DRAFT BACKGROUND REVIEW DOCUMENT

CURRENT STATUS OF FIVE *IN VITRO* PYROGENICITY TEST METHODS

- **THE HUMAN WHOLE BLOOD (WB)/IL-1 *IN VITRO* PYROGEN TEST: APPLICATION OF CRYOPRESERVED HUMAN WB**
- **AN ALTERNATIVE *IN VITRO* PYROGENICITY TEST USING THE MONOCYTOID CELL LINE MONO MAC 6 (MM6)/IL-6**
- **THE HUMAN PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC)/IL-6 *IN VITRO* PYROGEN TEST**
- **THE HUMAN WB/IL-1 *IN VITRO* PYROGEN TEST**
- **THE HUMAN WB/IL-6 *IN VITRO* PYROGEN TEST**

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

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LIST OF ACRONYMS AND ABBREVIATIONS

3 R's	Replacement, Reduction, and Refinement
ANOVA	Analysis of variance
AWIPT	Adsorb, Wash, *In Vitro* Pyrogen Test
BET	Bacterial Endotoxin Test
BP	British Pharmacopoeia
BRD	Background Review Document
cAMP	Cyclic adenosine monophosphate
°C	Degrees centigrade
CAS	Chemical Abstract Service
CASRN	Chemical Abstracts service Registry Number
CBER	Center for Biologics Evaluation and Research
CDER	Center for Drug Evaluation and Research
CDRH	Center for Devices and Radiological Health
CEC	Commission of European Communities
CFR	Code of Federal Regulations
cm	Centimeter
Cryo-	Cryopreserved
CSF	Colony stimulating factor
CV	Coefficient of variation
CVM	Center for Veterinary Medicine
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EDQM	European Directorate for the Quality of Medicines
EEC	European Economic Community
ELC	Endotoxin limit concentration
ELISA	Enzyme-linked immunosorbent assay
EP	European Pharmacopoeia
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee

EU	European Union
EU/mL	Endotoxin units per milliliter
FDA	U.S. Food and Drug Administration
Fn	Fibronectin
FR	*Federal Register*
g	Gram
GLP	Good Laboratory Practice
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GSK	GlaxoSmithKline
Hb	Hemoglobin
hGH	Human growth hormone
HSA	Human serum albumin
IACAC	Institutional Animal Care and Use Committee
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-6	Interleukin-6
i.m.	Intramuscular
ISO	International Standards Organization
i.t.	Intrathecal
IU	International units (endotoxin)
i.v.	Intravenous
JP	Japanese Pharmacopoeia
K	Threshold pyrogen dose
Kg	Kilogram
LAL	*Limulus* Amebocyte Lysate
λ	Sensitivity of LAL reagent in EU/mL
LPS	Lipopolysaccharide

MAPK	Mitogen associated protein kinase
M	Maximum human or rabbit pyrogen test dose in kg/hr
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
mm	Millimeter
MM6	Mono Mac 6
MVD	Maximum valid dilution
NC	Negative control
NF	National Formulary
ng	Nanogram
nm	nanometer
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NPC	Negative product control
OECD	Organization for Economic Co-Operation and Development
OD	Optical density
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEI	Paul Ehrlich Institute
pg	Picogram
PG	Peptidoglycan
PGE_2	Prostaglandin E_2
PHA	Phytohaemagglutinin
P.L.	Public law
PMA	Phorbol myristate acetate
Poly (I:C)	Polyinosine:polycytidylic acid
PPC	Positive product control
PWG	Pyrogenicity Working Group
QA	Quality assurance

rec	Recombinant
RPT	Rabbit pyrogen test
s.c.	Subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
SOT	Society of Toxicology
SMT	Study Management Team
SHS	Stabilized human serum
SOP	Standard operating procedure
TG	Test Guideline
THP-1	Acute monocyte leukemia cell line
TLR-4	Toll-like receptor-4
TNF-α	Tumor necrosis factor-α
μg	Microgram
UK	United Kingdom
U.S.	United States
U.S.C.	United States Code
UN	United Nations
USP	U.S. Pharmacopeia
USPTO	United States Patent Office
WB	Whole blood
WHO	World Health Organization
xg	Times gravity

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PREFACE

Endotoxin, a bacterial pyrogen also known as lipopolysaccharide, is an integral component of the Gram-negative bacterial cell membrane. Endotoxin directly interacts with host monocytoid cells to induce the release of a variety of proinflammatory cytokines (e.g., interleukin [IL]-1β, IL-6, tumor necrosis factor [TNF]-α). In addition to an initial febrile reaction, excessive release of these cytokines during Gram-negative bacterial sepsis can lead to multiple organ failure and death. For this reason, it is critical that parenteral pharmaceuticals, fluids for injection, medical devices, and human biological products be properly and accurately evaluated for the presence of endotoxin prior to their clinical or veterinary use. The original pyrogen test, the rabbit pyrogen test (RPT), was developed in 1941 to limit to an acceptable level the risks of febrile reaction in the patient to administration of, or contact with, the product of concern. While the RPT continues to serve this purpose well today, an endotoxin test using an extract from the blood cells of the horseshoe crab (i.e., the bacterial endotoxin test [BET]) was developed in the early 1970's as an alternative to the RPT for the detection of this pyrogen. In 1980, the U.S. Food and Drug Administration published guidelines for use of the BET as an end product test for human and animal drug products. The U.S., European, and Japanese Pharmacopoeias currently recognize both test methods for pyrogen testing (i.e., RPT and BET). The BET is recognized for its sensitivity to the presence of Gram-negative endotoxins, but it has well documented limitations, including its inability to respond to non-endotoxin pyrogens, as well as its susceptibility to interference from certain types of materials (e.g., high protein and lipid levels; glucans). In contrast, the RPT is capable of detecting both endotoxin and non-endotoxin pyrogens, but requires the need for interspecies translation from rabbits to humans, and is associated with animal welfare concerns that discourage animal testing.

More recent efforts have focused on the development of an *in vitro* test system that combines the sensitivity of the BET with the wide range of pyrogens detectable by the RPT. With this intention, test systems based on the activation of human monocytes *in vitro* have been developed that take advantage of the role of these cells in the fever response. The European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection (IHCP) at the European Commission's Joint Research Centre, conducted a validation study to independently evaluate the usefulness of six *in vitro* pyrogenicity test methods. The study was financed by the European Commission within the 5th Framework Programme of DG Research and recently published (Hoffmann et al, 2005). Since two tests based on THP-1 cell lines did not meet the validation criteria, they are not included in the peer review. During Summer 2004, the University of Konstanz (Germany) carried out catch-up validation studies of two tests using cryo-preserved blood (WB/IL-1) or blood cells (PBMC-IL6), the results of which were recently published (Schindler et al. 2006).

Based on these studies, in June 2005, ECVAM submitted background review documents (BRDs) for five methods to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) for consideration as replacements for the RPT. The proposed test methods were:

- The Human Whole Blood (WB)/IL-1 *In Vitro* Pyrogenicity Test: Application of Cryopreserved Human WB
- An Alternative *In Vitro* Pyrogenicity Test Using the Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6
- The Human PBMC/IL-6 *In Vitro* Pyrogen Test
- The Human WB/IL-1 *In Vitro* Pyrogen Test
- The Human WB/IL-6 *In Vitro* Pyrogen Test.

For simplicity, the submitted studies are referred to collectively as the ECVAM validation study in this document.

ICCVAM, which is charged with coordinating the technical evaluations of new, revised, and alternative test methods with regulatory applicability (ICCVAM Authorization Act of 2000, Public Law [P.L.] 106-545), unanimously agreed that the five submitted *in vitro* test methods should have a high priority for evaluation. An ICCVAM Pyrogenicity Working Group (PWG) was established to work with NICEATM to carry out these evaluations. The PWG consists of knowledgeable scientists from ICCVAM member agencies. The PWG functions include the review of draft test method BRDs, recommending proposed performance standards, identifying and recommending scientists for expert scientific review panels, preparing questions for expert or peer review panels, developing draft ICCVAM test method recommendations regarding the usefulness and applicability of the alternative test methods for regulatory testing, and recommending necessary validation studies. ICCVAM and NICEATM also collaborate closely with ECVAM. Accordingly, an ECVAM liaison was designated for the ICCVAM PWG to ensure input and contributions during the evaluation and review process.

NICEATM, which administers the ICCVAM and provides scientific support for ICCVAM activities, subsequently prepared a comprehensive background review document (BRD) that provided information and data from the validation studies for each of the five *in vitro* test methods. A request for any other data and information on these test methods was made through a 2005 *Federal Register* (*FR*) request (Available: http://iccvam.niehs.nih.gov/methods/pyrogen.htm), through the ICCVAM electronic mailing list, and through direct requests to over 100 interested stakeholders. No additional data or information was submitted in response to these requests.

This BRD is publicly available on the ICCCVAM/NICEATM website (http:// iccvam.niehs.gov) or from NICEATM on request. Comments from the public and scientific community are welcome and will be provided to the panel experts and made available on the ICCVAM/NICEATM website (see *FR* notice TBD, published TBD, available at http://iccvam.niehs.nih.gov). The independent review of the usefulness and limitations of the five test methods will take place in a public meeting of the international expert scientific panel on February 6, 2007 at the National Institutes of Health campus in Bethesda, Maryland. The review panel's independent report will be published and made available for public comment following the panel's February meeting. The ICCVAM and the PWG will consider the report and public comments, and prepare final test method recommendations that will be provided to federal agencies and made available to the public. ICCVAM final test method recommendations are forwarded to U.S. Federal agencies for consideration, in accordance with the ICCVAM Authorization Act of 2000 (P.L. 106-545).

We want to acknowledge the excellent cooperation and contributions from the many organizations and scientists who provided critical data and information necessary for the original ECVAM BRD and for the subsequent ICCVAM review. The efforts of many individuals who contributed to the preparation of the ICCVAM BRD are also gratefully acknowledged. These include David Allen, Ph.D., Bradley Blackard, M.S.P.H., and James Truax, M.A. of Integrated Laboratory Systems (ILS, Inc.), the NICEATM Support Contractor, as well as the members of the ICCVAM PWG and ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this final draft version. We also want to thank Raymond Tice, Ph.D., Deputy Director of NICEATM, for his extensive efforts on this project. Finally, we want to recognize the excellent leadership of the PWG Chair, Dr. Richard McFarland (FDA).

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EXECUTIVE SUMMARY

This draft Background Review Document (BRD) provides a comprehensive review of available data and information regarding the usefulness and limitations of five alternative *in vitro* pyrogenicity test methods. The test methods are:

- The Human Whole Blood (WB)/IL-1 *In Vitro* Pyrogen Test: Application of Cryopreserved Human WB
- An Alternative *In Vitro* Pyrogenicity Test Using the Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6
- The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test
- The Human WB/IL-1 *In Vitro* Pyrogen Test
- The Human WB/IL-6 *In Vitro* Pyrogen Test

The test methods were reviewed for their ability to detect the presence of Gram-negative endotoxin when spiked into a variety of parenteral pharmaceuticals. The objective of this BRD is to describe the current validation status of the *in vitro* pyrogenicity test methods, including what is known about their relevance[2] and reliability[3], the scope of the substances tested, and the availability of a standardized test method protocol for each test method.

The information summarized in this BRD is based on data contained in five individual BRDs submitted by the European Centre for the Validation of Alternative Methods (see **Appendix A**) to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The ECVAM BRDs were prepared according to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods (ICCVAM 2003) to allow for an independent scientific peer review

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[2] The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the "accuracy" or "concordance" of a test method.

[3] A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reproducibility and intralaboratory repeatability.

panel to assess the validation status of the proposed test methods, and for agencies to assess the acceptability of the proposed test methods for providing useful information for hazard or risk assessment. Each ECVAM BRD summarizes the validation studies conducted with each *in vitro* pyrogenicity test method, which is in turn compiled into a single document in the current BRD, which compares and contrasts the five test methods.

This BRD also summarizes available information obtained from published studies as well as additional unpublished data provided by ECVAM (see **Section 9.0**, Other Scientific Reports and Reviews). This section discusses *in vitro* pyrogenicity test method studies that could not be included in the performance analyses because of the lack of appropriate study details or test method results and/or the lack of appropriate *in vivo* rabbit pyrogen test (RPT) reference data. An online literature search for additional data and information on the proposed *in vitro* pyrogenicity test methods identified eighteen published studies that contained relevant data. ECVAM also provided additional unpublished data in response to a request for additional information related to the validation studies.

The proposed *in vitro* pyrogenicity test methods are based on the measurement of proinflammatory cytokines (i.e., interleukin-1 [IL-1] or interleukin-6 [IL-6]), released from monocytoid cells contained in WB, from isolated PBMC, or from a monocytoid cell line in response to exposure to a pyrogen, namely, Gram-negative endotoxin. No data were provided from the validation studies supporting the usefulness of these test method for non-endotoxin-based pyrogens.

U.S. regulatory agencies were surveyed to determine whether any of the proposed test methods have been considered for regulatory use where submission of test data is required. General regulatory practice (e.g., in the US and EU) is to accept pyrogenicity test method data when product specific validation of the test method has been demonstrated. It was noted in the ECVAM BRD that the U.S. FDA has accepted data from the PBMC test developed by Novartis and Baxter Healthcare which, in conjunction with RPT and BET data, were used to support the safety testing of a single specific drug product (New Drug Application Number 16-267/S-037).

Although there are differences among the *in vitro* pyrogenicity test methods based predominantly on the cell type used, there are some basic steps that are consistent across all methods as follows:

- The test substance is applied to the specific human-derived cells used in the *in vitro* test method (i.e., mixed with a suspension of cells).
- The test substance is incubated with the cells for a specified period of time
- The concentration of pro-inflammatory cytokines (e.g., IL-1β, IL-6) is quantified via a cytokine-specific enzyme-linked immunosorbent assay (ELISA) by comparison to a standard curve
- Using an endotoxin standard curve, the endotoxin content of the product is calculated
- A product "passes" (i.e., is considered negative for endotoxin) if the endotoxin content is < 0.5 endotoxin units (EU)/mL.

A total of ten parenteral pharmaceuticals were used for the determination of the sensitivity and specificity of the five *in vitro* pyrogenicity test methods. The ability of the *in vitro* pyrogenicity test methods to correctly identify the presence of Gram-negative endotoxin was evaluated using parenteral pharmaceuticals spiked endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Each substance was spiked with five concentrations of endotoxin tested once in three different laboratories. As outlined in **Table ES-1**, this analysis indicated that concordance among the test methods ranged from 81% to 93%, sensitivity ranged from 89% to 97%, specificity ranged from 81% to 97%, false negative rates ranged from 3% to 27%, and false positive rates ranged from 3% to 19%.

Table ES-1 Performance Analysis for Five *In Vitro* Pyrogenicity Test Methods[1]

Test Method	Concordance[2]	Sensitivity	Specificity	False Negative Rate	False Positive Rate
cryo WB/IL-1	91.7% (110/120)	97.4% (75/77)	81.4% (35/43)	2.6% (2/77)	18.6% (8/43)
MM6/IL-6	93.2% (138/148)	95.5% (85/89)	89.8% (53/59)	4.5% (4/89)	10.2% (6/59)
PBMC/IL-6	93.3% (140/150)	92.2% (83/90)	95.0% (57/60)	7.8% (7/90)	5.0% (3/60)
PBMC/IL-6 (cryo)[3]	*91.9% (136/148)*	*88.8% (79/89)*	*96.6% (57/59)*	*11.2% (10/89)*	*3.4% (2/59)*
WB/IL-6	91.9% (136/148)	88.8% (79/89)	96.6% (57/59)	11.2% (10/89)	3.4% (2/59)
WB/IL-1	81.0% (119/147)	72.7% (64/88)	93.2% (55/59)	27.3% (24/88)	6.8% (4/59)
WB/IL-1 (96-well plate method)[4]	*92.8% (129/139)*	*98.8% (83/84)*	*83.6% (46/55)*	*1.2% (1/84)*	*16.4% (9/55)*

Abbreviations: cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin -6; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

[1]Based on results of 10 parenteral drugs tested in each of three different laboratories; samples of each drug were tested with or without being spiked with a Gram-negative endotoxin standard (0, 0.25, 0.5, 0.5, or 1.0 EU/mL).

[2]Percentage (Number of correct runs/total number of runs)

[3]A modification of the PBMC/IL-6 test method using cryopreserved PBMCs.

[4]A modification of the WB/IL-1 test method using 96-well plates instead of tubes for the test substance incubation.

It was not possible to make a direct comparison between the RPT and *in vitro* pyrogenicity test results without the availability of parallel testing data (i.e., same test substance tested using the *in vitro* and *in vivo* methods). Therefore, *in vitro* results that are discordant from the RPT could not be identified with these studies. Discordant results reflect a failure of the *in vitro* test method(s) to identify Gram-negative endotoxin spiked into a test substance at the threshold concentration (0.5 EU/mL) established based on historical data from the RPT.

The limitations of these test methods have not been fully explored and identified. For this reason, pre-testing product specific validation will be necessary to establish if a particular test substance/material is appropriate for evaluation using these *in vitro* test methods. One identified limitation of the *in vitro* methods is the lack of data to determine their responses to, and suitability for, non-endotoxin pyrogens that are known to be detected by the RPT. However, an advantage to these *in vitro* test methods is that they are derived from human tissues, and thus avoid potential uncertainty associated with cross-species extrapolation.

Intralaboratory repeatability of each test method was evaluated by testing saline and various endotoxin spikes (0.06 to 0.5 EU/mL) in saline and evaluating the closeness of agreement among optical density readings for cytokine measurements at each concentration. Up to 20 replicates per concentration were tested and results indicated that variability in OD measurements increased with increasing endotoxin concentration, but the variability was not so great to interfere with distinguishing the 0.5 EU/mL spike concentration (i.e., the threshold for pyrogenicity) from the lower concentrations.

Intralaboratory reproducibility was evaluated using three marketed pharmaceuticals spiked with various concentrations of endotoxin. Three identical, independent runs conducted in each of the three testing laboratories, with the exception of the cryo WB/IL-1 test method[4]. All three possible combinations were compared (i.e., run 1 vs. run 2; run 1 vs. run 3; run 2 vs. run 3) and a mean value calculated, intended to provide an overall proportion of inter-run agreement. Inter-run agreement between two runs within each laboratory ranged from 75% to 100%, with mean values ranging from 83% to 100%. Agreement across 3 runs within a single laboratory ranged from 75% to 100%. Interlaboratory reproducibility was evaluated in two different studies in which each run from one laboratory was compared to all other runs of another laboratory. The proportion of equally qualified samples provided a measure of reproducibility. In the first study, three marketed pharmaceutical products were spiked with a saline control or various concentrations of endotoxin and tested three times in three different laboratories, except for cryoWB/IL-1, which was tested once in each laboratory in the catch-up validation study. The agreement across the three laboratories for each test method ranged from 58% to 86%[5], depending on the test method used and 92% for the cryoWB/IL-1 test method.

In the second study, reproducibility was determined using the results from the ten substances used in the accuracy analysis. Each substance was spiked with five concentrations of endotoxin and tested once in each of three laboratories. The agreement across three laboratories for each test method ranged from 57% to 88%, depending on the test method

[4] The ECVAM cryo WB/IL-1 test method BRD states that there was no direct assessment of intralaboratory reproducibility because such an evaluation was performed in the WB IL-1 (fresh blood) test method, and the authors assume that variability is not affected by the change to cryopreserved blood.

used. The levels, and order of agreement among laboratories was the same for both studies; the WB/IL-1 test method showed the least agreement (57-58%) and the cryo WB/IL-1 test method showed the most (88-92%).

As stated above, this BRD provides a comprehensive summary of the current validation status of five *in vitro* pyrogenicity test methods, including what is known about their relevance and reliability, the scope of the substances tested, and the availability of a standardized test method protocol for each test method. Data for these IVPT methods will be maintained for future use, so that these performance statistics may be updated as additional information becomes available.

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[5] However a modification of the WB/IL-1 test method (using 96-well plates for the test substance incubation) tested once in each laboratory resulted in agreement among laboratories of 83% to 92%.

1.0 INTRODUCTION AND RATIONALE FOR THE PROPOSED USE OF *IN VITRO* PYROGENICITY TEST METHODS

1.1 Introduction

1.1.1 Historical Background of *In Vitro* Pyrogenicity Test Methods and the Rationale for Their Development

A brief summary of the historical development the five *in vitro* pryogenicity test methods was provided in Section 1.1.1 of each European Centre for the Validation of Alternative Methods (ECVAM) Background Review Document (BRD) provided to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and is included in **Appendix A**[6]. This section includes supplementary information and provides a context for U.S. regulatory considerations.

Pyrogenic substances increase body temperature by inducing leukocytes (i.e., neutrophils, monocytes/macrophages, lymphocytes) to release proinflammatory cytokines (e.g., interleukin [IL]-1, IL-6, and tumor necrosis factor-α [TNF-α]) that act as endogenous pyrogens (Dinarello et al. 1999). These pyrogenic substances may originate from a variety of biological or synthetic/manufacturing sources. They may also be released from microbiological organisms such as bacteria, viruses, and fungi during cell death or following immunological attack (i.e., cell damage or death due to a local or systemic immune response). One of the most potent pyrogenic materials is bacterial endotoxin, which is a component of the outer cell wall of Gram-negative bacteria. Pyrogens may also be found in processing and packaging materials, chemicals, raw materials, or equipment used during manufacturing of parenteral drugs or medical devices. The presence of endotoxins in otherwise sterile biological preparations such as parenteral drugs suggests the presence of current or past bacterial contamination.

