	CV
	0.50	1	1	1	1
	1.00	1	1	1	1
Glucose 5% (GL)	0.00	0	0	0	0
	0.25	0	0	1	0
	0.50	1	0	1	CV
	0.50	1	0	1	1
	1.00	1	0	1	1
"Drug A" 0.9% NaCl (LO)	0.00	0	0	0	nq
	0.25	0	0	0	nq
	0.50	1	0	1	nq
	0.50	1	0	1	nq
	1.00	1	1	1	nq
MCP (ME)	0.00	0	0	0	0
	0.25	0	0	1	0
	0.50	1	0	1	CV
	0.50	1	0	1	1
	1.00	1	1	1	1
"Drug B"	0.00	0	0	0	nq

drug (code)	spike EU/ml	“truth”	results		
			PEI	Qualis	Novartis
0.9% NaCl (MO)	0.25	0	0	0	nq
	0.50	1	0	1	nq
	0.50	1	0	1	nq
	1.00	1	1	1	nq
Orasthin (OR) Syntocinon	0.00	0	0	0	nq
	0.25	0	CV	CV	nq
	0.50	1	1	1	nq
	0.50	1	1	1	nq
1.00	1	1	1	nq	
Sostril (SO)	0.00	0	0	0	nq
	0.25	0	0	1	nq
	0.50	1	0	1	nq
	0.50	1	1	1	nq
	1.00	1	1	1	nq

“0” denotes “non-pyrogenic”; “1” denotes “pyrogenic”.

Grey shading indicates that for these drugs the PPCs did not qualify so that the PC was used in the PM.

CV = sample showed a variability resulting in exclusion, i.e. CV > 45% and no significant outlier present.

nq = not qualified according to quality criteria, i.e. failure of PPCs and PCs

False classifications are in bold/color type.

Of the 150 available data for the KN CRYO WB/IL-1 method, eleven sets of 4 replicates showed a high variability resulting in exclusion, i.e. CV > 45% and no significant outliers present. Therefore 139 data in total could be used to estimate the specificity and sensitivity of the KN CRYO WB/IL-1 method. The results are shown separately for each participating laboratory (table 13.5.1) as well as combined for all these laboratories (table 13.5.2).

The specificity (table 13.5.3) that can be estimated from the available results for DL, NL1 and NL2 is 94.1%, 80% and 77.8% respectively. The estimated sensitivity (table 13.5.3) of the KN CRYO WB/IL-1 assay was excellent of all three participating laboratories: 96%, 100% and 100% respectively (calculated from results in table 13.5.2).

Table 13.5.1: 2x2 contingency table. Prediction model applied to the KN CRYO WB/IL-1 test result of 10 different substances assessed in three different laboratories. Results of each laboratory separately (DL, NL1 and NL2= PEI, Qualis and Novartis respectively).

Results DL		True status of samples		Total
		+	-	
PM	+	18	0	18
	-	11	19	30
Total		29	19	48

Results NL1		True status of samples		Total
		+	-	
PM	+	30	4	34
	-	0	14	14
Total		30	18	48

Results NL2		True status of samples		Total
		+	-	
PM	+	13	1	14
	-	2	8	10
Total		15	9	24

Table 13.5.2: 2x2 contingency table. Prediction model applied to the KN CRYO WB/IL-1 test result of 10 different substances assessed in three different laboratories (from table 13.4.1).

		True status of samples		Total
		+	-	
PM	+	61	5	66
	-	13	41	54
Total		74	46	120

The overall specificity of the KN CRYO WB/IL-1 assay is 89.1% $(46/(46+14))*100$. The overall sensitivity equals 82.4% $(61/(61+13) *100\%)$. Within the laboratories, specificity varied from 77.8%, 88.9% and up to 100%, whereas the sensitivity varied from 62.1% up to 86.7% and 100%. respectively

Table 13.5.3: Specificity and sensitivity of the KN WB-CRYO /IL-1 assay as determined from table 13.5.2

	N total	N correctly identified	proportion	95% CI lower limit	95% CI upper limit
Specificity (Sp)	46	41	89.1%	0.764	0.964
Sensitivity (Se)	74	61	82.4%	0.718	0.903

13.6 Test Method Reliability (Reproducibility)

The interlaboratory reproducibility (table 13.6.1) of the KN CRYO WB/IL-1 method was assessed from the results of the validation test with 10 substances spiked with 5 separate spikes. The reproducibility varied from 68.1% to 82.6% between two laboratories. The estimated reproducibility between three laboratories (65.2%) was based on a very limited number of samples, because in one of the laboratories the results of many samples were not qualified for analysis.

