ICCVAM Test Method Evaluation Report:

Validation Status of Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

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

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U.S. Public Health Service
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May 2008
NIH Publication No. 08-6392

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This document is available electronically at:
http://iccvam.niehs.nih.gov/methods/pyrogen/pyrogen.htm

Table of Contents

Page Number

List of Tables ..... **iv**
List of Abbreviations and Acronyms ..... **v**
Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives ..... **vii**
Acknowledgements ..... **viii**
Preface ..... **xi**
Executive Summary ..... **xv**
1.0 Introduction **1**
2.0 ICCVAM Recommendations for *In Vitro* Pyrogen Test Methods **3**
2.1 ICCVAM Recommendations: Test Method Uses and Limitations 3
2.2 ICCVAM Recommendations: Test Method Protocols 4
2.3 ICCVAM Recommendations: Future Studies 6
2.4 ICCVAM Recommendations: Performance Standards 7
3.0 Validation Status of *In Vitro* Pyrogen Test Methods **9**
3.1 Test Method Description 9
3.2 Validation Database 10
3.3 Reference Test Method Data 11
3.4 Test Method Accuracy 11
3.5 Test Method Reliability 12
3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement 18
4.0 ICCVAM Consideration of Public and SACATM Comments **19**
4.1 Public Comments in Response to *FR* Notice (70FR74833, December 16, 2005): Peer Panel Evaluation of *In Vitro* Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of *In Vivo* and *In Vitro* Data 19
4.2 Public Comments in Response to *FR* Notice (71FR74533, December 12, 2006): Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments 19
4.3 Public Comments in Response to *FR* Notice (72FR26395, May 9, 2007): Peer Review Panel Report on Five *In Vitro* Pyrogen Test Methods: Availability and Request for Public Comments 20
4.4 Public and SACATM Comments: SACATM Meeting on June 12, 2007 21
5.0 References **23**

Appendix A Independent Scientific Peer Review Panel Assessment **A-1**
A1 Peer Review Panel Report: The Use of Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products A-3
A2 Summary Minutes from Peer Review Panel Meeting on February 6, 2007 ..... A-53
A3 Pyrogenicity Peer Review Panel Biographies A-65

Appendix B Relevant Federal Pyrogenicity Regulations and Testing Guidelines..... B-1
B1 U.S. Code of Federal Regulations (CFR)..... B-3
B1-1 21 CFR 211.167 - Current Good Manufacturing Practice for Finished Pharmaceuticals: Special Testing Requirements (April 1, 2007)..... B-5
B1-2 21 CFR 314.50(d)(1)(ii)(a) - Applications: Content and Format of an Application (April 1, 2007)..... B-6
B1-3 21 CFR 610.9 - General Provisions: Equivalent Methods and Processes (April 1, 2007)..... B-17
B1-4 21 CFR 610.13 - General Biological Products Standards: Purity (April 1, 2007)..... B-19
B2 International Organization for Standardization - ISO 10993-11 - Biological Evaluation of Medical Devices - Part 11: Tests for Systemic Toxicity (First Edition 1993-12-15)..... B-21
B3 U.S. Pharmacopeia (USP) 30-NF25..... B-23
B3-1 (85) - Bacterial Endotoxins Test..... B-23
B3-2 (151) - Pyrogen Test..... B-23
B3-3 (1041) - Biologics..... B-23
B4 European Pharmacopoeia 5.0..... B-25
B4-1 2.6.8. Pyrogens..... B-25
B4-2 2.6.14. Bacterial Endotoxins..... B-25
B5 FDA Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices (December 1987)..... B-27

Appendix C ICCVAM Recommended Test Method Protocols..... C-1
C1 The Human Whole Blood (WB)/Interleukin (IL)-1β *In Vitro* Pyrogen Test..... C-3
C2 The Human WB/IL-1β *In Vitro* Pyrogen Test: Application of Cryopreserved (Cryo) Human WB..... C-25
C3 The Human WB/IL-6 *In Vitro* Pyrogen Test..... C-47
C4 The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test..... C-71
C5 The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 *In Vitro* Pyrogen Test..... C-95

Appendix D *Federal Register* Notices, Public Comments, and Relevant SACATM Meeting Minutes..... D-1
D1 *Federal Register* Notices..... D-3
D1-1 Vol. 70, No. 241, pp. 74833-74834, December 16, 2005: Peer Panel Evaluation of *In Vitro* Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of *In Vivo* and *In Vitro* Data..... D-5

D1-2 Vol. 71, No. 238, pp. 74533-74534, December 12, 2006: Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments D-7
D1-3 Vol. 72, No. 89, pp. 26395-26396, May 9, 2007: Peer Review Panel Report on Five *In Vitro* Pyrogen Test Methods: Availability and Request for Public Comments D-9
D2 Public Comments Received in Response to *Federal Register* Notices D-11
D3 SACATM Comments: ICCVAM Evaluation of *In Vitro* Pyrogen Test Methods D-43

Appendix E ESAC Statement on the Validity of *In Vitro* Pyrogen Tests E-1

List of Tables

Page Number

Table 1* Accuracy of *In Vitro* Pyrogen Test Methods..... xvii
Table 2-1 Summary of ICCVAM Recommended *In Vitro* Pyrogen Test Method Protocols..... 5
Table 3-1 Parenteral Drugs Used in the Validation Studies for Determining Test Method Accuracy..... 10
Table 3-2 Parenteral Drugs Used in the Validation Studies for Determining Test Method Reproducibility..... 11
Table 3-3* Accuracy of *In Vitro* Pyrogen Test Methods..... 12
Table 3-4 Intralaboratory Reproducibility of *In Vitro* Pyrogen Test Methods..... 15
Table 3-5 Interlaboratory Reproducibility of *In Vitro* Pyrogen Test Methods: Study One..... 17
Table 3-6 Interlaboratory Reproducibility of *In Vitro* Pyrogen Test Methods: Study Two..... 18

*Tables 1 and 3-3 are identical. Table 1 provides supporting information for the Executive Summary, while Table 3-3 provides the information in Section 3.2, where test method accuracy is discussed in greater detail.

List of Abbreviations and Acronyms

APHIS	Animal and Plant Health Inspection Service (USDA)
BET	Bacterial Endotoxin Test
BRD	Background Review Document
CBER	Center for Biologics Evaluation and Research (FDA)
CFR	Code of Federal Regulations
Cryo	Cryopreserved
CV	Coefficient of variation
EC	Endotoxin control
ECVAM	European Centre for the Validation of Alternative Methods
ELC	Endotoxin limit concentration
ELISA	Enzyme-linked immunosorbent assay
EP	European Pharmacopeia
ESAC	ECVAM Scientific Advisory Committee
EU	European Union
EU/mL	Endotoxin Units per milliliter
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IL	Interleukin
ILS	Integrated Laboratory Systems
ISO	International Organization for Standardization
LAL	Limulus Amebocyte Lysate test method
LLNA	Murine local lymph node assay
LPS	Lipopolysaccharide
MM6	Mono Mac 6
MVD	Maximum valid dilution
MRC	Medical Research Council (U.K.)
NA	Not applicable
NC	Not calculated
ND	Not done
NI	Not included
NICEATM	National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NPC	Negative product control
NSC	Negative saline control
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PEI	Paul Ehrlich Institut
PPC	Positive product control

PWG	Pyrogenicity Working Group
RPT	Rabbit pyrogen test
RSE	Reference standard endotoxin
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SC	Standard curve
SOP	Standard operating procedure
TS	Test substance
TLR	Toll-like receptors
U.S.	United States
U.K.	United Kingdom
USP	U.S. Pharmacopeia
WB	Whole blood
WHO	World Health Organization

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Acknowledgements

The following individuals are acknowledged for their contributions to the in vitro pyrogen test method review process.

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Preface

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is charged by the ICCVAM Authorization Act of 2000 with evaluating the scientific validity of new, revised, and alternative toxicological test methods applicable to U.S. Federal agency safety testing requirements (ICCVAM 2000). ICCVAM is required to provide recommendations to U.S. Federal agencies regarding the usefulness and limitations of test methods based on this scientific evaluation. This Test Method Evaluation Report provides ICCVAM recommendations for five *in vitro* test methods proposed for assessing the potential pyrogenicity of pharmaceuticals and other products. These recommendations are based on a comprehensive evaluation of the current validation status of these test methods.

In March 2005, the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre, submitted background review documents (BRDs) to ICCVAM for five *in vitro* test methods, which were proposed as replacements for the rabbit pyrogen test. The information in the BRDs was based on validation studies financed by the European Commission within the 5th Framework Programme of Directorate General Research, the results of which were recently published (Hoffmann et al. 2005a; Schindler et al. 2006). The five test methods are:

- The Human Whole Blood (WB)/Interleukin (IL)-1β *In Vitro* Pyrogen Test
- The Human WB/IL-1β *In Vitro* Pyrogen Test: Application of Cryopreserved Human WB
- The Human WB/IL-6 *In Vitro* Pyrogen Test
- The Human Peripheral Blood Mononuclear Cell/IL-6 *In Vitro* Pyrogen Test
- The Monocytoid Cell Line Mono Mac 6/IL-6 *In Vitro* Pyrogen Test

In June 2005, ICCVAM initiated evaluation of the validation status of these five test methods. An ICCVAM Pyrogenicity Working Group (PWG) was established to work with the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to carry out this review. Dr. Marlies Halder was designated by ECVAM as their liaison to the PWG. Following a NICEATM pre-screen evaluation of the ECVAM BRDs, NICEATM, ICCVAM and the ICCVAM PWG requested additional information and clarification from ECVAM on a number of issues. In March 2006, ECVAM provided revised BRDs and responses addressing these issues.

NICEATM, in conjunction with the PWG, prepared a comprehensive BRD to combine the available data and information for each of the five *in vitro* test methods into one document. The ICCVAM BRD describes the current validation status of these test methods, including what is known about their reliability and accuracy, the scope of the substances tested, and the availability of standardized protocols for each test method. The ICCVAM BRD was based on the ECVAM BRDs, but also includes other relevant data and analyses, including data and information submitted to NICEATM in response to a *Federal Register* (*FR*) Notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005). The ICCVAM draft BRD was made available to the public on December 12, 2006 (announced in *FR* Vol. 71, No. 238, pp. 74533-

74534, December 12, 2006) for comment and a public peer review panel meeting on February 6, 2007 was announced.

The independent scientific peer review panel (Panel) met in public session on February 6, 2007 at the National Institutes of Health in Bethesda, Maryland. The Panel first reviewed the ICCVAM draft BRD for errors and omissions and then discussed the current validation status of the five *in vitro* test methods. The Panel also reviewed the extent that the information in the ICCVAM BRD supported the ICCVAM draft test method recommendations for proposed test method uses, standardized protocols, test method performance standards, and future studies. Throughout the review process, interested stakeholders from the public were provided opportunities to provide comments including oral comments at the Panel meeting. The Panel considered these comments as well as public comments submitted in advance of the meeting before concluding their deliberations. The final independent Panel report was made available to the public (http://iccvam.niehs.nih.gov/docs/pyrogen/PrRevPanFinRpt.pdf) for review and comment on May 9, 2007 (announced in *FR* Vol. 72, No. 89, pp. 26395-26396).

The ICCVAM draft BRD and draft recommendations, the Panel report, and all public comments were made available to ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and comments were provided at their meeting on June 12, 2007.

ICCVAM and the PWG considered the Panel report, all public comments, and the comments of SACATM in preparing the final ICCVAM test method recommendations provided in this report. This report will be made available to the public and provided to U.S. Federal agencies for consideration, in accordance with the ICCVAM Authorization Action of 2000 (ICCVAM 2000). Agencies must respond to ICCVAM within 180 days after receiving an ICCVAM test method recommendation. These responses will be made available to the public on the NICEATM/ICCVAM website (http://iccvam.niehs.nih.gov) as they are received.

The efforts of the many individuals who contributed to the preparation, review and revision of this report are gratefully acknowledged. We greatly appreciate the careful preparation of the BRDs by ECVAM and their prompt response to requests for additional information. We especially recognize all of the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Karen Brown for serving as the Panel Chair and to Drs. Jack Levin, Melvyn Lynn, Anthony Mire-Sluis, and Jon Richmond for their service as Evaluation Group Chairs. The efforts of the PWG were invaluable for assuring a meaningful and comprehensive review. We especially thank the Chair of the PWG, Dr. Richard McFarland (FDA, Center for Biologics Evaluation and Research) for his effective leadership. The efforts of the NICEATM staff and support contractor in preparing the BRD, organizing the Panel meeting, and preparing this final report are greatly appreciated. We acknowledge Drs. David Allen and Elizabeth Lipscomb, Catherine Sprankle, James Truax, and Doug Winters of Integrated Laboratory Systems, Inc., the NICEATM support contractor, for their assistance. We also thank Dr. Raymond Tice, Deputy Director of NICEATM, for his efforts on this project.

This comprehensive ICCVAM evaluation of the validation status of these five test methods and the accompanying recommendations should aid agencies in providing guidance on their future use for regulatory safety testing. The ICCVAM recommendations for future studies are expected to advance broader applicability of these methods, which may further reduce animal use while ensuring continued or better protection of human health.

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Executive Summary

This Test Method Evaluation Report, prepared by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), summarizes ICCVAM's evaluation of the validation status of five *in vitro* test methods proposed for assessing the potential pyrogenicity of pharmaceuticals and other products, as potential replacements for the *in vivo* rabbit pyrogen test (RPT). The five test methods are:

- The Human Whole Blood (WB)/Interleukin (IL)-1β *In Vitro* Pyrogen Test
- The Human WB/IL-1β *In Vitro* Pyrogen Test: Application of Cryopreserved (Cryo) Human WB
- The Human WB/IL-6 *In Vitro* Pyrogen Test
- The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test
- The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 *In Vitro* Pyrogen Test

This report also provides ICCVAM's recommendations for current uses and limitations for each test method, as well as recommendations for standardized protocols, future studies, and performance standards. In support of this evaluation, ICCVAM prepared a draft Background Review Document (BRD) and ICCVAM draft test method recommendations, which were provided to an independent scientific peer review panel (Panel) and the public for consideration and comment. The ICCVAM draft BRD was prepared using data from validation studies that had been conducted by the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre. The ECVAM submission, prepared according to the ICCVAM submission guidelines (ICCVAM 2003), included five individual BRDs (i.e., one BRD for each test method), which summarized the validation studies for each of the five *in vitro* test methods.

The Panel met on February 6, 2007 to review the ICCVAM draft BRD for errors and omissions and to discuss the current validation status of the five *in vitro* test methods. The Panel also reviewed the extent that the information contained in the ICCVAM draft BRD supported the ICCVAM draft test method recommendations. In finalizing the test method recommendations presented here, ICCVAM considered the conclusions and recommendations of the Panel as well as comments from the public and its Scientific Advisory Committee on Alternative Toxicological Methods.

ICCVAM Recommendations: Test Method Uses and Limitations

Based on this evaluation, ICCVAM recommends that, although none of these test methods can be considered a complete replacement for the RPT for all testing situations for the detection of Gram-negative endotoxin, they can be considered for use to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis, subject to validation for each specific product to demonstrate equivalence to the RPT, in accordance

with applicable U.S. Federal regulations (e.g., U.S. Food and Drug Administration [FDA] [*])[†]. When used in this manner, these methods should be able to reduce the number of animals needed for pyrogenicity testing. Pyrogenicity testing may involve more than slight or momentary pain or distress when a pyrogenic response occurs. Accordingly, alternative test methods must be considered prior to the use of animals for such testing, as required by U.S. Federal animal welfare regulations and policies. Therefore, these and other *in vitro* alternative test methods should be considered prior to the use of animals in pyrogenicity testing and should be used where determined appropriate for a specific testing situation. Use of these methods, once appropriately validated, will support improved animal welfare while ensuring the continued protection of human health.

ICCVAM developed a recommended standardized protocol for each test method based primarily on ECVAM standard operating procedures (SOPs). ICCVAM also provided recommendations for further research and development, optimization, and validation efforts. These recommendations should be helpful to various stakeholders (e.g., applicable U.S. Federal regulatory agencies, the international regulatory community, the pharmaceutical industry) for determining when these test methods might be useful.

The Panel concluded that the validation criteria were adequately addressed in the ICCVAM BRD to determine the usefulness and limitations of these test methods to serve as a substitute for the RPT to identify Gram-negative endotoxin on a case-by-case basis, subject to validation for that specific product. However, the Panel stated the performance of these test methods in terms of their reliability and relevance did not support this proposed use.

In March 2006, the ECVAM Scientific Advisory Committee (ESAC) endorsed a statement of validity for these five *in vitro* pyrogen test methods (see **Appendix E**). Like ICCVAM, ESAC concluded that these five methods can detect Gram-negative endotoxin in materials currently tested with the RPT, and, therefore, may be useful for regulatory decisions, subject to validation for that specific product. Both ICCVAM and ESAC also concluded that the currently available database does not support the use of these test methods to detect a wider range of pyrogens, as suggested in the original ECVAM submission. However, ESAC concluded that these tests "can currently be considered as full replacements for the evaluation of materials or products where the objective is to identify and evaluate pyrogenicity produced by Gram-negative endotoxins, but not for other pyrogens." ICCVAM has concluded that the current validation database for these test methods is inadequate to support such a definitive statement based on the ECVAM validation study design, which did not include biologics or medical devices and evaluated only a limited range and number of pharmaceutical products. Additionally, no RPT data were generated with the same test samples used in the *in vitro* test methods (i.e., parallel testing).

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[*]Mechanisms exist for test method developers to qualify their method on a case-by-case basis. The use of any recommended method will be subject to product-specific validation to demonstrate equivalence as recommended by the FDA (e.g., U.S. Code of Federal Regulations (CFR) 21 CFR 610.9 and 21 CFR 314.50(d)(1)(ii)(a)).

[†]Substances other than endotoxin may induce the cellular release of IL-1β and/or IL-6. For this reason, users of these test methods should be aware that the presence of other materials might erroneously suggest the presence of endotoxin and lead to a false positive result.

Accuracy and Reliability

The accuracy of *in vitro* pyrogen test methods for detecting Gram-negative endotoxin was based on the results for 10 parenteral pharmaceuticals, each spiked with four concentrations of endotoxin (0, 0.25, 0.5, or 1.0 Endotoxin Units [EU]/mL, with 0.5 EU/mL tested in duplicate). As shown in **Table 1**, accuracy among the test methods ranged from 81% to 93%, sensitivity ranged from 73% to 99%, specificity ranged from 77% to 97%, false negative rates ranged from 1% to 27%, and false positive rates ranged from 3% to 23%.

Table 1 Accuracy of *In Vitro* Pyrogen Test Methods[1]

Test Method	Accuracy[2]	Sensitivity[3]	Specificity[4]	False Negative Rate[5]	False Positive Rate[6]
Cryo WB/IL-1β	92% (110/120)	97% (75/77)	81% (35/43)	3% (2/77)	19% (8/43)
MM6/IL-6	93% (138/148)	96% (85/89)	90% (53/59)	5% (4/89)	10% (6/59)
PBMC/IL-6	93% (140/150)	92% (83/90)	95% (57/60)	8% (7/90)	5% (3/60)
PBMC/IL-6 (Cryo)[7]	87% (130/150)	93% (84/90)	77% (46/60)	7% (6/90)	23% (14/60)
WB/IL-6	92% (136/148)	89% (79/89)	97% (57/59)	11% (10/89)	3% (2/59)
WB/IL-1β (Tube)	81% (119/147)	73% (64/88)	93% (55/59)	27% (24/88)	7% (4/59)
WB/IL-1β (96-well plate)[8]	93% (129/139)	99% (83/84)	84% (46/55)	1% (1/84)	16% (9/55)

Abbreviations: Cryo = Cryopreserved; EU/mL = Endotoxin units per milliliter; IL = Interleukin; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

[1]Data shown as a percentage (number of correct runs/total number of runs), 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, or 1.0 EU/mL, with 0.5 EU/mL tested in duplicate).

[2]Accuracy = the proportion of correct outcomes (positive and negative) of a test method.

[3]Sensitivity = the proportion of all positive substances that are classified as positive.

[4]Specificity = the proportion of all negative substances that are classified as negative.

[5]False negative rate = the proportion of all positive substances that are falsely identified as negative.

[6]False positive rate = the proportion of all negative substances that are falsely identified as positive.

[7]A modification of the PBMC/IL-6 test method that uses Cryo PBMCs.

[8]A modification of the WB/IL-1β test method that uses 96-well plates instead of tubes for the test substance incubation.

Repeatability within individual laboratories was determined for each *in vitro* test method, using saline and various endotoxin spikes to evaluate the closeness of agreement among optical density (OD) readings for cytokine measurements at each concentration. The results indicated that the variability in OD measurements increased with increasing endotoxin concentration. However, the variability was low enough that the threshold for pyrogenicity could still be detected (i.e., the 0.5 EU/mL spike concentration could still be distinguished from the lower concentrations).

Reproducibility within individual laboratories was evaluated using three marketed pharmaceuticals spiked with various concentrations of endotoxin. Three identical, independent runs were conducted in each of the three testing laboratories, with the exception

of the Cryo WB/IL-1β test method[‡]. The correlations (expressed as percentage of agreement) between pairs of the independent runs (i.e., run 1 vs. run 2; run 1 vs. run 3; run 2 vs. run 3) were determined, and the mean of these three values was calculated. Agreement across three runs within a single laboratory ranged from 75% to 100%.

Reproducibility across all laboratories 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 reproducibility study, three marketed pharmaceutical products were spiked with either saline control or various concentrations of endotoxin, and each sample was tested in triplicate in each of three different laboratories, except for the Cryo WB/IL-1β. In the second study, reproducibility was determined using the results from the 10 substances used in the accuracy analysis. Each drug was spiked with four concentrations of endotoxin and tested once in each of three laboratories. The extent and order of agreement among laboratories were similar in both studies: the WB/IL-1β test method showed the least agreement (57% to 58%), and the Cryo WB/IL-1β test method showed the most (88% to 92%).