The U.S., European, and Japanese Pharmacopoeias currently recognize two test methods for pyrogen testing, the *in vivo* rabbit pyrogen test (RPT) and the *in vitro* bacterial endotoxin test (BET), commonly referred to as the *Limulus* amebocyte lysate (LAL) test. The BET is

[6] References to ECVAM BRD sections are in normal type to distinguish them from references to ICCVAM BRD sections which are in **boldface** type.

recognized for its sensitivity to the presence of Gram-negative endotoxins, but it has well documented limitations, including its inability to respond to non-endotoxin pyrogens, as well as its susceptibility to interference from certain types of materials (e.g., high protein and lipid levels; glucans). In contrast, the RPT is capable of detecting both endotoxin and non-endotoxin pyrogens. However, disadvantages of the RPT include the need for interspecies translation from rabbits to humans, along with animal welfare concerns that discourage animal testing.

An *in vitro* test system that combines the sensitivity of the BET with the wide range of pyrogens detectable by the RPT would be an obvious improvement for pyrogen testing. With this intention, test systems based on the activation of human monocytes *in vitro* were developed that take advantage of an increased understanding of the biological mechanisms responsible for the human fever reaction (Dinarello, 1999). Initial efforts focused on peripheral blood mononuclear cells (PBMC), which release proinflammatory cytokines when exposed to endotoxin (Duff and Atkins, 1982; Dinarello 1984). A number of similar test systems, using either whole blood, PBMCs, or monocytoid cell lines (e.g., MONO MAC 6 [MM6], THP-1) were subsequently developed (Poole et al., 1988; Ziegler-Heitbrock et al, 1988; Tsuchiya et al, 1980; Hartung and Wendel, 1996; Hartung et al, 2001; Poole et al, 2003). Five test systems developed from human monocytoid cells were selected by ECVAM for prevalidation and validation studies with the intent of comparing their effectiveness for replacing the RPT and thereby eliminating the need for using rabbits for pyrogen testing. The results of these studies have been published (Hoffmann et al. 2005). The five tests selected were:

- The Human Whole Blood/ IL-1 *In Vitro* Pyrogen Test Using Cryopreserved Human Whole Blood (cryo WB/IL-1)
- An Alternative *In Vitro* Pyrogen Test Using the Human Monocytoid Cell Line MONO MAC6 (MM6/IL-6)
- *In Vitro* Pyrogen Test Using Human PBMCs (PBMC/IL-6)[7]

[7] As indicated in the ECVAM BRDs for the WB/IL-1 and PBMC/IL-6 test methods, catch-up validation studies were conducted to evaluate the performance of the WB/IL-1 test method when using 96-well plates, and the PBMC/IL-6 test method when using cryopreserved PBMCs. The plating procedure (WB/IL-1) and the cryopreservation procedure (PBMC/IL-6) are the only differences in the test method protocols (see **Appendix A**). These modifications were not

- The Human Whole Blood/IL-1 *In Vitro* Pyrogen Test (WB/IL-1)[5]
- The Human Whole Blood/IL-6 *In Vitro* Pyrogen Test (WB/IL-6)

1.1.2 Peer Reviews of *In Vitro* Pyrogenicity Test Method Validation Studies

The ECVAM-sponsored validation studies of each of these *in vitro* methods have been the subject of a recent formal peer review convened by the ECVAM Scientific Advisory Committee (ESAC). Two members of the ESAC served as co-Chairpersons for the review panel, which consisted of five additional U.S. and European reviewers. These reviewers assessed the ability of each test method to serve as a complete replacement for the *in vivo* rabbit pyrogen test (RPT). Subsequent to this review, the ESAC declared that, "these tests have been scientifically validated for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of this pyrogen, in materials currently evaluated and characterized by rabbit pyrogen tests."

However, in their comments to the Study Management Team (SMT), two of the reviewers suggested that, because an adequate link between the *in vitro* pyrogenicity test methods and the RPT was not demonstrated, the validation studies fell short of providing a basis for recommendation of any of these methods as substitutes or replacements for the methods presently indicated in the U.S., European, and Japanese Pharmacopeias (i.e., the RPT and BET).

This BRD was prepared for use by an ICCVAM Expert Panel following the review of these *in vitro* pyrogenicity test methods. Because individual BRDs for each method were provided by ECVAM, this ICCVAM BRD serves to combine common information, and references the appropriate sections of the ECVAM BRDs for specifics related to the individual test methods. The results and recommendations of the Expert Panel report, combined with the analyses presented in the ECVAM BRDs, will be used to support ICCVAM recommendations on the usefulness and limitations of each test method, the proposed standardized test method protocols, performance standards, and any additional studies considered necessary to further develop or characterize any or all of these test methods.

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submitted by ECVAM as separate test methods, and are therefore not being considered as separate test methods in this BRD. However, where relevant, comparative information is provided (e.g., see **Table 2-1** and **Sections 6.1** and **7.2**).

1.2 Regulatory Rationale and Applicability

1.2.1 Current Regulatory Testing Requirements and ICCVAM Prioritization Criteria

This section reviews and summarizes the extent to which the five ICCVAM prioritization criteria (ICCVAM 2003) apply to the *in vitro* pyrogenicity test methods under consideration.

Criteria 1. The extents to which the proposed test methods are (a) applicable to regulatory testing needs and (b) applicable to multiple agencies/programs.

Pyrogenicity testing is used by regulatory authorities primarily for end product release of human and animal parenteral drugs, biological products, and medical devices. The results from these assays are used to limit, to an acceptable level, the risks of febrile reaction in the patient exposed to the product of concern by injection and/or implantation. The current U.S. legislation requiring the use of pyrogenicity testing is set forth in the Federal Food, Drug, and Cosmetic Act (U.S.C., Title 21, Chapter 9). In addition, the U.S. Pharmacopeia (USP) maintains sterility requirements for pharmaceuticals that include pyrogenicity testing. As detailed in **Table 1-1**, the U.S. Food and Drug Administration (FDA) is the principal U.S. regulatory agency that requires pyrogenicity testing, with different Centers within the FDA regulating the affected products. The Center for Biologics Evaluation and Research, the Center for Drug Evaluation and Research, the Center for Devices and Radiological Health, and the Center for Veterinary Medicine require that human injectable drugs (including biological products), animal injectable drugs, and medical devices be tested for the presence of pyrogenic substances. **Table 1-1** also shows the statutory protocol requirements used by each FDA Center, along with the comparable enabling legislation and statutory protocol requirements of the European Union (EU) member nations.

Table 1-1 Summary of U.S. and European Legislation and Statutory Protocol Requirements for Pyrogenicity Testing

Agency	Regulated Products	Legislation	Statutory Protocol Requirements	Non-Governmental Standards
United States				
FDA-CBER	Biological products	- Federal Food, Drug, and Cosmetic Act (U.S.C. Title 21, Chapter 9)	- 21 CFR 610.13	- USP28 NF23<85> - USP28 NF23<151> - ISO 10993-11
FDA-CDER	Human parenteral pharmaceuticals			
FDA-CDRH	Medical devices			
FDA-CVM	Veterinary pharmaceuticals			
Europe				
EDQM	Human/veterinary parenteral pharmaceuticals, biological products, medical devices	- Council Regulation (EEC) 230/9/93 - Council Directive 93/39/EEC - Council Directive 93/40/EEC	- EP5.0 2.6.8 - EP5.0 2.6.14	- ISO 10993-11
EMEA				
Regulatory Authorities for Individual EU Countries				

Abbreviations: CBER = Center for Biologics Evaluation and Research; CDER = Center for Drug Evaluation and Research; CDRH = Center for Devices and Radiological Health; CFR = Code of Federal Regulations; CVM = Center for Veterinary Medicine; EDQM = European Directorate for the Quality of Medicines; EMEA = European Medicines Agency; EP = European Pharmacopoeia; EU = European Union; FDA = U.S. Food and Drug Administration; US =: U.S. Pharmacopeia

Criteria 2. Warranted, based on the extent of expected use or application and impact on human, animal, or ecological health.

The proposed test methods are intended to replace a method that is used extensively in pharmaceutical, biological product, and medical device development and registration (i.e., the RPT).

Criteria 3: The potential for the proposed test methods, compared to current test methods accepted by regulatory agencies, to (a) refine animal use (decrease or eliminate pain and distress), (b) reduce animal use, or (c) replace animal use.[8]

The two most common pyrogen tests presently used (i.e., RPT, BET) require the use of animals. The RPT is performed in rabbits that can be maintained and used for additional pyrogen tests. Although the *in vitro* BET is performed using haemolymph (the equivalent of

[8] Refinement alternative is defined as a new or revised test method that refines procedures to lessen or eliminate pain or distress to animals, or enhances animal well-being; Reduction alternative is defined as a new or revised test method that reduces the number of animals required; Replacement alternative is defined as a new or revised test method that replaces animals with nonanimal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate) (ICCVAM 1997).

blood) drawn from *Limulus polyphemus* (horseshoe crabs), which are subsequently returned to the wild, there is some mortality associated with the procedure (which requires approximately 20% of the animal's total blood volume). The proposed methods will reduce and replace animal use because they rely on human white blood cells that can be obtained with minimal risk from volunteers, or a human white blood cell line that can be maintained by the test laboratory.

Criteria 4: The potential for the proposed test method to provide improved prediction of adverse health or environmental effects, compared to current test methods accepted by regulatory agencies.

Sufficient data are presented to allow an assessment of the performance of the proposed test methods relative to the RPT (see Section 6.0). Because these methods are conducted using cells of human origin, one might assume that they would better reflect the human physiological response than current, non-human-based methods (i.e., RPT, BET), and thus more effectively predict adverse effects.

Criteria 5: The extent to which the test method provides other advantages (e.g., reduced cost and time to perform) compared to current methods.

Specific costs for the various tests have not been provided, and therefore such a definitive determination cannot be made. Because the proposed test methods are reportedly more labor-intensive than the RPT or BET, the costs may be greater. These costs, however, may be offset by the costs of maintaining an animal facility for the rabbits used in the RPT. The possibility of adapting the proposed methods to high throughput procedures may make them more cost effective.

1.2.2 Intended Uses of the Proposed *In Vitro* Pyrogenicity Test Methods

The proposed test methods are intended as an end product release test for the identification of pyrogens in human and animal parenteral drugs, biological products, and medical devices. Results from pyrogenicity testing are used to limit, to an acceptable level, the risks of febrile reaction to the injection and/or implantation of the product of concern.

1.2.3 Similarities and Differences in the Endpoints Measured by the Proposed Test Methods and the *In Vivo* Reference Test Method

The endpoint measured in the *in vitro* pyrogenicity test methods is release of proinflammatory cytokines, either IL-1β or IL-6, in response to a test substance challenge, depending on the specific cell type employed. The RPT involves measuring the rise in body temperature evoked in rabbits by the intravenous injection of a test solution. Although there is no direct association between the endpoints measured in the *in vitro* blood cell assays and the *in vivo* rabbit assay, cytokine release is involved in the development of the inflammatory response, which can lead to an increase in body temperature. Therefore, the *in vitro* release of proinflammatory cytokines, such as IL-1β and IL-6, is intended to presage the onset of such a cascade of responses resulting in a fever response. The cell types used for the various *in vitro* methods include those that would be directly associated with an inflammatory response (i.e., monocytes/monocytoid cells) *in vivo*. Both the *in vitro* and *in vivo* tests provide quantitative data that can be applied to specific decision criteria to identify a pyrogenic reaction.

1.2.4 Use of the Proposed Test Methods in an Overall Strategy of Hazard or Safety Assessment

As detailed in **Table 1-1**, there are current U.S. and European regulatory requirements to test pharmaceutical products, biological products, and medical devices for pyrogenicity. The pyrogenicity tests that are currently acceptable to regulatory authorities require intact animals (rabbits) or an *in vitro* test that requires the use of horseshoe crab haemolymph (BET). According to ECVAM, the *in vitro* human blood cell test methods are intended to replace the RPT for the identification of pyrogens where: (a) the test material is incompatible with the BET; or (b) the test material contains a non-endotoxin mediated pyrogen (although as detailed in **Section 3.0**, only Gram-negative endotoxin was included in the validation study, suggesting that other types of pyrogens have not been adequately validated – see **Section 1.1.2**)[9].

[9] Additional information on testing of other types of pyrogens was also provided by ECVAM and is included in **Appendix B**.

1.3 Scientific Basis for the *In Vitro* Pyrogenicity Test Methods

1.3.1 Purpose and Mechanistic Basis of the *In Vitro* Pyrogenicity Test Methods

The proposed methods are intended to identify pyrogens in parenteral pharmaceuticals, medical devices, and human biological products. These methods provide an *in vitro* model of the initiation of the human fever response by measuring proinflammatory cytokine release (i.e., IL-1β or IL-6) from human monocytes/monocytoid cells exposed to pyrogens. These proinflammatory cytokines are associated with the initiation of the *in vivo* fever response.

1.3.2 Similarities and Differences of Modes of Action Between the *In Vitro* Pyrogenicity Test Methods and the Fever Response in Humans and/or Rabbits

As detailed in **Section 1.2.3**, each of the five proposed *in vitro* test methods do not measure actual fever induction, but use proinflammatory cytokine release (i.e., IL-1β or IL-6) from human monocytoid cells as an indicator of the presence of a pyrogenic substance. By comparison, the RPT involves measuring the effect of concern, i.e., a change in body temperature in rabbits over a specified time period following an intravenous injection of a test substance.

Proinflammatory cytokines such as IL-1β and IL-6 have been characterized as endogenous pyrogens that are released from monocytoid cells in response to the presence of endotoxin and other pyrogenic substances, and have been associated with the fever response induced by pyrogenic substances in both humans and rabbits (Dinarello et al. 1999). The pyrogenicity of endotoxin is believed to be mediated through activation of the Toll-like (TLR-4) receptor on the blood cells. TLR-4 activation induces the mitogen-activated protein kinase (MAPK) signal transduction cascade, which activates proinflammatory cytokine expression. There is no direct evidence that circulating cytokines penetrate the blood-brain barrier, but it is more likely that they produce effects on the rich vascular network or circumventricular organs of the hypothalamus, or induce release of other effectors from endothelial cells (e.g., prostaglandins such as PGE_2) that increase levels of neurotransmitters (e.g., cAMP) which modulate the thermoregulatory center of the hypothalamus and induce a fever response. (Dinarello 1999; Beutler and Rietschel 2003).

As indicated above, the proposed *in vitro* test methods use human cells whereas the RPT and BET procedures use rabbits and horseshoe crab haemolymph, respectively. Although the relative sensitivities of each species to Gram-negative endotoxins vary, the responses of humans, horseshoe crabs (via haemolymph gelation), and rabbits to these pyrogens have been studied extensively, and test methods based on blood products or blood cells from each of these species appear to be capable of responding to pyrogens (Cooper et al 1971; Greisman and Hornick, 1969; Hoffman et al. 2005). However, there are documented cases of medicinal products, interfering substances, and specific pyrogenic substances that yield false-positive or false-negative results in either the RPT or BET tests (Carlin and Viitanen 2003, 2005; Moesby et al. 2000; Hartung et al. 2001; Ochiai et al. 2001).

1.3.3 Range of Substances Amenable to the *In Vitro* Pyrogenicity Test Methods and Limits of These Methods

The proposed methods are intended to be used for the identification of pyrogenic substances in parenteral pharmaceuticals, biological products, and medical devices. Because they are based on isolated human monocytes/monocytoid cells, they are considered capable of detecting both Gram-negative endotoxin and non-endotoxin-based pyrogens. While **Section 9.0** summarizes a number of published studies that have measured cytokine release following exposure to non-endotoxin pyrogens (e.g., lipoteichoic acid), the ECVAM validation studies focused specifically on Gram-negative endotoxin due to the unavailability of standardized, non-endotoxin pyrogens (see **Section 3.0**). Because these test methods measure the release of proinflammatory cytokines, test substances that modify this response (e.g., anti-inflammatory or immunosuppressant drugs) are considered inappropriate for testing. Each test protocol includes an interference test for identifying such substances so that they can be excluded from testing.

1.4 Validation of the *In Vitro* Pyrogenicity Test Methods

The ICCVAM Authorization Act (Sec. 4(c) mandates that "[e]ach Federal Agency … shall ensure that any new or revised … test method … is determined to be valid for its proposed use prior to requiring, recommending, or encouraging [its use]." (Public Law [P.L.] 106-545).

Validation is the process by which the reliability and relevance of an assay for a specific purpose are established (ICCVAM 1997). Relevance is defined as the extent to which an assay will correctly predict or measure the biological effect of interest (ICCVAM 1997). For the *in vitro* pyrogenicity test methods described in this BRD, relevance is restricted to how well the assays detect the presence of Gram-negative endotoxin. Reliability is defined as the reproducibility of a test method within and among laboratories and should be based on performance with a diverse set of substances that are representative of the types of chemical and product classes that are expected to be tested and the range of responses that needs to be identified. The validation process is designed to provide data and information that will allow U.S. Federal agencies to develop guidance on the development and use of *in vitro* pyrogenicity test methods for the detection of Gram-negative endotoxins.

The first stage in the evaluation of a new test procedure is the preparation of a BRD that presents and evaluates the relevant data and information about the method, including its mechanistic basis, proposed uses, reliability, and performance characteristics (ICCVAM 1997). This BRD summarizes the available information on each of the five *in vitro* pyrogenicity test methods listed in **Section 1.1.1**.

Where adequate data are available, the qualitative and quantitative performances of the assays are evaluated, and the reliability of each new method is compared with the reliability of the other methods and the currently accepted method. This BRD will aid in identifying essential test method components that should be considered during the development and validation of the various methods, and determine whether there are sufficient data to support a recommendation that a standardized protocol be developed for use by the regulatory agencies.

1.5 Search Strategies and Selection of Citations for the *In Vitro* Pyrogenicity BRD

NICEATM conducted an online literature search for relevant information on the proposed test methods using multiple internet databases (i.e., PubMed, SCOPUS, TOXLINE, Web of Science). Specifically, records were sought using various combinations of the terms *in vitro*, WBC, whole blood, PBMN, MONO MAC6, MM6, endotoxin, LPS, pyrogen, LAL, BET, IL-1, and IL-6. This search was conducted to supplement and update the list of peer-reviewed publications related to *in vitro* pyrogen testing that was provided in the ECVAM BRDs. U.S., EU, and Japanese pyrogenicity test guidelines were obtained from relevant regulatory agencies via the internet or through direct requests. A resulting database of 315 references is currently maintained as an EndNote® v8.0 electronic file. This database confirmed that the lists of references included in the ECVAM BRDs are complete and up-to-date.

2.0 *IN VITRO* PYROGENICITY TEST METHOD PROTOCOL COMPONENTS

2.1 Overview of How the *In Vitro* Pyrogenicity Test Methods Are Conducted

Although there are differences among the *in vitro* pyrogenicity test methods considered in this BRD, the basic procedural steps are consistent across all five methods:

- Interference testing is performed to verify that a test substance does not interfere with either the cell system used or with the specific cytokine-specific enzyme-linked immunosorbent assay (ELISA).
- The test substance is mixed with a suspension of human-derived blood cells and incubated for a specified period of time (i.e., 16 to 24 hr).
- The concentration of the specific proinflammatory cytokine (e.g., IL-1β, IL-6) is measured using an ELISA, and is compared to the response curve of an endotoxin standard
- An internationally accepted endotoxin standard derived from *Escherischia coli* (*E. coli*) (i.e., WHO-lipopolysaccharide [LPS] 94/580 [*E. coli* 0113:h10:K-]), or an endotoxin standard that has been calibrated against this standard, is used to generate the standard response curve for the assay. The endotoxin activity of a test substance is calculated by comparing the induced cytokine release with that induced by the endotoxin standard.
- A product "passes" (i.e., is considered negative for endotoxin pyrogen activity) if the cytokine response to the test substance is less than that induced by 0.5 endotoxin units (EU)/mL.

2.2 Description and Rationale for the Test Method Components for Proposed Standardized Protocols

The standard operating procedures (SOP) for each method assessed in the ECVAM validation studies are provided as appendices to each BRD (see Appendix A of each BRD). As indicated in **Section 2.1**, the essential principles of each protocol are common among the five methods reviewed. These include isolating and/or culturing human monocytoid cells (either included in whole blood, separated as a fraction [i.e., PBMCs], or as cell line [i.e., MM6]), performing interference testing with each substance, treating the cells in suspension with a test substance, collecting cytokine release data, and evaluating the data in relation to the proposed prediction model. **Table 2-1** provides a more complete comparison of the similarities and differences among the five test protocols. No rationale was provided by ECVAM for use of the particular primary blood cells in the various test methods; in such cases, their selection is justified by historical use. The use of the MM6 cell line was justified based on mechanistic considerations and its response to endotoxins.