Table 13.6.1: Interlaboratory reproducibility: Assessed by testing of 10 substances, spiked 5 times. One run of 50 samples by three different laboratories.

Laboratories	Interlaboratory Reproducibility	Number of equal predictions
DL - NL1	68.1%	32 / 47
DL - NL2	70.8%	17 / 24
NL1 – NL2	82.6%	19 / 23
Mean	73.8%	
same result in all laboratories	65.2%	15 / 23

DL =PEI; NL1 = Qualis; NL2 = Novartis

13.7 Summary and conclusion

In this study the specificity of the KN CRYO WB/IL-1 is comparable with the CRYO WB/IL-1 (89.1% versus 83.6%; see section 6 of this BRD). However, the mean sensitivity shown for the KN CRYO WB/IL-1 (82.4%) is significantly less than achieved with the CRYO WB/IL-1 (98.8%). This can be fully explained by the false negatives in one of the laboratories. The KN CRYO WB/IL-1 method shows a lower interlaboratory reproducibility than was estimated for the CRYO WB/IL-1 method. In addition, it is noted that in one of the laboratories the results of many samples were not qualified for analysis. It appears that in this study the KN CRYO WB/IL-1 is not yet as robust as the CRYO WB/IL-1 method which is described in sections 1-12 of this BRD.

14 Supporting Materials (Appendices)

14.1 Standard operating procedure (SOP) of the proposed method

Provide the complete, detailed protocol for the proposed test method.

Appendix A includes the test method protocol *CRYO WB/IL-1: Human Whole Blood Pyrogen Test in 96-well Plates Using Fresh or Cryopreserved Blood* (electronic file name: *SOP CRYO WB IL 1*). It covers three variations to the preparation of the whole blood described under point 7: 7A - fresh blood using 96-well plates, 7B - cryopreserved blood according to the so called "PEI (Paul-Ehrlich-Institute, Langen, Germany)" method = CRYO WB/IL-1" and 7C - cryopreserved blood according to the so called "Konstanz" method KN CRYO WB/IL-1.

The trial plan of the catch-up validation study is also included in Appendix A.

14.2 Standard operating Procedure (SOP) of the reference method

Provide the detailed protocol(s) used to generate reference data for this submission and any protocols used to generate validation data that differ from the proposed protocol.

14.3 Publications

Provide copies of all relevant publications, including those containing data from the proposed test method, the in vivo reference test method, and if applicable, a comparable validated test method with established performance standards.

Not all of the publications cited in the BRD (see section 12) are included as hardcopies in Appendix B, e.g. publications on statistical methods are not given.

Remark: The same set of hardcopies was included into Appendix B of all of the 5 submitted BRDs. Therefore some of the publications in Appendix B might not be referenced in the current BRD nor included in the list of the publications in section 12. Three general publications were added, which are not cited in any BRD but might be useful as background information to the validation study: the ECVAM publications on validation (Balls et al, 1995; Curren et al, 1995; Hartung et al, 2004). Several publications were included, which either give more background information on the human fever reaction or report on specific studies using *in vitro* pyrogen tests.

List of hard copies

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Part 2:**List of Diploma theses, reports and/or PhDs etc. concerning the WB/IL-1 test (IPT: *In vitro* Pyrogen Test)**