ICCVAM Recommendations: Test Method Protocols

ICCVAM recommends standardized protocols for each test method that should be used for validation of specific products on a case-by-case basis for U.S. regulatory consideration. These recommended protocols, provided in **Appendix C**, are primarily based on ECVAM SOPs for each test method. ICCVAM has updated these protocols to address inadequacies identified by the Panel, including modifications to standardize essential test method components across the five *in vitro* test methods. These modifications are not expected to reduce or otherwise impact test method accuracy and reliability.

The Panel concluded that the information provided in the ICCVAM draft BRD supported the ICCVAM draft recommended protocols for these five *in vitro* test methods, as long as inadequacies identified by the Panel with respect to reliability and relevance are fully addressed.

ICCVAM Recommendations: Future Studies

ICCVAM recognizes that these test methods could be applicable for detection of a wider range of pyrogens (i.e., endotoxin and pyrogens other than endotoxin) and test materials, provided that they are adequately validated for such uses. Test materials that have been identified clinically as pyrogenic might be invaluable for use in future validation studies and might allow such studies to be conducted without the use of animals. Wherever possible, historical data generated with the same test samples in both *in vitro* and *in vivo* studies (i.e., parallel testing data) should be retrospectively evaluated, or *in vitro* testing should be performed in parallel with RPT and/or bacterial endotoxin tests (BET) conducted for

[‡]The ECVAM Cryo WB/IL-1β test method BRD stated that there was no direct assessment of intralaboratory reproducibility because such an evaluation was performed in the WB/IL-1 test method, and the authors assumed that variability would not be affected by the use of cryopreserved blood.

regulatory purposes[§]. Future validation studies should include the following considerations:

1. Both endotoxin-spiked and non-endotoxin spiked samples should be included. Non-endotoxin pyrogen standards should be characterized prior to their use in any study, if possible.
2. All aspects of the studies should comply with Good Laboratory Practices.
3. Future studies should include products that have intrinsic pro-inflammatory properties in order to determine if these tests can be used for such substances.
4. Optimally, a study that includes three-way parallel testing, with the *in vitro* assays being compared to the RPT and the BET, should be conducted to comprehensively evaluate the relevance and comparative performance of these test methods. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, any *in vivo* testing should be limited to those studies that will fill existing data gaps.
5. Test substances that better represent all categories of sample types (e.g., pharmaceuticals, biologicals, and medical devices) intended for testing by the methods should be included.
6. The hazards associated with human blood products should be carefully considered, and all technical staff should be adequately trained to observe all necessary safety precautions.
7. Formal sample size calculations should be made to determine the required number of replicates needed to reject the null hypothesis at a given level of significance and power. For reliability assessments, formal hypothesis testing is essential with the alternative hypothesis being no difference between groups.

The Panel agreed with ICCVAM that any future studies should be performed using the ICCVAM recommended test method protocols. The Panel also provided other suggestions and recommendations for future studies (see **Appendix A**). Like ICCVAM, the Panel also recognized that these test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.

ICCVAM Recommendations: Performance Standards

As indicated above, these test methods have not yet been adequately evaluated for their ability to detect Gram-negative endotoxin in parenteral pharmaceuticals, biological products, and medical devices compared to the RPT or the BET. For this reason, ICCVAM does not consider it appropriate at this time to develop performance standards that can be used to evaluate the performance of other test methods that are structurally and functionally similar.

[§]In order to demonstrate the utility of these test methods for the detection of non-endotoxin pyrogens, either an international reference standard is needed (as is available for endotoxin [i.e., WHO-LPS 94/580 *E. coli* O113:H10:K-]) or, when a positive non-endotoxin-mediated RPT result is encountered, this same sample should be subsequently tested *in vitro*.

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1.0 Introduction

In June 2005, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) initiated a review of the validation status of five *in vitro* pyrogen test methods proposed as replacements for the rabbit pyrogen test (RPT). The test methods were submitted by the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre. This submission was based on a validation study financed by the European Commission within the 5th Framework Programme of Directorate General Research and was recently published (Hoffmann et al. 2005a; Schindler et al. 2006). The proposed test methods are:

- The Human Whole Blood (WB)/Interleukin (IL)-1β *In Vitro* Pyrogen Test
- The Human WB/IL-1β *In Vitro* Pyrogen Test: Application of Cryopreserved (Cryo) Human WB
- The Human WB/IL-6 *In Vitro* Pyrogen Test
- The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test
- The Monocytoid Cell Line Mono Mac 6 (MM6)/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 2000), 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 the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to carry out these evaluations; Dr. Marlies Halder was the ECVAM liaison to the PWG. Following a NICEATM pre-screen evaluation of the comprehensive background review documents (BRDs) submitted by ECVAM, NICEATM, ICCVAM and the ICCVAM PWG requested additional information and clarification from ECVAM on a number of issues. In March 2006, in response to this request, ECVAM submitted revised BRDs and a list of responses to address these issues.

NICEATM, which administers ICCVAM and provides scientific support for ICCVAM activities, subsequently prepared a comprehensive draft BRD that provided information and data from the validation studies and scientific literature to enable a peer review of the validation status of each of the five *in vitro* test methods. A request for any other data and information on these test methods and for nominations to serve on an independent, scientific pyrogenicity review panel (Panel) was made through a 2005 *Federal Register* (*FR*) notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E5_7410.pdf), through the ICCVAM electronic mailing list, and through direct requests to over 100 stakeholders. Panel nominations were received, but no additional data or information was submitted in response to this request.

Announcement of a public Panel meeting to review the validation status of the five *in vitro* pyrogen test methods and availability of the ICCVAM BRD was made through a 2006 *FR* notice (Vol. 71, No. 238, pp. 74533-74534, December 12, 2006, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E6_21038.pdf). The draft BRD was made publicly available on the NICEATM/ICCVAM website (http://iccvam.niehs.nih.gov). Additional information provided by ECVAM in response to a request from Panel was appended to this BRD. All of the information provided to the Panel was also made publicly available. Comments from the public and scientific community are available on the NICEATM/ICCVAM website.

The adequacy of the data and information contained in the ICCVAM BRD to support the ICCVAM draft test method recommendations were discussed by the Panel in a public meeting on February 6, 2007 at the National Institutes of Health campus in Bethesda, MD. A report of the Panel's recommendations (see **Appendix A**; Panel Report, available at http://iccvam.niehs.nih.gov/docs/pyrogen/PrRevPanFinRpt.pdf) was made available for public comment on the NICEATM/ICCVAM website (see *FR* notice [Vol. 72, No. 89, pp. 26395-26396, May 9, 2007], available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_8896.pdf).

The ICCVAM draft BRD, the Panel report, and all public comments were made available to ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), for review and comment at their meeting on June 12, 2007.

ICCVAM and the PWG then considered the Panel report, all public comments, and the comments of SACATM in preparing the final BRD and the final test method recommendations that are provided in this ICCVAM Test Method Evaluation Report. This report will be made available to the public and provided to U.S. Federal agencies for consideration (ICCVAM 2000). The ICCVAM final BRD, revised in response to the Panel and PWG comments, will also be provided as background information and technical support for this report. Agencies with applicable testing regulations and guidelines (see **Appendix B**) are required by law to respond to ICCVAM within 180 days of receiving an ICCVAM test method recommendation. These responses will be made available to the public on the NICEATM/ICCVAM website (http://iccvam.niehs.nih.gov) as they are received.

2.0 ICCVAM Recommendations for *In Vitro* Pyrogen Test Methods

ICCVAM evaluated the validation status of the five *in vitro* pyrogen test methods as potential replacements for the RPT. ICCVAM was unable to evaluate these tests as possible replacements for the Bacterial Endotoxin Test (BET) because the validation studies were not designed for this purpose.

2.1 ICCVAM Recommendations: Test Method Uses and Limitations

The ability of the WB/IL-1β, Cryo WB/IL-1β, WB/IL-6, PBMC/IL-6, and MM6/IL-6 test methods to detect Gram-negative endotoxin in a limited number of human parenteral drugs have been tested in recent validation studies. The performance assessment for these five test methods, and the drugs included in the associated validation studies are detailed in **Section 3.0**. Based on a review of the available data, these test methods have not been adequately evaluated for their ability to detect Gram-negative endotoxin in parenteral pharmaceuticals, biological products, and medical devices compared to the RPT or the BET. This is based on the fact that the validation study only evaluated a limited range and number of pharmaceutical products and did not evaluate the potential to detect endotoxin in biologics or medical devices. Therefore, none of the test methods should be considered as a complete replacement for the RPT or the BET for the detection of Gram-negative endotoxin. However, these test methods can be considered for use to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis, subject to product-specific validation to demonstrate equivalence to accepted pyrogen tests in accordance with applicable U.S. Federal regulations (e.g., U.S. Food and Drug Administration [FDA] [*])[†]. Potential users should consider the false negative/false positive rates as well as ease of use in selecting any test method for possible use. In addition, while the scientific basis of these test methods suggests that they have the capability to detect pyrogenicity mediated by non-endotoxin sources, there is insufficient data to support this broader application. Users should be aware that the performance characteristics for these *in vitro* pyrogen test methods might be revised based on additional data. Therefore, ICCVAM recommends that test method users routinely consult the NICEATM/ICCVAM website (http://iccvam.niehs.nih.gov/) and other appropriate sources to ensure that the most current information is considered.

2.1.1 Independent Peer Review Panel Conclusions and Recommendations

The Panel agreed that the applicable validation criteria have been adequately addressed in the ICCVAM draft BRD in order to determine the usefulness and limitations of these test methods to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product-specific validation. However, the Panel generally agreed that the performance of these test methods in terms of their reliability and relevance did not support this proposed use (see **Appendix A**).

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[*]Mechanisms exist for test method developers to qualify their method on a case-by-case basis. The use of any recommended method will be subject to product-specific validation to demonstrate equivalence as recommended by the FDA (e.g., 21 CFR 610.9 and 21 CFR 314.50(d)(1)(ii)(a)).

[†]Substances other than endotoxin may induce the cellular release of IL-1β and/or IL-6. For this reason, users of these test methods should be aware that the presence of other materials might erroneously suggest the presence of endotoxin and lead to a false positive result.

While ICCVAM agreed with the Panel that these test methods cannot be considered complete replacements for the RPT, they did recommend their use to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis, subject to product-specific validation to demonstrate equivalence to the RPT.

2.1.2 ECVAM Scientific Advisory Committee (ESAC) Statement of Validity

In March 2006, the ESAC unanimously endorsed a statement of validity for these five *in vitro* pyrogen test methods, which describes their recommendations on test method uses (see **Appendix E**). Like ICCVAM, ESAC concluded that these five methods can detect pyrogenicity mediated by Gram-negative endotoxin in materials currently tested in the RPT, and that they may be useful for regulatory decisions, subject to product-specific validation. Both ICCVAM and ESAC also concluded that the currently available database does not support their use to detect a wider range of pyrogens, as was suggested in the original ECVAM submission.

However, ESAC concluded 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. In contrast, as described in **Section 2.1**, ICCVAM has concluded that the current validation database for these test methods is inadequate to support such a definitive statement based on the ECVAM validation study design, which did not include biologics or medical devices and evaluated only a limited range and number of pharmaceutical products and additionally did not include parallel testing with the RPT.

2.2 ICCVAM Recommendations: Test Method Protocols

ICCVAM recommends that when testing is conducted, the *in vitro* pyrogen test method protocols should be based on the standardized test method protocols provided in **Appendix C**. These ICCVAM recommended protocols, summarized in **Table 2-1**, are based primarily on ECVAM Standard Operating Procedures (SOPs) for each test method, with modifications made by NICEATM and ICCVAM in an effort to standardize essential test method components across protocols where possible. These modifications are not expected to reduce test method performance. A table summarizing the differences between the ICCVAM recommended protocol and the relevant ECVAM protocol/SOP is provided as an introduction to each protocol included in **Appendix C**.

By comparison, the Panel concluded that the information provided in the ICCVAM draft BRD supported the ICCVAM draft recommended protocols for these five *in vitro* test methods, providing that the list of inadequacies identified by the Panel with respect to reliability and relevance are fully addressed. The revised ICCVAM recommended protocols (see **Appendix C**) have been updated to address many of the Panel's concerns.

Using these recommended standardized protocols will facilitate collection of consistent data and expand the current validation database. Exceptions and/or changes to the recommended standardized test method protocols should be accompanied by a scientific rationale. Users should be aware that the test method protocols could be revised based on future optimization and/or validation studies. Therefore, test method users should consult the NICEATM/ICCVAM website (http://iccvam.niehs.nih.gov) or other appropriate sources to ensure use of the most current recommended test method protocol.

Table 2-1 Summary of ICCVAM Recommended *In Vitro* Pyrogen Test Method Protocols

Protocol Component	ICCVAM Recommended *In Vitro* Pyrogen Protocols				
	WB/IL-1β	**Cryo WB/IL-1β**	**WB/IL-6**	**PBMC/IL-6**	**MM6/IL-6**
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD				
Number of Blood Donors	Minimum of 3 (independent or pooled)				NA
Decision Criteria for Interference	Mean OD[1] of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.5 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.25 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate (The number of samples or controls measured in quadruplicate)	NSC (1)				
	EC (5)				
	TS (14)				
	PPC[2] (0)	PPC (0)	PPC (0)	PPC (0)	PPC[3] (0)
	NPC[2] (0)	NPC (0)	NPC (0)	NPC (0)	NPC (0)
ELISA Plate	Includes seven point IL-1β SC and blank in duplicate		Includes seven point IL-6 SC and blank in duplicate		
Assay Acceptability Criteria	Mean OD of NSC ≤0.15				
	Quadratic function of IL-1β SC r ≥0.95[3]		Quadratic function of IL-6 SC r ≥0.95		
	EC SC produces OD values that ascend in a sigmoidal concentration response				
	NA	NA	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded	High responder blood donors (i.e., > 200 pg/mL IL-6) or low responder blood donors (i.e., Mean OD of 1EU/mL EC is significantly less than that of 1000 pg/mL IL-6) may be excluded	NA
	Outliers rejected using Dixon's test[4]				
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC[5] TS				

Abbreviations: Cryo = Cryopreserved; EC = Endotoxin control; ELC = Endotoxin Limit Concentration; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL= Interleukin; MM6 = Mono Mac 6; MVD = Maximum valid dilution; NA = Not applicable; NPC = Negative product control; NSC = Negative saline control; OD = Optical density; PBMC = Peripheral blood mononuclear cell; PPC = Positive product control; SC = Standard curve; TS = Test substance; WB = Whole blood

[1]In WB/IL-1β and MM6/IL-6 test methods, the mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

[2]In the ICCVAM protocols (see **Appendix C**), PPC and NPC are assessed in the interference test described in Section 4.2, which is performed prior to the ELISA.

[3]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

[4]Dixon 1950.

[5]Where unknown, the ELC is calculated (see **Appendix C**).

2.3 ICCVAM Recommendations: Future Studies

ICCVAM recognizes that these test methods could be applicable for the detection of a wider range of pyrogens (i.e., endotoxin and non-endotoxin) and test materials, provided that they are adequately validated for such uses. Test materials identified clinically as pyrogenic might be invaluable for use in future validation studies and might allow such studies to be conducted without the use of animals. Wherever possible, historical data from parallel *in vivo/in vitro* studies should be retrospectively evaluated, or parallel *in vitro* testing should be conducted with RPT and/or BET tests that are performed for regulatory purposes[‡]. Future validation studies should include the following considerations:

1. Both endotoxin-spiked and non-endotoxin spiked samples should be included. Non-endotoxin standards should be characterized prior to their use in any study, if possible.
2. All aspects of the studies should be compliant with Good Laboratory Practice.
3. Future studies should include products that have intrinsic pro-inflammatory properties in order to determine if such substances are amenable to these tests.
4. Optimally, a study that includes 3-way parallel testing, with the *in vitro* assays being compared to the RPT and the BET, should be conducted to allow for a comprehensive evaluation of the relevance and comparative performance of these test methods. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, any *in vivo* testing should be limited to those studies that will fill existing data gaps.
5. Test substances that better represent all categories of sample types (e.g., pharmaceuticals, biologicals, and medical devices) intended for testing by the methods should be included.
6. The hazards associated with human blood products should be carefully considered, and all technical staff should be adequately trained to observe all necessary safety precautions.
7. Formal sample size calculations should be made to determine the required number of replicates needed to reject the null hypothesis at a given level of significance and power. For reliability assessments, formal hypothesis testing is essential with the alternative hypothesis being no difference between groups.

The Panel agreed that any future studies should be performed using the ICCVAM proposed protocols. Like ICCVAM, the Panel also recognized that these test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.

[‡]In order to demonstrate the utility of these test methods for the detection of non-endotoxin pyrogens, either an international reference standard is needed (as is available for endotoxin [i.e., WHO-LPS 94/580 *E. coli* O113:H10:K-]) or, when a positive non-endotoxin-mediated RPT result is encountered, this same sample should be subsequently tested *in vitro*.

The Panel also recommended other studies for consideration:

1. A proposed strategy for the Cryo WB/IL-1β test method is to retest if a test fails because of too much variability. The statistical properties of this multistage procedure should be characterized.
    - ICCVAM note: This comment, which pertains to the ECVAM Catch-Up Validation SOP for the Cryo WB/IL-1β pyrogen test, is not relevant to the ICCVAM recommended protocol.
2. The effects of direct administration of IL-1β and IL-6 to rabbits and the comparison of the resulting pyrogenic response with endotoxin-mediated pyrogenicity should be evaluated. In addition, the correlation of IL-1β and IL-6 levels in the *in vitro* tests with levels produced in rabbits using similar doses of endotoxin should be evaluated.
    - ICCVAM note: This information would certainly be interesting and possibly useful in the comparison of the responses of the *in vitro* human cells to that of the *in vivo* rabbit. However, ICCVAM did not consider that the information gained could justify the additional resources and animals that would be required to perform such studies, and therefore, ICCVAM has not included this specific recommendation.
3. The endotoxin-spike concentrations used for the performance assessment studies should not be so close to the positive test concentration limit, especially considering the relatively large enhancement and inhibition range permitted in the sample specific qualification investigations.
    - ICCVAM note: ECVAM has previously commented that, "The study design, using borderline spikes, aimed to profile differences in pyrogen tests (i.e., RPT, BET, and *in vitro* tests), but does not reflect routine test situations. Furthermore, the threshold chosen represents the endotoxin limit, where 50% of the rabbits using the most sensitive rabbit strain react with fever." Therefore, the validation study was designed to maximally challenge the sensitivity of the *in vitro* pyrogen tests. For this reason, and because the *in vitro* test methods are being recommended for consideration on a case-by-case basis, subject to product-specific validation, ICCVAM has not included this specific recommendation.
4. A 'limit' test design protocol and a 'benchmark reference lot comparison' test design protocol for each assay should be included.
    - ICCVAM note: Because these *in vitro* test methods are being recommended for consideration on a case-by-case basis, subject to product-specific validation, ICCVAM did not consider the additional resources required to perform both study designs practical.

2.4 ICCVAM Recommendations: Performance Standards

As indicated above, these five *in vitro* test methods have not been adequately evaluated for their ability to detect Gram-negative endotoxin compared to the RPT or the BET in a

sufficient number and range of parenteral pharmaceuticals, and in no biological products and medical devices. For this reason, it is not feasible at this time to develop performance standards that can be used to evaluate the performance of other test methods that are structurally and functionally similar.

3.0 Validation Status of *In Vitro* Pyrogen Test Methods

The following is a synopsis of the information in the ICCVAM BRD, which reviews the available data and information for each of the five test methods. The ICCVAM BRD describes the current validation status of the five *in vitro* pyrogen test methods, including what is known about their reliability and accuracy, the scope of the substances tested, and standardized protocols used for the validation study. The ICCVAM BRD may be obtained electronically from the NICEATM/ICCVAM website (http://iccvam.niehs.nih.gov/) or by contacting NICEATM via email at niceatm@niehs.nih.gov. A hard copy of the ICCVAM BRD may be requested by email or by mail to NICEATM, NIEHS, P.O. Box 12233, Mail Drop EC-17, Research Triangle Park, NC 27709.

3.1 Test Method Description

According to the ECVAM submission, these *in vitro* pyrogen test methods are intended for the detection of Gram-negative endotoxin contained in substances intended for parenteral use (e.g., pharmaceuticals, biologics, medical devices). These methods are based on the detection of the release of proinflammatory cytokines (i.e., IL-1β or IL-6) from human monocytes or monocytoid cells induced by exposure to a product contaminated with Gram-negative endotoxin.

3.1.1 General Test Method Procedures

The *in vitro* pyrogen test methods measure cytokine release from monocytes or monocytoid cells (i.e., WB, PBMCs, or the MM6 cell line) by using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for either IL-1β or IL-6. The amount of endotoxin present is determined by comparing the values of endotoxin equivalents produced by WB cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)[4] or an equivalent standard expressed in Endotoxin Units (EU)/mL. A product is considered to be pyrogenic if the endotoxin concentration exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

3.1.2 Protocol Similarities and Differences

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

- The test substance is mixed with a suspension of human-derived cells.
- The mix of cells and test product is incubated for a specific time.
- The concentration of pro-inflammatory cytokines (e.g., IL-1β, IL-6) is measured with an ELISA by comparison to a standard curve.

[4]RSEs are internationally harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; U.S. Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

- The endotoxin content is calculated by comparing the measured concentration of pro-inflammatory cytokines to an endotoxin standard curve.
- A test substance is considered pyrogenic if the estimated endotoxin concentration of the test substance exceeds the ELC for the test substance.