951 **Table** 2-1 ***In Vitro* Pyrogenicity Test Method Components**

<table>
<tr><th></th><th>Test Method Component</th><th>WB/IL-1[1]</th><th>cryo WB/IL-1</th><th>WB/IL-6</th><th>PBMC/IL-6[1]</th><th>MM6/IL-6</th></tr>
<tr><td>Materials required</td><td>Source of cells</td><td>Human whole blood</td><td>Human whole blood</td><td>Human whole blood</td><td>Human whole blood</td><td>MM6 cell bank (original cell line maintained by Prof. H. Ziegler-Heitbrock, U. Munich)</td></tr>
<tr><td></td><td>Laboratory equipment</td><td>• Incubator (37°C, 5% CO_2,)
• Adjustable pipetters (2-2000 µL)
• Multichannel pipetter (8- or 12-channel)
• Serological pipets
• Pyrogen-free tips
• Centrifuge
• Vortex mixer
• Heparinized blood tubes
• Pyrogen-free hypodermic needles
• Pyrogen-free microfuge tubes OR 96-well plates (depending on tube or plate method)
• Microtiter plate reader (450 nm and 600-690 nm)
• Data analysis software</td><td>• Incubator (37°C, 5% CO_2, Laminar flow hood (recommended)
• Adjustable pipetters (2-2000 µL)
• Multichannel pipetter (8- or 12-channel)
• Pyrogen-free tips
• Centrifuge
• Vortex mixer
• Pyrogen-free tissue culture plasticware (e.g., flasks, 96-well plates, centrifuge tubes)
• Microtiter plate reader (450 nm and 600-690 nm)
• Data analysis software</td><td>• Incubator (37°C, 5% CO_2, humidified)
• Laminar flow hood (Class II)
• Centrifuge
• Water bath
• pH meter
• Microtiter plate reader (450 nm and 600-690 nm capable)
• Data analysis software
• Adjustable pipetters (2-2000 µL)
• Multichannel pipetter (8- or 12-channel)
• Heparinized blood tubes
• Pyrogen-free pipet tips
• Vortex mixer
• Pyrogen-free hypodermic needles
• Pyrogen-free tissue culture plasticware (e.g., flasks, 96-well plates, centrifuge tubes)</td><td>• Incubator (37°C, 5% CO_2, humidified)
• Inverted microscope
• Hemacytometer
• Laminar flow hood (Class II)
• Centrifuge
• Water bath
• pH meter
• Microtiter plate reader (450 nm and 600-690 nm capable)
• Data analysis software
• Adjustable pipetters (2-2000 µL)
• Multichannel pipetter (8- or 12-channel)
• Heparinized blood tubes
• Pyrogen-free pipet tips
• Vortex mixer
• Pyrogen-free hypodermic needles
• Pyrogen-free tissue culture plasticware (e.g., flasks, 96-well plates, centrifuge tubes)</td><td>• Incubator (37°C, 5% CO_2, humidified)
• Inverted microscope
• Hemacytometer
• Laminar flow hood (Class II)
• Centrifuge
• Water bath
• pH meter
• Microtiter plate reader (450 nm and 600-690 nm capable)
• Data analysis software
• Adjustable pipetters (2-2000 µL)
• Multichannel pipetter (8- or 12-channel)
• Pyrogen-free pipet tips
• Vortex mixer
• Pyrogen-free tissue culture plasticware (e.g., flasks, 96-well plates, centrifuge tubes)</td></tr>
<tr><td></td><td>Culture medium</td><td>None - whole blood is diluted with 0.9% NaCl</td><td>RPMI Complete Medium
• RPMI 1640 (part of the Endosafe Kit for cryoblood)
• no specific additives needed</td><td>None - whole blood is diluted with 0.9% NaCl</td><td>RPMI Complete Medium
• RPMI 1640
• HSA
• L-Glutamine (2 mM)
• Penicillin/streptomycin</td><td>RPMI Medium[2]
• RPMI 1640 medium
• HIFCS (10% or 2%)
• L-glutamine (2mM)
• MEM non-essential amino acid solution (0.1 mM)
• HEPES (20 mM)
• Bovine insulin (0.23 IU/mL)
• Oxaloacetic acid (1 mM)</td></tr>
</table>

	Test Method Component	WB/IL-1[1]	cryo WB/IL-1	WB/IL-6	PBMC/IL-6[1]	MM6/IL-6
						• Sodium pyruvate (1 mM)
	Other reagents	• Validated IL-1β ELISA kit • 0.9% NaCl (pyrogen-free) • Water for injection • Endotoxin standard	• Validated IL-1β ELISA kit • 0.9% NaCl (pyrogen-free) • Water for injection • Liquid nitrogen • Endotoxin standard	• Validated IL-6 ELISA kit • 0.9% NaCl (pyrogen-free) • Water for injection • Endotoxin standard	• Validated IL-6 ELISA kit • 0.9% NaCl (pyrogen-free) • Water for injection • Trypan blue • Endotoxin standard	• Validated IL-6 ELISA kit • 0.9% NaCl (pyrogen-free) • Water for injection • Trypan blue • DMSO • Endotoxin standard
Dose selection procedures		Interference testing performed to determine the lowest dilution of the test product necessary to achieve an acceptable endotoxin spike recovery (i.e., 50% to 200% recovery)[3]	Interference testing performed to determine the lowest dilution of the test product necessary to achieve an acceptable endotoxin spike recovery (i.e., 50% to 200% recovery)[3]	Interference testing performed to determine the lowest dilution of the test product necessary to achieve an acceptable endotoxin spike recovery (i.e., 50% to 200% recovery)[3]	Interference testing performed to determine the lowest dilution of the test product necessary to achieve an acceptable endotoxin spike recovery (i.e., 50% to 200% recovery)[3]	Interference testing performed to determine the lowest dilution of the test product necessary to achieve an acceptable endotoxin spike recovery (i.e., 50% to 200% recovery)[3]
Endpoints measured		IL-1β release via ELISA	IL-1β release via ELISA	IL-6 release via ELISA	IL-6 release via ELISA	IL-6 release via ELISA
Exposure of the test substance	**Pre-test preparation of cells**	Collect whole blood, heparinize, and use within 4 hr Plate Method: same collection procedure	• Collect blood, heparinize, and cryopreserve according to the Konstanz or PEI method • Prior to testing, thaw blood at 37°C for 15 min	Collect whole blood, heparinize, and use within 4 hr	• Collect whole blood and isolate PBMCs by centrifugation • Resuspend PBMCs in RPMI-C (1x10^6 cells/mL) (use PBMCs within 4 hr of initial blood collection)	• Incubate MM6 cells (4x10^5 cells/mL media) for 24 hr • Resuspend cells (2.5x10^6 cells/mL)[3] prior to testing
	Application of the test substance	Tube method: In a microfuge tube mix 1000µL 0.9% NaCl+100µL sample+100µL blood Plate method: In a 96-well plate mix 200µL 0.9% NaCl+20µL sample+20µL blood	Konstanz method: In a 96-well plate mix 200µL RPMI+20µL sample+20µL blood PEI Method: In a 96-well plate mix 180µL RPMI + 20µL sample+40µL blood	In a 96-well plate: Mix 50 µL standards/samples+100 µL 0.9% NaCl+50 µL blood	In a 96-well plate: Mix 50 µL standards/samples+100 µL RPMI-C+100 µL PBMCs	In a 96-well plate: Mix 50 µL standards/samples+100 µL RPMI-C+100 µL cells in suspension
	Duration of exposure	10-24 hr	10-24 hr (NOTE: for Konstanz method, after incubation, freeze wells at -20°C or -80°C and then thaw at 37°C)	16-24 hr	16-24 hr	16-24 hr
	Material used for ELISA	Tube method: centrifuge 2 min @ 10,000g-test supernatant	Blood/RPMI/sample mixture	Blood/saline/sample mixture	Cell supernatant	Cell supernatant

	Test Method Component	**WB/IL-1[1]**	**cryo WB/IL-1**	**WB/IL-6**	**PBMC/IL-6[1]**	**MM6/IL-6**
		Plate method: mix each well be pipetting and test resuspended mixture				
Known limits of use		Intended for parenteral pharmaceuticals, biological products, and medical devices that have been qualified through interference testing	Intended for parenteral pharmaceuticals, biological products, and medical devices that have been qualified through interference testing	Intended for parenteral pharmaceuticals, biological products, and medical devices that have been qualified through interference testing	Intended for parenteral pharmaceuticals, biological products, and medical devices that have been qualified through interference testing	Intended for parenteral pharmaceuticals, biological products, and medical devices that have been qualified through interference testing
Nature of the response assessed		Pyrogenic substances induce the release of proinflammatory cytokines (e.g., IL-1β) from monocytoid cells present in human whole blood	Pyrogenic substances induce the release of proinflammatory cytokines (e.g., IL-1β) from monocytoid cells present in human whole blood	Pyrogenic substances induce the release of proinflammatory cytokines (e.g., IL-6) from monocytoid cells present in human whole blood	Pyrogenic substances induce the release of proinflammatory cytokines (e.g., IL-6) from PBMCs	Pyrogenic substances induce the release of proinflammatory cytokines (e.g., IL-6) from these immortalized monocytoid cells
Appropriate controls	**Positive control (PC)**	0.5 EU/mL WHO-LPS 94/580 [*E. coli* 0113:h10:K-][5]	0.5 EU/mL WHO-LPS 94/580 [*E. coli* 0113:h10:K-][5]	0.5 EU/mL WHO-LPS 94/580 [*E. coli* 0113:h10:K-][5]	0.5 EU/mL WHO-LPS 94/580 [*E. coli* 0113:h10:K-][5]	0.5 EU/mL WHO-LPS 94/580 [*E. coli* 0113:h10:K-][5]
	Negative control (NC)	0.9% NaCl	0.9% NaCl	0.9% NaCl	0.9% NaCl	0.9% NaCl
	Positive product control (PPC)	Test substance spiked with 0.5 EU/mL	Test substance spiked with 0.5 EU/mL	Test substance spiked with 0.5 EU/mL	Test substance spiked with 0.5 EU/mL	Test substance spiked with 0.5 EU/mL
	Negative product control (NPC)	Test substance spiked with 0.9% NaCl	Test substance spiked with 0.9% NaCl	Test substance spiked with 0.9% NaCl	Test substance spiked with 0.9% NaCl	Test substance spiked with 0.9% NaCl
Acceptable ranges of control responses	**Positive controls**	• PC OD 1.6-fold>NC OD • PPC OD 1.6-fold>NPC OD • PPC OD should be within 50% to 200% of the PC OD	• PC OD 1.6-fold>NC OD • PPC OD 1.6-fold>NPC OD • PPC OD should be within 50% to 200% of the PC OD	PPC OD should be within 50% to 200% of the PC OD	• PPC OD should be within 50% to 200% of the PC OD • 1 EU/mL standard OD>1000 pg/mL IL-6 standard	• PC OD±20% of the expected value (i.e., 0.5 EU/mL) • PPC OD should be within 50% to 200% of the PC OD
	Negative controls	NC OD≤0.100	NC OD≤0.100	NC OD<200 pg/mL IL-6 standard	NC OD<500 pg/mL IL-6 standard	• NC OD<0.200
Nature of data to be collected and methods used for data collection		• IL-1β release from monocytoid cells present in whole blood is quantified with an ELISA for all standards and samples. • The endotoxin content of a test substance is calculated by comparing the induced IL-1β release with that induced by the	• IL-1β release from monocytoid cells present in whole blood is quantified with an ELISA for all standards and samples. • The endotoxin content of a test substance is calculated by comparing the induced IL-1β release with that induced by the	• IL-6 release from monocytoid cells present in whole blood is quantified with an ELISA for all standards and samples. • The endotoxin content of a test substance is calculated by comparing the induced IL-6 release with that induced by the	• IL-6 release from PBMCs is quantified with an ELISA for all standards and samples. • The endotoxin content of a test substance is calculated by comparing the induced IL-6 release with that induced by the endotoxin standard curve concentrations	• IL-6 release from MM6 cells is quantified with an ELISA for all standards and samples. • The endotoxin content of a test substance is calculated by comparing the induced IL-6 release with that induced by the endotoxin standard curve concentrations

Test Method Component	WB/IL-1[1]	cryo WB/IL-1	WB/IL-6	PBMC/IL-6[1]	MM6/IL-6
	endotoxin standard curve concentrations	endotoxin standard curve concentrations	endotoxin standard curve concentrations		
Type of media in which data are stored	Electronic files	Electronic files	Electronic files	Electronic files	Electronic files
Measures of variability	Mean±SD of the OD for each test substance/standard	Mean±SD of the OD for each test substance/standard	Mean±SD of the OD for each test substance/standard	Mean±SD of the OD for each test substance/standard	Mean±SD of the OD for each test substance/standard
Decision criteria and basis for their selection	≥0.5 EU/mL indicates a pyrogenic response based on the pyrogen threshold value as detailed in **Section 4.2**	≥0.5 EU/mL indicates a pyrogenic response based on the pyrogen threshold value as detailed in **Section 4.2**	≥0.5 EU/mL indicates a pyrogenic response based on the pyrogen threshold value as detailed in **Section 4.2**[6]	≥0.5 EU/mL indicates a pyrogenic response based on the pyrogen threshold value as detailed in **Section 4.2**[7]	≥0.5 EU/mL indicates a pyrogenic response based on the pyrogen threshold value as detailed in **Section 4.2**

Abbreviations: DMSO = dimethylsulfoxide; EU = endotoxin unit; HIFCS = heat-inactivated fetal calf serum; HSA = human serum albumin; MEM = minimum essential medium; MM6 = MONO MAC-6; NC = negative control; NPC = negative product control; OD = optical density; PBS = phosphate buffered saline; PC = positive control; PPC = positive product control

[1]As described in **Section 1.1.1**, a catch-up validation studies were also conducted to evaluate the performance of the WB/IL-1 test method using 96-well plates, and the PBMC/IL-6 test method when using cryopreserved PBMCs. The plating procedure (WB/IL-1) and the cryopreservation procedure (PBMC/IL-6) are the only differences in the test method protocols (see **Appendix A**).

[2]Medium should be qualified for testing by a valid LAL test (e.g., USP28NF23<85>) indicating that the endotoxin contamination is <0.06 IU/mL); FBS concentration for MM6 cells varies based on whether it is for maintenance/propagation (10%) or assay (2%) conditions.

[3]Dilution of the test material should not exceed the maximum valid dilution (MVD), where MVD = (endotoxin limit concentration)/(detection limit of the assay)

[4]Cell numbers represent viable cells based on trypan blue exclusion

[5]Or another endotoxin calibrated against this standard

[6]Specifies that each substance must "pass" (i.e., be non-pyrogenic) in blood from three different donors.

[7]Includes a sequential decision strategy in which 3 to 4 donors are tested per substance. 1) If all donors show negative - product is non-pyrogenic; 2) If ≥2 donors show a positive - product is pyrogenic; 3) If only one donor shows a positive, an additional 3 to 4 donors are tested and if no more than one donor is positive (out of 6 to 8 donors) - product is non-pyrogenic; otherwise, product is pyrogenic**.**

2.2.1 Methods Used to Analyze the Data, Including Methods to Analyze for Interference With the Assay

Once a substance has been tested in the requisite number of donor samples (see **Section 2.2.2**), the resulting sample test medium (as indicated in **Table 2-1**) is assayed in quadruplicate in the relevant cytokine ELISA. Outliers are identified using Dixon's test ($p>0.05$), and are excluded from the calculations of endotoxin content. Endotoxin standard curves are included in each assay, from which the endotoxin content of each replicate is estimated using a 4-parameter logistic model.

As indicated in **Table 2-1**, mean optical density (OD) readings are calculated for the positive and negative control samples, as well as for the relevant positive and negative product controls. The acceptable range of the positive product control (50% to 200% of the positive control response) defines the threshold for interference with the test system. If the positive product control response falls outside of this range, the samples are then assayed at the lowest dilution that does not cause interference.

2.2.2 Decision Criteria and the Basis for the Prediction Model Used to Identify a Pyrogenic Substance

As described in **Section 4.2**, historical RPT data were used to establish a threshold pyrogen dose (i.e., the endotoxin dose at which fever was induced in 50% of the rabbits), which was determined to be 5 EU/kg. Based on the largest allowable volume for injection in rabbits (10 mL/kg), the limit of detection that the *in vitro* pyrogen tests must meet was defined as 0.5 EU/mL. Accordingly, the prediction model for each test method was established based on this limit of detection (i.e., a substance is considered pyrogenic if the mean response is greater than or equal to the 0.5 EU/mL standard).

For three of the test methods, results from multiple donors (cryo WB/IL-1 [n=5], WB/IL-6 [n=3], and PBMC/IL-6 [n=3 to 4]) are required to determine the pyrogenicity of a test substance. In contrast, a single donor sample is used for the WB/IL-1 test method, as is a single cell sample for the MM6/IL-6 test method. As outlined in **Table 2-2**, unlike the cryo WB/IL-1 test method, the WB/IL-6 and PBMC/IL-6 test methods employ a decision strategy that takes into account the individual responses of each donor sample.

Table 2-2 Prediction Model Used for *In Vitro* Pyrogenicity Test Methods

Test Method	No. Donors	No. Positive	No. Negative	Decision
PBMC/IL-6	4[1]	4	0	Pyrogenic
		3	1	Pyrogenic
		2	2	Pyrogenic
		1	3	Non-pyrogenic
		0	4	Non-pyrogenic
	3[1]	3	0	Pyrogenic
		2	1	Pyrogenic
		1	2	Non-pyrogenic
		0	3	Non-pyrogenic
WB/IL-6	3	3	3	Pyrogenic
		2	1	Pyrogenic
		1	2	Non-pyrogenic
		0	3	Non-pyrogenic
cryo WB/IL-1	5 (pooled)[2]	1	0	Pyrogenic
		0	1	Non-pyrogenic
WB/IL-1	1	1	0	Pyrogenic
		0	1	Non-pyrogenic
MM6/IL-6	NA[3]	1	0	Pyrogenic
		0	1	Non-pyrogenic

Abbreviations: cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin-6; MM6 = Mono Mac 6; NA = Not applicable; No. = Number; WB = Whole blood

[1]Samples are collected from four donors for the PBMC/IL-6 test method, one donor sample may be excluded based on quality criteria, in which case the prediction model may be applied to results from three donors.

[2]Samples are collect from five donors for the cryo WB/IL-1 test method and pooled prior to cryopreservation

[3]Not applicable, because source material is obtained from an immortalized cell line.

2.2.3 Information and Data to be Included in the Study Report and Availability of Standard Forms for Data Collection and Submission

The test report should include the following information, if relevant to the conduct of the study:

Test Substances and Control Substances

- Name, and type (e.g., pharmaceutical, biological product, medical device eluate, etc.) of test product
- Purity and composition of the test substance or preparation
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., vortexing, sonication, warming; solvent used)
- Stability, if known

Justification of the Specific Protocol(s) Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time
- If the test method employs proprietary components, documentation of the procedure used to ensure their integrity from "lot-to-lot" and over time

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data from the testing laboratory (which should be included in the report)
- Acceptable negative control data, including historical control ranges from the testing laboratory (which should be included in the report)

Test Conditions

- Cell system used; donor information, if relevant
- Calibration information for the equipment used for measuring cytokine release (e.g., spectrophotometer)
- Details of test procedure used
- Description of modifications of the test procedure made by the testing laboratory for the substance being tested
- Reference to the laboratory's historical data for the cell system and protocol
- Description of data and quality assurance evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Good Laboratory Practice (GLP) Quality Assurance Statement

- This statement addresses all GLP inspections and audits made during the study, and the dates the results were reported to the Study Director. This statement also serves to confirm that the final report reflects the raw data.

Reporting requirements for GLP-compliant studies are provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003).

2.3 Basis for Selection of the Test Method Systems

One of the difficulties associated with the currently required pyrogenicity test methods (i.e., RPT and BET) is that both require extrapolation of the response from a non-human system to the human. In contrast, and as discussed in **Section 1.1.1**, all five of these test methods employ human cells in an attempt to mimic the human fever response *in vitro*. Because these test methods are conducted using cells of human origin, it is proposed that they will better reflect the human physiological response than current, non-human methods, and thus more effectively predict human adverse effects.

The whole blood test methods (i.e., WB/IL-1, cryo WB/IL-1, WB/IL-6) offer the convenience of performing the assay directly on a human blood sample, with minimal pretest preparation. The cryo WB/IL-1 test method was developed to offer the convenience of an increased time interval between the time of blood collection and the time a test is initiated (since the fresh blood methods require testing within four hours of collection), as well as increased standardization through the pooling of five donor samples to produce a larger sample bank of cells to use in the test. The MM6/IL-6 test method provides increased standardization by using an immortalized cell line that may be maintained in the laboratory indefinitely, and transferred among laboratories. Finally, the PBMC/IL-6 test method was developed in an attempt to pyrogen detection sensitivity by using the monocyte fraction of whole blood, which is considered to be the most sensitive human blood cell type to the presence of endotoxin.

2.4 Proprietary Components

Data from the test methods that use the IL-6 endpoint (i.e., WB/IL-6, PBMC/IL-6, MM6/IL-6) were obtained using a Novartis-developed IL-6 ELISA assay. The monoclonal anti-IL-6 antibody used in this ELISA was developed using an in-house IL-6 clone at Novartis (i.e., clone 6). Unless the Novartis assay is made publicly available, alternative, commercially available IL-6 ELISA assays will be necessary, and would have to be individually validated for use in these procedures.

The original MM6 cell line used in the MM6/IL-6 test method is maintained by Prof. H. Ziegler-Heitbrock at the University of Munich. According to Section 2.4 of the ECVAM MM6/IL-6 BRD (see **Appendix A**), these cells are available for research purposes by request to Prof. Ziegler-Heitbrock directly, or the German Collection of Microorganisms and Cell Cultures in Germany. Once obtained, the testing laboratory may establish a working cell-bank. However, the BRD also states that conditions for licensing of the MM6 cells are to be negotiated with Prof. Ziegler-Heitbrock directly.

According to the US Patent and Trademark Office (USPTO), patents are held for "Test for determining pyrogenic effect of a material" (U.S. 5,891,728, Apr 6, 1999), and "Pyrogenicity test for use with automated immunoassay systems" (U.S. 6,696,261 B2, Feb 24, 2004). These patents cover the WB/IL-1 and WB/IL-6 test methods, respectively. In addition, and related to the cryo WB/IL-1 test method, there is a patent pending for "Test procedure with biological system - Preparations containing deep-frozen blood are used for determining blood response" (USPTO 436518000).

There are several measures in the study validity criteria that may be used to verify the integrity of proprietary components. As outlined in **Table 2-1**, an endotoxin standard curve is established for each assay, which is in turn used to define the endotoxin activity of the test substances. In addition, positive and negative controls, along with positive and negative product controls, are used for interference testing, and serve as internal controls for each assay.

2.5 Number of Replicates

2.5.1 Number of Donors

There is no rationale provided for the number of donors included for each test method. As described in **Section 2.2.2**, samples from multiple donors are for three of the test methods. The cryo WB/IL-1 test method uses blood from five different donors and the WB/IL-6 and PBMC/IL-6 test methods use blood from at least three donors, which are tested individually.

In contrast, a single donor sample is used for the WB/IL-1 test method, as is a single cell culture for the MM6/IL-6 test method.