1. Final report for the BMBF (Bundesministerium für Bildung und Forschung) (University of Konstanz, 2000). „Evaluierung und Prävalidierung eines Vollblutmodelles zum Ersatz des Pyrogentests am Kaninchen (DAB10)“, Phase I, (“Evaluation and prevalidation of a whole blood assay for the replacement of the pyrogentest with rabbits”), July 1th, 1997 – June 30th, 2000, No. 0311424
2. Final report for the BMBF (Bundesministerium für Bildung und Forschung) (Langen, Paul-Ehrlich-Institut, 2000). „Evaluierung und Prävalidierung eines Vollblutmodelles zum Ersatz des Pyrogentests am Kaninchen (DAB10)“, Phase I, (“Evaluation and prevalidation of a whole blood assay for the replacement of the pyrogentest with rabbits”), July 1th, 1997 – June 30th, 2000, No. 0311425
3. PhD-Thesis from Markus Weigandt at the Ruprecht-Karls-University of Heidelberg, institute of hygiene (Director: Prof. H.-G. Sonntag): „Der humane Vollblut-Pyrogentest: Optimierung, Validierung und Vergleich mit den Arzneibuchmethoden“ (The human whole blood pyrogen test: optimization, validation and comparison with methods regulated in the pharmacopoeias), 2000
4. Master Thesis (Master of Science: MSc), Karin Kullmann: „Adaptation des *In vitro* Pyrogen Tests (IPT) für prothetische Materialien“ (“Adaptation of the *in vitro* pyrogen test (IPT) to medical devices”), Technical University of Furtwangen, July 2002
5. Final report for the BMBF (Bundesministerium für Bildung und Forschung) (Langen, Paul-Ehrlich-Institut, 2004). „Ersatz des Pyrogentests am Kaninchen durch einen Vollbluttest“, Phase II, (“replacement of the rabbit experiment with the whole blood test”), October 1th, 2000 – September 30th, 2003, No. 0311424A
6. Final report for the BMBF (Bundesministerium für Bildung und Forschung) (University of Konstanz, 2004). „Ersatz des Pyrogentests am Kaninchen durch einen Vollbluttest“, Phase II, (“replacement of the rabbit experiment with the whole blood test”), September 1th, 2000 – August 31th, 2003, No. 0311424A
7. Brazil/Germany Cooperation Project: final report for the BMBF (Bundesministerium für Bildung und Forschung). „Validation of *in vitro* Cytokine Release Assay (Whole Blood Assay) for Controlling the Quality of Human Injectable Products“ for bilateral Cooperation in Science and Technology (Germany – Brazil), April 1th 2002-March 31th 2004, No. BRA 02/004
8. Cuba/Germany Cooperation Project: final report for the BMBF (Bundesministerium für Bildung und Forschung). „Pyrogenicity Testing by Human Whole Blood“ for bilateral Cooperation in Science and Technology (Germany – Cuba), January 1th, 2001- December 31th, 2003, No. CUB 00/022
9. Final report for the BMWa (Bundesministerium für Wirtschaft und Arbeit): “Entwicklung einer humanrelevanten Messtechnik für luftgetragene Toxine mit humanem Vollblut“ (development of a human relevant measurement for air-borne

toxins with human whole blood), Sept 3th 2001– Sept. 30th 2003, No. KF 0317101KRF1

10. Postdoctoral lecture qualification (Habilitation), Bert Zucker, “Luftgetragene Endotoxine in Tierställen“ (“air-borne pyrogens in a stable”), Institut für Tier- und Umwelthygiene an der freien Universität Berlin, Berlin, 2004
11. Manuscript for the DIF (Deutsches IndustrieForum, DIF-Fachtagung), Stefan Fennrich: “Pyrogenverunreinigungen an medizinischen Oberflächen. *In vitro* pyrogen-Test (IPT) als humanrelevantes Prüfverfahren“ (Contamination with pyrogens on medical surfaces: the *in vitro* pyrogen test (IPT) as a human specific method), Würzburg, June 21th -22th, 2004, No. DIF 21/78/FE

Part 3: Further publications concerning the WB/IL-1 test (IPT)

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14.4 Original data

Include all available non-transformed original data for both the proposed test method, the in vivo reference test method, and if applicable, a comparable validated test method with established performance standards.

NOTE: The original data of the ELISA-plate reader were collected by S.Hoffman and ECVAM. These are available on the CD which goes with the BRD.

14.5 Performance standards

If appropriate performance standards for the proposed test method do not exist, performance standards for consideration by NICEATM and ICCVAM may be proposed. Examples of established performance standards can be located on the ICCVAM / NICEATM web site at <http://iccvam.niehs.nih.gov>.

APPENDIX A

Trial plan “Catch-up Validation of Novel Pyrogen Tests Based on the Human Fever Reaction”

Detailed protocol CRYO WB/IL-1: *Human Whole Blood Pyrogen Test in 96-well Plates Using Fresh or Cryopreserved Blood(electronic file name: SOP CRYO WB IL 1).*

APPENDIX B

Not all of the publications cited in the BRD (see section 12) are included as hardcopies in Appendix B, e.g. publications on statistical methods are not given.

Remark: The same set of hardcopies was included into Appendix B of all of the 5 submitted BRDs. Therefore some of the publications in Appendix B might not be referenced in the current BRD nor included in the list of the publications in section 12. Three general publications were added, which are not cited in any BRD but might be useful as background information to the validation study: the ECVAM publications on validation (Balls et al, 1995; Curren et al, 1995; Hartung et al, 2004). Several publications were included, which either give more background information on the human fever reaction or report on specific studies using *in vitro* pyrogen tests.