3.2 Validation Database

The test substances selected for use in the validation studies were marketed parenteral pharmaceuticals. No biological or medical device products were included in the validation study. A total of 13 test substances were included in the performance analysis of each of the five *in vitro* test methods. Ten substances (**Table 3-1**), each spiked with four concentrations of endotoxin (0, 0.25, 0.5, and 1.0 EU/mL, with 0.5 EU/mL tested in duplicate), were used to evaluate accuracy. Three substances (**Table 3-2**), each spiked with three concentrations of endotoxin (0, 0.5, and 1.0 EU/mL, with 0 EU/mL tested in duplicate), were used to assess intralaboratory reproducibility. Interlaboratory reproducibility was evaluated in two different studies. The first study tested the substances listed in **Table 3-2** in triplicate in each of three laboratories. In the second study, interlaboratory reproducibility was tested using the substances in **Table 3-1**, which were tested once in each of three laboratories.

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

Test Substance[2]	Active Ingredient	Source	Lot Number(s)	Indication	MVD (-fold)
Beloc®	Metoprolol tartrate	Astra Zeneca	DA419A1	Heart dysfunction	140
Binotal®	Ampicillin	Grünenthal	117EL2	Antibiotic	140
Ethanol 95%	Ethanol	B. Braun	2465Z01	Diluent	35
Fenistil®	Dimetindenmaleat	Novartis	21402 26803[3]	Antiallergic	175
Glucose 5%	Glucose	Eifelfango	1162 3132[3]	Nutrition	70
MCP®	Metoclopramid	Hexal	21JX22	Antiemetic	350
Orasthin®	Oxytocin	Hoechst	W015	Initiation of delivery	700
Sostril®	Ranitidine	Glaxo Wellcome	1L585B 3H01N[3]	Antiacidic	140
Syntocinon®	Oxytocin	Novartis	S00400	Initiation of delivery	-
Drug A - 0.9% NaCl	0.9% NaCl	-	-	-	35
Drug B - 0.9% NaCl	0.9% NaCl	-	-	-	70

Abbreviations: MVD = Maximum valid dilution

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

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

[3]Indicates the lot numbers used in the catch-up validation study for the Cryopreserved whole blood/Interleukin-1β test method.

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

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

[1]Each substance was tested in all five *in vitro* pyrogen test methods.
[2]Each test substance was spiked with 0, 0.5, or 1.0 Endotoxin Units (EU)/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]), with 0 EU/mL tested in duplicate. Each sample contained the appropriate spike concentration when tested at its maximum valid dilution.

3.3 Reference Test Method Data

The historical RPT studies were conducted at the Paul Ehrlich Institut (PEI), which supports regional German regulatory authorities, provides marketing approval of certain marketed biological products (e.g., sera, vaccines, test allergens), and functions as a World Health Organization (WHO) collaborating center for quality assurance of blood products and *in vitro* diagnostics. The unit for pyrogen and endotoxin testing of the PEI is accredited following the International Organization for Standardization (ISO) and the International Electrotechnical Commission 17025 (ISO 2005). In a request for additional information from ECVAM, it was stated that the RPT data was generated according to the European Pharmacopeia (EP) monograph, but the detailed protocol used by this laboratory was not provided.

These data were generated for internal quality control studies from 171 rabbits (Chinchilla Bastards). Chinchilla Bastards are reported to be a more sensitive strain than the New Zealand White rabbit strain for pyrogenicity testing (Hoffmann et al. 2005b). However, the USP (USP 2007) and the EP (EP 2005) do not prescribe a specific rabbit strain for the RPT.

3.4 Test Method Accuracy

The ability of the *in vitro* pyrogen test methods to correctly identify the presence of Gram-negative endotoxin was evaluated using parenteral pharmaceuticals spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). As described in **Section 3.2**, 10 substances (see **Table 3-1**) spiked with four concentrations of endotoxin (i.e., 0, 0.25, 0.5, or 1.0 EU/mL, with 0.5 EU/mL tested in duplicate) 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). Outliers were identified using Dixon's test (i.e., significance level of α = 0.01) and subsequently excluded from the evaluation, which resulted in fewer than a total of 150 runs per evaluation (Dixon 1950; Barnett et al. 1984). A comparison of the results for the *in vitro* test methods indicates that the number of runs excluded was greatest for the Cryo WB/IL-1β and WB/IL-1β (plate method) test methods, which had 30 and 11 runs excluded, respectively. No other test method had more than three runs excluded.

As described in **Section 3.3**, no RPTs were conducted in parallel with the *in vitro* pyrogen 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). This historical data were 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 method performance values.

The accuracy of each *in vitro* pyrogen test method for correctly identifying samples spiked with 0.5 or 1.0 EU/mL endotoxin as positive and samples spiked with 0 or 0.25 EU/mL endotoxin as negative was evaluated. As provided in **Table 3-3**, accuracy ranged from 81% to 93%, sensitivity ranged from 73% to 99%, specificity ranged from 77% to 97%, false negative rates ranged from 1% to 27%, and false positive rates ranged from 3% to 23%.

Table 3-3 Accuracy of *In Vitro* Pyrogen Test Methods[1]

Test Method	Accuracy[2]	Sensitivity[3]	Specificity[4]	False Negative Rate[5]	False Positive Rate[6]
Cryo WB/IL-1β	92% (110/120)	97% (75/77)	81% (35/43)	3% (2/77)	19% (8/43)
MM6/IL-6	93% (138/148)	96% (85/89)	90% (53/59)	5% (4/89)	10% (6/59)
PBMC/IL-6	93% (140/150)	92% (83/90)	95% (57/60)	8% (7/90)	5% (3/60)
PBMC/IL-6 (Cryo)[7]	87% (130/150)	93% (84/90)	77% (46/60)	7% (6/90)	23% (14/60)
WB/IL-6	92% (136/148)	89% (79/89)	97% (57/59)	11% (10/89)	3% (2/59)
WB/IL-1β (Tube)	81% (119/147)	73% (64/88)	93% (55/59)	27% (24/88)	7% (4/59)
WB/IL-1β (96-well plate)[8]	93% (129/139)	99% (83/84)	84% (46/55)	1% (1/84)	16% (9/55)

Abbreviations: Cryo = Cryopreserved; EU/mL = Endotoxin units per milliliter; IL = Interleukin; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

[1]Data shown as a percentage (number of correct runs/total number of runs), 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, or 1.0 EU/mL, with 0.5 EU/mL tested in duplicate).

[2]Accuracy = the proportion of correct outcomes (positive and negative) of a test method.

[3]Sensitivity = the proportion of all positive substances that are classified as positive.

[4]Specificity = the proportion of all negative substances that are classified as negative.

[5]False negative rate = the proportion of all positive substances that are falsely identified as negative.

[6]False positive rate = the proportion of all negative substances that are falsely identified as positive.

[7]A modification of the PBMC/IL-6 test method that uses Cryo PBMCs.

[8]A modification of the WB/IL-1β test method that uses 96-well plates instead of tubes for the test substance incubation.

3.5 Test Method Reliability

Intralaboratory repeatability was evaluated by testing saline spiked with various concentrations of endotoxin (0, 0.06, 0.125, 0.25, 0.5, and 1.0 EU/mL) and then evaluating the closeness of agreement among OD readings for cytokine measurements at each concentration. For each test method, each experiment was conducted up to three times. From 5 to 32 replicates per concentration were tested and results indicated that variability in OD measurements increased with increasing endotoxin concentration. However, the variability

did not 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 three concentrations of endotoxin (i.e., 0, 0.5, and 1.0 EU/mL, with 0 EU/mL tested in duplicate). Three identical, independent runs were conducted in each of the three testing laboratories, with the exception of the Cryo WB/IL-1β test method[5]. The correlations (expressed as a percentage of agreement) between pairs of the independent runs (i.e., run 1 vs. run 2; run 1 vs. run 3; run 2 vs. run 3) were determined and the mean of these three values was calculated. In all reproducibility analyses, a single run consisted of each of the products assayed in quadruplicate. Acceptability criteria for each run included a Coefficient of Variation (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. As an 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 3-4**, the agreement across three runs in an individual lab ranged from 75% to 100%.

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, the interlaboratory reproducibility was evaluated using results from three marketed pharmaceuticals spiked with endotoxin and tested in triplicate in each of the three laboratories. As shown in **Table 3-5**, 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 (excludes the Cryo WB/IL-1β test method, which used only one run per laboratory). However, if the WB/IL-1β tube method is excluded, the range of agreement across laboratories is 72% to 86%. In comparison, the agreement across three laboratories for the Cryo WB/IL-1β test method, for which only one run per laboratory was conducted, was 92%.

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[5]The ECVAM Cryo WB/IL-1β test method BRD stated that there was no direct assessment of intralaboratory reproducibility because such an evaluation was performed in the WB/IL-1β test method, and the authors assumed that variability would not be affected by the use of cryopreserved blood.

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Table 3-4 Intralaboratory Reproducibility of *In Vitro* Pyrogen Test Methods

Run Comparison[1]	WB/IL-1β			Cryo WB/IL-1β			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)	NI[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%	NC	95%	ND	ND	ND	83%	92%	100%	95%	100%	95%	100%	95%	95%
Agreement[2] across 3 runs	83%	NC	92%	ND	ND	ND	75%	92%	100%	92%	100%	92%	100%	92%	92%

Abbreviations: Cryo = Cryopreserved; IL = Interleukin; MM6 = Mono Mac 6; NC = Not calculated; ND = Not done; NI = Not included; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

[1]Comparison among 3 individual runs within each laboratory.

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

[3]Not done. The ECVAM Cryo WB/IL-1β BRD states that an assessment of intralaboratory reproducibility was performed using the WB IL-1β (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 included due to lack of sufficient data. The sensitivity criteria were not met for 1 of 3 substances in run 2, and 1 of 3 substances in run 3.

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Table 3-5 Interlaboratory Reproducibility of *In Vitro* Pyrogen Test Methods: Study One

Lab Comparison[1]	Agreement Between Laboratories[1]				
	WB/IL-1β (Tube)	Cryo WB/IL-1β	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 = Interleukin; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; 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, 0.5 and 1.0 EU/mL, with 0 EU/mL spiked in duplicate, were tested three times in three different laboratories, with the exception of Cryo WB/IL-1β (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-1β 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-1β, which was only tested once in each laboratory, resulting in only one possible combination per substance).

In the second study, interlaboratory reproducibility was evaluated with the same 10 substances used for evaluating accuracy. In this study, each of the substances was spiked with four concentrations of endotoxin (0, 0.25, 0.5, and 1.0 EU/mL, with 0.5 EU/mL spiked in duplicate) and tested once in each of three laboratories. As shown in **Table 3-6**, the agreement across three laboratories for each test method ranged from 57% to 88%, depending on the test method considered. The extent and order of agreement among laboratories was the same for both studies; the WB/IL-1β test method showed the least agreement (57-58%) and the Cryo WB/IL-1β test method showed the most (88-92%).

Table 3-6 Interlaboratory Reproducibility of *In Vitro* Pyrogen Test Methods: Study Two

Lab Comparison[1]	Agreement Between Laboratories[1]						
	WB/IL-1β (Tube)	WB/IL-1β (Plate)	Cryo WB/IL-1β	WB/IL-6	PBMC/IL-6	PBMC/IL-6 (Cryo)	MM6/IL-6
1 vs 2	73% (35/48)	88% (37/42)	84% (38/45)	85% (41/48)	84% (42/50)	96% (48/50)	90% (45/50)
1 vs 3	82% (40/49)	90% (35/39)	88% (21/24)	85% (41/48)	86% (43/50)	76% (38/50)	90% (43/48)
2 vs 3	70% (33/47)	92% (43/47)	100% (25/25)	88% (44/50)	90% (45/50)	80% (40/50)	83% (40/48)
Mean	75%	90%	91%	86%	87%	84%	88%
Agreement across 3 labs	57% (27/47)	85% (33/39)	88% (21/24)	79% (38/48)	80% (40/50)	76% (38/50)	81% (39/48)

Abbreviations: Cryo = Cryopreserved; IL = Interleukin; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; 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, and 1.0 EU/mL, with 0.5 EU/mL spiked in duplicate, were tested once in three different laboratories.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The currently accepted pyrogen test methods require the use of rabbits or horseshoe crab hemolymph. The proposed *in vitro* pyrogen test methods use monocytoid cells of human origin, obtained either from WB donations or from an immortalized cell line. The capability of these five *in vitro* assays to detect Gram-negative endotoxin suggests that they may reduce or eventually replace the use of rabbits and/or horseshoe crab hemolymph for pyrogen testing. However, at the present time, the RPT detects classes of pyrogens that have neither been examined nor validated with the *in vitro* pyrogen test methods and thus, the RPT will still be required for most test substances.

Human blood donations are required for four of the five *in vitro* test methods (WB/IL-1β, WB/IL-6, Cryo WB/IL-1β, and PBMC/IL-6) proposed as replacements for the RPT, and as such, no animals will be used when these assays are appropriate for use. While the collection of human blood is a common medical procedure, the many aspects of human blood collection must be considered to ensure that human donors are treated appropriately, and that such collection and use is in accordance with all applicable regulations, policies, and guidelines.

4.0 ICCVAM Consideration of Public and SACATM Comments

In response to three *FR* notices that were released between December 2005 and May 2007, eight public comments were received (see **Appendix D**). Comments received in response to or related to the *FR* notices are also available on the NICEATM/ICCVAM website (http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm). The following sections, delineated by *FR* notice, provide a brief discussion of the public comments received.

4.1 Public Comments in Response to *FR* Notice (70FR74833, December 16, 2005): Peer Panel Evaluation of *In Vitro* Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of *In Vivo* and *In Vitro* Data

NICEATM, in an *FR* notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005), requested (1) public comments on the appropriateness and relative priority of convening an independent peer review panel (Panel) to evaluate the validation status of five *in vitro* pyrogen test methods, (2) the nomination of scientists with relevant knowledge and experience to potentially serve on the Panel should it be convened, and (3) submission of data from the RPT, the BET, and *in vitro* pyrogenicity testing using any of the five *in vitro* pyrogen test methods under consideration by NICEATM.

In response to this *FR* notice, NICEATM received two comments. No additional data or information was submitted in response to this request. One nomination requested consideration of three potential panelists.

One commenter provided a reference for an *in vitro* pyrogen test method that measured TNF-α (Martinez et al. 2004). The comment and article were provided to the Panel. However, the reference was not included in the ICCVAM BRD because the *in vitro* pyrogen methods being evaluated by NICEATM measured only IL-1β and IL-6.

A second commenter requested an expeditious review of the *in vitro* pyrogen test methods and described limitations of the currently used *in vivo* pyrogen test methods (i.e., the RPT and the BET). This commenter also stated that the peer review of the *in vitro* test methods is appropriate, necessary, and should be given extremely high priority.

4.2 Public Comments in Response to *FR* Notice (71FR74533, December 12, 2006): Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments

NICEATM, in an *FR* notice (Vol. 71, No. 238, pp. 74533-4, December 12, 2006), announced (1) an independent scientific peer review meeting to evaluate the validation status of five *in vitro* pyrogen test methods proposed as replacements for the RPT, and (2) the availability of an ICCVAM draft BRD on five *in vitro* pyrogen test methods, which describes the current validation status of these methods and contains all of the data and analyses supporting their current validation status, and ICCVAM draft recommendations on the proposed use of these test methods, draft test method protocols, and draft performance standards. NICEATM invited the submission of written comments on the ICCVAM draft BRD and on the ICCVAM draft test method recommendations. In response to this *FR* notice, NICEATM received four comments.

One commenter expressed that it was not clear why ICCVAM was neither considering the *in vitro* pyrogen test methods for detection of non-endotoxin pyrogens nor for replacement of both the RPT and the BET. The commenter suggested that exclusion of these broader uses would minimize the impact of these test methods on reduction in animal use and urged ICCVAM "to significantly revise its recommendations and BRD to more accurately reflect the potential use of these methods as full replacements for both the {BET} and RPT." Furthermore, they "strongly encouraged ICCVAM to delete the recommendation regarding the conduct of *de novo* RPTs to further demonstrate *in vivo/in vitro* concordance." ICCVAM appreciates the concern for the proposed limited use of these test methods. However, neither data comparing the *in vitro* test methods to the BET nor data directly comparing non-endotoxin pyrogens to the BET or the RPT were included in the validation studies submitted by ECVAM. Therefore, ICCVAM was unable to consider the *in vitro* test methods as replacements for the BET or to propose the use of these test methods for non-endotoxin pyrogens. However, ICCVAM did identify and recommend future studies that could fill these data gaps and in turn, potentially broaden the applicability of these test methods to that suggested by the commenter.

Several commenters argued that the scope of the test substances was limited and the data provided were inadequate to support the intended use of the *in vitro* test methods (i.e., as a complete replacement for the RPT). These commenters emphasized that additional testing is needed before these test methods can be recommended for this broader application. ICCVAM agreed with these comments, which are reflected in the ICCVAM recommended future studies.

One commenter provided data on an alternative *in vitro* pyrogen test method that is based on the measurement of reactive oxygen species from the human HL-60 promyelocytic leukemia cell line (Blatteis 2006; Timm et al. 2006). The comment and articles were provided to the Panel. However, these data were not included in the ICCVAM BRD because the *in vitro* pyrogen methods being evaluated by NICEATM measured only IL-1β and IL-6.

4.3 Public Comments in Response to *FR* Notice (72FR26395, May 9, 2007): Peer Review Panel Report on Five *In Vitro* Pyrogen Test Methods: Availability and Request for Public Comments

NICEATM, in an *FR* notice (Vol. 72, No. 89, pp. 26395-26396, May 9, 2007), announced the availability of the Panel report and invited the submission of written comments on the report. In response to this *FR* notice, NICEATM received two comments.

One commenter indicated that several of the Panel's observations and recommendations were "nonsensical, irrelevant, or inappropriate." This commenter also expressed concern about the “random” selection of Panel members and recommended both simplification of the questions posed to the Panel and an orientation meeting to provide the panelists with background information and focus. It was recommended that "ICCVAM coordinate with the pharmaceutical and medical devices industry to conduct product-specific validation on a set of pre-selected products and devices to serve as further validation work." ICCVAM appreciates comments related to the evaluation process of new alternative test methods. ICCVAM notes that Panel members were selected from nominations received in response to an *FR* notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005), in conjunction with recommendations from the ICCVAM PWG, which includes a liaison from ECVAM.

Additionally, orientation sessions are routinely convened for the Panel to provide background information on the ICCVAM test method evaluation process.

A second commenter outlined responses to specific comments and/or recommendations made in the Panel report. These comments provided rationale for the design of the ECVAM validation study and summarized existing data to address many of the Panel's concerns. ICCVAM appreciates these written responses and clarifications to specific Panel comments. ICCVAM considered all comments prior to finalization of the ICCVAM BRD and in preparation of the ICCVAM test method evaluation report.

4.4 Public and SACATM Comments: SACATM Meeting on June 12, 2007

The June 12, 2007 SACATM Meeting included a discussion of the ICCVAM review of the *in vitro* pyrogen test methods. At this meeting, three public comments and four SACATM comments were presented.

One public commenter reiterated the written comments submitted in response to the *FR* notice announcing the availability of the Panel report (see **Section 4.3**, first commenter).

A second public commenter (who was also the Chair of the ICCVAM peer review panel) stated that, "given more time to discuss these methods, the Panel might have been able to provide a stronger recommendation for one or more of the assays." ICCVAM appreciates comments related to the evaluation process and now intends to extend the time allocated for Panel meetings to ensure that sufficient time is allotted.

A third public commenter noted that the long list of future studies recommended by the Panel were impractical and not feasible to complete, particularly considering the expense that had already been invested in the validation effort. This commenter also provided additional comments relevant to the criticisms of these *in vitro* test methods made by the Panel (e.g., the limitations of the *in vitro* methods were not fairly compared to the limitations of the RPT and BET; only endotoxin was included in the validation study because no non-endotoxin reference standard is available; and false positives were recorded because the assays are too sensitive). ICCVAM considered many of these comments in the revisions of the ICCVAM BRD and in the preparation of the ICCVAM test method evaluation report.

One SACATM member expressed concern with the high false negative rates reported for some of the assays, the proprietary issues associated with using the Novartis IL-6 ELISA, the lack of concordance assessment between the RPT and the *in vitro* data, and the range of substances included in the validation studies. A second SACATM member provided comments on the statistical analyses used to assess the *in vitro* data. ICCVAM agrees with many of these concerns, which are reflected in the ICCVAM test method recommendations.

A third SACATM member recommended that multiple test methods not be reviewed simultaneously. As stated above, ICCVAM plans to allocate additional time for deliberation at Panel meetings.

A fourth SACATM member suggested the concept of "core panelists" who are knowledgeable about the ICCVAM evaluation process for ICCVAM reviews with the addition of *ad hoc* experts for specific methods. ICCVAM also appreciates this suggestion and makes every effort to include in each panel individuals with direct experience with the ICCVAM evaluation process as well as experts in the subject matter being evaluated.

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Appendix A

Independent Scientific Peer Review Panel Assessment

A1 Peer Review Panel Report: The Use of Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products A-3

A2 Summary Minutes from Peer Review Panel Meeting on February 6, 2007 ..... A-53

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Appendix A1

Peer Review Panel Report: The Use of Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products

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Independent Peer Review Panel Report: Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products

April 2007

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

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

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Services
Department of Health and Human Services

This document is available electronically at:
http://iccvam.niehs.nih.gov/docs/pyrogen/PrRevPanFinRpt.pdf

The findings and conclusions of this report are those of the Independent Scientific Peer Review Panel and should not be construed to represent the official views of ICCVAM or its member agencies.