2.5.2 Number of Assay Replicates

Once each substance has been tested in the requisite number of donor samples (see **Section 2.2.2**), the resulting sample test medium is assayed in quadruplicate in the relevant cytokine ELISA. As indicated in **Section 2.2.1**, Dixon's test is used to detect outliers among the replicates. Section 2.5 of the ECVAM BRDs states that four replicates were chosen at it is considered the minimum number for inclusion in Dixon's test.

2.6 Modifications to the Test Method Protocols Based on ECVAM Validation Study Results

In the MM6/IL-6 test method, prevalidation studies demonstrated that pre-incubation of the cells at a defined initial concentration of 2 x 10^7 cells/50 mL RPMI-C for 24 hr greatly improved test method performance. Therefore, this modification was included in the validation study, and subsequently carried forward to the recommended MM6/IL-6 test method protocol.

For the PBMC/IL-6 test method, a single blood donor was initially used as a source of PBMCs. However, the use of PBMCs from four separate donors (assayed individually) was shown to reduce variability, and this modification was carried forward in the recommended PBMC/IL-6 test method protocol.

No modifications were made to the WB/IL-1, cryo WB/IL-1, and WB/IL-6 test method protocols as a result of the prevalidation or validation testing experiences.

2.7 Differences with Comparable Validated Test Methods with Established Performance Standards

The differences between the *in vitro* pyrogenicity test methods and the currently accepted pyrogenicity test methods (i.e., BET and RPT) are described in **Sections 1.2.3** and **1.3.2**.

3.0 SUBSTANCES USED FOR THE VALIDATION OF *IN VITRO* PYROGENICITY TEST METHODS

3.1 Rationale for the Substances or Products Selected for Testing

In vitro pyrogenicity test method validation studies should evaluate an adequate sample of substances and products of the types that are intended to be tested with these methods. The list of test substances selected for inclusion in the ECVAM validation studies consists solely of marketed parenteral pharmaceuticals that have been labeled as free from detectable pyrogens. No specific rationale was provided for the selection of these test substances. For evaluating test method performance, each test substance was spiked with a Gram-negative endotoxin standard (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Endotoxin was selected as a "model" pyrogen for inclusion based on its availability in a standardized form and because of the known ability of these cells to respond to endotoxin-based pyrogens.. No non-endotoxin-based pyrogenic substances are presently available in a standardized form.

Each sample contained the appropriate endotoxin spike concentration when tested at its Maximum Valid Dilution (MVD). The MVD takes into account the endotoxin limit concentration (ELC) and the detection limit of the particular test method. The U.S. and European Pharmacopoeias assign ELCs for drugs based on their specific administered dose, route of administration, and dosing regimen. Based on the selected threshold pyrogen dose of 0.5 EU/mL (see **Section 4.0**), and the decision criteria used in the validation studies to identify a pyrogenic response (≥0.5 EU/mL, see **Section 5.0**), a concentration of 0.5 EU/mL was used as the detection limit for the *in vitro* test methods when calculating the MVDs for each of the test substances.

3.2 Number of Substances

A total of 13 substances were included in the performance analysis of each of the five *in vitro* test methods. Ten substances, each spiked with five concentrations of endotoxin, were used to evaluate accuracy. For the evaluation of reproducibility, three substances were each spiked with four concentrations of endotoxin.

3.3 Identification and Description of Substances Tested

As indicated in **Section 3.1**, the test substances selected for use in the validation studies were marketed parenteral pharmaceuticals. **Table 3-1** lists the 10 test substances used to evaluate accuracy, and **Table 3-2** lists the three test substances used to evaluate reproducibility.

Table 3-1 Test Substances (Parenteral Drugs) Used in the Validation Studies for Determining Test Method Accuracy[1]

Test Substance[2]	Source	Active Ingredient	Indication	MVD (-fold)
Beloc®	Astra Zeneca	Metoprolol tartrate	Heart dysfunction	140
Binotal®	Aventis	Ampicillin	Antibiotic	140
Ethanol 13% (w/w)	B. Braun	Ethanol	Diluent	35
Fenistil®	Novartis	Dimetindenmaleat	Antiallergic	175
Glucose 5% (w/v)	Eifel	Glucose	Nutrition	70
MCP®	Hexal	Metoclopramid	Antiemetic	350
Orasthin®	Aventis	Oxytocin	Initiation of delivery	700
Sostril®	GSK	Ranitidine	Antiacidic	140
Drug A - 0.9% NaCl	-	0.9% NaCl	-	35
Drug B - 0.9% NaCl	-	0.9% NaCl	-	70

Abbreviations: MVD = Maximum valid dilution; GSK = GlaxoSmithKline; NaCl = Sodium chloride; w/w = Weight/weight; w/v = Weight/volume

[1]Each substance was tested in all five *in vitro* pyrogenicity test methods.

[2]Each test substance was spiked with 0, 0.25, 0.5, 0.5, or 1.0 EU/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Each sample contained the appropriate spike concentration when tested at its Maximum Valid Dilution (MVD).

Table 3-2 Test Substances (Parenteral Drugs) Used in the Validation Studies for Determining Test Method Reproducibility[1]

Test Substance[2]	Source	Agent	Indication
Gelafundin®	Braun Melsungen	Gelatin	Transfusion
Jonosteril®	Fresenius	Electrolytes	Infusion
Haemate®	Aventis	Factor VIII	Hemophilia

[1]Each substance was tested in all five *in vitro* pyrogenicity test methods.

[2]Each test substance was spiked with 0, 0, 0.5, or 1.0 EU/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Each sample contained the appropriate spike concentration when tested at its Maximum Valid Dilution (MVD).

3.4 Sample Coding Procedure

According to the ECVAM BRDs (Section 3.4), the ten test substances and the five spike concentrations used for the evaluation of accuracy were blinded to the testing laboratories.

For the reproducibility analyses, although the four spike concentrations were blinded to the participating laboratories, the identities of the three test substances were not.

3.5 Rationale for the Selection of the Recommended Reference Substances

Reference substances are used to assess the accuracy and reliability of a proposed, mechanistically and functionally similar test method and are a representative subset of those used to demonstrate the reliability and the accuracy of the validated reference test method (in this case, the RPT). These substances should:

- represent the range of responses that the validated test method is capable of measuring or predicting
- have produced consistent results in the validated test method
- produce responses that reflect the accuracy of the validated test method
- have well-defined chemical structures and/or compositions
- be readily available
- not be associated with excessive hazard or prohibitive disposal costs

Pyrogenicity test methods provide a unique situation with regard to reference substances for use in validation studies because the only available standardized reference pyrogen is the international reference standard endotoxin (WHO-LPS 94/580 [*E. coli* 0113:h10:K-]). This reference standard was used to spike each of the test substances used in the ECVAM validation studies (see **Tables 3-2** and **3-3**). This reference standard is also used as a positive control and for qualifying the test methods during interference testing, and is also used when performing the BET.

As described in **Section 4.0**, the response of the reference test method (i.e., RPT) to endotoxin is well documented. For this reason, the threshold pyrogen dose used for establishing the decision criteria for the *in vitro* test methods was based on historical RPT data for rabbits treated with endotoxin in the laboratory that supplied the RPT data.

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4.0 *IN VIVO* REFERENCE DATA FOR THE ASSESSMENT OF TEST METHOD ACCURACY

4.1 Description of the Protocol Used to Generate *In Vivo* Data

4.1.1 The Rabbit Pyrogen Test (RPT)

The RPT protocols most widely accepted by regulatory agencies are outlined in the USP, (USP23 NF28<151>), the U.S. Code of Federal Regulations (21CFR610.13), the European Pharmacopoeia (EP, EP5.0 2.6.8), and the Japanese Pharmacopoeia (JP, JP XIV), as summarized in **Table 4-1**. The RPT involves measuring the temperature increase in rabbits following an intravenous injection (via the ear vein) of a test substance in a dose not to exceed 10 mL/kg injected within a period of not more than 10 minutes. Initially, three rabbits are injected and the increase (or decrease) in temperature relative to the baseline value is measured at 30-min intervals for up to three hours. The resulting data are used to calculate an overall temperature increase by summing the results from all three animals, which is then used to assign a label of pyrogenic or non-pyrogenic.

Table 4-1 Test Guidelines for the Rabbit Pyrogen Test

RPT Protocol Component	Reference			
	21CFR610.13	**EP5.0 2.6.8**	**JP XIV**	**USP28 NF23 <151>**
Number of rabbits	3 or 8[1]	3, 6, 9, or 12[1]	3 or 8[1]	3 or 8[1]
Rabbit species/strain	Not specified	Not specified	Not specified	Not specified
Exclusion criteria for rabbits during the initial selection of rabbits	• Used in a negative pyrogen test in the preceding 2 days • Used in a pyrogen test in which its temperature rose ≥0.6°C in the preceding 2 weeks	• Weight<1.5 kg • Decreased weight in the preceding week • Used in a negative pyrogen test in the preceding 3 days • Used in a positive pyrogen test in the preceding 3 weeks	• Weight<1.5 kg • Decreased weight in the preceding week • Previously used in a positive pyrogen test • Rabbits from negative pyrogen tests may be reused only when a "as a long a resting period as possible is taken"	• Used in a negative pyrogen test in the preceding 2 days • Used in a pyrogen test in which its temperature rose ≥0.6°C in the preceding 2 weeks
Testing room conditions	20 to 23°C	Within 3°C of the housing quarters (temperature not specified)	20 to 27°C and constant humidity	20 to 23°C
Food/water during test	Food withheld during the test, but water available at all times	Food withheld overnight and until end of the test. Water withheld during the test.	Food withheld beginning several hrs. prior to first temperature recording and until the end of the test.	Food withheld during the test period, but water available at all times
Depth of temperature probe in rectum	Not less than 7.5 cm	Approximately 5 cm	60-90 mm	Not less than 7.5 cm
Preliminary test	≤7 days prior to main test, perform all procedures used for the main test except the injection.	• 1-3 days prior to main test, treat test animals with an injection of warmed (38.5°C) pyrogen-free saline • Record temperature at 90 min prior to injection and every 30 min thereafter up to 3 hr. • Exclude any rabbits with an increase of >0.6°C	Not specified	≤7 days prior to main test, perform all procedures used for the main test except the injection.
Baseline temperature	• Record temperature ≤ 30 min prior to injection • For any group of rabbits, use only if baseline temperatures do not vary>1°C among rabbits • Exclude rabbits with baseline temperature>39.8°C	• Mean of two temperature recordings at 40 minutes and 10 minutes prior to injection • Exclude rabbits if variation >0.2°C between measurements noted • Exclude rabbits with initial temperature >39.8°C or <38.0°C	• Record temperature three times at one-hr intervals prior to injection • Assuming no appreciable variability among recordings, use the last recording as the baseline value. • Exclude animals if 2nd and 3rd temperature measurements exceed 39.8°C	• Record temperature ≤30 min prior to injection • For any group of rabbits, use only if baseline temperatures do not vary >1°C among rabbits • Exclude rabbits with baseline >39.8°C
Injection volume	≥3 mL/kg <u>*BUT*</u> ≤10mL/kg	≥0.5 mL/kg <u>*BUT*</u> ≤10mL/kg	10 mL/kg, unless otherwise specified	≤10 mL/kg

Injection time	≤10 min	≤4 min, unless otherwise indicated	Not specified, but injection should occur within 15 min of the third pretest temperature recording	≤10 min
Injection site	Marginal ear vein	Marginal ear vein	Marginal ear vein	Marginal ear vein
Pre-warming of test material	37°C±2°C	38.5°C	37°C	37°C±2°C
Temperature recording intervals after injection	30 min intervals for 1 to 3 hr	≤30 min intervals for 3 hr	1 hr intervals for 3 hr	30 min intervals for 1 to 3 hr

Abbreviations: CFR = U.S. Code of Federal Regulations; EP = European Pharmacopoeia; JP = Japanese Pharmacopoeia; RPT = Rabbit pyrogen test; USP = United States Pharmacopoeia

[1]Each test is initially conducted with three animals and additional animals are tested to resolve equivocal results in the first three animals

4.1.2 Current *In Vivo* Pyrogenicity Test Method Protocols

As indicated in **Table 4-1**, U.S. and international regulatory agencies have tailored the RPT test method protocol to suit their specific needs and goals in protecting human health. The current test method protocols (i.e., 21CFR610.13; EP5.0 2.6.8; JP XIV; USP28NF23<151>) recommend using healthy, adult rabbits with no specific breed/strain requirements. Rabbits are to be adequately acclimated to their surroundings and housed in an environment free from excessive external stimuli. Each rabbit is conditioned prior to the test with a sham test that includes all of the procedural steps except the injection. Reuse of test rabbits is permitted only after an appropriate withdrawal period has been completed (2-3 days for a negative result, 2-3 weeks for a positive result).

The test is conducted in a room that is designated solely for pyrogen testing, in which the temperature is within 3°C of the uniform temperature of the housing room (i.e., 20°C±3°C). Food is withheld during the test, but access to water is continuous. The baseline temperature, which is used to calculate the increase in temperature during the test, is measured 30-40 minutes prior to injection of the test substance. In each group of rabbits tested, the variation in baseline temperature among the rabbits should not vary more than 1°C, and rabbits with an initial temperature greater than 39.8°C are excluded from testing.

The test substance is pre-warmed to approximately 37°C and injected (≤10 mL/kg) into the marginal ear vein, completing each injection within 10 minutes. The rectal temperature is recorded at 30-minute intervals for up to three hours after the injection. The decision criteria outlined in **Table 4-2** are then used to determine a pyrogenic response. As shown in **Table 4-2**, these decision criteria by which labels of pyrogenic or non-pyrogenic are assigned vary among the USP, CFR, EP, and JP test guidelines.

Table 4-2 Decision Criteria for Determining a Pyrogenic Response in the Rabbit Pyrogen Test

RPT Protocol	No. Rabbits	Product passes if:	Product fails if:
USP28 NF23<151>	3	0/3 rabbits show an increase of ≥0.5°C	NA[1]
	5[1]	≤3/8 rabbits show an increase of ≥0.5°C ***AND*** the summed responses ≤3.3.	>3/8 rabbits show an increase of ≥0.5°C ***AND/OR*** the sum of all responses >3.3
21CFR610.13	3	0/3 rabbits show an increase of ≥0.5°C	NA[1]
	5[1]	≤3/8 rabbits show an increase of ≥0.6°C ***AND*** the summed responses ≤3.7.	>3/8 rabbits show an increase of ≥0.6°C ***AND/OR*** the summed responses >3.7
EP5.0 2.6.8	3	Summed responses ≤1.15°C	Summed responses >2.65º
	6[2]	Summed responses ≤2.80°C	Summed responses >4.30º
	9[2]	Summed responses ≤4.45°C	Summed responses >5.95º
	12	Summed responses ≤6.60°C	Summed responses >6.60º
JP XIV	3	3/3 rabbits show an increase of <0.6°C ***AND*** the summed responses ≤1.4°C	≥2/3 rabbits show an increase ≥0.6°C
	5[3]	≥4/5 rabbits show an increase <0.6°C	≥2/5 rabbits show an increase ≥0.6°C

CFR = U.S. Code of Federal Regulations; EP = European Pharmacopoeia; JP = Japanese Pharmacopoeia; USP = United States Pharmacopoeia; NA = Not applicable

[1]If ≥1/3 rabbits show an increase of ≥0.5°C, continue test with an additional five rabbits.

[2]Three additional animals are tested when the summed responses falls in between the previous range.

[3]Five additional animals are tested when neither criteria is met, and results are based on these five animals only.

4.2 Reference Data Used to Assess *In Vitro* Test Method Accuracy

There were no direct comparisons using the same test substances in the proposed *in vitro* test methods and the RPT. Rather, historical data from 171 rabbits tested with endotoxin (0, 5, 10, 15, 20 EU/kg in 1 mL/kg) were obtained from a single laboratory. Two different sources of endotoxin have been used: *E. coli* EC5; and *E. coli* EC6 that were reported to be identical to the WHO standard used in the validation studies). These historical data were used to establish a threshold pyrogen dose (i.e., the endotoxin dose at which fever was induced in 50% of the rabbits), which was determined to be 5 EU/kg. Based on the largest allowable volume for injection in rabbits (10 mL/kg), the limit of detection that new pyrogen tests must meet was defined as 0.5 EU/mL.

4.3 Availability of Original Records for the *In Vivo* Reference Data

Section 4.1 of the ECVAM BRDs indicates that the Paul-Ehrlich Institute, Germany, provided the historical RPT data.

4.4 *In Vivo* Data Quality

Ideally, all data supporting the validity of a test method should be obtained and reported from studies conducted in accordance with GLP guidelines (OECD 1998; EPA 2003a, 2003b; FDA 2003). These guidelines provide an internationally standardized approach for the reporting requirements of studies designed for regulatory submissions, internal audits of laboratory records and data summaries, the archive of study data and records, and information about the test protocol and laboratory personnel, to provide assurances regarding the integrity, reliability, and accountability of the study. According to Section 4.4 of the ECVAM BRDs (with the exception of the WB/IL-6 BRD, which states that this section is "not applicable"), all RPT procedures were GLP-compliant.

4.5 Availability and Use of Toxicity Information from the Species of Interest

As stated in **Section 1.2.1**, the major regulatory requirement for pyrogenicity testing is for end-product release of human and animal parenteral drugs, medical devices, and human biological products. Results from such testing are used to limit to an acceptable level the risks of febrile reactions to the injection and/or implantation of the product. Therefore, for protection of both human and veterinary health, it is vital that the test method employed provide an accurate estimation of the potential for a pyrogenic reaction in humans and other species.

The estimation of the potential for a pyrogenic reaction has traditionally been obtained in rabbits using the RPT, and more recently in horseshoe crabs with the BET (for Gram-negative endotoxins only). A number of studies have compared febrile responses between rabbits and humans and showed that rabbits tended to be equal to, or more sensitive than humans with respect to their threshold pyrogenic responses, but higher doses were more pyrogenic in humans (Co Tui and Schrift 1942; Westphal 1956; Keene et al. 1961). Greisman and Hornick (1969) compared three purified endotoxin preparations in mature New Zealand white rabbits and in male volunteers and showed that the pyrogenic response induced was similar in both species. Based on these studies, the rabbit is considered to be predictive of the human response (and may often over predict the response).

4.6 Information on the Accuracy and Reliability of the *In Vivo* Test Method

Hoffmann et al. (2005) modeled the sensitivity and specificity of the RPT. They reviewed historical data from 171 rabbits challenged with endotoxin in a single laboratory in order to establish a threshold pyrogen dose (i.e., the endotoxin dose at which fever was induced in 50% of the rabbits). A threshold value of 0.5 EU/mL was defined by regression analysis of the data. The performance characteristics of the RPT (i.e., sensitivity and specificity) were then determined using a 2 x 2 contingency table, and incorporating the parameters obtained from the regression analysis. The authors considered the prevalence of the endotoxin spikes included in the ECVAM accuracy evaluations in the validation studies (i.e., 0 EU/mL: 20%; 0.25 EU/mL: 20%; 0.5 EU/mL: 40%; 1.0 EU/mL: 20%) and applied the threshold pyrogen dose of 0.5 EU/mL to calculate theoretical values for sensitivity (58%) and specificity (83%) of the RPT.

The accuracy and reliability of the RPT for endotoxin testing has been considered adequate for U.S. and international regulatory needs for many years. Since its inclusion in the USP in 1941, the RPT had been extensively used, and is the preferred method for detection of pyrogenicity for product development, because of the inability of the BET to detect non-endotoxin pyrogens.

5.0 TEST METHOD DATA AND RESULTS

5.1 Test Method Protocol

The standard operating procedures (SOPs) used during the ECVAM validation studies are included in **Appendix A**. As described in **Section 2.1**, there are many similarities among the protocols for each of the *in vitro* pyrogenicity test methods, with very few notable differences other than the type of cells used (i.e., whole blood cells, PBMCs, monocytoid cell line) and the proinflammatory cytokine assayed (i.e., IL-1β or IL-6). These similarities and differences are outlined in **Table 2-1**. An internationally accepted endotoxin standard (i.e., WHO-LPS 94/580 [*E. coli* 0113:h10:K-]) was used to spike samples of saline or marketed parenteral pharmaceuticals. The same pharmaceuticals were used to create the spiked samples for all five test methods (see **Table 3-1** and **3-2**). These samples were included in a series of studies designed to determine the relevance and reliability of each of the *in vitro* test methods.

5.2 Availability of Copies of Original Data Used to Evaluate Test Method Performance

ECVAM provided raw data from the validation studies in an electronic format (Excel® spreadsheets) that consisted of OD_{450} measurements for all replicates included in each of the validation studies.

NICEATM attempted to obtain additional *in vitro* and/or *in vivo* pyrogenicity test method data. A *Federal Register* (*FR*) notice (Vol. 70, No. 241, pp. 74833-4, December 16, 2005; available at http://iccvam.niehs.nih.gov/methods/pyrogen.htm) was published requesting original *in vitro* pyrogenicity test method data and reference data from the currently used pyrogenicity test methods (i.e., RPT and/or BET). In addition, the *FR* notice was sent directly to more than 100 interested stakeholders internationally. Despite these efforts, no additional data were submitted.

5.3 Description of the Statistical Approaches Used to Evaluate the Resulting Data

Details of the statistical approaches used to evaluate the accuracy and reliability of each of the five test methods are included in Section 5.3 of each ECVAM BRD. Briefly, as indicated in **Section 3.2**, ten substances (each spiked with five concentrations of endotoxin) were tested in each test method to evaluate accuracy, while three substances (each spiked with four concentrations of endotoxin) were used to evaluate test method reproducibility. Varying concentrations of endotoxin-spiked saline were tested for the analysis of intralaboratory repeatability.

The evaluation of intralaboratory repeatability included a coefficient of variation (CV) analysis of the OD_{450} measurements for the replicates of each endotoxin concentration. Boxplots were also generated to demonstrate variability among OD_{450} measurements for each concentration. Similar analyses were conducted for the three substances used to assess intra- and interlaboratory reproducibility.