List of hard copies

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APPENDIX C

List of abbreviations and definitions

Accuracy	The ability of a test system to provide a test result close to the accepted reference value for a defined property.
BET	The bacterial endotoxin test is used to detect or quantify endotoxins of gram-negative bacterial origin using amoebocyte lysate from horseshoe crab (<i>Limulus polyphemus</i> or <i>Tachypleus tridentatus</i>)
BRD	Background Review Document
CRYO WB/IL-1	Whole blood assay (using cryopreserved blood) with IL-1 as endpoint
CV	coefficient of variation
DL	Developing laboratory = laboratory which developed the method or the most experienced laboratory
ELC	Endotoxin limit concentration; maximum quantity of endotoxin allowed in given parenterals according to European Pharmacopoeia
Endotoxins	Endotoxins are a group of chemically similar cell-wall structures of Gram-negative bacteria, i.e. lipopolysaccharides
ELISA	Enzyme linked immunosorbent assay
EU/ml	European Units per ml
IL-1	interleukin 1
IL-6	interleukin 6
Intralaboratory reproducibility	A determination of the extent that qualified people within the same laboratory can independently and successfully replicate results using a specific protocol at different times.
Interlaboratory reproducibility	A measure of the extent to which different qualified laboratories, using the same protocol and testing the same substances, can produce qualitatively and quantitatively similar results. Inter-laboratory reproducibility is also referred to as between-laboratory reproducibility.
KN	University of Konstanz (Konstanz, Germany), developing laboratory WB/IL-1 and CRYO WB/IL-1
LPS	lipopolysaccharides
MM6	MONO MAC-6 cell line

MM6/IL-6	In vitro pyrogen test using MM6 cell line and IL-6 release as an endpoint
MVD	Maximum valid dilution; the MVD is the quotient of the ELC and the detection limit
NIBSC	National Institute for Biological Standards and Control (London, UK), developing laboratory for WB/IL-6
NL	naïve laboratory = laboratory with non or minor experience with the method
NPC	negative product control (clean, released lot of the nominated product under test)
Novartis	Novartis (Basel, Switzerland), developing laboratory PBMC/IL-6
OD	optical density
PBMC	Peripheral blood mononuclear cells
PBMC/IL-6	In vitro pyrogen test using fresh peripheral blood mononuclear cells and IL-6 release as endpoint
PBMC-CRYO/IL-6	In vitro pyrogen test using cryopreserved peripheral blood mononuclear cells and IL-6 release as endpoint
PEI	Paul-Ehrlich Institut (Langen, Germany), participating laboratory
PM	prediction model = is an explicit decision-making rule for converting the results of the in vitro method into a prediction of in vivo hazard
PPC	positive product control (product under test spiked with 0.5 EU/ml of WHO-LPS (code 94/580))
Prevalidation study	A prevalidation study is a small-scale inter-laboratory study, carried out to ensure that the protocol of a test method is sufficiently optimised and standardised for inclusion in a formal validation study. According to the ECVAM principles, the prevalidation study is divided into three phases: protocol refinement, protocol transfer and protocol performance (Curren et al, ATLA 23, 211-217).
Pyrogens	fever-causing materials
Pyrogens, endogenous	endogenous pyrogens are messenger substances released by blood cells reacting to pyrogenic materials; e.g. IL-1, IL-6, TNF- α , prostaglandin E ₂
Pyrogens, exogenous	exogenous pyrogens derive from bacteria, viruses, fungi or from the host himself
Reliability	Measures of the extent to which a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is

	assessed by calculating intra- and interlaboratory reproducibility and intra-laboratory repeatability.
Relevance	Relevance of a test method describes whether it is meaningful and useful for a particular purpose. It is the extent to which the measurement result and uncertainty can accurately be interpreted as reflecting or predicting the biological effect of interest.
Repeatability	Repeatability describes the closeness of agreement between test results obtained within a single laboratory when the procedure is performed independently under repeatability conditions, i.e. in a set of conditions including the same measurement procedure, same operator, same measuring system, same operating conditions and same location, and replicated measurements over a short period of time.
RIVM	National Institute of Public Health and the Environment (Bilthoven, The Netherlands), developing laboratory MM6/IL-6 method
Sensitivity	Sensitivity is the proportion of all positive/active substances that are correctly classified by a test method.
Specificity	Specificity is proportion of all negative/inactive substances that are correctly classified by a test method.
TMB	chromogenic substrate 3,3',5,5' -tetramethylbenzidine
TNF- α	tumour necrosis factor- α
USP	US Pharmacopoeia
Validation	Validation is the process by which the reliability and relevance of a procedure are established for a specific purpose
Validation study	A validation study is a large-scale interlaboratory study, designed to assess the reliability and relevance of an optimised method for a particular purpose
WB/IL-1	Whole blood assay (using fresh blood) with IL-1 release as endpoint
WB/IL-6	Whole blood assay (using fresh blood) with IL-6 release as endpoint
WHO	World Health Organization