TABLE OF CONTENTS

***IN VITRO* PYROGENICITY INDEPENDENT PEER REVIEW PANEL ..... iv**
PREFACE v
EXECUTIVE SUMMARY vii

A. REVIEW OF THE VALIDATION STATUS OF *IN VITRO* PYROGEN TEST METHODS 1

1.0 Introduction and Rationale for the Proposed Use of *In Vitro* Pyrogen Test Methods 19
1.1 Introduction 19
1.2 Regulatory Rationale and Applicability 20
1.3 Scientific Basis for the *In Vitro* Pyrogen Test Methods 22

2.0 *In Vitro* Pyrogen Test Method Protocol Components 24
2.1 Overview of How the *In Vitro* Pyrogen Test Methods are Conducted 24
2.2 Description and Rationale for the Test Method Components for Proposed Standardized Protocols 24
2.3 Basis for Selection of Test Method Systems 26
2.4 Proprietary Components 26
2.5 Number of Replicates 26
2.6 Modifications to the Test Method Protocols Based on ECVAM Validation Study Results 27

3.0 Substances Used for the Validation of *In Vitro* Pyrogen Test Methods 27
3.1 Rationale for the Substances or Products Selected for Testing 27
3.2 Number of Substances 27
3.3 Identification and Description of Substances Tested 28
3.4 Sample Coding Procedure 28

4.0 *In Vivo* Reference Data for the Assessment of Test Method Accuracy 28
4.1 Description of the Protocol Used to Generate *In Vivo* Data 28
4.2 Reference Data Used to Assess *In Vitro* Test Method Accuracy 29
4.3 Availability of Original Records for the *In Vivo* Reference Data 29
4.4 *In Vivo* Data Quality 29
4.5 Availability and Use of Toxicity Information from the Species of Interest 29
4.6 Information on the Relevance and Reliability of the *In Vivo* Test Methods 30

5.0 Test Method Data and Results 12
5.1 Test Method Protocol 30
5.2 Availability of Copies of Original Data Used to Evaluate Test Method Performance 31
5.3 Description of the Statistical Approaches Used to Evaluate the Resulting Data 31
5.4 Summary of Results 32

5.5 Use of Coded Chemicals and Compliance with GLP Guidelines..... 33
5.6 Lot-to-Lot Consistency of Test Substances..... 33

6.0 Relevance of the *In Vitro* Pyrogen Test Methods..... 34
6.1 *In Vitro* Pyrogen Test Method Relevance..... 34
6.2 Summary of the Performance Statistics for *In Vitro* Pyrogen Test Methods..... 34

7.0 Reliability of the *In Vitro* Pyrogen Test Methods..... 35
7.1 Selection Rationale for the Substances Used to Evaluate the Reliability of *In Vitro* Pyrogen Test Methods..... 35
7.2 Analysis of Repeatability and Reproducibility..... 35
7.3 Historical Positive and Negative Control Data..... 37

8.0 Test Method Data Quality..... 37
8.1 Adherence to National and International GLP Guidelines..... 37
8.2 Data Quality Audits..... 37
8.3 Impact of Deviations from GLP Guidelines..... 37
8.4 Availability of Laboratory Notebooks or Other Records..... 38

9.0 Other Scientific Reports and Reviews..... 38
9.1 Have Relevant Data Identified in Other Published or Unpublished Studies Conducted Using the *In Vitro* Test Methods Been Adequately Considered?..... 38
9.2 Are the Conclusions Published in Independent Peer-Reviewed Reports or Other Independent Scientific Reviews of the *In Vitro* Test Methods Adequately Discussed and Compared?..... 38
9.3 Are There Other Comparative *In Vitro* Test Method and RPT Data That Were Not Considered in the ICCVAM Draft BRD, But are Available for Consideration?..... 38

10.0 Animal Welfare Considerations (Refinement, Reduction, and Replacement)..... 39
10.1 How the Five *In Vitro* Pyrogen Test Methods Will Refine, Reduce, or Replace Animal Use..... 39
10.2 Requirement for the Use of Animals..... 39

11.0 Practical Considerations..... 39
11.1 Transferability of the *In Vitro* Pyrogen Test Methods..... 39
11.2 Personnel Training Considerations..... 40
11.3 Cost Considerations..... 40
11.4 Time Considerations..... 40

12.0 Recommended Additional References..... 23

13.0 Summary of Validation Status of the *In Vitro* Pyrogen Test Methods..... 25

B. REVIEW OF ICCVAM DRAFT TEST METHOD RECOMMENDATIONS 26
1.0 Proposed Test Method Usefulness and Limitations 26
2.0 Proposed Test Method Standardized Protocols 26
3.0 Proposed Test Method Performance Standards 29
4.0 Proposed Additional Studies 30
C. OVERALL PEER REVIEW OUTCOMES 33

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PREFACE

This document is an independent report of the *In Vitro* Pyrogenicity Peer Review Panel ('Panel') evaluation of the validation status of five *in vitro* test methods for pyrogenicity testing. The Panel was convened as a National Institutes of Health (NIH) Special Emphasis Panel by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to provide advice to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). This report summarizes the discussions, conclusions, and recommendations of the Panel's public meeting convened at the NIH in Bethesda, MD on February 6, 2007. ICCVAM and the ICCVAM Pyrogenicity Working Group (PWG) will consider the Panel report, along with comments from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and prepare final test method recommendations for U.S. Federal agencies. ICCVAM test method recommendations will be forwarded to U.S. Federal agencies for consideration and action, in accordance with the ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*-3, available at http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf).

The Panel considered five *in vitro* test methods submitted to ICCVAM by 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. ECVAM submitted background review documents (BRDs) for these test methods to ICCVAM for consideration as replacements for the rabbit pyrogen test (RPT) in June 2005. The proposed test methods are:

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

ICCVAM established an ICCVAM PWG to work with NICEATM to carry out the evaluation of these test methods. The ICCVAM PWG developed draft test method recommendations and questions for consideration by the Panel. The ICCVAM PWG also collaborated closely with ECVAM throughout the evaluation process to obtain additional information for consideration by the Panel and ICCVAM.

The Panel was provided a comprehensive draft BRD prepared by NICEATM in conjunction with the PWG and ICCVAM. The draft BRD provided all available data and information related to the five *in vitro* pyrogen test methods. The five ECVAM submitted BRDs (one for each test method), the ECVAM response to PWG questions, and other supplemental information (i.e., key references and testing guidelines/regulations for pyrogenicity testing) were appended to the draft BRD. All of the information provided to the Panel was also made

publicly available, and public comments were requested via a *Federal Register* (*FR*) notice (Vol. 71, No. 238, pp. 74533-74534, 12/12/06). The *FR* notice also announced the public ICCVAM independent peer Panel review meeting scheduled for February 6, 2007.

The Panel was charged with:

- Reviewing the ICCVAM draft BRD for completeness and to identify any errors or omissions in the draft BRD
- Evaluating the information in the draft BRD to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM 2003[1]) have been appropriately addressed
- Considering the ICCVAM draft test method recommendations for the following and commenting on the extent to which they are supported by the information in the draft BRD:
    - proposed test method uses
    - proposed recommended standardized protocols
    - proposed test method performance standards
    - proposed additional studies

At the Panel's public meeting on February 6, 2007, the Panel made recommendations for corrections and additions to the draft BRD and then discussed the current validation status of these five *in vitro* test methods. The Panel also commented on the ICCVAM draft test method recommendations for proposed test method uses, recommended standardized protocols, test method performance standards, and additional studies. The public was provided the opportunity to comment several times during the meeting. The Panel considered these comments as well as public comments submitted in advance of the meeting before concluding their deliberations.

The Panel gratefully acknowledges the efforts of NICEATM staff in coordinating the logistics of the peer review Panel meeting and in preparing materials for the review. The Panel also thanks Dr. Thomas Hartung (Head of ECVAM) for providing an overview of the test methods and for additional clarifications at the meeting. Finally, as Panel Chair, I want to thank each Panel member for their thoughtful and objective review of these test methods.

Karen Brown, Ph.D.
Chair, *In Vitro* Pyrogenicity Peer Review Panel
April 2007

[1]ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508. Research Triangle Park, NC. NIEHS. The guidelines can be obtained at http://iccvam.niehs.nih.gov/SuppDocs/SubGuidelines/SD_subg034508.htm.

EXECUTIVE SUMMARY

This report describes the conclusions and recommendations of the *In Vitro* Pyrogenicity Peer Panel ('Panel') regarding the validation status of five *in vitro* pyrogen test methods[1], and the ability of these test methods to individually serve as a substitute for the Rabbit Pyrogen Test (RPT) for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation. The test methods are:

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

Panel Recommendations for the ICCVAM Background Review Document

The Panel stated that, in general, the information presented in the ICCVAM draft Background Review Document (BRD) was sufficient for its purpose. Exceptions are included within the body of the Panel report. The Panel identified a number of sections where clarification or a more comprehensive explanation would improve the ICCVAM draft BRD. For example, the extent to which the RPT is currently performed when risk assessments and regulatory decisions are concerned only with the presence of endotoxin should be provided. Likewise, a more detailed review of the various mechanisms and processes thought to be involved in the actual induction of fever itself, and a more detailed description of the statistical approaches used to evaluate the resulting data would be helpful. The Panel stated that the rationale for the selected test substances was neither appropriate nor acceptable and they recommended the inclusion of non-endotoxin pyrogens, protein- and lipid-containing materials that are used parenterally, and 'classical' examples of biological products and medical devices. The Panel also requested that the formal validation statement from the ECVAM Scientific Advisory Committee (ESAC) (and the supporting documents) be appended to the ICCVAM BRD. The Panel agreed that a comprehensive summary of findings on overall conclusions about the usefulness and limitations of each of the *in vitro* pyrogen tests compared to the Bacterial Endotoxin Test (BET) or the RPT should be included in the ICCVAM final BRD.

With regard to animal welfare, the Panel suggested that the ICCVAM final BRD provide information on the number of rabbits used for pyrogenicity testing to permit an accurate assessment of the actual impact on animal use. The Panel recommended that the ICCVAM

[1]These test methods are referred to in this report as *in vitro* pyrogen tests in order to maintain consistency with the designation provided by the test methods' submitter (ECVAM). However, the Panel noted that this designation may be inappropriate because the usefulness and limitations for these test methods have been defined only for their ability to detect bacterial endotoxin and not other pyrogens.

final BRD discuss the practice of, and the U.S. Federal restrictions on, the reuse of rabbits in pyrogenicity testing, as well as the availability and use of the recombinant clotting factor C (rFC) that could replace the need for horseshoe crab hemolymph. The Panel also felt that the lack of direct parallel testing in rabbits with the products tested in the validation study was a significant limitation to the study design.

The Panel concluded that the cost and logistical considerations involved in conducting a study using the *in vitro* test methods were incompletely stated. The Panel recommended that a more detailed cost comparison for conducting the RPT and the *in vitro* test methods be performed. The Panel also commented that both the cost and logistical problems associated with the need to harvest and use human blood in four of the test methods were understated.

Validation Status of the *In Vitro* Pyrogen Test Methods

The Panel agreed that the applicable validation criteria have been adequately addressed in the ICCVAM draft BRD in order to determine the usefulness and limitations of these test methods to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation. However, the Panel generally agreed that the performance of these test methods in terms of their reliability and relevance did not support this proposed use. A minority opinion (Dr. Peter Theran) suggested that the qualification in the above statement (i.e., that uses were subject to product specific validation) should allow for these test methods to be used for the specified purpose. A second minority opinion (Drs. Karen Brown, Albert Li, and Jon Richmond) expressed concern that it is not clear that the qualification included in the above statement would preclude the use of the *in vitro* test methods as replacements for the RPT in those circumstances where the BET is currently serving to replace the RPT.

Review of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Draft Recommendations for Test Method Usefulness and Limitations

The Panel concluded that the available data and demonstrated performance of these five *in vitro* test methods, in terms of their relevance[2] and reliability[3], did not support the ICCVAM draft recommendations in terms of their usefulness and limitations. The Panel felt that the usefulness of these test methods for detecting Gram-negative endotoxin has not been properly assessed for concordance with the RPT or for relevance in comparison to the BET, and therefore, it was not possible to truly assess their usefulness and limitations.

One minority opinion stated (Dr. Peter Theran): This Panel has considered the failure to undertake additional RPTs a significant flaw in this validation study and therefore proposed that, in the future, similar validation studies should use the RPT to provide concordance data. I have no objection to the performance of *in vitro* tests in parallel with rabbit tests, which are already scheduled to be performed, in order to achieve concordance data. But, it is my

[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 inter-laboratory reproducibility and intralaboratory repeatability.

opinion, that any recommendation for *de-novo* parallel RPT should be accompanied by a statement, as follows: "The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)." The inclusion of this statement would reinforce the importance of the 3R's and would serve as a reminder of U.S. Federal law.

Review of the ICCVAM Draft Recommendations for Test Method Standardized Protocols

The Panel agreed that the information provided in the ICCVAM draft BRD supported the ICCVAM draft recommended protocols for these five *in vitro* test methods, providing that the list of inadequacies[4] identified by the Panel with respect to reliability and relevance are fully addressed.

Review of the ICCVAM Draft Recommendations for Test Method Performance Standards

The Panel did not support the statement that the available data and demonstrated performance in terms of relevance and reliability supported the ICCVAM draft recommendations for these *in vitro* test methods in terms of their performance standards. The Panel noted several inadequacies with regard to the essential test method components for each *in vitro* test method and agreed that the demonstrated performance of certain aspects of several of the assays, particularly in terms of relevance, yielded some concern. With regard to the minimum list of reference substances, the Panel agreed that if the intent of the proposal was to replace the RPT with one or more of the *in vitro* test methods under consideration, then the *in vitro* test methods must be validated for all classes of substances (e.g., pharmaceuticals, biologicals, and implants) and medical devices that are tested with the RPT.

The same minority opinion directed towards the issue of parallel testing using the RPT as detailed above was expressed.

Review of the ICCVAM Draft Recommendations for Future Studies

The Panel agreed that to better determine the relevance of these *in vitro* test methods, the proposed additional studies should be performed using the ICCVAM proposed protocols, taking into account the Panel's comments and recommendations. The Panel also agreed that if the intended use of the *in vitro* assays were only to detect Gram-negative endotoxin, it would seem critical to include parallel studies with the BET in any future validation efforts. However, if the intended use of the *in vitro* methods is to evaluate substances containing endotoxin that are unable to be evaluated with the BET, then the parallel testing studies should include the RPT. The Panel recognized that these test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.

[4]Based on the list of 20 separate inadequacies outlined in this report, three Panel members felt that this list would be better described as a list of "many and substantial" inadequacies.

The same minority opinion directed towards the issue of parallel testing using the RPT as detailed above was expressed.

OVERALL PEER REVIEW OUTCOMES

This international independent Peer Review Panel, consisting of 13 expert scientists from five different countries, provided comments and recommendations on the usefulness and limitations of five *in vitro* pyrogen test methods for the detection and quantification of Gram-negative endotoxin and on the ICCVAM draft test method recommendations on the use of these *in vitro* methods as partial replacements for the RPT. These remarks are summarized below.

- In general, the information presented in the ICCVAM draft BRD was sufficient for the purpose of determining the usefulness and limitations of these test methods for their proposed use and for adequately addressing the applicable validation criteria on the basis of the currently available evidence.
- The available data and demonstrated performance in terms of their reliability and relevance do not at this time support the ICCVAM draft proposed use for these test methods (i.e., as a partial substitute or replacement for the RPT, for the identification of Gram-negative endotoxin, on a case-by-case basis, subject to product specific validation). To better characterize the test methods and more clearly define their reliability and relevance, the Panel recommended that specific additional studies be performed using the ICCVAM proposed protocols, taking into account the Panel's comments and recommendations.
    - The lack of parallel testing in the *in vitro* tests and the RPT, and the resulting lack of concordance data, was considered to be a major limitation of the validation study design. For this reason, the Panel recommended that future studies include parallel testing. A minority opinion (Dr. Peter Theran) associated with parallel testing was expressed as follows: “The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)".
- The available data and demonstrated performance in terms of their reliability and relevance does not support the ICCVAM draft performance standards for these *in vitro* test methods for regulatory purposes.
- The information provided in the ICCVAM draft BRD supports the ICCVAM draft recommended protocols for these five *in vitro* test methods, providing that the list of inadequacies[5] identified by the Panel with respect to reliability and relevance are fully addressed.
- These test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.
- It is critical to recognize, despite concerns about the performance of these five *in vitro* test methods, that a formal process exists for materials regulated under 21 CFR 610.9

[5]Based on the list of 20 separate inadequacies outlined in this report, three Panel members felt that this list would be better described as a list of "many and substantial" inadequacies.

to qualify these *in vitro* methods for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation.

A. REVIEW OF THE VALIDATION STATUS OF *IN VITRO* PYROGEN TEST METHODS

1.0 INTRODUCTION AND RATIONALE FOR THE PROPOSED USE OF *IN VITRO* PYROGEN TEST METHODS[1]

1.1 Introduction

1.1.1 Is the historical background provided for the *in vitro* pyrogen test methods and the rationale for their development adequate?

Yes, the Preface, the Executive Summary and **Section 1.1.1** of the ICCVAM draft Background Review Document (BRD) are all informative, clear, and concise with the following exceptions:

1. The action of pyrogens on circulating cells and the mechanism by which the pro-inflammatory cytokines produce pyrexia should be considered in **Section 1.1.1** instead of in **Section 1.3.2**).
2. The reduction in the use of animals to test medicinal products produced under current Good Manufacturing Practices (GMP) is an obvious goal. However, no information is provided on the current use of the Rabbit Pyrogen Test (RPT) or the bacterial endotoxin test (BET) (i.e., the approximate number of rabbits and horseshoe crabs used each year for pyrogen testing), or of anticipated trends in their use, or of the extent to which the RPT is currently used in contexts where risk assessments deem endotoxin to be the only relevant contaminant.
3. On lines 694-696 of the ICCVAM draft BRD (December 1, 2006), it is stated that the proposed *in vitro* tests were selected for their ability to replace the RPT. In the previous paragraph, it is stated that the RPT is capable of detecting both endotoxin and non-endotoxin pyrogens. Elsewhere, it is noted that these *in vitro* tests have not been validated for detecting non-endotoxin pyrogens. If the aim of testing these materials with the RPT is to detect a range of pyrogens, then these assays cannot, on the basis of information supplied in the validation dossier, completely replace the RPT.
4. A more detailed review of the various mechanisms and processes thought to be involved in the actual induction of fever itself, particularly in the case of drugs that are not administered intravenously, would have been useful. A number of reviews on this subject describe a far more complex picture than presented. These additional references include:

   Netea et al. (2000) and Saper and Breder (1994).

[1]These test methods are referred to in this report as *in vitro* pyrogen tests in order to maintain consistency with the designation provided by the test methods' submitter (ECVAM). However, the Panel noted that this designation may be inappropriate because the usefulness and limitations for these test methods have been defined only for their ability to detect bacterial endotoxin and not other pyrogens.

1.1.2 Is the previous review of the ECVAM validation studies adequately summarized?
Yes, the previous review of the ECVAM validation studies was adequately summarized. The questions resulting from the initial review have been answered and included in the ICCVAM draft BRD. However, it would have been better if the actual ESAC validation statement in full had been appended, as well as any documents used to support the ESAC conclusion. The ECVAM BRDs (though not the ESAC statement) contain inconsistent text relating to the possible practical uses of the novel tests that the validation tests were intended to support.

1.2 Regulatory Rationale and Applicability

1.2.1 Are the current regulatory testing requirements and ICCVAM prioritization criteria adequately discussed and up-to-date?
Yes, the current United States (U.S.) and European Union (EU) regulatory testing requirements are properly referenced and the relevant documents have been supplied. The previous product specific acceptance of peripheral blood mononuclear cell (PBMC) data by the U.S. Food and Drug Administration (FDA) is also mentioned in the Executive Summary.

Inclusion of the following information would have been useful:

1. It should be stated whether the acceptance of the PBMC data by the FDA was a replacement for the BET or the RPT. The document 21 CFR 610.9 provides for the use of alternative methods to test for pyrogenic substances as long as the use of these methods does not compromise the safety, purity or potency of the product. The 1987 FDA guideline on the validation of the BET as an end-product endotoxin test for human and animal parenteral drugs also sets forth acceptable conditions for the use of the test *in lieu* of the RPT. However, no mention is made of the fact that the European Directorate for the Quality of Medicines (EDQM) also has a working party of experts (apparently independent of ECVAM and ESAC) reviewing the whole area of *in vitro* pyrogens tests and their potential use.
2. The ICCVAM final BRD should discuss the availability and use of the rFC that could replace the need for horseshoe crab hemolymph.
3. The ICCVAM draft BRD gives few insights into how any recommendations, following acceptance by the relevant agencies, would be incorporated into U.S. Pharmacopeia (USP) and European Pharmacopeia (EP) test requirements.

Specific comments on the five ICCVAM prioritization criteria outlined in the ICCVAM draft BRD:

Criterion 1 (Applicability to regulatory testing needs and multiple agencies/programs): It is clear that the test methods are relevant to the end-product testing of a variety of healthcare products (for endotoxin) and that the FDA is the principal U.S. regulator for such products.

Criterion 2 (Warranted, based on extent of expected use or application and impact): It is clear from the documents that this criterion is only met with respect to the detection of endotoxin.

Criterion 3 (Potential to address any/all of the 3Rs): The tests have the potential to reduce or replace animal use and the associated morbidity and mortality. However, no information is provided in the ECVAM BRDs or in the ICCVAM draft BRD to permit the actual impact on animal use to be accurately assessed.