The reproducibility analysis procedures used incorporated the decision criteria that were developed to differentiate between pyrogenic and non-pyrogenic materials (using a threshold value of 0.5 EU/mL). In all reproducibility analyses, a single run consisted of each of the substances (as described above and in **Section 3.2**) assayed in quadruplicate. Acceptability criteria for each run included a CV analysis to remove highly variable samples from the analyses. This criterion ranged from $CV<0.25$ to $CV<0.45$, depending on the test method being considered. For the measurement of intralaboratory reproducibility, pair-wise comparisons between the runs were determined and the correlations between runs expressed as a percentage of agreement between two individual laboratories. It should be noted that this analysis takes into account the agreement of the resulting pyrogenicity call (i.e., pyrogenic or non-pyrogenic), but does not consider whether the call is correct. All three possible combinations were compared (i.e., run 1 vs. run 2; run 1 vs. run 3; run 2 vs. run 3) and a mean value calculated, which was intended to provide an overall proportion of inter-run agreement.

Similar analyses were conducted for an assessment of interlaboratory reproducibility, in which pairwise comparisons between laboratories were determined and the correlations were expressed as a percentage of agreement. This analysis included each run from each laboratory (n=3 per laboratory) and all possible interlaboratory combinations were compared. Similar to the intralaboratory analysis, this analysis takes the resulting pyrogenicity call from each run in each laboratory into consideration, but does not consider whether the call is correct. **Section 7.0** provides additional details and the resulting data from these analyses.

For the accuracy analysis, 2 x 2 contingency tables were constructed using the decision criteria defined in **Table 4-2** to assign a pyrogenicity call. Each run for each sample from each laboratory was considered independently. Accordingly, the *in vitro* call was compared to the "true status" (based on the known endotoxin spike concentration) of the sample. The resulting accuracy statistics were calculated based on the overall database for each test method. Similar to the reproducibility analyses, acceptability criteria for each run included a CV analysis to remove highly variable samples from the analyses, for which a range of CV<0.25 to CV<0.45 was used, depending on the test method being considered. **Section 6.0** provides additional details and the resulting data from these analyses.

5.4 Summary of Results

Graphical representations of the repeatability and reproducibility analyses are provided in Section 5.2 of each ECVAM BRD (see **Appendix A**). The tabulated results from which the intra- and interlaboratory reproducibility analyses and accuracy analyses can be conducted are provided in Section 5.4 of the ECVAM BRDs. The tables in that section include the test substance name, the endotoxin spike concentration, the pyrogenicity call for each *in vitro* run, and the "true status" of each test substance.

5.5 Use of Coded Chemicals and Compliance with GLP Guidelines

Ideally, all data supporting the validity of a test method should be obtained using coded chemicals and reported in accordance with GLP guidelines (OECD 1998; EPA 2003a, 2003b; FDA 2003). As described in **Section 8.1**, all studies were carried out in accordance with GLP guidelines based on the information available in the ECVAM BRDs (**Appendix A**), **Section 3.4** indicates that the ten test substances and the five spike concentrations used for the accuracy evaluation were blinded to the testing laboratories. However, although the four spike concentrations were blinded to the participating laboratories for the reproducibility studies, the identity of the three test substances was not.

5.6 Lot-to-Lot Consistency of Test Substances

Lot-to-lot consistency of test substances is evaluated to ensure that the same substance, with the same physicochemical properties, is being evaluated over the duration of the study. In these studies, the test substances used were from released clinical lots of parenteral pharmaceuticals, which would imply that they had been subjected to rigorous chemical manufacturing control analyses to verify that the compositions are consistent. In addition, the international standard for Gram-negative endotoxin, WHO-LPS 94/580 (*E. coli* O113:H10:K-), is used as the spike solution, which provides a measure of consistency for the positive control substance, and the spike substance.

5.7 Availability of Data for External Audit

As described in **Section 8.4**, all records are stored and archived by the participating laboratories and are available for inspection.

6.0 RELEVANCE OF THE *IN VITRO* PYROGENICITY TEST METHODS

6.1 Accuracy of the *In Vitro* Pyrogenicity Test Methods

A critical component of an ICCVAM evaluation of the validation status of a method is an assessment of its relevance. The measure of relevance used in this evaluation is the performance of the new test in identifying pyrogens as compared to the performance of the current reference method (ICCVAM 2003). This aspect of assay performance is typically evaluated by calculating:

- Concordance (also referred to as Accuracy): the proportion of correct outcomes (positive and negative) of a test method
- Sensitivity: the proportion of true positive substances that are correctly classified as positive
- Specificity: the proportion of true negative substances that are correctly classified as negative
- Positive predictivity: the proportion of correct positive responses among substances testing positive
- Negative predictivity: the proportion of correct negative responses among substances testing negative
- False positive rate: the proportion of true negative substances that are falsely identified as positive
- False negative rate: the proportion of true positive substances that are falsely identified as negative.

The ability of the *in vitro* pyrogenicity test methods to correctly identify the presence of Gram-negative endotoxin was evaluated using parenteral pharmaceuticals spiked endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). As described in **Section 3.2**, ten substances (see **Table 3-1**) spiked with five concentrations of endotoxin were used for the evaluation. The individual spike concentrations in each substance were tested once, using each test method, in three different laboratories, providing a total of 150 runs (i.e., 10 substances x 5 spike solutions x 3 laboratories = 150) for evaluation in each test method. The quality criteria outlined in **Table 2-1** were used to identify run outliers that were subsequently excluded from the evaluation resulting in fewer than 150 runs included in the evaluation.

As described in **Section 4.2**, no RPT assays were conducted in parallel with the *in vitro* pyrogenicity test methods during the ECVAM validation studies. Instead, historical RPT data from rabbits tested with endotoxin were used to establish a threshold pyrogen dose (i.e., the endotoxin dose at which fever was induced in 50% of the rabbits), which was subsequently used to establish the limit of detection (i.e., 0.5 EU/mL) that the *in vitro* test methods being validated must meet. Accordingly, the *in vitro* call was compared to the "true status" (based on the known endotoxin spike concentration) of the sample. The resulting calls were used to construct 2x2 contingency tables, which were used to calculate the resulting test performance values.

6.1.1 Relevance of the Cryo WB/IL-1 Test Method

Of the 150 available runs for the cryo WB/IL-1 test method, 10 showed excessive variability among the four replicates (i.e., CV >45%), resulting in their exclusion from the analysis. An additional 20 runs did not qualify according to one or more of the criteria outlined in **Table 2-1**. Therefore a total of 120 runs were used in the performance analysis which showed that the cryo WB/IL-1 test method has a concordance of 92% (110/120), a sensitivity of 97% (75/77), a specificity of 81% (35/43), a false negative rate of 3% (2/77), and a false positive rate of 19% (8/43) (see **Table 6-1)**.

Table 6-1 Performance Analysis for Five *In Vitro* Pyrogenicity Test Methods[1]

Test Method	Concordance[2]	Sensitivity	Specificity	False Negative Rate	False Positive Rate
cryo WB/IL-1	91.7% (110/120)	97.4% (75/77)	81.4% (35/43)	2.6% (2/77)	18.6% (8/43)
MM6/IL-6	93.2% (138/148)	95.5% (85/89)	89.8% (53/59)	4.5% (4/89)	10.2% (6/59)
PBMC/IL-6	93.3% (140/150)	92.2% (83/90)	95.0% (57/60)	7.8% (7/90)	5.0% (3/60)
PBMC/IL-6 (cryo)[3]	91.9% (136/148)	88.8% (79/89)	96.6% (57/59)	11.2% (10/89)	3.4% (2/59)
WB/IL-6	91.9% (136/148)	88.8% (79/89)	96.6% (57/59)	11.2% (10/89)	3.4% (2/59)
WB/IL-1	81.0% (119/147)	72.7% (64/88)	93.2% (55/59)	27.3% (24/88)	6.8% (4/59)
WB/IL-1 (96-well plate method)[4]	92.8% (129/139)	98.8% (83/84)	83.6% (46/55)	1.2% (1/84)	16.4% (9/55)

Abbreviations: cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin -6; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

[1]Based on results of 10 parenteral drugs tested in each of three different laboratories; samples of each drug were tested with or without being spiked with a Gram-negative endotoxin standard (0, 0.25, 0.5, 0.5, or 1.0 EU/mL).

[2]Percentage (Number of correct runs/total number of runs)

[3]A modification of the PBMC/IL-6 test method using cryopreserved PBMCs.

[4]A modification of the WB/IL-1 test method using 96-well plates instead of tubes for the test substance incubation.

6.1.2 Relevance of the MM6/IL-6 Test Method

Of the 150 available runs for the MM6/IL-6 test method, two showed excessive variability among the four replicates (i.e., CV >25%), resulting in their exclusion from the analysis. No runs were excluded based on the criteria outlined in **Table 2-1**. Therefore a total of 148 runs were used in the performance analysis. Based on this analysis, the MM6/IL-6 test method has a concordance of 93% (138/148), a sensitivity of 96% (85/89), a specificity of 90% (53/59), a false negative rate of 4% (4/89), and a false positive rate of 10% (6/59) (see **Table 6-1)**.

6.1.3 Relevance of the PBMC/IL-6 Test Method

None of the 150 available runs for the PBMC/IL-6 test method showed excessive variability (i.e., CV >40%) and all runs met the criteria outlined in **Table 2-1**. Therefore all 150 runs were included in the performance analysis. Based on this analysis, the PBMC/IL-6 test method has a concordance of 93% (140/150), a sensitivity of 92% (83/90), a specificity of 95% (57/60), a false negative rate of 8% (7/90), and a false positive rate of 5% (3/60) (see **Table 6-1)**.

6.1.3.1 *Relevance of the PBMC/IL-6 Method When Using Cryo PBMCs*

As indicated in **Table 2-1**, the PBMC/IL-6 test method protocol was also conducted using a modified protocol that included cryo PBMCs. None of the 150 available runs for this modification of the PBMC/IL-6 test method showed excessive variability (i.e., CV >40%) and all runs met the criteria outlined in **Table 2-1**. Therefore all runs were included in a performance analysis. Based on this analysis, the PBMC/IL-6 test method, when using cryo PBMCs, has a concordance of 87% (130/150), a sensitivity of 93% (84/90), a specificity of 77% (46/60), a false negative rate of 7% (6/90), and a false positive rate of 23% (14/60). The high false positive rate can be attributed to a large number of false positives (50% [10/20]) in one of the three laboratories (the false positive rate in the remaining two laboratories is 10%).

6.1.4 <u>Relevance of the WB/IL-6 Test Method</u>

None of the 150 available runs for the WB/IL-6 test method showed excessive variability (i.e., CV >45%) and all runs met the criteria outlined in **Table 2-1**. However, two samples were mishandled by one of the testing laboratories, and thus the two associated runs were excluded from the analysis. As a result, 148 runs were included in the performance analysis for the detection of Gram-negative endotoxin. Based on this analysis, the WB/IL-6 test method has a concordance of 92% (136/148), a sensitivity of 89% (79/89), a specificity of 97% (57/59), a false negative rate of 11% (10/89), and a false positive rate of 3% (2/59) (see **Table 6-1)**.

6.1.5 <u>Relevance of the WB/IL-1 Test Method</u>

Of the 150 available runs for the WB/IL-1 test method, three showed excessive variability among the four replicates (i.e., CV >45%), resulting in their exclusion from the analysis. No runs were excluded based on the criteria outlined in **Table 2-1**. Therefore a total of 147 runs were used in the performance analysis. Based on this analysis, the WB/IL-1 test method has a concordance of 81% (119/147), a sensitivity of 73% (64/88), a specificity of 93% (55/59), a false negative rate of 27% (24/88), and a false positive rate of 7% (4/59) (see **Table 6-1)**.

6.1.5.1 *Relevance of the WB/IL-1 Test Method When Using 96-Well Plates*

As indicated in **Table 2-1**, the WB/IL-1 test method protocol was also conducted using a modified protocol that used 96-well plates instead of individual tubes. Of the 150 available runs for this modification of the WB/IL-1 test method, 11 showed excessive variability (i.e., CV >45%). No runs were excluded based on the criteria outlined in **Table 2-1**. Therefore, a total of 139 runs were included in a performance analysis. Based on this analysis, the WB/IL-1 test method, when using 96-well plates, has a concordance of 93% (129/139), a sensitivity of 99% (83/84), a specificity of 84% (46/55), a false negative rate of 1% (1/84), and a false positive rate of 16% (9/55).

6.2 Summary of the Performance Statistics for *In Vitro* Pyrogenicity Test Methods

The performance of five *in vitro* pyrogenicity test methods for the detection of Gram-negative endotoxin (based on 10 parenteral pharmaceuticals, each spiked with five concentrations of endotoxin) was evaluated. As outlined in **Table 6-1**, this analysis indicated that concordance among the test methods ranged from 81% to 93%, sensitivity ranged from 89% to 97%, specificity ranged from 81% to 97%, false negative rates ranged from 3% to 27%, and false positive rates ranged from 3% to 19%[10].

6.2.1 Discordant Results

It was not possible to make a direct comparison between the RPT and *in vitro* pyrogenicity test results without the availability of parallel testing data (i.e., same test substance tested using the *in vitro* and *in vivo* methods). Therefore, *in vitro* results that are discordant from the RPT could not be identified with these studies. Discordant results reflect a failure of the *in vitro* test method(s) to identify Gram-negative endotoxin spiked into a test substance at the threshold concentration (0.5 EU/mL) established based on historical data from the RPT (see **Section 4.2**).

6.2.2 Strengths and Limitations of *In Vitro* Pyrogenicity Test Methods

The limitations of these test methods have not been fully explored and identified. For this reason, pre-testing product specific validation will be necessary to establish if a particular test substance/material is appropriate for evaluation using these *in vitro* test methods. A

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[10] Including the cryopreservation modification in the PBMC/IL-6 test method protocol resulted in a false positive rate of 23% and a false negative rate of 7%. Including the 96-well plate modification in the WB/IL-1 test method resulted in a false positive rate of 16% and a false negative rate of 1%.

recognized limitation of the *in vitro* methods is the lack of data to determine their responses to, and suitability for, non-endotoxin pyrogens that are known to be detected by the RPT. However, an advantage to these *in vitro* test methods is that they are derived from human tissues, and thus avoid potential uncertainty associated with cross-species extrapolation.

7.0 RELIABILITY OF THE *IN VITRO* PYROGENICITY TEST METHODS

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement among test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 2003). Intralaboratory reproducibility refers to the determination of the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the determination of the extent to which different laboratories can replicate results using the same protocol and test chemicals, and indicates the extent to which a test method can be transferred successfully among laboratories. A reliability assessment includes a quantitative and/or qualitative analysis of repeatability and intra- and interlaboratory reproducibility. In addition, measures of central tendency and variation are summarized for historical control data (negative, vehicle, positive), where applicable.

An evaluation of intralaboratory repeatability and reproducibility could be conducted because *in vitro* pyrogenicity test data were available from replicate wells within individual experiments, and from replicate experiments within the individual laboratories. In addition, comparable data were available from each of the three laboratories that performed the validation studies, which allowed an evaluation of interlaboratory reproducibility.

7.1 Selection Rationale for the Substances Used to Evaluate the Reliability of *In Vitro* Pyrogenicity Test Methods

The quality of a reliability evaluation depends on the extent to which the substances tested adequately represent the range of physicochemical characteristics and response levels that the test method should be capable of evaluating.

The rationale for selecting the substances used in the validation studies was discussed in **Section 3.0**. In brief, substances that were used in the ECVAM validation studies were marketed parenteral pharmaceuticals, labeled as free from detectable pyrogens (i.e., they had passed the RPT or BET test). No rationale for the selection of these specific test substances was provided. Each test substance was spiked with a series of concentrations of Gram-negative endotoxin standard (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Endotoxin was selected as a "model" pyrogen for inclusion based on its availability in a standardized form. No other pyrogenic substances are presently available in a standardized form.

7.2 Analysis of Intralaboratory Repeatability and Reproducibility

Intralaboratory repeatability analyses were performed using the OD values obtained for each test with each spiked sample. All analyses of intra- and interlaboratory reproducibility were performed on the classifications of pyrogenic or non-pyrogenic, rather than on the absolute OD values generated in each run. Analyses of intralaboratory reliability typically include a coefficient of variation (CV) analysis, which is a statistical measure of the deviation of a variable from its mean (e.g., Holzhütter et al. 1996).

7.2.1 Intralaboratory Repeatability

In the ECVAM validation study, intralaboratory repeatability of each test method was evaluated by testing saline and various endotoxin spikes (0.06 to 0.5 EU/mL) in saline and evaluating the closeness of agreement among optical density readings for cytokine measurements at each concentration. Each experiment was conducted up to three times for each test method. Up to 20 replicates per concentration were tested and results indicated that variability in OD measurements increased with increasing endotoxin concentration, but the variability was not so great to interfere with distinguishing the 0.5 EU/mL spike concentration (i.e., the threshold for pyrogenicity) from the lower concentrations. **Table 7-1** details the study designs for each of these evaluations. At least four different study designs were employed for each test method with the exception of the cryo WB/IL-1. In the ECVAM cryo WB/IL-1 BRD (see **Appendix A**) Appendix D indicates that because intralaboratory reliability was extensively evaluated in the WB/IL-1, only a subset (n=2) of these studies was conducted as a part of a "catch-up validation" study. Based on the "acceptable" intralaboratory performance in this subset of studies, additional studies were not considered necessary.

Table 7-1 Intralaboratory Repeatability Assessed with Saline Spiked with WHO-LPS 94/580

Experiment	Study Design	Test Method				
		MM6/IL-6	PBMC/IL-6	WB/IL-1	WB/IL-6	cryo WB/IL-1[1]
1A	Endotoxin concentration (EU/mL)	0, 0.25, 0.5	0, 0.25, 0.5	0, 0.5	0, 0.5	0, 0.5
	N (per spike)	20	20	32	20	32
	Repetitions of experiment	1	1	1	1	1
1B	Endotoxin concentration (EU/mL)	0, 0.063, 0.125, 0.25, 0.5	0, 0.063, 0.125, 0.25, 0.5	0, 0.063, 0.125, 0.25, 0.5	0, 0.063, 0.125, 0.25, 0.5	0, 0.063, 0.125, 0.25, 0.5
	N (per spike)	12	12	12	10	12
	Repetitions of experiment	1	1	1	1	1
2A	Endotoxin concentration (EU/mL)	0, 0.25, 0.5	0, 0.5	0, 0.5	0, 0.25, 0.5	ND
	N (per spike)	20	8	12	8	ND
	Repetitions of experiment	3	3	3	3	ND
2B	Endotoxin concentration (EU/mL)	0, 0.25, 0.5	0, 0.063, 0.125, 0.25, 0.5	0, 0.25, 0.5	0, 0.5	ND
	N (per spike)	20	8	8	5	ND
	Repetitions of experiment	3	3	3	8	ND
2C	Endotoxin concentration (EU/mL)	ND	0, 0.125, 0.25, 0.5	0, 0.5	ND	ND
	N (per spike)	ND	8	5	ND	ND
	Repetitions of experiment	ND	8	8	ND	ND

Abbreviations: cryo = Cryopreserved; EU = Endotoxin unit; IL-1 = Interleukin-1; IL-6 = Interleukin-6; LPS = Lipopolysaccharide; MM6 = Mono Mac 6; N = number of replicates; ND = Not done; PBMC = Peripheral blood mononuclear cells; WB = Whole blood; WHO = World Health Organization

[1]Assessed using saline spiked with WHO-LPS 94/580 endotoxin.

[2]The cryo WB/IL-1 test method was included in a catch-up validation study to assess intralaboratory reliability in a subset of experiments (n=2).

7.2.2 Intralaboratory Reproducibility

Intralaboratory reproducibility was evaluated using three marketed pharmaceuticals spiked with various concentrations of endotoxin (see **Table 3-2**). Three identical, independent runs conducted in each of the three testing laboratories, with the exception of the cryo WB/IL-1 test method[11]. All three possible combinations were compared (i.e., run 1 vs. run 2; run 1 vs. run 3; run 2 vs. run 3) and a mean value calculated, intended to provide an overall proportion of inter-run agreement. In all reproducibility analyses, a single run consisted of each of the substances assayed in quadruplicate. Acceptability criteria for each run included a CV analysis to remove highly variable responses from the analyses. The criterion used to identify outliers ranged from CV <0.25 to CV <0.45, depending on the method being considered, and was arbitrarily set based on results using saline spiked with endotoxin. For example, for the MM6/IL-6 test method, the CV for any single spike concentration was ≤ 0.12, and therefore, the outlier criterion was set at 0.25.

Agreement between different runs was determined for each substance in three laboratories. As shown in **Table 7-2**, the agreement across three runs in an individual lab ranged from 75% to 100%.

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[11] The ECVAM cryo WB/IL-1 test method BRD states that there was no direct assessment of intralaboratory reproducibility because such an evaluation was performed in the WB IL-1 (fresh blood) test method, and the authors assume that variability is not affected by the change to cryopreserved blood assayed in 96-well plates.

Table 7-2 Intralaboratory Reproducibility of *In Vitro* Pyrogenicity Test Methods

Run Comparison[1]	WB/IL-1			Cryo WB/IL-1			WB/IL-6			PBMC/IL-6			MM6/IL-6		
	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3
1 vs 2	92% (11/12)	100% (8/8)	100% (12/12)	ND[3]	ND	ND	75% (9/12)	92% (11/12)	100% (12/12)	92% (11/12)	100% (12/12)	100% (12/12)	100% (12/12)	92% (11/12)	100% (12/12)
1 vs 3	83% (10/12)	88% (7/8)	92% (11/12)	ND	ND	ND	100% (12/12)	92% (11/12)	100% (12/12)	100% (12/12)	100% (12/12)	92% (11/12)	100% (12/12)	92% (11/12)	92% (11/12)
2 vs 3	92% (11/12)	NA[4]	92% (11/12)	ND	ND	ND	75% (9/12)	92% (11/12)	100% (12/12)	92% (11/12)	100% (12/12)	92% (11/12)	100% (12/12)	100% (12/12)	92% (11/12)
Mean	89%	-	95%	ND	ND	ND	83%	92%	100%	95%	100%	95%	100%	95%	95%
Agreement[2] across 3 runs	83%	-	92%	ND	ND	ND	75%	92%	100%	92%	100%	94%	100%	92%	92%

Abbreviations: Cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin-6; MM6 = Mono Mac 6; NA = Not assessed; ND = Not done; WB = Whole blood

[1]Comparison between 3 individual runs within each laboratory

[2]All possible combinations of runs among the 3 laboratories were compared

[3]Not done. The cryo WB/IL-1 BRD states that an assessment of intralaboratory reproducibility was performed using the WB IL-1 (fresh blood) test method, and it was assumed that intralaboratory variability would not be affected by the change to cryopreserved blood assayed in 96-well plates.