Appendix D

Experiments performed regarding the assessment of the intralaboratory reproducibility of CRYO WB/IL-1, KN CRYO WB/IL-1 and 96-wells WB/IL-1

The intralaboratory reproducibility of WB/IL-1 was extensively assessed and the studies carried out for this purpose are described in Section 5 and table 5.1.1 of the BRD.

Table 5.1.1 of WB/IL-1 BRD

Experiment	spikes	n (per spike)	repetitions	
1A	0; 0.5	32	1	64
1B	0; 0.063; 0.125; 0.25; 0.5	12	1	60
2A	0; 0.5	12	3	72
2B	0; 0.25; 0.5	8	3	72
2C	0; 0.5	5	8	80

1A: replicates of the negative and the positive control

1B: limit of detection

2A: behaviour of one fresh blood donor on three successive days

2B: Influence of the operator

2C: Robustness with regard to different donors

For the catch-up validation study, only a few of the experiments described in Table 5.1.1 were carried out (1A and 1B) with some of the methods. The results are given in the following:

1. Comparison of the WB/IL-1 and 96-wells WB/IL-1 (Fig. 1a/b).

Numbers: coefficient of variation (%) as determined using GraphPadPrism Software

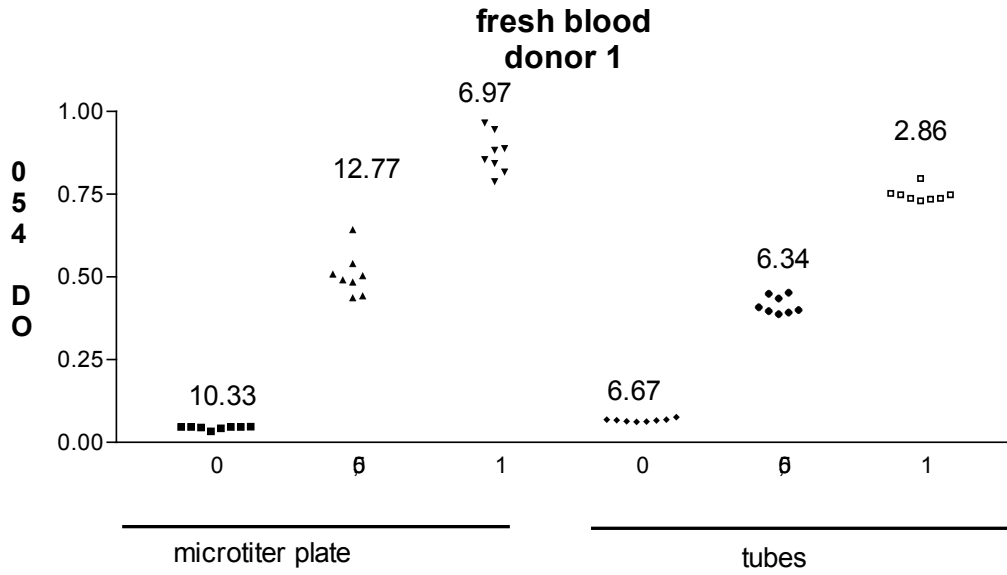


Fig. 1a: Comparison of 96-wells WB/IL-1 with fresh blood of two donors with WB/IL-1 (donor 1)