Criterion 4 (Potential to provide improved prediction): The documents indicate that the level of protection provided by each of the *in vitro* test methods is equivalent to that provided by the RPT. However, in the original ECVAM BRDs, it is recognized that sensitivity may have been underestimated and specificity overestimated as a consequence of having one of the spiked-sample points set at the regulatory limit. On lines 777-784 of the ICCVAM draft BRD (December 1, 2006), the statement that these methods would better predict the human pyrogenic response than the RPT because they use human cells is not supported by test results in the ICCVAM draft BRD. In contrast, it is stated on lines 1299-1303 of the ICCVAM draft BRD (December 1, 2006) that the pyrogenic response to endotoxin in rabbits and humans is "similar in both species. Based on these studies, the rabbit is considered to be predictive of the human response (and may often overpredict the response)."

Criterion 5 (Other advantages): The new test methods clearly take longer to produce definitive results. However, no animal facility is required. It was a surprise (in the absence of definitive cost information) that the novel tests were considered to be potentially more expensive than the RPT. Contract research organizations should be consulted on potential cost comparisons, as wide acceptance of these methods may in part be cost-dependent.

1.2.2 Is the description of the intended uses of the *in vitro* pyrogen tests complete?

These methods are proposed as partial replacements for the RPT. The RPT detects both endotoxin and non-endotoxin pyrogens, but the *in vitro* pyrogen tests have not been validated for non-endotoxin pyrogens. Therefore, they cannot be considered complete replacements for the RPT.

It is not clear when, or in which situations, the *in vitro* pyrogen test methods would be appropriate for use. The BET detects endotoxin in most cases and is used instead of the RPT for this purpose. The application of the *in vitro* test methods for the detection of endotoxin in sample types that cannot be measured in the BET is plausible; however, this proposed use would represent a very limited application for the *in vitro* pyrogen tests.

1.2.3 Are the similarities and endpoints measured by the proposed test methods and the reference (RPT) test method adequately described and discussed?

Yes, although the exact causes of the endpoint of the RPT (i.e., fever) are relatively complex and unclear, it has been known for many years that cytokines, especially those involved in the inflammatory response (i.e., IL-1, IL-6, and TNF) can induce febrile reactions. The development of tests based on the production of such cytokines from human white blood cells or cell lines appears to correlate well with the induction of fever in both the RPT and humans. However, the RPT detects a whole organ/body fever response; whereas, the proposed test methods detect only cytokine secretion. Evidence to suggest that detection of IL-1 or IL-6 is necessarily an indication of a febrile reaction is lacking. Additional information should be included in the ICCVAM final BRD on the relationship between IL-1

or IL-6 levels produced in cultures of monocytes and the development of fever in humans. The fact that the cytokine profiles for different endotoxins may vary between rabbits and humans should also be considered.

1.2.4 Is the description of the use of the proposed test methods in an overall strategy of hazard or safety assessment adequate?

Yes, the utility of the *in vitro* pyrogen methods as an addition to the current RPT, especially where non-endotoxin pyrogens are involved, has been clearly discussed. No specific claims are made for an immediate replacement of the RPT, although future studies may lead to such an event. The overall demonstration of the applicability of the methods to non-endotoxin detection is a stated goal. However, this goal does not appear to adequately match the methods employed since non-endotoxin standards were not used. One information gap (in the ICCVAM draft BRD and ECVAM BRDs) is the extent to which the RPT is currently performed when risk assessments and regulatory decisions are concerned only with the presence of endotoxin (that is clearly the intention when only the BET is used). Product-by-product validation will be required and the full extent of materials for which the new tests are not suited remains to be defined.

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

1.3.1 Is the purpose and mechanistic basis of the *in vitro* test method(s) adequately described and compared to known and/or suspected mechanisms/modes of action for fever production in humans?

Yes, the purpose and mechanistic basis of the *in vitro* test methods appears to be adequate while acknowledging that, at this point, the reference standard included in the validation study was Gram-negative endotoxin only. The mechanisms underlying fever induction, including the production of cytokines involved in the inflammatory cascade, appear to be important. The administration of such cytokines can directly induce fevers and their levels have been shown to dramatically increase during fevers. However, the known and suspected mechanisms/mode of action of fever may be far more complex than that described (see also response to **Section 1.1.1**).

The claim in **Section 1.3.1** to 'identify pyrogens' should perhaps be restated to 'detect pyrogens.'

1.3.2 Are the known similarities and differences of modes of action between the *in vitro* pyrogen test methods and the fever response in human and/or rabbits adequately considered?

Yes, an extensive literature search was performed that covered a wide range of cases illustrating the similarities and differences between the modes of action between the RPT, the *in vitro* pyrogen tests, and the induction of fever in humans. The correlation, or lack thereof, between the tests and human fever induction has been discussed in a scientifically valid manner. It should be noted that the RPT has served as a good predictor of human pyrogen response. Although there are false positives and false negatives associated with the RPT, it is not clear that these proposed *in vitro* assays provide better, similar, or worse results. A major concern is the lack of validation of these new assays directly compared to the RPT.

The mode of action is oversimplified. See response to **Section 1.1.1**, especially the reference to Netea et al. (2000) that provides an excellent review on the multiple-pathway mechanisms

that link cytokine responses (some of which are monitored by the proposed *in vitro* assays) and fever production. Furthermore, no description of the mode of action at either the molecular or cellular level is presented, which may prevent an adequate comparison between the methods. Specific questions that should be addressed include:

1. Are there any scientific data that compare IL-1 and IL-6 production and fever response between humans and rabbits?
2. Is the induction of IL-1 and IL-6 (or even fever) similar or different between endotoxin and other known TLR-4 ligands?
3. What is the mechanism of action for pyrogens that do not utilize TLR-4?
4. TLR-4-mediated IL-1 mRNA induction and the consequent release of mature IL-1 from cells by stimuli other than pyrogens are regulated by different molecular mechanisms. Are these mechanisms similar or different *in vitro* and *in vivo*, or between humans and rabbits?

1.3.3 Is the range of substances amenable to the *in vitro* pyrogen test methods, and are the limits of the test methods adequately characterized?

Yes, given what is known of materials with the potential to interfere with the test system supplemented by the need for product-by-product validation and the exclusion of interference. More work will have to be carried out to understand the types of materials that could be tested in these assays and how they would be handled (e.g., cell therapies and implants). However, it must be considered that a manufacturer of a medicinal product would have to validate the *in vitro* method they have selected specifically for their particular product before it would acceptable to any regulatory authority. Thus, comprehensive testing of a wide variety of substances may not be necessary to introduce these tests into general use. Insufficient information exists at present to be confident that all types of materials that will demonstrate interference have been identified (e.g., materials that are cytotoxic, contain immunological adjuvants, or have antipyretic properties) but case-by-case evaluation provides the necessary safeguards. In addition, although the test methods have been shown to have the potential to identify non-Gram negative pyrogens, the validation study only presented detailed data and analysis with respect to the tests' potential to detect Gram-negative endotoxins (see also **Section 1.3.1**).

However, with respect to the limits of the test methods, no mention is made of the wide range of drugs that are toxic to blood cells or that induce a substantial pro-inflammatory response and consequently are not amenable to testing by these methods. Many pure, well-established non-endotoxin compounds have been shown to activate blood cells, including monocytic cells, to produce pro-inflammatory cytokines *in vitro* and *in vivo* (see suggested additional references [Ishii et al., 2005; Ishii and Akira, 2006] in **Section 12.0**).

On page 1-5, line 770 of the ICCVAM draft BRD (December 1, 2006) states, "Although the *in vitro* BET is performed using hemolymph (the equivalent of 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)". This concern has been largely solved with the commercial introduction of rFC, which was originally cloned from the horseshoe crab. This commercial product is currently being compared to the BET for submission for inclusion in the USP. A need for a

replacement for the RPT for early compound development testing and testing of biologics that have some propensity to harbor non-endotoxin pyrogens remains to be fulfilled. Thus, the goals of the overall effort need further refinement. Endotoxin is, of course, the important standard for validation purposes but non-endotoxin standards need to be characterized to further such a test for non-endotoxin testing; this concept is referred to on page 1-7, lines 821-822 of the ICCVAM draft BRD (December 1, 2006).

2.0 *IN VITRO* PYROGEN TEST METHOD PROTOCOL COMPONENTS

2.1 Overview of How the *In Vitro* Pyrogen Test Methods are Conducted

Are there gaps or missing information in the overview of how the tests are conducted?

This section seems adequate and complete. The overview of how the *in vitro* pyrogen tests are carried out is brief and to the point. The assays essentially expose human blood cells (either primary or cell line derived) to a test substance that may or may not induce cytokine release. Any cytokine release is subsequently detected with an immunoassay.

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

Are the description and rationale for each of the following test method protocol components for the recommended versions of the *in vitro* test methods adequately described and appropriate? Should any protocol components be modified, and, if so, why? Are additional protocol components needed, and, if so, why?

2.2.1 Materials, equipment and supplies

Specific concerns with respect to human blood donors include: diurnal variation, genetic polymorphisms (i.e., in genes coding for Toll-like receptors [TLRs], cytokine receptors, response elements, etc.), and number of donors required.

The effect of components in the blood and their effects on the assay systems are not clear (i.e., the effect of variations in the number of monocytes in peripheral blood, which range from 2 to 10%, as well as the effect of neutrophil or lymphocyte presence on the cytokine response).

2.2.2 Endpoint(s) measured

The viability of the human blood cells should be monitored before and after incubation with the test samples. Cytotoxic substances should not be tested with these methods.

2.2.3 Duration of exposure

A fixed exposure time rather than a broad range of exposure times (e.g., 16 to 24 hours) should be defined.

2.2.4 Known limits of use

It is suggested that the *in vitro* pyrogen tests are suitable for the testing of medical devices and materials by direct contact rather than testing extracts. However, direct contact may not adequately permit the solubilization or leaching of potential pyrogens.

2.2.5 Nature of the response assessed

The nature of the response assessed is accurately summarized. However, a description of the blood cell types known to respond to pyrogens by producing IL-1 and/or IL-6 should be included.

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

The ECVAM BRDs do not discuss why high quality Gram-positive material (Lipoteichoic acid [LTA]) available from the University of Konstanz was not also used as a 'model' pyrogen. The inclusion of such non-endotoxin positive controls would be useful in future validation studies to further characterize the usefulness and limitations of these methods for the detection of such substances.

2.2.7 Acceptable ranges of negative, vehicle, and positive control responses and the basis for the acceptable ranges, or procedures for establishing acceptable ranges

The ECVAM BRDs indicate that (refer to **Sections 6.1.1**), with hindsight, the use of an endotoxin spike solution at the threshold pyrogen dose (marking the pass/fail level for regulatory purposes) was not wise. See above (response to Criterion 4, **Section 1.2**) regarding possible relevance to determination of sensitivity and specificity of the novel test methods.

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

The description of the nature of the data to be collected and the methods used for data collection is accurate.

2.2.9 Type of media in which data are stored

The type of data storage media seems to fit the purpose. However, one printed version of the data should be stored.

2.2.10 Measures of variability

The description of the measures of variability reflects the current state of knowledge. Other relevant physiological variables may exist but the main sources of potential variation seem to have been addressed.

2.2.11 Statistical or nonstatistical methods used to analyze the resulting data

Generally adequate, but additional clarification is desired. It would seem appropriate to use a consistent approach across assays. For example, in some places, Dixon's test was used to identify outliers, while in others Grubb's test was used; the reasons and contexts for these differences are not apparent. However, it is accepted that minor problems arise with the calculation of sensitivity and specificity of the novel test methods from using a spike-point coincident with the regulatory limit.

The statement that "using an endotoxin curve, the endotoxin content of the product is calculated" is not true. The *in vitro* pyrogen test is not specific for Gram-negative endotoxin

and therefore, it is impossible to know whether the response measured is due to endotoxin or another pro-inflammatory response reactive substance in the sample.

2.2.12 Decision criteria and the basis for the prediction model used to classify a test substance as positive or negative for the presence of a pyrogenic material

The RPT data used to set the pass/fail criteria were produced in one rabbit strain in one laboratory and were not obtained concurrently within the validation study.

It is not clear that the criteria used to assign test results as positive or negative are based on the precise criteria set out in the USP. The significance of any deviations from these criteria is also not clear.

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

The descriptions provide a good overview of each test for the purposes of comparing and contrasting them with one another and with current methods.

2.3 Basis for Selection of Test Method Systems

Is the description of the basis for selection of the test method systems complete and appropriate?

A brief description of the advantages of each test method have been provided and are appropriate for considering the limitations of the existing tests for pyrogens, namely the RPT and the BET.

2.4 Proprietary Components

Are proprietary components appropriately identified (if applicable), and are the procedures adequate for ensuring their integrity from 'lot-to-lot' and over time?

The licensing procedure and availability of the Mono Mac 6 (MM6) cell line is unclear. Variations in the MM6 cell line (and primary cells) must be properly controlled. A direct comparison of the commercially available enzyme-linked immunosorbent assay (ELISA) kits should also be included in the ICCVAM final BRD.

2.5 Number of Replicates

Are the numbers of replicate and/or repeat experiments appropriate for each test method?

The appropriate number of donors from which to collect blood cells is unclear. Furthermore, some of the test methods permit pooling of blood donors while others do not. The rationale for these differences is unclear.

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

Are the protocol modifications based on ECVAM validation study results appropriate for each modified test method?

Yes, only minor modifications were made to two of the five assays, both to improve assay performance, and therefore the limited explanations are appropriate.

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

3.1 Rationale for the Substances or Products Selected for Testing

Is the rationale for the selected test substances appropriate and acceptable?

No, the only rationale given for the choice of test substances is that they represent marketed parenteral pharmaceuticals that were readily available at reasonable cost. According to their USP monographs, seven of the ten test substances are currently tested in the BET, not in the RPT. No USP monographs exist for the remaining three because pyrogen testing is not required. The inclusion of test substances that may interfere with the *in vitro* responses should be tested.

Although the test materials spiked with endotoxin are described as having been initially pyrogen-free and having been approved for clinical use, all that can be said with confidence is that they did not contain a level of pyrogen above the permissible or tolerable limit. As a result, in describing the concentration of endotoxin in the spiked sample, it is more correct to state the minimum level of endotoxin they were known to contain rather than offering an absolute value.

Non-endotoxin pyrogens should be evaluated because these pyrogens must be tested in the RPT and they cannot be tested in the BET. The list of test substances should also include protein- and lipid-containing materials that are used parenterally. No 'classical' examples of biological products or medical devices were included; thus, the validation for either of these categories has not been provided.

Although it is stated that endotoxin was chosen as a model pyrogen, insufficient information exists in the ICCVAM draft BRD or in the supporting ECVAM BRDs to support this claim. The validation study documents, the ESAC validation statement and the ICCVAM draft BRD claim only that the test methods are suited for the detection/qualification of Gram-negative endotoxin for regulatory testing.

3.2 Number of Substances

Please comment on the adequacy of the number of substances used in the performance analyses.

The total number of substances included in the validation study is adequate only for validation of a specific class of products. Replacement of the RPT would require a much larger number of substances because of the wide range of product classes that would require testing. Moreover, the test substances should have represented each of the major classes of

products normally tested in the RPT (e.g., medical devices, biologicals, implants, and those substances known to interfere with the RPT, the BET, and/or the *in vitro* pyrogen tests) as positive controls for interference testing.

3.3 Identification and Description of Substances Tested

Are the test substances adequately identified and described?

The samples included in the validation process are adequately identified and described such that they could be readily obtained for future studies. However, more information on their purity and batch/lot numbers is needed in order to adequately demonstrate that the same substances were tested throughout the validation studies. In response to a request for additional information, ECVAM did provide the lot numbers used in the validation study, which demonstrated that they were identical. However, some differences in the lots tested in the catch-up validation study were noted (e.g., two of the ten substances had different lot numbers due to the lack of availability; one was a different substance with the same active ingredient).

3.4 Sample Coding Procedure

Were the coding procedures used in the validation studies appropriate?

The coding procedures were adequate for the assessment of relevance during the validation studies. However, the identity of the substances used in the reproducibility analyses was not blinded (although the spike concentrations were). A reason was not given.

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

Are the *in vivo* reference data used in the validation study appropriate to allow for adequate assessment of test method relevance[2] (accuracy/concordance, sensitivity, specificity, false positive and false negative rates) of these *in vitro* pyrogen test methods as a partial replacement for the RPT, for materials which may be contaminated with gram-negative endotoxin, but which cannot be tested by the BET?

No, a summary of the reference data demonstrating whether substances that were shown to be pyrogenic in humans either passed or failed the RPT, BET or *in vitro* pyrogen tests would have been useful.

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

Is the RPT protocol used to generate reference data for the cited studies appropriate?

[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.

The RPT protocol and the pass-fail criteria used would not meet the current USP requirements. The significance of these deviations is not clear. The data are derived from a single study carried out at the Paul Ehrlich Institute (PEI) where historical controls tested over five years were accumulated and analyzed. The protocols used at the PEI were based on the EP monograph for the RPT, although this fact is not explicitly stated in the publications.

Furthermore, the detailed protocol used by this laboratory was not provided.

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

Is the interpretation of the reference data used to assess *in vitro* test method accuracy correct? Is any other data or information needed to determine the accuracy of the test methods?

The reference data were previously and separately generated by one protocol, in one laboratory, using one strain of rabbit, and two sources of endotoxin. A second study, undertaken in Brazil, is cited. The response criteria of the Brazilian study do not match those of the PEI study. It is not clear why the Brazilian study was not relied upon for the validation study.

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

Are there any concerns with the availability of the original reference data records as described?

The data were derived from a single study at the PEI and presented in graphical form. No additional data were available for analysis. Archived records have not been audited by ECVAM or ICCVAM.

4.4 *In Vivo* Data Quality

Are there any concerns with the RPT data quality?

The ECVAM documentation is not sufficiently specific and in the absence of the primary data, the quality of the RPT data is unknown. The ICCVAM draft BRD does not clearly indicate the GLP status of the laboratory or of the study. However, the PEI did not have formal GLP accreditation (refer to **Section 5.5**, ECVAM response to a request for additional information).

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

Is the discussion of the availability of relevant pyrogenicity information for humans adequate and appropriate? Are there other sources of quality human data for pyrogenicity that should be considered? Would human data be compatible with regulatory needs (e.g., exposure duration, individual sensitivity)?

The available data are limited. However, the availability of information on clinical adverse events resulting from the administration of medical products producing pyrogenic effects, and the relevant pre-clinical test data, would be an excellent source of human data. See

suggested additional reference (McKinney et al. 2006). The data would reflect responses seen using appropriate human exposure; thus, it should be compatible with regulatory needs.

A discussion of relevant pyrogenicity information for humans is present in the ICCVAM draft BRD, but additional information is needed. An extensive literature on acute human pyrogenicity responses exists and this data should be better reviewed. Effects of longer exposure and individual sensitivity are available in Rylander (2002).

The data in the cited paper by Greisman and Hornick (1969)[3] are not accurately described (page 4-6, lines 1299-1301 of the ICCVAM draft BRD [December 1, 2006]).

4.6 Information on the Relevance and Reliability of the *In Vivo* Test Methods

Is what is known about the relevance and reliability[4] of the RPT adequately discussed and appropriately considered?

The appropriateness of the theoretical assumption model is unclear. It is not clear how the sensitivity and specificity values have been derived using this model. Therefore, reference to these values as accurate figures (particularly with respect to the 58% sensitivity) is a concern.

The theoretical sensitivity and specificity for the RPT that has been supplied does not seem to reflect its performance in practice or the regulatory decisions and level of patient safety that RPT data currently supports.

The 'correct' figures for the theoretical specificity of the RPT are confusing. It was stated to be 83% in **Section 4.6** of the ICCVAM draft BRD (December 1, 2006) but given as 88.3% in the ECVAM response to ICCVAM questions (page 24).

However, this difference has little bearing on the overall interpretation of the results.

5.0 Test Method Data and Results

5.1 Test Method Protocol

Are the *in vitro* test method protocols used to generate each set of data considered in the ICCVAM draft BRD appropriately described?

The following problems with all five *in vitro* test method protocols were noted:

1. Quality control (QC) testing of cell viability is not performed. Viability testing of the human cells before and after incubation should be performed.
2. No microscopic examination for anticipated levels of cell fragments and debris is described.
3. Substances should not be tested at cytotoxic concentrations by these methods.

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[3] Greisman SE, Hornick RB. 1969. Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. Proc Soc Exp Bio Med 131(4):1154-1158.

[4] 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 inter-laboratory reproducibility and intralaboratory repeatability.

4. More detailed source information and the pyrogen status (i.e., pyrogen-free) should be required for all protocol components.
5. A description of the procedure used for donor recruitment and donor selection is not provided.
6. A description of the protocols used for preparation of blood samples for the cytokine assays is not found.

The following problems with specific test method protocols were noted:

1. In the Cryo WB IL-1 assay, the incubation of the test sample is performed in the presence of 10% DMSO (methods for its removal after thawing of the cryopreserved cell preparation and before its use are not described). DMSO is known to effect the detection of certain cytokines. In response to a request for additional information, ECVAM indicated that the DMSO is not removed.
2. A limit to the passage number should be defined for the MM6 cell cultures.
3. The use of the terms RPMI-M and RPMI-C (described in the ECVAM MM6/IL-6 Standard Operating Procedure [SOP]) is confusing in the ICCVAM protocol.
4. A typographical mistake appears in the ICCVAM MM6/IL-6 protocol (lines 285 and 286 of the ICCVAM draft BRD [December 1, 2006]) where 'FBS' is stated instead of 'PBS'.

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

Has the availability of the original data use in the test method performance evaluation been adequately described?

Yes, the availability of the original source data has been adequately described.