[4]Not assessed due to lack of sufficient data. The sensitivity criteria were not met for 1/3 substance in run 2, and 1/3 substance in run 3.

7.2.3 Interlaboratory Reproducibility

Interlaboratory reproducibility was evaluated in two different studies. In both studies, each run from one laboratory was compared with all runs of another laboratory. The proportions of similarly classified samples provide a measure of reproducibility. In the first study, in a similar manner as the evaluation of intralaboratory reproducibility, the interlaboratory reproducibility was evaluated using results from three marketed pharmaceuticals spiked with endotoxin and tested three times in each of the three laboratories. As shown in **Table 7-3**, the agreement across three laboratories for each test method (where three runs per laboratory were conducted) ranged from 58% to 86%, depending on the test method considered. In comparison, the agreement across three laboratories for the cryo WB/IL-1 test method, for which only one run per laboratory was conducted, was 92%.

Table 7-3 Interlaboratory Reproducibility of *In Vitro* Pyrogenicity Test Methods

Lab Comparison[1]	Agreement Between Laboratories[1]				
	WB/IL-1	**Cryo WB/IL-1**	**WB/IL-6**	**PBMC/IL-6**	**MM6/IL-6**
1 vs 2	92% (77/84)[2]	92% (11/12)[3]	72% (78/108)	81% (87/108)	97% (105/108)
1 vs 3	77% (83/108)	92% (11/12)[3]	75% (81/108)	86% (93/108)	89% (96/108)
2 vs 3	68% (57/84)[2]	92% (11/12)[3]	97% (105/108)	89% (96/108)	86% (93/108)
Mean	79%	92%	81%	85%	90%
Agreement across 3 labs[4]	58% (167/288)[2]	92% (11/12)[3]	72% (234/324)	78% (252/324)	86% (279/324)

Abbreviations: Cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin-6; MM6 = Mono Mac 6; WB = Whole blood

[1]Data from three substances (see **Table 3-2**) spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]) at 0, 0, 0.5 and 1.0 EU/mL tested three times in three different laboratories, with the exception of cryo WB/IL-1 (only the preliminary run from each laboratory used for analysis)

[2]Some of the runs did not meet the assay acceptance criteria and therefore were excluded from the analysis.

[3]For the cryo WB/IL-1 test method, each substance tested only once in each laboratory.

[4]All possible combinations of runs among the 3 laboratories were compared (with the exception of cryo WB/IL-1, which was only tested once in each laboratory, resulting in only one possible combination per substance).

In the second study, reproducibility was evaluated with the same ten substances used for evaluating accuracy. In this study, each of the substances was spiked with five concentrations of endotoxin and tested once in each of three laboratories. As shown in **Table 7-4**, the agreement across three laboratories for each test method ranged from 57% to 88%, depending on the test method considered. The levels, and order of agreement among laboratories was the same for both studies; the WB/IL-1 test method showed the least agreement (57-58%) and the cryo WB/IL-1 test method showed the most (88-92%)[12].

Table 7-4 Interlaboratory Reproducibility of *In Vitro* Pyrogenicity Test Methods

Lab Comparison[1]	Agreement Between Laboratories[1]				
	WB/IL-1[2]	Cryo WB/IL-1	WB/IL-6	PBMC/IL-6[3]	MM6/IL-6
1 vs 2	73% (35/48)	84% (38/45)	85% (41/48)	84% (42/50)	90% (45/50)
1 vs 3	82% (40/49)	88% (21/24)	85% (41/48)	86% (43/50)	90% (43/48)
2 vs 3	70% (33/47)	100% (25/25)	88% (44/50)	90% (45/50)	83% (40/48)
Mean	75%	91%	86%	87%	88%
Agreement across 3 labs	57% (27/47)	88% (21/24)	79% (38/48)	80% (40/50)	81% (39/48)

Abbreviations: Cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin-6; MM6 = Mono Mac 6; WB = Whole blood
[1]Data from 10 substances spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]) at 0, 0.25, 0.5, 0.5, and 1.0 EU/mL tested once in three different laboratories
[2]Interlaboratory reproducibility was also evaluated for the modified WB/IL-1 test method protocol (i.e., 96-well plates, see **Table 2-1**) in which the reproducibility between laboratories ranged from 83% to 92% (mean=89%) and the agreement across three labs was 83%.
[3]Interlaboratory reproducibility was also evaluated for the modified PBMC/IL-6 test method protocol (i.e., cryo PBMCs, see **Table 2-1**) in which the reproducibility between laboratories ranged from 76% to 96% (mean=84%) and the agreement across three labs was 76%.

7.3 Historical Positive and Negative Control Data

No historical control data were provided for any of the five *in vitro* pyrogenicity test methods. However, the intralaboratory repeatability analysis described in **Section 7.2.1** included repeat testing of both spiked (0.5 EU/mL endotoxin) and unspiked saline, and the accumulated positive and negative control values, respectively for each of the methods. As a result, the database that was accumulated during the ECVAM validation studies provides an indication of the range and variability in responses for the positive and negative controls.

[12] Interlaboratory reproducibility was also evaluated for the modified PBMC/IL-6 test protocol (i.e., cryo PBMCs, see **Table 2-1**) in which the reproducibility between laboratories ranged from 76% to 96% (mean=84%) and the agreement across three labs was 76%.

8.0 TEST METHOD DATA QUALITY

8.1 Adherence to National and International GLP Guidelines

Ideally, all data supporting the validity of a test method should be obtained and reported in accordance with GLP guidelines, which are nationally and internationally recognized rules designed to produce high-quality laboratory records. GLPs provide a standardized approach to report and archive laboratory data and records, and information about the test protocol to insure the integrity, reliability, and accountability of a study (OECD 1998: U.S. EPA 2003a, 2003b; FDA 2003).

The ECVAM validation studies for the five proposed methods were carried out in accordance with GLP guidelines and all deviations from GLP compliance are noted in the BRDs. Although no direct statement of GLP compliance was provided for the historical RPT data provided in the ECVAM BRDs, the studies were conducted at the Paul Ehrlich Institute (PEI), which is a German Federal Agency for Sera and Vaccines that provides regional support for German regulatory authorities, is qualified for granting marketing approval of certain marketed biological products (e.g., sera, vaccines, test allergens), and is a WHO collaborating center for quality assurance of blood products and *in vitro* diagnostics (http://www.pei.de).

8.2 Data Quality Audits

Formal assessments of data quality, such as a quality assurance (QA) audit, generally involve a systematic and critical comparison of the data provided in a study report with the laboratory records generated for the study. No attempt was made to formally audit the quality of the data presented in the five ECVAM BRDs. However, as indicated in **Section 5.2**, the raw data from the validation studies are available from the participating laboratories for a quality analysis.

8.3 Impact of Deviations from GLP Guidelines

The impact of the deviations from the GLP guidelines, as reported in the ECVAM BRDs, was not evaluated.

8.4 Availability of Laboratory Notebooks or Other Records

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

8.5 Need for Data Quality

Data quality is a critical component of the validation process. To ensure data quality, ICCVAM recommends that all data generated during the validation of a method be available, along with the detailed protocol(s) under which the data were produced. Original data should be available for examination, as should supporting documentation such as laboratory notebooks. Ideally, the data should adhere to GLP guidelines (ICCVAM 1997). Data protocols for the validation studies summarized here are available from ECVAM (see **Appendix A**), and the data from the individual laboratories are available for inspection, as indicated in **Section 8.4**.

9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS

9.1 Summaries of *In Vitro* Pyrogenicity Test Methods and Data from Published and Unpublished Studies

Data from the *in vitro* pyrogenicity methods proposed in the ECVAM submission are provided in **Appendix A** and were used in the performance analyses described in **Section 6.0** and **Section 7.0**. A *FR* notice (Vol. 70, No. 241, pp. 74833-74834, Dec 16, 2005) was published requesting the submission of data from the RPT, the BET, and from *in vitro* pyrogenicity testing with the five test methods described in this BRD. No data were received in response to this notice.

NICEATM conducted a prescreen evaluation of the ECVAM BRDs to verify that the information contained fulfilled the requirements prescribed in the ICCVAM submission guidelines (ICCVAM 2003). Based on this evaluation, the PWG requested additional data for the proposed methods to support many of the claims in the ECVAM BRD (e.g., the ability to detect both endotoxin and non-endotoxin pyrogens). In response to this request, ECVAM provided supplemental information that included unpublished data from *in vitro* pyrogenicity tests as an attempt to address these issues (see **Appendix B**).

As indicated in **Section 1.5**, NICEATM conducted an online literature search for relevant information on the proposed test methods using multiple internet databases (i.e., PubMed, SCOPUS, TOXLINE, Web of Science). This search revealed nine additional scientific publications that contained data from *in vitro* pyrogenicity product testing. Some of these studies contained comparisons of the results obtained in the *in vitro* method(s) with those obtained in the RPT and/or BET (see **Tables 9-1** to **9-8**). These studies were not included in previous sections of the BRD because they used a different method or protocol, or because they lacked sufficient information for an evaluation of accuracy and reliability (e.g., an adequate validation study design was not included, a standardized reference pyrogen was not used). Summaries of these published studies and available data from the *in vitro* pyrogenicity methods are presented below.

9.1.1 Andrade et al. (2003)

The authors evaluated the utility of human PBMCs and diluted WB for *in vitro* pyrogenicity tests and compared the responses to those obtained in the BET and RPT for the same diverse sampling of parenteral pharmaceuticals and biological products (see **Tables 9-1** and **9-2**). Interference testing of each substance was performed with spikes of the international endotoxin standard (i.e., WHO-LPS 94/580). These studies established an endotoxin detection limit of 0.06 EU/mL for both *in vitro* assays, and the results were consistent with those from the BET and RPT. The authors concluded that both the PBMC and WB methods were comparable to the BET and the RPT in their ability to detect and quantify the presence of endotoxin. In addition, the WB test method was able to detect concentration-dependent IL-6 release on exposure of WB to non-endotoxin pyrogens and pyrogens from Gram-positive organisms (i.e., *Candida albicans* and *Staphylococcus aureus*).

Table 9-1 Results of Pyrogenicity Testing of Pharmaceutical/Biological Products in the Human PBMC Assay, the BET, and the RPT[1]

Product	Number of Batches[2]	PBMC (EU/mL)	BET (EU/mL)	RPT
Ampicillin - 1000 mg/5 ml A	1	<6	<0.06	Pass
Ampicillin - 1000 mg/5 ml A	1	<6	<0.06	Pass
Gentamycin - 80 mg/2 mL	2	<3	<0.06	Pass
Oxacillin - 500 mg/5 mL	2	<3	<0.06	Pass
Enoxaparin - 100 mg/mL	3	<1.2	<0.06	Pass
Insulin 100 - U/mL	2	<3	<0.06	Pass
Tenoxican - 40mg/2 mL	1	<6	<0.06	Pass
Metoclopramide - 10 mg/2 mL	4	<3	<0.06	Pass
Calcium folinate - 50 mg/5 mL	1	<2.4	<0.06	Pass
Ranitidine - 25 mg/mL	2	<6	1.2-2.4	Pass
Pantoprazol - 40 mg/10 mL	1	<3	<0.06	Pass
Human serum albumin - 20%	1	<4.8	0.48-0.96	Pass
Erythropoietin - 4000 IU/vial A	1	<1.2	0.48-0.96	Pass
Erythropoietin - 2000 IU/vial B	1	112 ± 10[1]	491-983	Fail
Erythropoietin - 4000 IU/vial C	1	<1.2	<0.06	Pass
recG-CSF - 200 µg/vial A	3	<0.6	<0.06	Pass
Saline solution - 0.9% A	1	<0.3	<0.06	Pass

Abbreviations: BET = Bacterial Endotoxin Test; CSF = Colony Stimulating Factor; PBMC = Peripheral blood mononuclear cells; rec = Recombinant; RPT = Rabbit pyrogen test; SD = Standard deviation

[1]From Andrade et al. (2003)

[2]Batch results were combined; PBMC and BET study values represent a mean±SD value or consensus detection limits (n=3 donors; 4 replicates from each donor).

Table 9-2 Results of Pyrogenicity Testing of Pharmaceutical/Biological Products by the Human WB Culture Assay, the BET, and the RPT[1]

Product	Number of Batches[2]	WB Culture (EU/mL)	BET (EU/mL)	RPT
Dipyrone - 500 mg/mL	3	<24	<0.06	Pass
Amikacin - 500 mg/2 mL	2	<12	<0.06	Pass
Ampicillin - 1000 mg/5 ml A	1	<6	<0.06	Pass
Ampicillin - 1000 mg/5 ml A	1	<6	<0.06	Pass
Gentamycin - 80 mg/2 mL	2	<6	<0.06	Pass
Oxacillin - 500 mg/5 mL	2	<6	<0.06	Pass
Vancomycin - 500 mg/5 mL	2	<6	<0.06	Pass
Enoxaparin - 100 mg/mL	3	<0.6	<0.06	Pass
Heparin - 5000 IU/mL	2	<0.6	<0.06	Pass
Insulin - 100 U/mL	3	<6	<0.06	Pass
Ketoprofen - 100 mg/2mL	1	<6	<0.06	Pass
Diclofenac - 75 mg/3 mL	1	<12	<0.06	Pass
Tenoxicam - 40 mg/2 mL	2	<6	<0.06	Pass
Metoclopramide - 10 mg/2 mL	3	<3	<0.06	Pass
Cytarabine - 100 mg/5mL	1	<1.2	<0.06	Pass
Calcium folinate - 50 mg/5 mL	1	<0.6	<0.06	Pass
Ranitidine - 25 mg/mL	1	<6	1.2-2.4	Pass
Pantoprazol - 40 mg/10 mL	1	<6	<0.06	Pass
Furosemide - 10 mg/mL	2	<0.6	<0.06	Pass
rec-hGH - 4 IU/vial A	2	<0.2	<0.06	Pass
rec-hGH - 4 IU/vial B	1	12.4±2.5[1]	15.84-31.68	Pass
Human serum albumin - 20%	1	<2.4	0.48-0.96	Pass
Erythropoietin - 4000 IU/vial A	1	0.76	0.48-0.96	Pass
Erythropoietin - 2000 IU/vial B	1	141±2.8[1]	491-983	Fail
Erythropoietin - 4000 IU/vial C	1	<0.6	<0.06	Pass
recG-CSF - 300 μg/vial	3	<0.6	<0.06	Pass
Saline solution 0.9% A	2	<0.3	<0.06	Pass
Saline solution 0.9% B	1	44.8±5[1]	48-96	Fail
Glucose - 0.5%	1	2054±95[1]	1920-3840	Fail
Vitamin K - 10 mg/mL	2	<6	<0.06	Pass

Abbreviations: BET = Bacterial Endotoxin Test; CSF = Colony Stimulating Factor ; hGH = Human growth hormone; rec = recombinant; RPT = Rabbit pyrogen test; SD = Standard deviation; WB = Whole Blood

[1]From Andrade et al. (2003)

[2]Batch results were combined; PBMC and BET study values represent a mean±SD value or consensus detection limits (n= 3 donors; 4 replicates from each donor).

9.1.2 Bleeker et al. (1994)

This study measured IL-6 release from PBMCs as an indicator of pyrogenicity for *in vitro* safety testing of hemoglobin (Hb) solutions. The authors demonstrated that pure, polymerized Hb produced under aseptic conditions did not induce or inhibit IL-6 production, whereas production under non-aseptic conditions led to IL-6 release, which was also seen with the BET. Based on these findings, the authors concluded that IL-6 release from isolated PBMCs provides a sensitive indicator of endotoxin contamination in Hb solutions. The observed detection limit for endotoxin in Hb solutions (below 0.4 EU/mL), led the authors to suggest that this test method would be more sensitive to the presence of endotoxin than the RPT.

9.1.3 Carlin and Viitanen (2003)

Using WB and MM6-based *in vitro* pyrogenicity methods, this study evaluated the pyrogenic potential of a multivalent vaccine, Infanrix® (GlaxoSmithKline) that contains protein and polysaccharide components from both Gram-positive and Gram-negative bacteria. The five Infanrix® vaccines studied (e.g., Infanrix®, Infanrix® Hep B, Infanrix® polio, Infanrix® hexa, and Infanrix® polio Hib) contain Gram-positive bacterial components that are potentially pyrogenic but not detectable in the BET. IL-6 production in the WB/IL-6 test method varied among the seven donor blood samples in response to each of the five vaccines. Some donor samples produced a weak or no IL-6 release and others produced a large release (**Table 9-3**). However, IL-6 production from any single donor was similar to all vaccines when tested at various times. The variability in the magnitude of response to each vaccine among donors and the consistency of the response of any single donor was also seen when IL-1β was used as a marker. IL-6 release from WB was also examined following exposure to three concentrations of endotoxin standard (0.2, 2, and 20 pg/1.2 mL). All donor WB samples released IL-6 in a concentration responsive manner.

The IL-6 release from MM6 cells (**Table 9-4**) exposed to the five Infanrix® vaccines was measured using an ELISA assay and compared to the responses induced by three concentrations of endotoxin standard (0.2, 2, and 20 pg/1.2 mL) in three separate experiments. The MM6 cells produced minimal responses to the vaccines when compared to

WB, but released significant amounts of IL-6 in response to high concentrations of endotoxin. However, IL-6 induction by two different endotoxin standards in MM6 cells was strongly attenuated (>80% inhibition) when either of two vaccines (Infanrix® and Infanrix® Hep-B) was present (data not included in **Table 9-4**). Based on these studies, the authors suggested that a BET or RPT result might not correlate with the human fever response one might expect in humans immunized with such vaccines, because the production of proinflammatory cytokines may be compromised by various components in the vaccine product, and because Gram-positive components in the vaccines would not be detected in the BET.

Table 9-3 IL-6 Production from WB after Exposure to Endotoxin or Five Infanrix® Vaccines[1,2]

Experiment (Blood Donor)	Endotoxin		Vaccine (Absorbance in ELISA; n=4[3])					
	Endotoxin (pg/1.2 mL)	Absorbance (ELISA)	Dilution (µL vaccine/1.2 mL	Infanrix®	Infanrix® Hep-B	Infanrix® Hexa	Infanrix® Polio	Infanrix® Polio Hib
1	0.2	0.47	0.03	0.945	1.052	1.069	0.869	1.082
	2	0.971	0.3	1.826	2.055	2.014	1.832	1.919
	20	1.116	3	2.826	2.587	2.638	2.609	2.2
2	0.2	0.001	0.03	0.149	0.256	0.231	NT	0.284
	2	0.127	0.3	0.869	0.847	1.095	NT	0.933
	20	0.764	3	1.998	1.986	2.187	NT	1.685
3	0.2	-0.007	0.03	0.005	0.037	0.009	0.007	0.208
	2	0.09	0.3	0.275	0.457	0.282	0.321	0.261
	20	0.811	3	0.941	1.057	0.795	1.284	1.325
4	0.2	0.006	0.03	0.056	0.053	0.028	0.088	0.104
	2	0.043	0.3	0.165	0.312	0.44	0.309	0.533
	20	0.458	3	1.229	1.489	1.476	1.181	1.242
5	0.2	0.043	0.03	-	0.071	-0.003	-0.003	0.011
	2	0.024	0.3	0.007	0.014	0.004	0.03	0.05
	20	0.435	3	0.042	0.164	0.008	0.08	0.12
6	0.2	0.013	0.03	-0.009	-0.018	-0.01	-0.022	0.012
	2	0.022	0.3	-0.007	-0.008	0.005	-0.019	-0.007
	20	0.569	3	0.132	0.411	0.042	0.132	0.188
7	0.2	0.036	0.03	-0.012	-0.012	-0.01	-0.014	0.07
	2	0.014	0.3	-0.01	-0.01	-0.012	-0.011	-0.013
	20	0.436	3	0.183	0.274	0.045	0.183	0.525

Abbreviations: ELISA = Enzyme-linked immunosorbent assay; Hep = Hepatitis; IL-6 = Interleukin-6; NT = Not tested; WB = Whole blood

[1]From Carlin and Viitanen (2003)

[2]WB was challenged with endotoxin standard or vaccine in pyrogen-free water to provide the final concentration and incubated overnight at 37°C.

[3]Duplicate samples were run in two separate experiments.

Table 9-4 IL-6 Production by MM6 Cells after Exposure to Endotoxin or Five Infanrix® Vaccines[1,2]

MM6 Batch	Endotoxin			Vaccine (Absorbance in ELISA; 250,000 MM6 cells); n=4[3]				
	Endotoxin (pg/1.2 mL)	Absorbance in IL-6 ELISA	Dilution (µL vaccine/1.2 mL	Infanrix®	Infanrix® Hep-B	Infanrix® Hexa	Infanrix® Polio	Infanrix® Polio Hib
1	0.2	-0.001	0.3	0.013	0.014	0.001	0.002	-0.001
	2	0.026	3	0.078	0.158	0.06	0.105	0.07
	20	0.383	30	0.054	0.052	0.053	0.106	0.089
2	0.2	-0.001	0.3	0.004	0.01	0.001	0.003	0.004
	2	0.025	3	0.033	0.062	0.019	0.037	0.032
	20	0.4	30	0.013	0.012	0.018	0.038	0.038
3	0.2	-0.009	0.3	-0.012	-0.017	-0.021	-0.014	-0.019
	2	0.03	3	0.019	0.05	0.01	0.043	0.026
	20	0.192	30	-0.018	-0.012	-0.007	0	0.005

Abbreviations: ELISA = Enzyme-linked immunosorbent assay; IL-6 = Interleukin-6; MM6 = Mono Mac 6
[1]From Carlin and Viitanen (2003)
[2]MM6 cells were stimulated with endotoxin standard or vaccine in pyrogen-free water to provide the final concentration and incubated overnight at 37°C.
[3]n = Duplicate samples were run in two separate experiments.