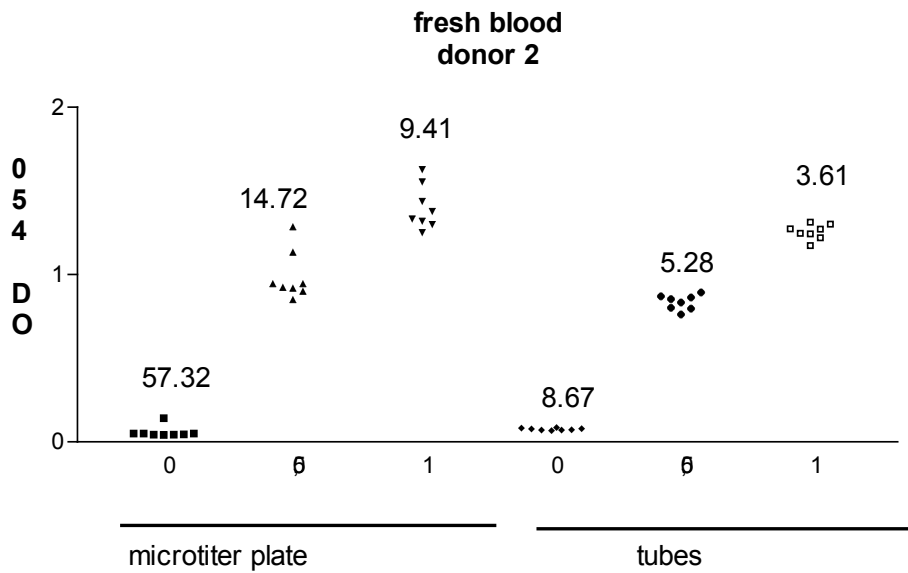


Fig. 1b: Comparison of 96-wells WB/IL-1 with fresh blood of two donors with WB/IL-1 (donor 2)

2. With several lots of cryopreserved blood, experiments concerning the variability of results and the detection of the required 0.5 EU/ml stimulus were made (8fold values) and are given in the following figures:

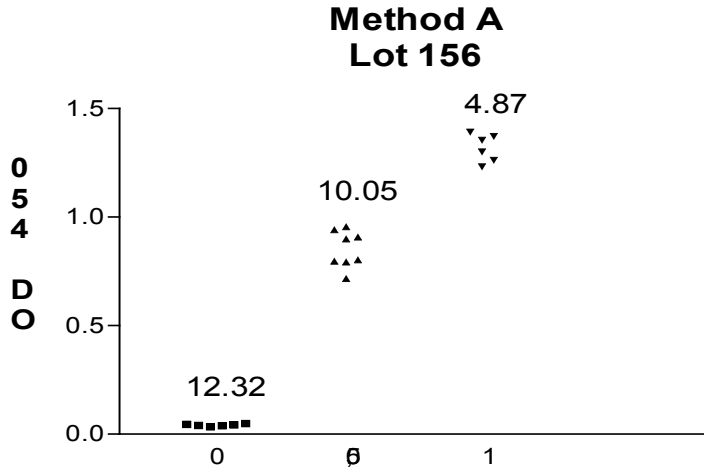


Fig 2a: Method A = CRYO WB/IL-1: blood frozen at -80°C;
Lot 156 8fold tested, 3 spikes

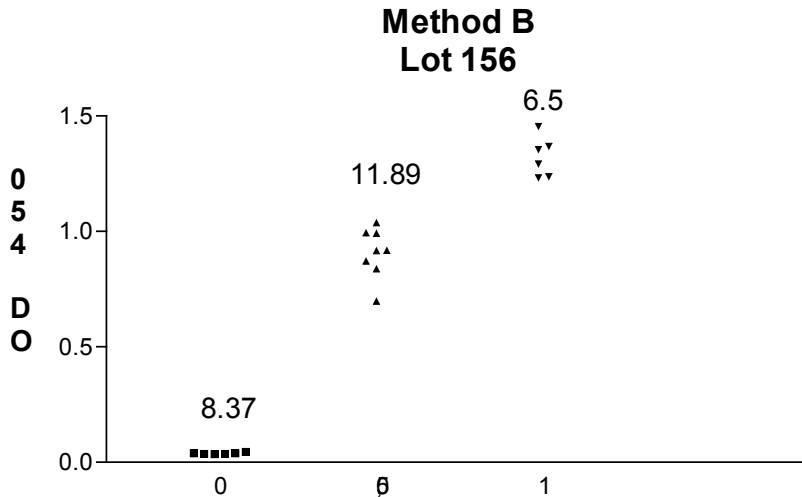


Fig 2b: Method B = KN CRYO WB/IL-1: blood frozen in the vapour phase of liquid nitrogen; Lot 156 8fold tested, 3 spikes