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

Are the statistical and non-statistical approaches used in each cited study to evaluate the *in vitro* test results appropriate? What other approaches could have been used?

The statistical approaches appear adequate. However, it is suggested that more emphasis should have been placed on a quantitative estimate of pyrogen concentrations rather than dichotomizing results based upon a hypothesis test. One would have expected to see *a priori* criteria for successful validation in terms of acceptable performance statistics.

The term 'correlation' appears to be used colloquially (e.g., lines 1365 and 1373 of the ICCVAM draft BRD [December 1, 2006]); a correlation is not a percentage. Therefore, 'correlation' should be replaced with 'association' everywhere, except when Pearson's correlation is being referenced.

Information on the identification and elimination of aberrant data from **Section 4.2** of the Trial Data report should be included in the ICCVAM final BRD.

5.4 Summary of Results

Is the summary of the results for each test method appropriate and adequate?

No data were presented to confirm that results in the *in vitro* tests reflect human physiological responses or that production of IL-1 or IL-6 *in vitro* correlates with pyrogenicity *in vivo*. A quantitative link between IL-1 and IL-6 concentrations and their donor-to-donor variation with physiological effects was not presented. It should be mentioned that according to Schindler et al. (2006)[5], which describes the validation of the Cryo WB/IL-1 method, testing problems existed with many of the products included in the study (up to 9 of 10). This is evident by failure of the positive product control (PPC), which under normal circumstances would invalidate the test. Instead, when the PPC failed, the authors report that the saline control was used in place of the PPC and the experiment was still considered acceptable. This practice is unacceptable.

The lack of direct parallel testing in rabbits with the products tested in the validation study prevents an evaluation of actual physiological effects. It also would have been of assistance to the Panel if information had been provided to document that the use of human cells could partially replace the BET and RPT for the detection of substances that are pyrogenic in humans.

Some of the data (or lack thereof) indicate significant limitations of the *in vitro* assays. Specific examples are listed below:

1. In the ICCVAM draft BRD (December 1, 2006), page 2-7, line 989: The use of a single donor in the WB/IL-1 assay is inadequate.
2. In the ICCVAM draft BRD (December 1, 2006), page 2-10, line 1050: There are no data offered to document that the use of human cells will better reflect human physiological responses or that production of IL-1 or IL-6 *in vitro* correlates with pyrogenicity *in vivo*.
3. In the ICCVAM draft BRD (December 1, 2006), page 6-2, line 1456: 20 of 150 runs in the Cryo WB/IL-1 assay were not usable. Even then, the false positive rate of the remaining 120 assays was 18.6%.
4. In the ICCVAM draft BRD (December 1, 2006), page 6-4, line 1493: 1 of the 3 validation laboratories had a 50% false positive rate for the PBMC/IL-6 assay.
5. In the ICCVAM draft BRD (December 1, 2006), page 7-7, Table 7-4: Agreement across three validation laboratories was only 57% for the WB/IL-1 assay.

[5] Schindler S, Spreitzer I, Löschner B, Hoffmann S, Hennes K, Halder M, Brügger P, Frey E, Hartung T, Montag T. 2006. International validation of pyrogen tests based on cryopreserved human primary blood cells. J Immunol Methods 316:42-51.

5.5 Use of Coded Chemicals and Compliance with GLP Guidelines

For each set of data for each test method, is whether coded substances were tested and whether experiments followed GLP Guidelines adequately documented?

The use of coded substances is adequately documented, but the rationale for not blinding the identity of the three substances tested in the reliability analyses is not known. The *in vitro* pyrogen test studies were conducted 'in the spirit of' GLP requirements. However, gaps and lapses in the information supplied by ECVAM would indicate that none of the testing laboratories were audited in real-time. In response to a request for additional information from ECVAM, it was stated that:

> "The initial validation study has been carried out to large extent in laboratories such as National Control laboratories, which do not operate under GLP. It was, however, agreed to comply with the requirements of GLP, especially with regard to the creation and management of SOPs. The partner laboratories have received presentations on the requirements. No auditing was done but various quality checks and blinding mainly under the responsibility of ECVAM were included. In the catch-up validation two GLP laboratories and two National Control laboratories participated."
>
> "Raw data: In both studies, the laboratories were asked to transfer readings into Excel sheets provided by the statistician. This was mostly done by directly inserting the ASCII files created by the plate reader. However, reader printouts are available and can be provided on request."

5.6 Lot-to-Lot Consistency of Test Substances

Is the information on the 'lot-to-lot' consistency of the test substances, the time frame of the various studies, and the laboratory in which the study or studies were conducted, adequately described?

Information on specific lots used in the validation studies was not provided in the ICCVAM draft BRD and therefore, lot-to-lot consistencies cannot be evaluated. Additional information has been received to demonstrate that the same lots were tested in the validation study, but there were lot differences in 2 of 10 substances used in the catch-up validation study. In addition, because one of the substances used in the original validation was no longer available, a different substance (with the same active ingredient) was used in the catch-up validation.

Unfortunately, little or no high concentration protein samples (e.g., Factor VIII concentrates or 5-25% human albumin samples), where lot-to-lot inconsistencies might be expected, were tested in the validation studies. This exclusion was explained to some extent by ECVAM in the responses that they provided to the ICCVAM/PWG questions. Interference testing for all sample types should be tested on multiple lots (also see the specific inadequacy [No. 10, lines 1361-1362] noted in the proposed test method standardized protocols).

6.0 RELEVANCE OF THE *IN VITRO* PYROGEN TEST METHODS

6.1 *In Vitro* Pyrogen Test Method Relevance

Has the relevance (e.g., accuracy/concordance, sensitivity, specificity, positive, and negative predictivity, false positive and false negative rates) of the *in vitro* test methods for detection of pyrogens, as defined by statutes in the United States Code (see Section 1), or for sterility testing defined by the U.S. Pharmacopeia or the International Standards Organization, been adequately evaluated? Are the discussions of the relevance of each *in vitro* test method and the reference test method appropriate and accurate?

In general, the evaluation of the relevance of the *in vitro* pyrogen tests appears to have been appropriately demonstrated and discussed, but limited by the ability to judge a positive versus negative response using a cut-off at 0.5 endotoxin units (EU)/mL. Furthermore, because only endotoxin-spiked samples were tested, relevance has been demonstrated only for the detection of bacterial endotoxin.

This section is entirely focused on comparisons between the *in vitro* pyrogen test methods since the RPT was not carried out in parallel, but rather estimates of the RPT performance were modeled statistically. The validity of this approach remains in question due to the nature of the RPT, where a definitive cut-off point does not exist, but was defined based on the results generated from the historical database. Therefore, no data exist with which to establish concordance with the RPT and thus, the discussion on concordance with the RPT is speculative.

Discrepancies between Table 6-1 and the accompanying text of the ICCVAM draft BRD (December 1, 2006) for the cryopreserved PBMC assay prevented assessment of this method.

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

Is the summary of the performance of the test methods adequately described? Are the strengths and limitations of each *in vitro* test method adequately identified?

A more critical description and explanation are needed (i.e., a failure of the prediction model or a failure of the assay to correctly detect the pyrogen concentration) for the cases where the test failed to correctly classify the pyrogen concentration.

The discussion of the strengths and limitations of each of the test methods should be expanded. Specific points include:

1. Inadequate performance is noted for: a) Cryo WB/IL-1 (false positive rate = 18.1%); b) WB/IL-1 (false negative rate = 27.3%); c) WB/IL-1 (false positive rate = 16.4%). High false positive rates are clearly a concern for manufacturers since lots may be unnecessarily withheld from release.
2. The high exclusion rate for individual runs in the case of the Cryo WB/IL-1 test (20% - 30% out of 150 runs) due to excessive variability among the four replicates, even with a relatively high coefficient of variation (CV) criteria (CV > 45%).

3. The low sensitivity (only 72%) for the WB/IL-1 assay, resulting in an extremely high false negative rate (27.3 %). High false negative rates would obviously be a major concern, as endotoxin-contaminated lots would be released.

Taken together, these statements could indicate that the WB/IL-1 assays (WB/IL-1 Cryo WB/IL-1, and WB/IL-1 96-well plate method) do not, in general, perform as well as the other assays that measure an IL-6 response.

It would have been very interesting to have had the opportunity to compare performance analysis data for the BET, since only endotoxin spiked samples were used in the validation and endotoxin testing is now the intended use for the *in vitro* pyrogen tests. Unfortunately, the BET was not performed in the validation so no direct comparison can be made between it and the new *in vitro* assays.

7.0 RELIABILITY OF THE *IN VITRO* PYROGEN TEST METHODS

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

Is the selection rationale and the number and types of substances used to evaluate the reliability of the *in vitro* test methods (intralaboratory repeatability and intra- and inter-laboratory reproducibility) as well as the extent to which the chosen set of substances represent the range of possible test outcomes appropriate?

The use of a standard material such as the endotoxin control (WHO-LPS, 94/580) is a valid choice for conducting the experiments described since it is a well-characterized, well-documented material. However, the rationale for the selection of the drugs used in the studies for evaluating reproducibility versus sensitivity/specificity is not clear, except that they were manufactured under GMP, were licensed products, were reported not to be contaminated with unacceptable levels of endotoxin, and were all available at reasonable cost. It would have been more appropriate to evaluate reliability using a subset of the drugs used in the sensitivity/specificity studies.

7.2 Analysis of Repeatability and Reproducibility

Are the analyses and conclusions regarding the intralaboratory repeatability and reproducibility and the intra- and inter-laboratory reproducibility of each test method appropriate? Should other analyses be considered?

The experiments performed to evaluate intralaboratory repeatability and intra- and inter-laboratory reproducibility were overly complicated. However, the analysis based on 'positive or negative' calls suggests that the reliability of these *in vitro* test methods are generally acceptable both within and between laboratories, although a more critical description is needed to explain the lack of agreement among some test results.

It is interesting that the variability of the cell line-based MM6 assay is much reduced compared to that obtained for the whole blood assays, although this observation did not translate into an improved ability to assign a negative or positive status to a sample.

The following deficiencies were noted:

1. More discussion is needed about the use of a coefficient of variation (CV) analysis to evaluate the reliability of the *in vitro* test methods, including how an 'acceptable' CV was identified (e.g., 45% in the WB/IL-1 assay) and why the criteria for an acceptable CV was inconsistent among the different *in vitro* test methods.
2. It is not clear which statistical test(s) was used to detect outliers and whether the test(s) was based on original or log-transformed data. Furthermore, it is not clear how many data points were identified as outliers and how they were subsequently handled during data analysis. The information provided by ECVAM addressing these concerns should be integrated into the ICCVAM final BRD.
3. A quantitative assessment of the intra- and inter-laboratory variability would have been more informative than an assessment based on dichotomizing the test results. The assessment should have included estimates of the amount of inter- and intra-laboratory variability and the number of replicates needed to estimate the sources of variability. Consistent with general practice, acceptable levels of variability should have been identified *a priori*, and it should have been recognized that formal hypothesis testing is essential with the alternative hypothesis being no different between groups.
4. Potential problems related to plate-to-plate variation and/or other plate design issues should be addressed in the ICCVAM final BRD.
5. The use of the term 'mean value calculated' needs to be clarified.
6. It is misleading to state that the test substances were spiked at four concentrations when two of the spikes are at the same concentration. The concentrations should be noted explicitly, even in summaries if this is their first reference.
7. The ICCVAM final BRD should state whether or not the data were log-transformed prior to analysis (as was stated in the ECVAM BRDs). Furthermore, in the ECVAM BRDs, the decision rationale for performing a log transformation versus a square-root transformation of the data should be provided. In all ECVAM BRDs, it is not clear whether all analyses used log-transformed data or if transformed data were used only for the *t*-test in the classification phase of the analysis (e.g., ECVAM BRD for WB/IL-1, page 25).
8. The ECVAM BRDs state that all data are log-transformed, but the y-axis on the graphs is labeled OD 450 (e.g., ECVAM BRD for Cryo WB/IL-1, Appendix D). The data should be log-transformed if this has not yet been done. The CV after transformation is of most interest; however, the figures appear to give data before the transformation indicating that the variance increases with the mean. Data after the transformation should also be plotted to show that the relationship of the mean and the variance is well suited to the log transformation. The analysis with respect to the transformation needs to be

clarified. The values on the x-axis are unreadable and need to be given in the legends or in the description that accompanies each figure.

9. The notation used in the *t*-test (e.g., the subscripts on the population and sample means) needs to be defined. In the standard two-sample *t*-test, the groups are assumed to be independent. However, it looks like one group is a collection of subgroups and the other group is one of these (i.e., the data from one group are used in the calculation of both means). This point needs to be clarified.

7.3 Historical Positive and Negative Control Data

Is the availability of historical negative and positive control data adequately considered?

The fact that the *in vitro* pyrogen test methods are not in routine use except for the two manufacturers cited (who are unlikely to provide what would be considered proprietary data) leads to a paucity of historical data.

8.0 TEST METHOD DATA QUALITY

8.1 Adherence to National and International GLP Guidelines

Is the extent of adherence to national and international GLP guidelines for all submitted *in vitro* and *in vivo* test data and the use of coded substances and coded testing adequately presented?

It is clear that SOPs exist and that protocols were developed for all *in vitro* experiments performed. However, the precise GLP status of the studies and the test laboratories is not clearly stated and the ICCVAM final BRD should be revised to clarify this information. The *in vivo* data are derived from a single published study.

8.2 Data Quality Audits

Are the results of any data quality audits, if conducted, adequately summarized?

From the information provided, it would seem that no audits were undertaken while the studies were in progress. However, the ECVAM BRDs state that 'deviations' were recorded but no further details or information is provided. A summary of the GLP deviations that occurred would have been useful for determining their overall significance to the experimental outcome.

8.3 Impact of Deviations from GLP Guidelines

Does the lack of an evaluation of the impact of deviations from GLP guidelines affect the data analysis?

This question cannot be answered, as no data have been provided on any deviations from GLP guidelines.

8.4 Availability of Laboratory Notebooks or Other Records

Is the availability of laboratory notebooks or other records for an independent audit adequately discussed?

Yes, the study authors state that all raw data are available for inspection and have been archived appropriately.

9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS

9.1 Have Relevant Data Identified in Other Published or Unpublished Studies Conducted Using the *In Vitro* Test Methods Been Adequately Considered?

Although an extensive literature has been cited and discussed, no attempt at a comprehensive summary of findings or overall conclusions about the relevance of the *in vitro* pyrogen tests compared to the BET or the RPT, or the advantages/capabilities or disadvantages/limitations of the individual *in vitro* assays, has been presented in the ICCVAM draft BRD.

The following additional references should be included (see **Section 12.0** for full citations**)**:

1. Marth and Kleinhappl (2002). The studies described here indicate the importance of monitoring multiple pro-inflammatory cytokine responses. In the specific case cited, the TNF-α pro-inflammatory cytokine response appeared to correlate best with fever.
2. Norata et al. (2005), van Deventer et al. (2000), von Aulock et al. (2003) are relatively new studies that evaluate the effects of genetic polymorphisms on TLR-4 responses.
3. Martis et al. (2005). This paper describes a situation where the PBMC/IL-6 assay was used to help resolve a non-febrile adverse drug reaction issue with a licensed product.

9.2 Are the Conclusions Published in Independent Peer-Reviewed Reports or Other Independent Scientific Reviews of the *In Vitro* Test Methods Adequately Discussed and Compared?

Yes, the conclusions are adequate for the published data.

The formal ESAC validation statement and other EU validation expert/panel process documents should be appended to the ICCVAM final BRD.

9.3 Are There Other Comparative *In Vitro* Test Method and RPT Data That Were Not Considered in the ICCVAM draft BRD, But are Available for Consideration?

It is known that manufacturers have parallel test result data for the BET and RPT for specific products, which unfortunately are not published or peer reviewed. As a consequence, a number of companies are now advocating that they should be permitted to use the BET as an alternative to the RPT to detect the presence of Gram-negative endotoxin on a case-by-case basis, such as for testing of established products with documented proof that safe, reliable and consistent GMP production and QC procedures are in place.

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

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

Is the extent to which the *in vitro* test methods will refine (reduce or eliminate pain or distress), reduce, or replace animal use in the RPT adequately described?

No numbers are included regarding the current number of rabbits used and/or killed with this test. These estimates would be helpful when assessing the potential impact of these *in vitro* tests. However, given that the proposed use for these test methods is very limited, it is not clear that their application would have a significant impact on animal numbers.

The ICCVAM final BRD should discuss the practice of, and the U.S. Federal restrictions on, the reuse of rabbits in pyrogenicity testing.

A discussion on the ethical cost of conducting concurrent RPT testing should be added.

10.2 Requirement for the Use of Animals

Is the discussion of the use of cultured human cells and the need for volunteers for donations of peripheral blood used in the *in vitro* test methods appropriate and adequate?

No, the licensing arrangements and the maintenance of the MM6 cell line are unclear.

The discussion that reduction of the use of animals (i.e., rabbits) will be associated with the increased use of another animal (i.e., humans) is inadequate.

11.0 PRACTICAL CONSIDERATIONS

11.1 Transferability of the *In Vitro* Pyrogen Test Methods

Are the following aspects of *in vitro* test method transferability, including an explanation of how this compares to the transferability of the RPT, adequately described with regard to the:

11.1.1 Facilities and major fixed equipment needs?

Yes, either a sterile tissue culture facility or a laboratory animal facility is needed.

11.1.2 General availability of other necessary equipment and supplies?

Yes, equipment and supplies for both *in vitro* and *in vivo* studies are routinely available. In general, the skills and kits required are available in most diagnostic and testing facilities.

The availability (in ready to use kit form), the convenience, and the lower costs of the BET will mitigate against widespread use of the *in vitro* pyrogen tests that are far more work intensive (e.g., cytokine and endotoxin standard curves must be established, tests must be performed in quadruplicate, multiple donors are required), less convenient (as yet only one of the assays is available in kit form), and probably associated with higher costs.

11.1.3 Nature of the drug substance tested?
Yes, the drug substances are adequately described. The overall requirements for the assays are comparable with most other types of *in vitro* QC diagnostic assays.

11.2 Personnel Training Considerations

Are the following aspects of the *in vitro* test method training adequately considered? Is the explanation of how this compares to the level of training required to conduct the RPT adequate with respect to:

11.2.1 The required level of training and expertise needed to conduct the test method?
Yes, the individual technical steps and competencies are common to many other laboratory activities.

11.2.2 Any training requirements needed for personnel to demonstrate proficiency and any laboratory proficiency criteria that should be met?
The training required for adequate conduct of biological assays cannot be overestimated. Aseptic tissue culture techniques are essential, as is the ability to accurately serially dilute material. It is necessary to maintain the MM6 cell line and functional and non-activated monocytes obtained from whole blood. Activation can be caused by physical disruption or contaminants. Competency in each of these techniques should be demonstrated prior to allowing personnel to carry out these tests on medicinal products intended for human use or for certification. It should also be noted that the required expertise needed does not typically reside in the laboratories that conduct the test (i.e., RPT) targeted for replacement by the proposed *in vitro* tests.

11.3 Cost Considerations

Is the cost involved in conducting a study using the *in vitro* test method, as compared to the cost of conducting the RPT, adequately evaluated, and is this considered to be cost-effective compared to the *in vivo* method?

No, the direct and indirect costs of operating an animal facility that would be needed to house rabbits are incompletely stated. The *in vitro* pyrogen tests would seem to be considerably more cost effective than the RPT. It would be interesting to see pricing costs from contract research organizations for both classes of tests, mindful that cost considerations will impact on the level of use.

11.4 Time Considerations

Is the amount of time needed to conduct a study using the *in vitro* test method as compared to the time it takes to conduct the RPT adequately evaluated, and is the *in vitro* test method considered to be time-effective compared to the *in vivo* method?

The *in vitro* pyrogen test methods require two days to complete (twice as long as the BET and RPT under normal circumstances). Furthermore, the *in vitro* pyrogen test methods are dependent on the availability of donors or blood supplies, which might further restrict the frequency to which these tests can be performed.

12.0 RECOMMENDED ADDITIONAL REFERENCES

Are all relevant publications referenced in the ICCVAM draft BRD? If not, what additional references should be included?

The following references should be included:

Barnett V, Lewis T. 1984. Outliers in Statistical Data, 3rd ed. In: Wiley Series in Probability and Mathematical Statistics. (V Barnett, T Lewis, eds). New York:John Wiley & Sons.

Brunson KW, Watson DW. 1974. Pyrogenic specificity of Streptococcal exotoxins, Staphylococcal enterotoxin, and Gram-negative endotoxin. Inf Immun 10(2):347-351.

Burrell R. 1994. Human responses to bacterial endotoxin. Circ Shock 43(3):137-153.

Dinarello CA. 2004. Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. J Endotoxin Res 10(4):201-222.

Dixon WJ. 1950. Analysis of extreme values. Ann Math Stat 21(4):488-506.

Gaines Das RE, Brügger P, Patel M, Mistry Y, Poole S. 2004. Monocyte activation test for pro-inflammatory and pyrogenic contaminants of parenteral drugs, test design and data analysis. J Immunol Methods 288(1-2):165-177.

Grubbs FE. 1950. Sample criteria for testing outlying observations. Ann Math Stat 21(1):27-58.

Hochstein HD, Fitzgerald EA, McMahon FG, Vargas R. 1994. Properties of US standard endotoxin (EC-5) in human male volunteers. J Endotoxin Res 1(1):52-56.

Ishii KJ, Coban C, Akira S. 2005. Manifold mechanisms of toll-like receptor-ligand recognition. 2005. J Clin Immunol 25(6):511-521.

Ishii KJ, Akira S. Innate immune recognition of, and regulation by, DNA. 2006. Trends Immunol 27(11):525-532.