9.1.4 Carlin and Viitanen (2005)

This study provides support for the findings from a previous study (Carlin and Viitanen (2003) in which the authors demonstrated IL-6 release by a WB method in response to pyrogenic or spiked multivalent vaccine preparations that were inactive in the BET. It also confirms that IL-6 was released from WB of some, but not all donors. The present study demonstrates that IL-6 release in susceptible donors was caused by toxoids from Gram-positive diphtheria, and to a lesser extent, from tetanus bacterial components of the vaccines. The WB donors were studied for two years and their responses to the individual vaccines, whether responsive or non-responsive, were consistent. The responses of these donors to Gram-negative endotoxin or LTA from Gram-positive bacteria were consistent and confirmed the findings of Fennrich et al. (1999) with respect to the consistency of responses among several hundred blood donors to endotoxin. The authors concluded that individual donor-specific differences in IL-6 release from WB exposed to the multivalent vaccines resulted from toxoids present in the diphtheria or tetanus component, and noted that these donor-specific responses to the vaccines were not observed in the BET.

9.1.5 Daneshian et al. (2006)

This study describes the development of a modification to the WB/IL-1 method termed AWIPT (Adsorb, Wash, *In Vitro* Pyrogen Test). The authors indicate that this modification is intended to increase sensitivity to the presence of endotoxin contamination by isolating endotoxin from WB. To accomplish this, the sample containing endotoxin (naturally occurring or spiked) is treated with HSA covalently linked to macroporous acrylic beads. The HSA-treated beads bind the endotoxin, which is subsequently eluted from the beads. The WB/IL-1 test method is therefore performed using a slightly modified protocol in which the diluted WB is incubated overnight with the sample in the bead suspension.

The results showed that HSA-coated beads bind endotoxin in a concentration-dependent manner (when spiked with 0, 25, 50, and 100 pg/mL LPS), but little or none was bound to unmodified beads. The test showed a detection limit of 25 pg/mL LPS (i.e., 0.25 EU/mL), which is less sensitive than the BET (3 pg/mL) and slightly lower than the RPT (50 pg/mL). IL-1β secretion in response to either LPS or LTA was generally higher using the AWIPT procedure, but the concentrations of LPS or LTA needed to induce a response were similar;

thus the sensitivity of this test modification was comparable to that of the unmodified WB/IL-1 test method (*Note: Additional data received from ECVAM suggests that modification of the AWIPT is capable of increasing the sensitivity for detection of endotoxin from the current 0.25 EU/mL to 0.0001 EU/mL [see **Appendix B**]).*

Daneshian et al. studied the kinetics of cytokine release from WB in response to a challenge with 2 pg/mL endotoxin. IL-1β release in the AWIPT-treated samples lagged slightly behind that of the standard WB/IL-1 test in the 0 to 8 hr time period, whereas more IL-1β was produced in the AWIPT-treated samples in the 10 to 30 hr time period. Some immunomodulatory or toxic cancer drug samples tested in the WB/IL-1 method interfered with the WB/IL-1 assay and required a higher dilution (1/10 to 1/100) to detect IL-1β. Detection of endotoxin spiked into these test samples (measured as IL-1β release) generally occurred at lower dilutions in AWIPT than in the WB/IL-1 test method, suggesting that the interfering substances were removed by the procedure. For example, five dilutions (ranging from 1/3 to 1/316) of liposomal daunorubicin were spiked with 25 pg/mL of endotoxin and detection of IL-1β was compared between the two methods. This cytokine was not detectable in the WB/IL-1 method (< 30% of the IL-1β released by endotoxin) at any drug dilution, whereas in the AWIPT, IL-1β was detected at drug dilutions of 1/32, 1/100, and 1/316 (>78% of the IL-1β released by endotoxin).

The authors concluded that the inclusion of endotoxin adsorption and washing steps in the WB/IL-1 method (i.e., the AWIPT) to remove potentially interfering substances improved the detection of pyrogenic contaminants in immunomodulatory and toxic cancer drug samples. They suggest that the AWIPT method offers an improvement for safety testing of products administered to patients, and for batch control in pharmaceutical processing.

9.1.6 Eperon et al. (1996, 1997)

Eperon and colleagues developed an *in vitro* test system for measuring pyrogenic substances using two clones derived from MM6 cells (H.W.L. Ziegler-Heitbrock, University of Munich) and one from a THP-1 cell line (European Collection of Animal Cell Cultures (Porton Down, Salisbury, United Kingdom). These clones are reported to be phenotypically stable over time with respect to their superior responsiveness to endotoxin than the parent cell lines. Endotoxin content was measured by the release of TNF-α using an immunoassay. These clones demonstrate high LPS sensitivity when non-pyrogenic fetal calf serum is used in the assay as a serum supplement. Enhanced expression of the cell-surface endotoxin receptor CD14 was obtained by pretreatment of the cells for two days with calcitrol. Purified endotoxin (i.e., LPS; smooth strain and rough mutant), other cellular components from Gram-negative or Gram-positive bacteria, and Mycobacteria were tested. The MM6 clones responded to these pyrogenic products in an order of potency of detection equivalent to that found in the RPT and similar to that observed in the BET (i.e., Gram-negative endotoxin > Gram-positive material > non-endotoxin pyrogens). The response of the THP-1 clone was similar to that of the MM6 clones, except that the THP-1 clone did not respond to diphosphoryl lipid A, a structural component of LPS.

Pyrogenicity testing of a panel of stable blood products including albumin and Immunoglobulin G (IgG) for parenteral use produced similar results in the RPT and MM6 or THP-1 clones when tested as received (i.e., free of detectable pyrogens) or positive results when spiked with 20 EU/mL endotoxin (**Table 9-5**) with a few exceptions. For example, in the cell-based test, there was one borderline, but significant positive result in an unspiked sample (i.e., false positive relative to the RPT). In the BET, 4 of 13 (31%) unspiked samples tested positive (i.e., false positive). The results suggest that the cell-based assays may produce fewer false positives than the BET.

When ten bacterial and viral vaccine preparations were evaluated, the monocytoid cell-based test method (e.g., combined results from two experiment with each cell line) correlated well with the RPT (positive or negative for endotoxin) with the exception of one preparation that produced nearly 10-fold less TNF-α than the other samples, and was near the limit of detection, but not statistically significant different from the negative control (**Table 9-6**). The authors suggest that these cloned monocytoid cell-based test methods are valid *in vitro* alternatives for detection of endotoxin in commercial preparations, and produce results comparable to the RPT and BET.

Table 9-5 Pyrogenic Activity of Blood Preparations for Parenteral Use[1]

Preparation	Endotoxin Spike	RPT[2]	BET[3,4]	Cell Test[5,6]
IgG for i.v. use	20 EU/mL	+	+	+
	20 EU/mL	+	+	+
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
Albumin	20 EU/mL	+	+	+
	20 EU/mL	+	+	+
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
Test threshold[7]		*Δt = 1.5 °C*	*300 pg/mL LPS*	*50 pg/mL TNF*

Abbreviations: BET = Bacterial Endotoxin Test; ELISA = Enyme-linked immunosorbent assay; EU = Endotoxin Units; IgG = Immunoglobulin G; i.v. = Intravenous; LPS = lipopolysaccharide; RPT = Rabbit pyrogen test; TNF = Tumor necrosis factor

[1]From Eperon et al. (1997)

[2]n=3

[3]n=2

[4]Haemachem BET assay (St. Louis)

[5]n=4 *[Note: cell type not specified, although author's claim that either MM6 or THP-1 are equally capable of endotoxin detection]*

[6]TNF induction was determined using a commercial TNF ELISA.

[7]RPT threshold was obtained from the European Pharmacopoeia; the threshold for the BET and cell-based test methods was considered to be equal to 2 standard deviations from the mean of a set of negative samples.

Table 9-6 Pyrogenic Activity of Vaccine Preparations[1]

Vaccine Preparation[2]	Batch[3]	Cell Test[4]	Pyrogenicity
IgG for i.m. use	A-1	n.d.[5]	-
	A-2	n.d.	-
Bacterial vaccines	B-1	10.8±0.3	+
	C-1	6.0±3.6	+
	D-1	1.4±1.8	-
Viral vaccines	E-1	n.d.	-
	E-2	n.d.	-
	F-1	n.d.	-
	F-2	n.d.	-
	G-1	21.2±3.2	+

Abbreviations: IgG = Immunoglobulin G; i.m. = Intramuscular; n.d. = Non-detectable
[1]From Eperon et al. (1997)
[2]Vaccine solutions were tested at 1/20 (v/v)
[3]Letters refer to distinct types of vaccine preparations; numbers to different lots
[4]TNF-α production in ng/mL±S.E.M (n=3) *[Note: cell type not specified, although author's claim that either MM6 or THP-1 are equally capable of endotoxin detection]*
[5]No measurable quantity of cytokine was detected.

9.1.7 Pool et al. (1998)

This study describes a WB assay for the detection of pyrogens in blood products. IL-6 release from WB in response to endotoxin is used to define a pyrogenic response. This assay was highly responsive to *Escherichia coli* endotoxin (i.e., the limit of detection of endotoxin was 1.25 EU/mL), and also responded to whole bacteria (*E. coli* and *Bacillus subtilis [B.subtilus]*). There was considerable variation in IL-6 levels released from WB between donors following exposure to endotoxin, but each donor response was always linear. The pyrogenicity of production batches of human serum albumin (HSA), fibronectin (Fn), and stabilized human serum (SHS) solutions were evaluated using the WB method and compared to the BET and RPT. Spike recovery in batches of these samples varied between 90 and 116% for *E. coli* endotoxin, 74 to 111% for *B. subtilis*, and 61 to 99% for *E. coli* and the products tested did not interfere with the IL-6 assay system. Good correlations were found among the WB, BET, and RPT assay results (**Table 9-7**). Of 22 products tested, the WB assay and the RPT were in agreement (i.e., pass or fail) for all tests, while one sample was classified as negative in the BET, but positive in both the WB method and the RPT. The detection limit for endotoxin by the WB method was 1.25 EU/mL, which is lower than the established pyrogen cut-off level (as stated in the European Pharmacopoeia) for the products under investigation (i.e., 2 EU/mL for HSA and SHS; 4.5 EU/mL for Fn). The authors concluded that the WB assay was able to detect both Gram-negative and a Gram-positive pyrogens and exhibited greater sensitivity to endotoxin than the RPT.

Table 9.7 Comparison of the WB test, BET, and the RPT for Detecting the Pyrogenicity of Production Batches of Biological Products[1]

Product	**Batch**	**WB (EU/mL)[2]**	**BET**	**RPT**
Fibronectin - 0.5 mg/mL	Fn3195	<0.05	Pass	Pass
	Fn3296	<0.05	Pass	Pass
	Fn3596	1.28	Pass	Pass
Human serum albumin - 200 mg/mL	B274	29.4	Fail	Fail
	B291	<0.05	Pass	Pass
	B293	<0.05	Pass	Pass
	B294	<0.05	Pass	Pass
	B295	<0.05	Pass	Pass
	B296	<0.05	Pass	Pass
	B297	<0.05	Pass	Pass
	B298	1	Pass	Pass
	B299	1.1	Pass	Pass
	B300S	1	Pass	Pass
	B301	<0.05	Pass	Pass
	B302	>20	Pass[3]	Fail
Stabilized human serum - 50 mg/mL	SS349	0.7	Pass	Pass
	SS350	<0.05	Pass	Pass
	SS351	<0.05	Pass	Pass
	SS352	0.5	Pass	Pass
	SS353	<0.05	Pass	Pass
	SS354	0.6	Pass	Pass
	SS355	0.5	Pass	Pass

Abbreviations: BET = Bacterial Endotoxin Test; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin Units; IL-6 = Interleukin-6; WB = Whole blood; RPT = Rabbit pyrogen test

[1]From Pool et al. (1998)

[2]Result based on IL-6 secretion in human WB using an ELISA calibrated to an *E. coli* endotoxin standard (Kabi Diagnostica).

[3]False negative relative to the RPT response

9.1.8 Taktak et al. (1991)

This paper summarizes the development of an *in vitro* pyrogenicity test method based on IL-6 release from MM6 cells. A detectable level of IL-6 was released in response to 2.5 pg/mL of endotoxin, yielding a level of sensitivity of 25 pg/mL when testing 5% HSA at a 1/10 dilution for the presence of endotoxin. Three batches of a therapeutic HSA that caused fever in humans were positive in the MM6/IL-6 method, whereas the same substances were negative in the BET and the RPT (**Table 9-8**). As in the BET, the samples required a 1/10 dilution to remove interfering substances, and had assay sensitivity equal to that of the BET (25 pg/mL) and 40-fold greater than the RPT (1000 pg/mL). The authors suggest that the MM6/IL-6 method represents an important alternative to the existing pyrogen tests and may be a more appropriate end product test for the detection of pyrogens in parenteral products, such as HSA, that cannot be detected in the BET.

Table 9-8 Results of Pyrogenicity Testing of Batches of Therapeutic HSA Using the MM6/IL-6, BET, and RPT[1]

Batch of HSA	Endotoxin Quantitation by IL-6 Release (pg/mL)	Endotoxin Quantitation by the BET		RPT Result
		IU/mL	pg/mL[6]	
1[2]	97±2.3[3,4]	1.0-2.0	140-280	Pass
2[2]	30±2.8[4]	2.4-3.2	336-448	Pass
3[2]	31±2.3[4]	0.5-0.75	70-105	Pass
4	<25[5]	<0.24	<34	Pass
5	<25[5]	3.6-4.8[7]	504-762	Pass
6	<25[5]	<0.26	<36	Pass

Abbreviations: BET = Bacterial Endotoxin Test; HSA = Human serum albumin; IL-6 = Interleukin-6; IU = International units; pg = Picograms; RPT = Rabbit pyrogen test

[1]From Taktak et al. (1991)

[2]Batch of HSA used that caused fever in humans.

[3]Mean±S.E.M.

[4]Values are significantly different from subthreshold concentrations of endotoxin (<2.5 pg/mL; p<0.001).

[5]Values below the detection limit of the test system (25 EU/mL); preparations of HSA were tested at a dilution of 1/10 and 2.5 pg/mL endotoxin was the lowest concentration of endotoxin tested that evoked a significant release of IL-6.

[6]1.0 IU=0.14 ng for preparation used.

[7]False positive relative to RPT.

9.2 Conclusions from Scientific Literature Based on Independent Peer-Reviewed Reports and/or Reviews

Hartung et al. (2001) provided a summary report of an ECVAM-sponsored workshop to review the current status of pyrogen testing, to review the capabilities of new pyrogen tests, and to provide recommendations for their continued development. The need for alternatives to the RPT and the BET was discussed, and their respective limitations were highlighted. The workshop compared the utility of the various methods (i.e., *in vitro* pyrogenicity test methods, BET, RPT) for testing a variety of pyrogenic materials. Workshop conclusions indicated a need for alterative test methods to address the limitations of the BET and RPT, but stressed the need for appropriate validation of any new method.

An additional nine reports describing studies of cell-based *in vitro* pyrogenicity methods were obtained from the literature search described in **Section 9.1** and representative of the alternative *in vitro* pyrogenicity methods considered in the ECVAM workshop**.** Although these reports did not include data on test substances that could be used in the performance analysis in **Section 6.0** and **Section 7.0**, they did evaluate the use of the *in vitro* pyrogenicity test methods for sensitivity to endotoxin (i.e., endotoxin detection limit), specificity of the response to endotoxin and/or non-endotoxin pyrogens (i.e., spectrum and relative potency of various pyrogens detected), and/or the impact of interfering substances. A summary of each study is presented below.

9.2.1 De Groote et al. (1992)

The authors measured the release of various cytokines (IL-1β, IL-6, TNF-α, IL-2, IFN-γ, and granulocyte-macrophage colony stimulating factor [GM-CSF]) in response to endotoxin or phytohaemagglutinin (PHA) stimulation of WB and PBMC cultures. Endotoxin stimulated IL-1β, TNF-α, and IL-6 release, while PHA stimulated IL-2, IFN-γ, and GM-CSF release. There was a significant correlation between production of the three endotoxin-induced cytokines and the number of monocytes in the challenged culture, suggesting that monocytes are the major source of these cytokines: the other cytokines did not correlate with any of the cell types. The data also suggested that WB produced less variable levels of cytokines than

PBMC on exposure to endotoxin. Consistent results were obtained with the WB test using more than 50 different blood donors. The authors suggest that WB is a more appropriate choice for studying cytokine production *in vitro* and its modulation by exogenous or endogenous factors, because natural cell-to-cell interactions are preserved, immune mediators are available, and cytokine levels obtained with PBMC were more variable.

9.2.2 Fennrich et al. (1999)

Fennrich and colleagues compared a commercially available human WB/IL-1 pyrogenicity assay (PyroCheck® from DPC Biermann, Bad Nauheim distributed by Millenia, UK) to the BET and RPT. There was a concentration-dependent IL-1β release in WB that was incubated with nitrocellulose filters containing live *E. coli* bacteria and *E. coli* killed by heat or by antibiotics. The authors also tested air conditioning filters from a veterinary sheep facility and identified filters to be contaminated with bacteria that were later confirmed by microbiological tests (the data and the identity of the organisms identified were not presented). The authors compared the PyroCheck®, BET, and the RPT (**Table 9-9**) and concluded that PyroCheck® is a simple, accurate test that detects a wider range of pyrogens than the BET.

Table 9-9 Comparison of the Application Spectra of the RPT, the BET, and the Human WB Assay (PyroCheck®)[1]

Test		Applications		
		PyroCheck®	**BET**	**RPT**
Pyrogens	Gram-negative	+	+	+
	Gram-positive	+	-	+
	Fungi	+	-	+
Product pyrogenicity	Biologicals	+	-	+
	Pharmaceuticals	+	+	+
	Medical devices	+	+[2]	-
	Air quality	+[2]	+[2]	-
	Blood products	+	-	-

Abbreviations: BET = Bacterial Endotoxin Test; RPT = Rabbit pyrogen test; WB = Whole blood
[1]From Fennrich et al. (1999)
[2]Based on preliminary data

9.2.3 Hansen and Christensen (1990)

This study compared the results from PBMC exposed to endotoxin or ultraviolet light-killed *S. aureus* as an index of pyrogenicity, and then compared these results to the BET and the RPT. The authors used human PBMC obtained from heparinized peripheral blood and measured IL-1-like material in culture supernatants by evaluating co-mitogenic activity on PHA-stimulated murine thymocytes (measured in units of IL-1β where 1 unit is defined as the concentration that gives 50% of the maximal incorporation of ^{3}H-thymidine in the thymocyte assay). The endpoint is referred to as an IL-1-like material because other cytokines such as IL-2, IL-6, and TNFα may also stimulate the proliferative response of the thymocytes. When exposed to endotoxin, PBMC secreted cytokines in a concentration-dependent manner that provided a limit of detection of 200 pg/mL of endotoxin. In comparison, the BET can normally detect 10 to 100 pg/mL of endotoxin, while the RPT can detect 500 pg/mL. Therefore, the PBMC procedure had a level of detection of endotoxin 2.5-fold lower than that of the RPT and 2-fold higher than the BET. The PBMCs also responded with greater sensitivity to the Gram-positive pyrogen *S. aureus* (10^5 cells/mL), which was not detected in the BET (10^9 cells/mL). Based on these results, the authors proposed that the PBMC test be used as an alternative *in vitro* test to the BET and RPT.

9.2.4 Hartung and Wendel (1996)

The authors stimulated human WB with various inflammatory agents to release endogenous cytokines (IL-1β, TNFα) and inflammatory mediators (PGE_2) as an *in vitro* method for the detection of pyrogenic materials. Cytokines were released in a concentration-dependent manner following exposure to endotoxin or LTA. Heat-killed Gram-positive bacteria (*S. aureus*) or components of these organisms (i.e., muropeptides, LTA, enterotoxins, streptolysin O) and plant mitogens such as phorbol myristate acetate (PMA) and PHA also produced a cytokine response. Higher concentrations (three orders of magnitude) of the Gram-positive pyrogens were needed to elicit a response as compared to Gram-negative pyrogenic material.

Studies to determine the variability among the responses of different donor WB samples were also performed. Only two of the 18 donor samples released IL-1β in response to 1 pg/mL of endotoxin, but all responded to 10 pg/mL endotoxin. The release of IL-1β from the WB samples of 45 individual donors exposed to 100 ng/mL of endotoxin was also consistent. Based on these results, the authors suggested using the WB/IL-1 test method as an *in vitro* alternative to the RPT.

9.2.5 Moesby et al. (1999)

Moesby and colleagues compared pyrogenicity testing using MM6 cells, isolated PBMC, and the BET. LPS and ultraviolet light (UV)-killed Gram-negative *Staphylococcus. typhimurium (S. typhimurium)* or Gram-positive *S. aureus* produced concentration-dependent increases in IL-6 production in MM6 or PBMC cultures. PBMC, but not MM6 cells, were able to differentiate UV-irradiated yeast (*C. albicans*) and mold (*Aspergillus niger*) pyrogens, as evidenced by statistically significant increases in IL-6 production. The BET can detect Gram-negative endotoxin, but not Gram-positive endotoxin or LTA (the pyrogenic component of Gram-positive bacteria), and it may weakly detect yeast or viral pyrogens that the MM6 assay could not detect. Therefore, the authors suggest that pyrogen testing using MM6 cells would be a useful supplement to the BET for the detection of both Gram-negative and Gram-positive bacteria.