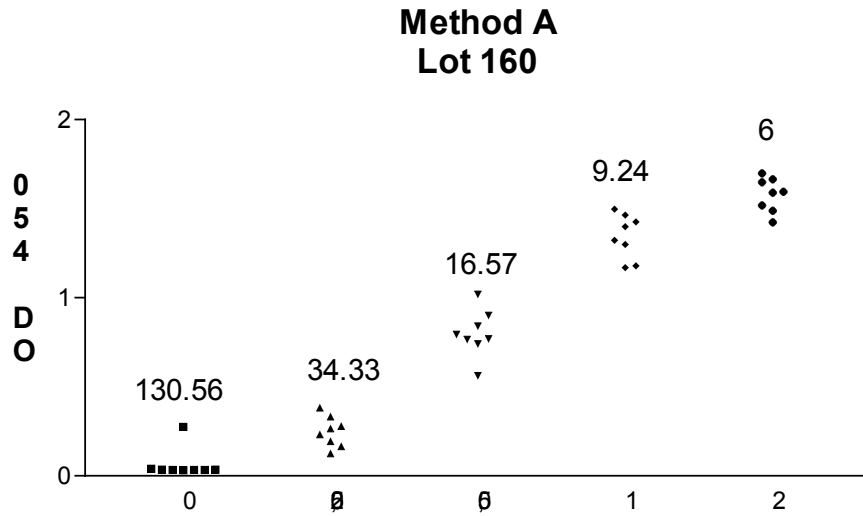


Fig 2c: Method A = CRYO WB/IL-1: blood frozen at -80°C;
Lot 160 8fold tested, 5 spikes

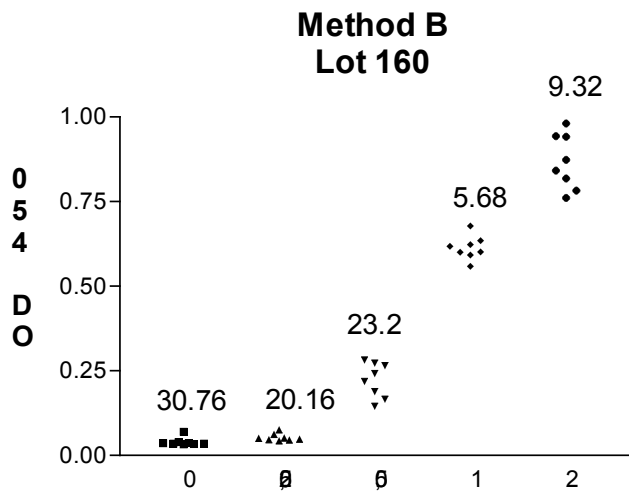


Fig 2d: Method B = KN CRYO WB/IL-1: blood frozen in the vapour phase of liquid nitrogen Lot 160 8fold tested, 5 spikes

3. Since KN CRYO/WB/IL-1 showed a higher variance and a lower limit of detection than the other two methods, a set of experiments was performed:

- reproducibility of saline and 0.5 EU/ml (corresponds to 1A in above Table 5.1.1)
- limit of detection experiment (corresponds to 1B in above Table 5.1.1)

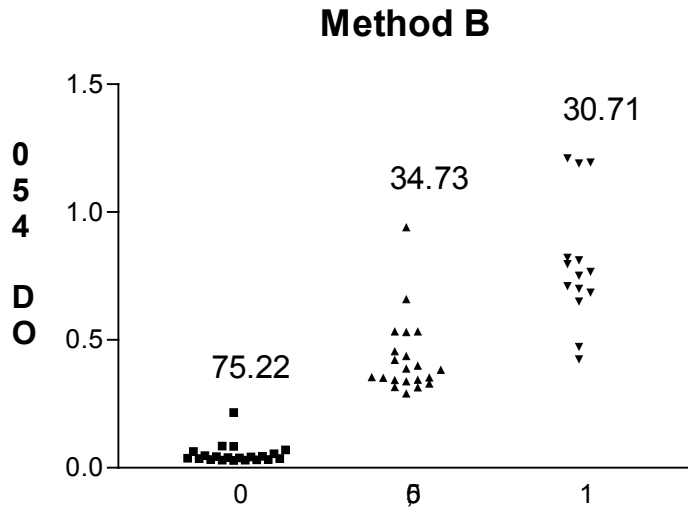


Fig 3: Method B = KN CRYO WB/IL-1: blood frozen in the vapour phase of liquid nitrogen; Variance of the method at spikes of 0 and 0.5 EU/ml: 18fold values; 1 EU/ml: 12fold values.