Marth E, Kleinhappl B. 2002. Albumin is a necessary stabilizer of TBE-vaccine to avoid fever in children after vaccination. Vaccine 20(3-4):532-537.

Martich GD, Boujoukos AJ, Suffredini AF. 1993. Response of man to endotoxin. Immunobiol 187(3-5):403-416.

Martin MA, Roberts S. 2006. An evaluation of bootstrap methods for outlier detection in least squares regression. J Appl Stat 33(7):703-720.

Martis L, Patel M, Giertych J, Mongoven J, Tammine M, et al. 2005. Aseptic perontonitis due to peptidoglycan contamination of pharmacopoeia standard dialysis solution. Lancet 365:588-594.

McKinney BA, Reif DM, Rock MT, Edwards KM, Kingsmore SF, Moore JH, Crowe Jr JE. 2006. Cytokine expression patterns associated with systemic adverse events following smallpox vaccination. J Inf Dis 194(4):444-453.

Mullington J, Korth C, Hermann DM, Orth A, Galanos G, Holsboer F, Pollmächer T. 2000. Dose-dependent effects of endotoxin on human sleep. Am J Physiol Regulatory Integrative Comp Physiol 278(4):947-955.

Netea MG, Kallberg BJ, van der Meer JWM. 2000. Circulating cytokines as mediators of fever. Clin Inf Dis 31(Suppl 5):S178-S184.

Norata GD, Garlaschelli K, Ongari M, Raselli S, Gigore L, Benenuto F, Maggi FM, Catapano AL. 2005. Effect of Toll-like receptor 4 (TLR-4) variants on intima-media thickness and monocyte-derived macrophage response to LPS. J Int Med 258(1):21-27.

Rylander R. 2002. Endotoxin in the environment. J Endo Res 8(4):241-252.

Saper CB, Breder CD. 1994. The neurological basis of fever. New England J Med 330(26):1880-1886.

van Deventer SJH, Buller HR, ten Cate JW, Aarden LA, Hack EC, Sturk A. 1990. Experimental endotoxemia in humans: Analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. Blood 76(12):2520-2526.

van Deventer SJH. 2000. Cytokine and cytokine receptor polymorphisms in infectious disease. Intensive Care Med 26(Suppl 1):S98-S102.

von Aulock S, Schroder NWJ, Gueninzius K, Traub S, Hofffmann S, Graf K, Dimmeler S, Hartung T, Schumann RR, Hermann C. 2003. Heterozygous Toll-like receptor 4 polymorphism does not influence lipopolysaccharide-induced cytokine release in human whole blood. J Inf Dis 188(6):938-943.

13.0 Summary of Validation Status of the *In Vitro* Pyrogen Test Methods

Does the Panel agree that the applicable validation criteria have been adequately addressed in order to determine the usefulness and limitations of these *in vitro* test methods, to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation?

Yes, the information is adequate with which to make an informed decision.

Does the Panel agree that the performance of these test methods in terms of their reliability and relevance support the proposed use of these test methods (i.e., the detection of Gram-negative endotoxin in materials that are currently tested in the RPT, subject to product specific validation to demonstrate equivalency to the RPT)?

No, refer to the reasons indicated in the responses to **Sections 1.0** to **12.0**.

Minority Opinion # 1 (Drs. Karen Brown, Albert Li, and Jon Richmond): The qualification in the above statement 'subject to product specific validation' should allow for a vote of yes.

Minority Opinion #2 (Dr. Peter Theran): It is not clear that the qualification included in the above statement would preclude the use of the *in vitro* test methods as replacements for the RPT in those circumstances where the BET is currently serving to replace the RPT.

B REVIEW OF ICCVAM DRAFT TEST METHOD RECOMMENDATIONS

1.0 Proposed Test Method Usefulness and Limitations

Does the Panel agree that the available data and demonstrated performance in terms of relevance (i.e., accuracy/concordance, sensitivity, specificity, positive, and negative predictivity, false positive and false negative rates) and reliability (i.e., intralaboratory repeatability and intra- and inter-laboratory reproducibility) support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the proposed test method usefulness and limitations[6]?

The Panel does not agree with this statement for the following reasons:

The usefulness of these *in vitro* test methods for detecting Gram-negative endotoxin has not been properly assessed for concordance with the RPT or for relevance in comparison to the BET. Therefore, it is not possible to truly assess their usefulness and limitations. It is regrettable that their ability to detect non-endotoxins could not be demonstrated and validated due to the limitations of the validation and performance evaluation studies conducted.

Test materials in pure form may directly promote the formation and release of cytokines and thus, they may not be suited to evaluation by the *in vitro* methods.

As much effort as possible should be placed on truly demonstrating that these assays can be reliably used to detect non-endotoxin pyrogens in actual manufacturing settings for a wide variety of products. Otherwise, these assays have little advantage over the already established and widely used BET.

Mechanisms exist for test method developers to qualify their method on a case-by-case basis (i.e., 21 CFR 610.9). Therefore, the use of any recommended method should be subject to product specific validation to demonstrate equivalence as regulated by 21 CFR 610.9.

Minority Opinion (Dr. Peter Theran): This Panel has considered the failure to undertake additional RPTs a significant flaw in this validation study and therefore proposed that, in the future, similar validation studies should use the RPT to provide concordance data. I have no objection to the performance of *in vitro* tests in parallel with rabbit tests, which are already scheduled to be performed, in order to achieve concordance data. But, it is my opinion, that any recommendation for *de-novo* parallel RPT should be accompanied by a statement, as follows: "The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)." The inclusion of this statement would reinforce the importance of the 3R's and would serve as a reminder of U.S. Federal law.

2.0 Proposed Test Method Standardized Protocols

Does the Panel agree that the available data and demonstrated performance in terms of relevance (i.e., accuracy/concordance, sensitivity, specificity, positive, and negative

[6]The ICCVAM draft recommendations state that there is sufficient information, based on validation studies with a limited number of pharmaceuticals, to substantiate the use of these test methods for the detection of pyrogenicity mediated by Gram-negative endotoxin in materials that are currently tested in the RPT, subject to product specific validation to demonstrate equivalency.

predictivity, false positive and false negative rates) and reliability (i.e., intralaboratory repeatability and intra- and inter-laboratory reproducibility) support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the proposed test method standardized protocols?

The Panel agrees with this statement, provided that the following list of inadequacies[7] within the proposed standardized protocols are fully addressed:

1. Donor-to-donor inflammatory response variation is problematic and therefore multiple donors should be used and the number used appropriately justified.
2. Restricting the protocols to a 'limits' test design, based on the intravenous fever threshold, for all test materials independent of administration route could be considered inappropriate. The threshold concentration for intrathecally-administered materials would be lower because of the reduced permissible endotoxin limit associated with these types of products. The use of a 'benchmark reference lot comparison' test design would alleviate the necessity to use such strict permissible 'limits'. Interestingly, in the two known examples where *in vitro* pyrogen test data have been considered by the FDA for release testing (cited in the ICCVAM draft BRD), 'benchmark reference lot comparison' test design protocols have been used.
3. The protocols do not include sufficient descriptions of donor selection criteria (e.g., volunteer or paid, recruitment process, etc.) and conditions for venipuncture (e.g., qualified phlebotomists, number and frequency of venipunctures, etc.). In practice, the requirement for blood donors to have taken no medication and the recommended CO_2 concentrations are more stringent than the provisions suggested in the draft recommendations.
4. The protocols are inconsistent in their acceptance criteria with respect to the number of blood donors. The IL-6 primary cell assays require four donors to be used for each test with acceptance criteria applied to each donor. The IL-1 assays show equal variability between donors, but do not require these acceptance criteria.
5. The suggested dilution scheme for the initial endotoxin standard and for the subsequent dilutions should not be recommended. The initial dilution of the endotoxin standard in two of the assays uses 20 μL into 1980 μL. The margin of error with a micropipette is such that even the smallest error at this initial dilution could affect the whole assay and is often the cause of a substantial proportion of assay variability. To reduce this potential problem, it is recommended that alternative dilution schemes be developed based on the accuracy of the micropipetters.
6. The use of in-house ELISA assays should not be recommended due to poor transferability and the potential for poor interlaboratory reproducibility associated with these assays.

[7]Based on the list of 20 separate inadequacies outlined in this report, three Panel members felt that this list would be better described as a list of "many and substantial" inadequacies.

7. The protocols should clearly specify the need for resonication and/or vortexing of any reference endotoxin solution prior to each use.
8. To adequately test for interference, spiked test samples containing endotoxin must be pre-incubated for a specified time prior to addition to the blood cells.
9. The following should be included in the revised protocols: a consistent number of donors to be used for all test methods; the acceptable range of cytokine response for each test method; the rules and the rationale for exclusion of low and/or high responders.
10. Three separate lots should be included in the pre-qualification of any test material, similar to the protocol used for the BET.
11. The protocol for the MM6 cell line describes procedures that would be used for collecting blood from donors. This point obviously is not required for this particular protocol.
12. The ECVAM protocols are very complete as to sources for all solutions, equipment, etc. required for testing. The ICCVAM protocols are less specific. More specific details on all test method protocol components should be included.
13. Intellectual property issues, as identified in the ICCVAM draft BRD, should be addressed in the ICCVAM protocols.
14. To prevent inactivation of LPS binding protein, it should be specified that FBS is heat inactivated at 55°C.
15. The symbols for correlation coefficient (r and r^2) are interchanged inappropriately.
16. On pages 14 and 21, lines 298 and 450 respectively in the PBMC/IL-6 protocol, the basis for the definition of low responders must be justified.
17. On page 15, line 325 in the PBMC/IL-6 protocol, the performance of monocyte counts using a hemocytometer is inaccurate compared to modern flow cytometric methods.
18. If a hemocytometer is used, specifications for the number of replicate determinations (e.g., at least duplicate), the minimum number of cells counted, and the magnification used must be stated.
19. On page 14, line 295 in the WB/IL-1 protocol, the statement "not taken any drug" is not sufficiently inclusive. This statement must also specify no over-the-counter medications or recreational drugs.
20. On page 20, line 411 in the WB/IL-6 protocol, the statement “If necessary, . . . endotoxin concentration can be modified” is insufficient. The modification of endotoxin concentration must be defined.

3.0 Proposed Test Method Performance Standards

Does the Panel agree that the available data and demonstrated performance in terms of relevance (i.e., accuracy/concordance, sensitivity, specificity, positive, and negative predictivity, false positive and false negative rates) and reliability (i.e., intralaboratory repeatability and intra- and inter-laboratory reproducibility) support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the proposed test method performance standards?

The Panel does not agree with this statement, based on the inadequacies within the proposed performance standards outlined below.

Essential Test Method Components

1. A uniform CV criterion should be defined, which is adequately stringent. The reported range of 20% - 45% is inappropriate.
2. The number of individual blood donors used and/or the number of donors to be included in a pool of multiple donors should be defined, if deemed appropriate.
3. The stringency by which the endotoxin curves are validated should be defined (either by using a four-parameter logistic model or by checking that the OD concentration values ascend in a sigmoidal manner).
4. The use of CVs or any other measure of variability should be appropriately justified. If the data have been log-transformed, then CVs are not informative.
5. The following issues may overestimate the performance of the test methods: a) The nature of the prediction model used for dichotomizing the results; b) Experimental design and data analysis that might lead to overestimation of the sensitivity of the tests; c) The nature and interpretation of the *in vivo* data used in the study; d) The nature and cause of incorrect results and the lack of agreement within and between laboratories; e) Whether the tests accurately estimate the actual concentration of the pyrogen and whether results met some pre-defined criteria of success.
6. In **Section 2.3.3.1**, a 'significant increase' is not defined. In **Section 2.3.6**, consideration should be given to adding Quality Assurance data and known biological properties under the 'test substances and control substances' heading.

Accuracy and Reliability Values

The demonstrated performance of certain aspects of several of the assays, particularly in terms of accuracy or relevance, yields some concern. Two of the assays have false positive rates in excess of 16%, which essentially means that approximately 1 in every 6 production lots could be unnecessarily prevented from being released, a rate unlikely to be accepted by manufacturers. A number of these performance characteristic issues can probably be explained by the fact that some of the spike concentrations used were very close to the 'limit' concentration criterion set.

If the intended use of the *in vitro* assays were only to detect Gram-negative endotoxin, it would seem very important to compare their performance in parallel validation studies that should include the BET. If the intended use of the *in vitro* methods is to evaluate substances containing endotoxin that are unable to be evaluated with the BET, then the parallel testing studies should include the RPT. This type of comparison has neither been made from the RPT (2-way parallel testing was also not performed on the endotoxin-spiked sample sets included in the validation studies cited in the ICCVAM draft BRD) nor the BET standpoint. The last thing one wants to recommend is an inferior performing assay to the one that is already established; similar or superior is fine.

Minimum List of Reference Substances

If the intent of the proposal was to replace the RPT with one or more of the *in vitro* test methods under consideration, then the *in vitro* test methods must be validated for all classes of substances (e.g., pharmaceuticals, biologicals, and implants) and medical devices that can be tested with the RPT. Validation of the *in vitro* test methods with pyrogens (e.g., LTA, components of viruses and fungi) other than endotoxin also needs to be conducted.

***Minority Opinion (Dr. Peter Theran)*:** This Panel has considered the failure to undertake additional RPTs a significant flaw in this validation study and therefore proposed that, in the future, similar validation studies should use the RPT to provide concordance data. I have no objection to the performance of *in vitro* tests in parallel with rabbit tests, which are already scheduled to be performed, in order to achieve concordance data. But, it is my opinion, that any recommendation for *de-novo* parallel RPT should be accompanied by a statement, as follows: "The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)." The inclusion of this statement would reinforce the importance of the 3R's and would serve as a reminder of U.S. Federal law.

4.0 Proposed Additional Studies

Does the Panel agree that the available data and demonstrated performance in terms of relevance (i.e., accuracy/concordance, sensitivity, specificity, positive, and negative predictivity, false positive and false negative rates) and reliability (i.e., intralaboratory repeatability and intra- and inter-laboratory reproducibility) support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the proposed additional studies?

The Panel agrees that to better determine the potential of these test methods, the proposed additional studies should be performed using the test methods described in the ICCVAM draft BRD, taking into account the comments and recommendations detailed previously. The Panel recognizes that these test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses. Wherever possible, either historical data from parallel studies conducted concurrently should be retrospectively evaluated or parallel testing should be conducted concurrently with RPT data generated for regulatory purposes.

The following additional recommendations are given:

1. A repository of test materials that have been identified clinically as pyrogenic would be invaluable for use in future validation studies and may allow such studies to be conducted without the further use of animals.
2. A 'limit' test design protocol and a 'benchmark reference lot comparison' test design protocol for each assay should be included.
3. Both endotoxin-spiked and non-endotoxin spiked samples should be included.
4. The non-endotoxin standards should be characterized as completely as possible prior to their use in any study and should satisfy the requirements set forth by ICCVAM for reference standards that are stated in the ICCVAM draft BRD.
5. The endotoxin-spike concentrations used for the performance assessment studies should not be so close to the positive test concentration limit, especially considering the relatively large enhancement and inhibition range permitted in the sample specific qualification investigations.
6. All aspects of the studies should be completely GLP compliant and importantly, the laboratories and results should be independently audited. This would include pre- and post-study audits of the laboratories.
7. The substances tested in the studies should also include products that have intrinsic pro-inflammatory properties.
8. A prospective study that includes 3-way parallel testing, with all of the *in vitro* assays (using both of the above mentioned protocol designs) being compared to the RPT and the BET, should be included to allow for complete concordance analysis and comparative performance assessment. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, the design of any side-by-side studies should be limited only to those that can gain more data than already available in the literature (i.e., data from parallel testing), most likely on the ability of the RPT and the *in vitro* pyrogen tests to detect non-endotoxin pyrogens.
9. Test substances that better represent all categories of sample types intended for testing by the methods (e.g., pharmaceuticals, biologicals, and medical devices) should be included. If relevant, extraction procedure protocols for the detection of pyrogens in medical device materials should be included.
10. The effects of direct administration of IL-1 and IL-6 to rabbits and the comparison of the resulting pyrogenic response with endotoxin-mediated pyrogenicity should be evaluated.
11. The correlation of IL-1and IL-6 levels in the *in vitro* tests with levels produced in rabbits using similar doses of endotoxin should be evaluated.

The following statistical recommendations are noted:

1. For reliability assessments, formal hypothesis testing is essential with the alternative hypothesis being no different between groups.
2. For any additional studies, formal sample size calculations for equivalence testing should be made to determine that the required number of replicates needed to reject the null hypothesis (i.e., that there is a difference in reliability) at a given level of significance and power. If the study is not prospectively powered, the posterior power should be provided along with the observed significance level.
3. The proposed strategy for the Cryo WB/IL-1 test method is to retest if a test fails because of too much variability. The statistical properties of this multistage procedure should be characterized.

Minority Opinion (Dr. Peter Theran): This Panel has considered the failure to undertake additional RPTs a significant flaw in this validation study and therefore proposed that, in the future, similar validation studies should use the RPT to provide concordance data. I have no objection to the performance of *in vitro* tests in parallel with rabbit tests, which are already scheduled to be performed, in order to achieve concordance data. But, it is my opinion, that any recommendation for *de-novo* parallel RPT should be accompanied by a statement, as follows: "The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)." The inclusion of this statement would reinforce the importance of the 3R's and would serve as a reminder of U.S. Federal law.

C. OVERALL PEER REVIEW OUTCOMES

This international independent Peer Review Panel, consisting of 13 expert scientists from five different countries, provided comments and recommendations on the usefulness and limitations of five *in vitro* pyrogen test methods for the detection and quantification of Gram-negative endotoxin and on the ICCVAM draft test method recommendations on the use of these *in vitro* methods as partial replacements for the RPT. These remarks are summarized below.

- In general, the information presented in the ICCVAM draft BRD was sufficient for the purpose of determining the usefulness and limitations of these test methods for their proposed use and for adequately addressing the applicable validation criteria on the basis of the currently available evidence.
- The available data and demonstrated performance in terms of their reliability and relevance do not at this time support the ICCVAM draft proposed use for these test methods (i.e., as a partial substitute or replacement for the RPT, for the identification of Gram-negative endotoxin, on a case-by-case basis, subject to product specific validation). To better characterize the test methods and more clearly define their reliability and relevance, the Panel recommended that specific additional studies be performed using the ICCVAM proposed protocols, taking into account the Panel's comments and recommendations.
    - The lack of parallel testing in the *in vitro* tests and the RPT, and the resulting lack of concordance data, was considered to be a major limitation of the validation study design. For this reason, the Panel recommended that future studies include parallel testing. A minority opinion (Dr. Peter Theran) associated with parallel testing was expressed as follows: “The use of rabbits in new parallel tests for the validation of an *in-vitro* test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)".
- The available data and demonstrated performance in terms of their reliability and relevance does not support the ICCVAM draft performance standards for these *in vitro* test methods for regulatory purposes.
- The information provided in the ICCVAM draft BRD supports the ICCVAM draft recommended protocols for these five *in vitro* test methods, providing that the list of inadequacies[8] identified by the Panel with respect to reliability and relevance are fully addressed.
- These test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.
- It is critical to recognize, despite concerns about the performance of these five *in vitro* test methods, that a formal process exists for materials regulated under 21 CFR 610.9 to qualify these *in vitro* methods for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation.

[8]Based on the list of 20 separate inadequacies outlined in this report, three Panel members felt that this list would be better described as a list of "many and substantial" inadequacies.

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Appendix A2

Summary Minutes from Peer Review Panel Meeting on February 6, 2007

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Meeting Summary

Independent Peer Review Panel Meeting
Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products
National Institutes of Health (NIH), Natcher Conference Center
Bethesda, MD

February 6, 2007
8:30 a.m. – 5:00 p.m.

Panel Members:

Karen Brown	DRL Pharma and Pair O'Doc's Enterprises
Brian Crowe	Baxter Vaccine AG
Nancy Flournoy	University of Missouri-Columbia
Ihsan Gursel	Bilkent University
Ken Ishii	ERATO, Japan Science & Technology Agency, Osaka University
Jack Levin	University of California-San Francisco
Albert Li	*In Vitro* ADMET Laboratories
David Lovell	University of Surrey
Melvyn Lynn	Eisai Medical Research
Anthony Mire-Sluis	AMGEN, Inc.
Jon Richmond	UK Home Office
Peter Theran	MSPCA
Kevin Williams	Eli Lilly

ICCVAM and ICCVAM Pyrogenicity Working Group (PWG) Members:

Mustafa Akkoyunlu	FDA/CBER
Peter Amin	FDA/CBER
Kimberly Benton	FDA/CBER
Joseph George	FDA/CBER
David Hussong	FDA/OPS
Abigail Jacobs	FDA/CDER
Jodie Kulpa-Eddy (ICCVAM Vice Chair)	USDA/APHIS
Robert Mello	FDA/CDER
Richard McFarland (PWG Chair)	FDA/CBER
Penelope Rice	FDA/CFSAN
William Stokes	NIEHS
Raymond Tice	NIEHS
Daniela Verthelyi	FDA/CDER
Marilyn Wind (ICCVAM Chair)	CPSC
Jiaqin Yao	FDA/CDER

Public Attendees:

Allen Dearry	NIEHS
Basil Golding	FDA/CBER
Thomas Hartung	ECVAM
Coty Huang	FDA/CBER
Sue Leary	ARDF
Thomas Montag	ECVAM
Michael Myers	FDA/CVM
Steven Myers	Becton, Dickinson & Company
Seishiro Naito	NIID
Michael Scott	FDA/CVM
Kristie Stoick	PCRM
Michael Timm	University of Copenhagen
Rachel Waltman	USDA/APHIS

NICEATM Staff:

David Allen	ILS, Inc.
Elizabeth Lipscomb	ILS, Inc.
Linda Litchfield	ILS, Inc.
Debbie McCarley	NIEHS
James Truax	ILS, Inc.
Douglas Winters	ILS, Inc.