9.2.6 Nakagawa et al. (2002)

Nakagawa and colleagues describe an *in vitro* pyrogen test system based on pro-inflammatory cytokine release from a sub-clone of MM6 cells (i.e., MM6-CA8) and compare this response to a human WB culture system and the RPT. Similar to MM6 cells, MM6-CA8 were developed for superior reactivity to both endotoxin and peptidoglycan (PG). The MM6-CA8 cells release IL-6, TNF-α, and IL-1, but in greater quantities than MM6 cells in the range of 1 to 1000 pg/mL of endotoxin (up to 4-fold greater) or to 1 to 1000 ng/mL PG (up to 10-fold greater) compared to MM6 cells. The range of responses of human WB to the various pyrogens was similar to that of the MM6-CA8 cells. The relative potencies of the various pyrogens in the RPT were similar to those of the cytokine-induction potencies in the WB and MM6-CA8 methods, except for polyinosinic:polycytidylic acid (Poly [I:C]), which was reported to be 10,000-fold more potent as a pyrogen injected in rabbits when compared to humans. The authors conclude that these results suggest MM6-CA8 cells can detect a variety of pyrogens using IL-6 as the marker, and that these responses are highly relevant to the prediction of human fever reactions.

9.2.7 Pool et al. (1999)

This article describes a method to differentiate between endotoxin and non-endotoxin pyrogens when testing HSA solutions in a WB culture assay. Detection limits for four Gram-positive (*Bacillus stearothermophilus [B. stearothermophilus]*, *B. subtilis*, *Micrococcus luteus*, and *S. aureus*) and four Gram-negative bacteria (*E. coli*, *Kleibsiella pneumoniae*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*) were expressed as the number of whole bacteria required to produce a pyrogenic response equal to that of 1.25 EU/mL endotoxin. *B. stearothermophilus* and *E. coli* produced concentration-dependent increases in IL-6 production. The cationic antibiotic Polymyxin B, which inhibits the binding of endotoxin to the CD14 receptor, produced concentration-dependent inhibition of IL-6 release following exposure to 10 EU/mL endotoxin in the WB assay at concentrations up to 1 EU/mL and completely inhibited IL-6 release at concentrations above 2 EU/mL. In contrast, Polymyxin B had no effect on IL-6 release following exposure to *B. subtilis*. These data suggest that Polymyxin B may be useful for differentiating endotoxin and non-endotoxin pyrogenic contaminants. The data also suggests that binding of endotoxin to Polymyxin B (e.g., by linkage to an affinity column) may be used in the depyrogenation process.

9.2.8 Poole et al. (2003)

This paper describes a rapid single-plate *in vitro* test for the presence of pyrogenic substances based on monocyte activation. The assay uses polyclonal antibodies to IL-6 or TNFα cytokines, coated and stabilized onto 96-well plates. Monocytoid cells (e.g., PBMC, MM6 or THP-1 cells), endotoxin standard (LPS), test sample, and a second biotinylated antibody specific for the cytokine (e.g., either IL-6 or TNF-α) are incubated for 2 to 4 hr in the antibody-coated wells. An ELISA for one of the cytokines is then performed on the washed plate. IL-6 is preferred and provides a limit of detection of 0.015 EU/mL with PBMC, 0.05 EU/mL in MM6 cells, and 0.03 EU/mL with diluted WB. The amount of TNF-α released in WB in response to endotoxin was approximately 50 to 70% lower than IL-6, but was released earlier (i.e., 2 hr vs. 4 hr). The amount of IL-6 released on exposure to endotoxin tended to be greater in this single plate test when compared to the traditional two plate test (i.e., in which the supernatant from one plate is transferred to a second plate for the ELISA) using PBMCs, MM6 cells, THP-1 cells, or WB. The authors report that this single plate assay using IL-6 release as the endpoint can be completed in 5 hr, and that this time could be reduced to 3 hr using TNFα as the endpoint (because it is released earlier from the cells). The authors also suggest that this single plate test method is readily adaptable to high throughput assays.

9.2.9 Schindler et al. (2004)

The authors optimized conditions for use of cryopreserved human WB in pyrogen testing to obviate the need for fresh WB. The release of IL-1β from fresh and cryo WB collected from five donors was used as the measure of endotoxin presence. Challenge with 0.5 or 1.0 EU/mL endotoxin resulted in IL-1β release in bloods from all donors, although kinetic studies suggested that IL-1β release was delayed one hour in the cryopreserved samples. Cryopreservation did not appear to alter the spectrum of detectable pyrogens or immune stimuli when results were compared to that of fresh WB, and no cytokine release was measured in materials that fresh WB did not respond to. Seven clinical-grade (i.e., endotoxin-free) parenteral products spiked with 0.5 EU/mL of endotoxin revealed that there was less interference in cryo WB than in fresh WB based on lower minimal interference dilutions that were always at or below the maximal valid dilution (MVD) for each product. The data showed that a broad variety of drugs could be tested for pyrogenic contaminants using cryo

WB while maintaining the endotoxin limit concentration (ELC) established in the various Pharmacopoeias.

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10.0 ANIMAL WELFARE CONSIDERATIONS (REFINEMENT, REDUCTION, AND REPLACEMENT)

10.1 How the Five *In Vitro* Test Methods Will Refine, Reduce, or Replace Animal Use

ICCVAM promotes the scientific validation and regulatory acceptance of new methods that refine, reduce, or replace animal use where scientifically feasible. Refinement, Reduction, and Replacement are known as the three "Rs" of animal protection. These principles of humane treatment of laboratory animals are described as:

- refining experimental procedures such that animal suffering is minimized;
- reducing animal use through improved science and experimental design; and
- replacing animal models with non-animal procedures (e.g., *in vitro* technologies), where possible (Russell and Burch 1959)

The *in vitro* pyrogenicity test methods address each of these animal welfare considerations. The assays use monocytoid cells of human origin, obtained either from whole blood donations or from an immortalized cell line. The currently accepted pyrogenicity test methods require the use of either rabbits (RPT) or horseshoe crab haemolymph (BET). Because isolated cells are treated in these human cell assays, treatment-related pain and suffering are avoided in live animals. The capability of these five *in vitro* assays to detect Gram-negative endotoxin, suggests that they may reduce or replace the use of rabbits and/or horseshoe crabs for pyrogen testing. However, the RPT will detect classes of pyrogens that have not been examined/validated in the human cell tests, and therefore may still need to be used in certain circumstances.

10.2 Requirement for the Use of Animals

10.2.1 <u>Rationale for the Use of Animals</u>

Non-human animal species are not used for these *in vitro* tests. As indicated above, the *in vitro* pyrogenicity methods use monocytoid cells of human origin obtained from either an immortalized cell line (MM6/IL-6) or whole blood donations (WB/IL-1, WB/IL-6, cryo WB/IL-1, and PBMC/IL-6) within a short time before the test is to be performed. Therefore, human volunteers are required for four of the five methods. Standard phlebotomy techniques are used to obtain the blood samples. The only pain and distress experienced by the donor would be associated with the collection procedure (i.e., needle stick), which is commonplace in medical procedures. The use of an experienced phlebotomist perform the collection should minimize pain and distress. As indicated in **Section 2.2.2**, blood samples from up to five donors are required for a single assay, depending on the method under consideration.

11.0 PRACTICAL CONSIDERATIONS

Several issues are taken into account when assessing the practicality of using an *in vitro* test method in place of an *in vivo* test method. In addition to reliability and accuracy evaluations, assessments of the laboratory equipment and supplies needed to carry-out the *in vitro* test method, level of personnel training, labor costs, and the time required to complete the test method relative to the *in vivo* test method are necessary. The time, personnel cost, and effort required to conduct the proposed test method(s) must be considered to be reasonable when compared to the *in vivo* test method it is intended to replace.

11.1 Transferability of the *In Vitro* Pyrogenicity Test Methods

Test method transferability addresses the ability of a method to be accurately and reliably performed by multiple laboratories (ICCVAM 2003), including those experienced in the particular type of procedure, and otherwise competent laboratories with less or no experience in the particular procedure. The degree of transferability of a test method can be evaluated by its interlaboratory reproducibility. ECVAM measured the transferability (i.e., interlaboratory reproducibility) of each assay among experienced laboratories. The results obtained, and presented in **Tables 7-3** and **7-4**, provide an estimate of the minimum variability to be expected; interlaboratory variability is anticipated to be greater (i.e., lower transferability) among laboratories having less experience with the assays.

11.1.1 Facilities and Major Fixed Equipment

A standard laboratory facility for sterile tissue culture is necessary for performing the *in vitro* pyrogenicity methods described here. The major equipment necessary to conduct the tests are readily available and include, a laminar flow hood, tissue culture incubator, water bath, and spectrophotometric microplate reader.

In contrast, the RPT requires a facility that meets applicable State and Federal regulations for the care and housing of laboratory animals. The primary expense for equipping a facility to conduct the RPT would be the acquisition of an adequate animal room and associated housing (e.g., cages, bedding, food, water, etc.) for boarding animals during the study, and specifically trained animal care support personnel.

11.1.2 General Availability of Other Necessary Equipment and Supplies.

The equipment and supplies necessary to conduct the *in vitro* pyrogenicity test methods (e.g., micropipetters, sterile tissue culture vessels, disposable plastic ware, assay reagents) are readily available in most scientific laboratories, or can be obtained from any of several scientific laboratory equipment vendors.

The RPT requires fewer general laboratory supplies. Those that are needed are readily available in most toxicity testing laboratories, or could be readily obtained from any of a number of scientific laboratory equipment vendors.

11.2 Personnel Training Considerations

Training considerations are defined as the level of instruction needed for personnel to conduct the test method accurately and reliably (ICCVAM 2003). Evaluation of the levels of training and expertise needed to conduct the test method, as well as the training requirements needed to insure that personnel are competent in the test procedures, are discussed below.

11.2.1 Required Training and Expertise Needed to Conduct the *In Vitro* Pyrogenicity Test Methods

Laboratory personnel require training in the relevant enzyme immunoassay protocols and the aseptic techniques associated with mammalian tissue culture. The quality criteria associated with each *in vitro* test method may be used to ensure that personnel are competent in the performance of the various procedures. When a technician has mastered all aspects of the protocol, and can independently conduct the assay such that the quality criteria have been met, the individual is considered to have demonstrated proficiency in the assay.

The RPT requires training in the care and handling of laboratory animals, and the collection of accurate rectal temperature measurements at the appropriate time intervals from each rabbit. The laboratory personnel must be adequately trained to maintain the animals, and to accurately and consistently record the proper body temperature. It is not known what, if any, proficiency requirements are in place for the RPT.

11.3 Cost Considerations

In addition to the major fixed equipment, there are three additional factors that contribute to the cost of the proposed *in vitro* methods: 1) cost of the monocytoid cell line (i.e., Mono Mac 6); 2) cost of the reagents for the ELISA procedure; and 3) labor costs for laboratory personnel.

Because the proposed *in vitro* test methods are relatively more labor-intensive than the RPT, it is estimated that the cost of any these *in vitro* methods would be more than that of the BET or the RPT. However, because these test methods are amenable to high throughput screening in a properly equipped laboratory, these increased costs could be considerably reduced.

Another consideration is the need for a laboratory animal veterinarian, the housing and care of the rabbits before, during, and after the test, and the cost of replacement rabbits, where necessary. These costs could offset the one-time costs of the tissue culture equipment and microplate reader.

11.4 Time Considerations

The *in vitro* pyrogenicity methods require two working days for completion. On the first day, blood is drawn and the test materials are prepared and incubated with the monocytoid cells. On the second day, the cytokine release from the cells is determined by immunoassay. The BET and RPT can both be completed within one working day. However, depending on the specific protocol employed, the RPT could require additional testing in up to 12 animals, which would extend the time to completion.

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12.0 REFERENCES

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13.0 GLOSSARY[13]

Amebocytes: The blood cells of the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*) that contain the active components of the reagent used in the bacterial endotoxin test (i.e., amebocyte lysate).

Assay[2]: The experimental system used. Often used interchangeably with "test" and "test method."

Bacterial Endotoxin Test (BET)[3]: A test used to quantify endotoxins of Gram-negative bacterial origin using amebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are two types of techniques for this test: the gel-clot techniques, which are based on gel formation, and the photometric techniques. The photometric techniques include the turbidimetric technique, which is based on the development of turbidity after cleavage of an endogenous substrate, and a chromogenic method, which is based on the development of color after cleavage of a synthetic peptide-chromogen complex.

Coded substances: Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Coefficient of variation: A statistical representation of the precision of a test. It is expressed as a percentage and is calculated as follows:

$$\left(\frac{standard\ deviation}{mean}\right) \times 100\%$$

[13] The definitions in this Glossary are restricted to their uses with respect to the Rabbit Pyrogen Test, the *in vitro* pyrogenicity test methods included in this BRD, and the Limulus Amebocyte Lysate (LAL) assay.
[2] From ICCVAM (2003)
[3] From USP (2005)

Concordance[2]: The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of "relevance." The term is often used interchangeably with "accuracy" (see also "two-by-two" table). Concordance is highly dependent on the prevalence of positives in the population being examined.

Endogenous pyrogens: Various cytokines including interleukins (e.g., IL-1α, IL-1β), tumor necrosis factor (i.e., TNF-α, TNF-β), and interferon (IFN-γ) released from leukocytes in response to external stimuli (e.g., endotoxin) capable of causing an increase in body temperature above the normal level.

Endotoxin Limit Concentration (ELC): The concentration at which endotoxin is considered to be pyrogenic. It is expressed as the ratio of the threshold pyrogen dose (K) and the rabbit pyrogen test dose or the maximum human dose administered on a weight (kg) basis in 1 hr (M) defined as K/M. The ELC varies based on M.

- The FDA ELC for non-intrathecal medical devices is 0.5 EU/mL
- The FDA ELC for intrathecal medical devices is 0.06 EU/mL

Endpoint[2]: The biological or chemical process, response, or effect assessed by a test method.

False negative[2]: A substance incorrectly identified as negative by a test method.

False negative rate[2]: The proportion of all positive substances falsely identified by a test method as negative (see "two-by-two" table). It is one indicator of test method accuracy.

False positive[2]: A substance incorrectly identified as positive by a test method.

False positive rate[2]: The proportion of all negative substances that are falsely identified by a test method as positive (see "two-by-two" table). It is one indicator of test method accuracy.

Fever: Elevation of body temperature above the normal level.

Good Laboratory Practices (GLP)[2]: Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organization for Economic Cooperation and Development and

Japanese authorities that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

Hazard[2]: The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Interlaboratory reproducibility[2]: A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes and indicates the extent to which a test method can be transferred successfully among laboratories.

Intralaboratory repeatability[2]: The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

Intralaboratory reproducibility[2]: The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

In Vitro: In glass. Refers to assays that are carried out in an artificial system (e.g., in a test tube or Petri-dish) and typically use single-cell organisms, cultured cells, cell-free extracts, or purified cellular components.

In Vivo: In the living organism. Refers to assays performed in multi-cellular organisms.

Lipopolysaccharide (LPS): A complex of lipid and carbohydrate (endotoxin) released from the cell walls of Gram-negative organisms that is pyrogenic and capable of producing septic shock.

Lipoteichoic acid (LTA): A polyol phosphate polymer bearing a strong negative charge that is covalently linked to the peptidoglycan in Gram-positive bacteria. It is strongly antigenic, but is generally absent in Gram-negative bacteria and therefore is considered to be the primary pyrogenic component of Gram-positive bacteria.

Minimum Valid Concentration (MVC): The concentration of a product when it is diluted to the maximum valid dilution (MVD) expressed as λM/K, where:

- **λ =** The sensitivity of the LAL reagent used expressed as EU/mL. The value varies with the method used. For the gel-clot method it is the labeled LAL sensitivity (EU/mL). For the chromogenic, turbidometric, or kinetic-turbidometric LAL test methods it is the lowest point used in the standard curve.
- **M =** The maximum human dose for pyrogenicity administered on a weight basis (kg) in 1 hr, or the rabbit pyrogen test dose (whichever is larger). It is one of the variables used to define the Endotoxin Limit Concentration (ELC) defined as the ratio of K/M, where K is the threshold pyrogen dose in rabbits or humans.
- **K** = See **threshold pyrogen dose**.

Maximum Valid Dilution (MVD): When a USP Endotoxin Limit Concentration (ELC) is defined, the MVD is the ratio of the product of the ELC and the product potency to the LAL reagent sensitivity (λ) expressed as ([ELC x Product Potency]/λ). If there is no official USP ELC defined, then the MVD is the ratio of the Product Potency/Minimum Valid Concentration (MVC).

Monocytoid cells: Cells obtained from peripheral blood or grown in culture that phenotypically resemble monocytes or macrophages.

Negative control: An untreated sample containing all components of a test system, except the test substance solvent, which is replaced with a known non-reactive material, such as water. This sample is processed with test substance-treated samples and other control samples to determine whether the solvent interacts with the test system.

Negative predictivity[2]: The proportion of correct negative responses among substances testing negative by a test method (see "two-by-two" table). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

Parenteral: Introduction into the body by some means other than through the gastrointestinal tract; referring particularly to intravenous (i.v.), intramuscular (i.m.), subcutaneous (s.c.), or intrathecal (i.t.) injection.

Performance[2]: The accuracy and reliability characteristics of a test method (see "accuracy, reliability").

pH: A measure of the acidity or alkalinity of a solution. A pH of 7.0 is neutral; higher pHs are alkaline, lower pHs are acidic.

Positive control: A sample containing all components of a test system and treated with a substance known to induce a positive response, which is processed with the test substance-treated and other control samples to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay over time.

Positive predictivity[2]: The proportion of correct positive responses among substances testing positive by a test method (see "two-by-two" table). It is one indicator of test method accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

Prevalence[2]: The proportion of positives in the population of substances tested (see "two-by-two" table).

Protocol[2]: The precise, step-by-step description of a test method, including a listing of all necessary reagents, criteria and procedures for evaluation of the test data.

Pyrogen: A substance that causes a rise in body temperature above normal or that produces a fever. Gram-negative, Gram-positive, and acid-fast bacteria, molds, viruses, and yeast and some of their cellular constituents are pyrogenic.

Quality assurance[2]: A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures is assessed independently by individuals other than those performing the testing.

Rabbit Pyrogen Test (RPT)[3]: A test designed to limit to an acceptable level the risks of febrile reaction in the patient to the administration, by injection, or the product concerned.

The test involves measuring the rise in temperature of rabbits following the intravenous injection of a test solution.

Reduction alternative[2]: A new or modified test method that reduces the number of animals required.

Reference test method[2]: The accepted *in vivo* test method used for regulatory purposes to evaluate the potential of a test substance to be hazardous to the species of interest.

Refinement alternative[2]: A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

Relevance[2]: The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the "accuracy" or "concordance" of a test method.

Reliability[2]: A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reproducibility and intralaboratory repeatability.

Replacement alternative[2]: A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility[2]: The consistency of individual test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol and test substances (see intra- and interlaboratory reproducibility).

Sensitivity[2]: The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy (see "two-by-two" table).

Specificity[2]: The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy (see "two-by-two" table).

Test[2]: The experimental system used; used interchangeably with "test method" and "assay."

Test method[2]: A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with "test" and "assay." See also "validated test method" and "reference test."

Test method component: Structural, functional, and procedural elements of a test method that are used to develop the test method protocol. These components include unique characteristics of the test method, critical procedural details, and quality control measures.

Threshold pyrogen dose: The dose level at which a product is considered to be pyrogenic or non-pyrogenic. It is one of the variables (K) used to calculate the Endotoxin Limit Concentration (ELC) defined as K/M, where M is the rabbit pyrogen test dose or the maximum human dose administered in 1 hr (whichever is larger).

- The threshold pyrogen dose for non-intrathecal use in rabbits and humans is 5.0 EU/kg
- The threshold pyrogen dose for intrathecal use in rabbits and humans is 0.2 EU/kg

Tiered testing: A testing strategy where all existing information on a test substance is reviewed, in a specified order, prior to *in vivo* testing. If the irritancy potential of a test substance can be assigned, based on the existing information, no additional testing is required. If the irritancy potential of a test substance cannot be assigned, based on the existing information, a step-wise animal testing procedure is performed until an unequivocal classification can be made.

Transferability[2]: The ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories.

Two-by-two table[2]: The two-by-two table can be used for calculating accuracy (concordance) ([a+d]/[a+b+c+d]), negative predictivity (d/[c+d]), positive predictivity (a/[a+b]), prevalence ([a+c]/[a+b+c+d]), sensitivity (a/[a+c]), specificity (d/[b+d]), false positive rate (b/[b+d]), and false negative rate (c/[a+c]).

		NEW TEST OUTCOME		
		Positive	**Negative**	**Total**
Reference Test Outcome	**Positive**	a	c	a + c
	Negative	b	d	b + d
	Total	a + b	c + d	a + b + c + d

Validated test method[2]: An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Validation[2]: The process by which the reliability and relevance of a procedure are established for a specific purpose.

Weight of evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

Appendix A

Individual BRDs Submitted by ECVAM on Five *In Vitro* Pyrogenicity Test Methods

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Appendix B

ECVAM Response to ICCVAM Questions

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Appendix C

Supplemental Information

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Appendix C1

Key References

List of key references (see **Section 12.0** for a complete list of references):

Bleeker WK, de Groot EM, den Boer PJ, BiesselsPTM, Asrden LA, Bakker JC. 1994. Measurement of interleukin-6 production by monocytes for *in vitro* safety testing of hemoglobin solutions. Art Cells Blood Subs Immob Biotech 22(3):835-840.

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APPENDIX C2

Guidelines for Pyrogenicity Testing

List of included guidelines:

United States Pharmacopeia
- Bacterial Endotoxins Test
- Pyrogen Test
- Biologics

European Pharmacopoeia
- Pyrogens
- Bacterial Endotoxins
- Parenteral Preparations

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