4. Due to the obvious lower sensitivity of KN CRYO WB/IL-1, an experiment was performed concerning the limit of detection according to 1B of the above table (Fig. 4a/b). This experiment was done twice with different pools of cryopreserved blood

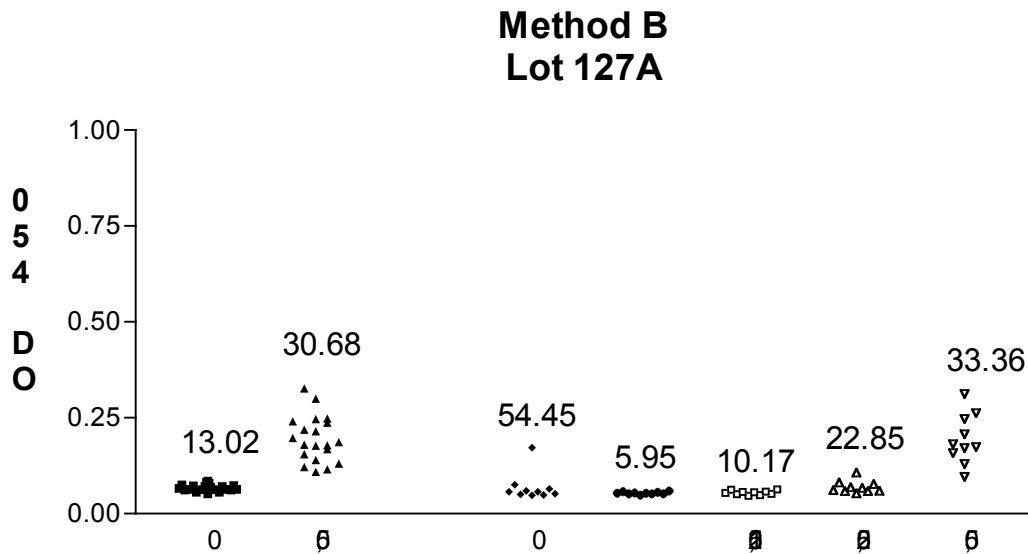


Fig. 4a: Method B = KN CRYO WB/IL-1: Limit of detection of the frozen blood (nitrogen) Lot Number 127A. Stimulus: E. coli O113: H10:

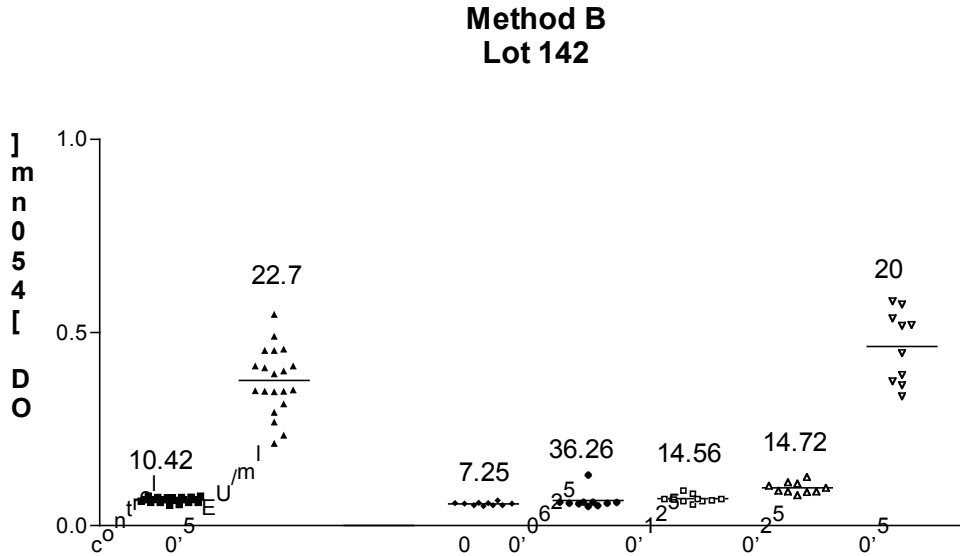


Fig. 4b: Method B = KN CRYO WB/IL-1: Limit of detection of the frozen blood (nitrogen), lot 142. Stimulus: E. coli O113: H10

All in all, these above experiments indicated no need for extensive studies concerning the intralaboratory performance and variability of the three methods. The only method that appeared problematic was KN CRYO WB/IL-1, whose characteristics were further explored with the above experiments. Nevertheless, KN CRYO WB/IL-1 proved to reliably retrieve the 0.5 EU/ml control as positive, despite a higher variability.

It was concluded, that the data from the different laboratories performing the methods in the prevalidation would suffice, taking the experiences of the former validation into account.