Abbreviations: APHIS = Animal and Plant Health Inspection Service; ARDF = Alternatives Research and Development Foundation; CBER = Center for Biologics Evaluation and Research; CDER = Center for Drug Evaluation and Research; CFSAN = Center for Food Safety and Applied Nutrition; CPSC = Consumer Product Safety Commission; CVM = Center for Veterinary Medicine; ECVAM = European Centre for the Validation of Alternative Methods; ERATO = Exploratory Research for Advanced Technology; FDA = U.S. Food and Drug Administration; ILS = Integrated Laboratory Services; MSPCA = Massachusetts Society for the Prevention of Cruelty to Animals; NIEHS = National Institute of Environmental Health Sciences; NIID = National Institute for Infectious Diseases; OPS = Office of Pharmaceutical Science; PCRM = Physicians Committee for Responsible Medicine; USDA = U.S. Department of Agriculture

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Call to Order

Dr. Karen Brown (Panel Chair) called the meeting to order at 8:30 a.m. and introduced herself. She then asked all Peer Panel members, National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, members of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the ICCVAM Pyrogenicity Working Group (PWG) in attendance, the European Centre for the Validation of Alternative Methods (ECVAM) liaison to the PWG, and members of the public to state their name and affiliation for the record. Dr. Brown asked all individuals to identify themselves when they spoke and to use the provided microphones. Dr. Brown stated that three public comment sessions were scheduled during the meeting and she reminded individuals who wished to speak to register at the registration table. Dr. Brown emphasized that there was no need to repeat the same comments at each comment session.

Welcome from the ICCVAM Chair

Dr. Marilyn Wind, Consumer Product Safety Commission and Chair of ICCVAM, welcomed everyone to the Peer Review Panel meeting and thanked the Panel members for their participation. Dr. Wind stressed the importance of an independent scientific peer review to the ICCVAM test method evaluation process.

Welcome from the Director, NICEATM, and Conflict of Interest Statements

Dr. William Stokes, Director of NICEATM, welcomed everyone and reiterated Dr. Wind's appreciation to the participants for agreeing to serve on the Panel. Dr. Stokes stated that he would be serving as the Designated Federal Official for the public meeting. He stated this meeting was being held in accordance with the Federal Advisory Committee Act regulations and that the Panel was constituted under the NIH Special Emphasis Panel charter. Dr. Stokes read the conflict of interest statement and asked the Panel members to declare if they had any direct or indirect conflicts, and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict. None of the Panel members declared a conflict of interest.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes provided an overview of the ICCVAM test method evaluation process. He stated that the international Panel was made up of 13 scientists from five different countries (Austria, Japan, Turkey, United Kingdom, and United States). Dr. Stokes described that the purpose of the Panel was to assist ICCVAM by carrying out an independent scientific peer review of the information provided in the ICCVAM Background Review Document (BRD) on the validation studies of five *in vitro* test methods proposed for assessing the potential pyrogenicity of pharmaceuticals and other products. He stated that Panel members were experts selected and appointed by the National Institute of Environmental Health Sciences (NIEHS) to ensure sufficient scientific expertise to carry out a comprehensive review of these test methods.

Dr. Stokes listed the 15 ICCVAM member agencies and provided a brief review of ICCVAM's history. He summarized the ICCVAM Authorization Act of 2000 (available at: http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf) and detailed the purpose and duties of ICCVAM as mandated in the Act. He noted that one of ICCVAM's duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available from the ICCVAM/NICEATM website or directly from NICEATM. Dr. Stokes pointed out that ICCVAM does not carry out research, development, or validation studies, but instead, facilitates these processes by convening scientific symposia, workshops, and expert Panel reviews such as this one.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public. Given sufficient regulatory applicability, sufficient data, resources, and priority, a method will move forward to a formal evaluation. A draft BRD is prepared by NICEATM in conjunction with an ICCVAM working group for the relevant toxicity testing area (e.g., pyrogenicity), which provides a

comprehensive review of all available data and information. ICCVAM then considers all of the available information and prepares draft recommendations for 1) proposed usefulness and limitations of the test methods, 2) test method protocol, 3) performance standards, and 4) future studies. The draft BRD is then made publicly available for review and comment. An independent peer review Panel is then convened to provide comments and recommendations on the draft BRD, public comments, and ICCVAM draft test method recommendations. A *Peer Review Panel Report* is published and considered by ICCVAM, along with public and SACATM comments, when their final recommendations are forwarded to the appropriate ICCVAM agencies.

Dr. Stokes reviewed the criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance.

ICCVAM Charge to the Panel

Dr. Stokes reviewed the charge to the Panel, which was to: 1) review the draft BRD for completeness and identify any errors or omissions; 2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been addressed for the proposed use; and 3) consider and provide comment on the extent to which the ICCVAM draft test method recommendations including the proposed use, recommended protocols, performance standards, and recommended additional studies are supported by the information provided in the BRD.

Dr. Stokes thanked the PWG, ICCVAM, and NICEATM for their work on this project, and he acknowledged the NICEATM staff for organizing the Panel meeting and preparing the materials being reviewed.

Overview of Pyrogenicity Testing Requirements and Current Pyrogenicity Testing Procedures

Dr. Richard McFarland, Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER) and Chair of the PWG, thanked the PWG members for their efforts in producing the draft BRD, and thanked the Panel members for their participation in the peer review process. Dr. McFarland discussed the scientific need for pyrogenicity testing and its relationship to the regulatory mandate for protection of public health. He discussed the need for risk management, especially the detection of endotoxin and non-endotoxin pyrogen-contaminated products, and he noted the need for classification and labeling of products as pyrogen-free (i.e., the product does not exceed established endotoxin limits). Dr. McFarland then summarized the U.S. and European legislation and statutory protocol requirements for pyrogen testing.

Overview of the Five *In Vitro* Pyrogen Test Method Protocols

Dr. Thomas Hartung, Head of ECVAM and invited test method expert, remarked that he has been closely involved in the ECVAM validation studies and as such recognized his considerable conflict of interest. Dr. Hartung summarized the disadvantages of the rabbit pyrogen test (RPT) and the bacterial endotoxins test (BET), and related these limitations to the development of the *in vitro* pyrogen test methods.

Dr. Hartung indicated that a typical *in vitro* pyrogen test method consists of two parts: 1) incubation of the test sample in a cellular cytokine release system (i.e., whole blood [WB], Mono Mac 6 [MM6] cells, Peripheral blood mononuclear cells [PBMC]); and 2) cytokine detection using a specific enzyme-linked immunosorbent assay (ELISA) (e.g., Interleukin [IL]-1β or IL-6). He stated that the European Commission granted $2.5 million for the validation of these novel test methods, but that this sum was only sufficient to cover "the basics". Dr. Hartung then made the following comments regarding the design of the ECVAM validation study:

- For the validation study, the endotoxin threshold was set at 0.5 Endotoxin Units (EU)/mL, based on the positive response of 50% of the most sensitive rabbit strain to 50 pg of endotoxin. A substance was considered pyrogen-free if the endotoxin level in an *in vitro* test method corresponded to less than 0.5 EU/mL. A positive product control (PPC) was used in a pretest to insure that there is no interference. Specific criteria were used to minimize assay variability (e.g., blood donors, coefficient of variation).
- In 1988, Dr. Stephen Poole described an IL-6 cytokine assay using isolated leukocytes. The PBMC test method evolved from this study and has subsequently been used by Novartis for U.S. Food and Drug Administration (FDA) release of one product (i.e., after product-specific validation and in conjunction with the rabbit pyrogen test).
- Two of the assays included in the validation exercise, WB/IL-1β and WB/IL-6, utilize human WB. Many research studies have described using these test systems for routine pyrogen testing of up to 80 pharmaceutical products against a variety of pyrogens. A commercial kit has been developed using theWB/IL-1β test system.
- A catch-up validation study was performed using the Cryo-WB/IL-1β test method, which was not available during the original validation study. This assay utilizes cryopreserved WB pooled from several donors. Although the cells remain in diluted dimethyl sulfoxide, an effect on cell morphology or viability is not observed.

Overview of the Draft *In Vitro* Pyrogen Test Method Background Review Document (BRD)

Dr. David Allen, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), presented an overview of the ICCVAM draft BRD. Dr. Allen indicated that five BRDs were submitted by ECVAM in June 2005. A *Federal Register* notice was used to request data from over 100 interested stakeholders, but no additional data were submitted. Following this request, a comprehensive ICCVAM draft BRD, which describes the current validation status of the five *in vitro* test methods based on U.S. Federal regulatory standards, was compiled and made available to the public on December 1, 2006.

Dr. Allen briefly summarized the performance characteristics of the *in vitro* test methods, which are detailed in the ICCVAM draft BRD (available at: http://iccvam.niehs.nih.gov/methods/pyrogen/pyrodocs/pyroBRDUdocs/PyroBRD01Dec06.pdf).

Dr. Allen noted that Dr. Marlies Halder, ECVAM liaison to the PWG, provided additional information requested by the Panel, including data audits, evidence of Good Laboratory Practice (GLP) compliance of testing laboratories, information on the protocol used for the historical RPT studies, and lot numbers of the test substances. He also stated that a request was made for the ECVAM Science Advisory Committee (ESAC) peer-review documents, but that these documents are not available to the public.

Peer Review Panel Evaluation:
Dr. Brown introduced the relevant Panel Group Leaders for each BRD Section: (Dr. Melvyn Lynn - Sections 1, 2, and 11; Dr. Jack Levin - Sections 3, 5, and 6; Dr. Anthony Mire-Sluis - Sections 7 and 8; Dr. Jon Richmond - Sections 4, 9, and 10). The Group Leaders presented the draft responses to the Evaluation Guidance Questions for consideration by the entire Panel. The Panel discussion and their recommended revisions to each section of the ICCVAM BRD are reflected in the *Independent Peer Review Panel Report: Five In Vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products*, published in April 2007 (hereafter, the Panel report, available at: http://iccvam.niehs.nih.gov/docs/pyrogen/PrRevPanFinRpt.pdf).

Public Comments (Session 1)
Ms. Mary Lou Chapek - President and Chief Executive Officer of MBP Laboratories, Inc.
Dr. David Allen read the written comments submitted by Ms. Chapek to ICCVAM/NICEATM. Her comments are summarized as follows:

- Ms. Chapek expressed disappointment in the number of test methods reviewed by ICCVAM and accepted by federal agencies over the past 15 years. She commented that the pyrogenicity BRD and recommendations currently under discussion indicate a lack of focus. Ms. Chapek noted that substantial work remains to be done for validation of these test methods and she suggested the phased strategy outlined below.
- Phase I should concentrate on replacement of the BET, not the RPT. A large array of test substances compatible with the BET could be spiked with endotoxin to determine their accuracy and sensitivity and to determine the level of interference, if any, with each of these test systems.
- Phase II should consist of validation of one or two *in vitro* test methods for replacement of the RPT. Cell-based assays that do not depend on blood, which has an impractical limited time domain, would be preferable and could be compared directly to RPT data. The task would still be complex, but with a smaller focus. Phase II would also require evaluation and validation of all materials currently tested in the RPT, as well as the pyrogens detected in the RPT. Some of these standards would have to be developed. Although these studies may take years for completion, replacement of the RPT by one or two of the *in vitro* pyrogen tests in Phase II would constitute an achieved goal by ICCVAM.

Dr. Thomas Montag - Paul Ehrlich Institute (PEI) - Germany
Dr. Montag provided the following comments:

- He stated that the PEI is responsible for the quality and safety of biological drugs in general and that his laboratory has been involved in alternative pyrogen testing with Dr. Hartung for over 12 years. While the data is proprietary, he confirmed that he has used the WB/IL-1β assay for approximately two years.
- Dr. Montag commented that donors are now pooled (up to 10 at a time) to minimize variability, especially for detection of non-endotoxin pyrogens. For the Cryo WB/IL-1β pyrogen test, the blood is typically pretested for reactivity after pooling. In response to the PPC issue mentioned previously, he remarked that this was a design flaw that had been corrected in the ECVAM Standard Operating Procedure. He also stated that an expert Panel from the EDQM is now in the process of creating a draft of this alternative test method for publication.

Final Review of the BRD for Errors and Omissions

Dr. Brown asked the Panel to review the recommended revisions for each BRD section, taking into account the public comments, and to decide if additional changes are necessary. No changes were made to the draft recommendations based on the public comments.

Validation Status of the *In Vitro* Test Methods

Dr. Brown asked the Panel if they agreed that the applicable validation criteria had been adequately addressed in the ICCVAM BRD in order to determine usefulness and limitations of these *in vitro* test methods, to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis subject to product-specific validation.

The Panel agreed that the information was adequate with which to make an informed decision.

Dr. Brown asked the Panel if they agreed that the performance of these test methods in terms of their relevance and reliability support the proposed use for the detection of Gram-negative endotoxin in materials that are currently tested in the RPT, subject to product-specific validation to demonstrate equivalency to the RPT.

The Panel did not agree with this statement based on the reasons indicated in the responses to the questions related to Sections 1.0 to 12.0 of the ICCVAM BRD. Two minority opinions were expressed. Responses to these questions, and the associated minority opinions are detailed in the Panel Report.

Public Comments (Session 2)

Dr. David Hussong - FDA, Center for Drug Evaluation and Research (CDER)

Dr. Hussong commented that the Code of Federal Regulations (CFR), Section 211.167, states that if a drug is to be labeled as pyrogen-free, an appropriate test is required. The U.S. Pharmacopeia (USP) provides guidelines for the RPT and the BET. While the BET is not considered equivalent to the RPT, data from the BET is accepted. The USP states that use of alternative tests is permitted and that they may be used *in lieu* of the BET, provided that the alternative test uses a reference standard for comparison. It should be noted that the FDA CDER approves drugs, not test methods, but welcomes the use of alternative test methods.

Dr. Thomas Hartung - Head, ECVAM - Italy

Dr. Hartung stated that the *in vitro* pyrogen tests were designed to determine the threshold level of endotoxin in the most sensitive rabbit strain. This design was ambitious and consequently, resulted in the low sensitivity (58%) and specificity (83%) observed. It should be noted that some assays had values of 80% or 90% at this critical concentration and performed better than the RPT.

ICCVAM Draft Recommendations for *In Vitro* Pyrogen Test Methods
Presentation of Draft ICCVAM Recommendations
Dr. Brown asked the Panel to evaluate the extent to which the ICCVAM draft recommendations are supported by the information and data provided in the ICCVAM draft BRD. Dr. Brown reminded the Panel that the purpose is not to approve or disapprove of the ICCVAM draft recommendations, but rather to comment on the extent to which they are supported by the information contained in the ICCVAM BRD. The Panel discussion and associated conclusions relevant to each of the ICCVAM recommendations are reflected in the Panel Report.

Public Comments (Session 3)
Ms. Kristie Stoick - Physicians Committee for Responsible Medicine
Ms. Stoick reviewed written comments that she previously submitted to ICCVAM/NICEATM. She stated that the pace of acceptance of alternative methods, such as these *in vitro* pyrogen tests, in the opinion of the animal protection community, is unacceptably slow. She continued to state that too much time is spent debating every scientific detail and that the ultimate goal is lost. She closed by asking ICCVAM to take into account her comments when considering the Panel's recommendations for the validation of these assays.

Final Review of the ICCVAM Draft Recommendations
Dr. Brown asked the Panel to review the ICCVAM draft recommendations, taking into account the public comments, and to decide if additional changes are necessary. No changes were made to the draft recommendations based on the public comments.

Concluding Remarks
Dr. Brown thanked the Panel and ICCVAM/NICEATM for their help. She expressed hope that this peer review process helped to establish a focus for ICCVAM and that the reduction in animal use would be the ultimate outcome. Dr. Stokes thanked the Panel for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes stated that the ICCVAM PWG and ICCVAM would consider these recommendations as they move forward with this process and the results of this meeting would culminate in a Peer Review Panel Report that would be released to the public toward the end of March for additional comment.

Adjournment
The meeting was adjourned at 5:47 p.m.

William S. Stokes, D.V.M.
NIEHS
P.O. Box 12233
MD-EC17
Research Triangle Park, NC 27709

Dear Dr. Stokes:

The Meeting Summary, Peer Review Panel Public Meeting, Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products, dated February 6, 2007, accurately summarizes the Peer Review Panel meeting of February 6, 2007, in Bethesda, MD.

Sincerely,

Signature	Printed Name	Date
	KAREN K. BROWN	3/4/08

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Appendix A3

Pyrogenicity Peer Review Panel Biographies

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Karen Brown, Ph.D. (Panel Chair)

Dr. Brown received her Ph.D. in Microbiology and Biochemistry at Oklahoma State University in Stillwater. She is President, Pair O' Docs Enterprises, consulting with companies and with the U.S. Department of Agriculture Animal and Plant Health Inspection Service (APHIS) Center of Veterinary Biology on development of *in vitro* assays to replace animal tests for release of veterinary vaccines and is a Consultant, sharing the CEO position for MVP Laboratories. Dr. Brown's resume indicates her broad expertise in *in vitro* and *in vivo* pyrogen testing and thorough knowledge of regulatory requirements for drug and product development. Early in her career, Dr. Brown developed bench and supervisory experience working in Quality Control conducting animal testing, including the rabbit pyrogen test and *in vitro* Limu endotoxin testing. She initiated an *in vitro* development group at Bayer as Head of Biological Research and Development that specialized in developing and validating ELISAs for Limulus Amebocyte Lysate (LAL) testing and antigen quantitation for release of biological products. Dr. Brown remains involved in endotoxin testing by consulting with various companies to determine correlations in endotoxin levels in various veterinary products to reactions produced by some of these products when used to vaccinate animals. She has expertise in microbiology, veterinary medicine, vaccine and biologicals development and safety testing, *in vitro* methods development, and technical government relations (European Union [EU] and United States [U.S.]). Dr. Brown was Chairman of the *In Vitro* Working Group of the Veterinary Biologics Section of the Animal Health Institute (AHI) and APHIS liaison (regulatory) for registration of new vaccine and diagnostic products. Dr. Brown has conducted or managed research and development to register 44 new drug products, pharmaceuticals, vaccines, or diagnostic products or technologies and she has 44 publications and presentations and 23 U.S. patents. She is a member of the AHI, Veterinary Biologics Section, the Association of Veterinary Biological Companies, the American Society for Microbiology, and the U.S. Animal Health Association.

Brian Crowe, Ph.D.

Dr. Crowe received his Ph.D. in Microbiology from Trinity College in Dublin, Ireland. He is the Director of Immunology (Vaccines) at Baxter Vaccine AG in Austria and has responsibility for two research departments (Molecular Immunology and Humoral Immunology) and a quality control department (Biological Control) comprised of three quality control laboratories (Bacteriology, *in vitro* and *in vivo* testing). Dr. Crowe's resume demonstrates a significant and broad level of expertise in pyrogen test methodology and knowledge of laboratory, manufacturing, and validation procedures. Dr. Crowe has responsibility for general safety and toxicity testing and heads the Rabbit Pyrogen Testing and Endotoxin (LAL) Testing Units for Baxter Bioscience in Austria with testing rates of 3,000 to 26,000 samples per year. Dr. Crowe has extensive experience with high throughput screening, cytokine response assays, cytotoxicity testing, inflammatory response assays, complement testing, and other molecular, cellular, and humoral immunological response testing. He is also well versed in Good Manufacturing Practice and Good Laboratory Practice (GLP) standards and in issues of validation and audit requirements and procedures. Dr. Crowe has authored or coauthored 25 publications and 4 patents. His research interests are focused on bacterial and viral vaccines.

Nancy Flournoy, M.S., Ph.D.

Dr. Flournoy received a M.S. degree in Biostatistics from the University of California at Los Angeles, and a Ph.D. in Biomathematics from the University of Washington. She is Professor and Chair of the Department of Statistics at the University of Missouri-Columbia. Her research interests include adaptive designs, bioinformatics, chemometrics, clinical trials, and environmetrics. She has an extensive list of edited volumes and papers on statistical theory, statistical genetics and immunology, epidemiology in immune suppressed subjects, clinical trials for prevention and treatment of viral infection, transplantation biology and its effects on digestion, lungs, eyes, mouth, and central nervous system, optimization of statistical processing, and additional papers, interviews, and technical reports. She has editorial responsibilities for numerous statistical journals, serves on numerous advisory boards, and nominating committees. She is a member and past Chair of the Council of Sections of the American Statistical Association, and served in various other statistical, medical and toxicological societies or programs as Chair or as a member of the Board of Directors. She is a former member of the Scientific Advisory Committee on Alternative Toxicological Methods. She also served on the Expert Panels for the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) that evaluated the Revised Up-and-Down Procedure; the Current Validation Status of *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants; and Five *In Vitro* Pyrogen Test Methods.

Ihsan Gursel, MSc, Ph.D.

Dr. Gursel received his MSc. and Ph.D. degrees from the Middle East Technical University, Department of Biological Sciences in Ankara, Turkey. He is an Assistant Professor in the Department of Molecular Biology and Genetics at Bilkent University in Ankara. Dr. Gursels' resume indicates that he has significant experience studying the receptor family believed to mediate pyrogenic responses (i.e., Toll-like receptors [TLR]). Dr. Gursel's research interests include studies on the roll of TLR and TLR-ligand interactions in an innate immune response, gene expression and transcriptional profiling of immune cells via high throughput screening methods, design of controlled release systems for oligodeoxynucleotide targeting and delivery, and application of biodegradable natural polymers for biomaterials, tissue engineering, and drug delivery. Dr. Gursel has received numerous awards and grants to support his work and has authored or coauthored more than 45 publications, 7 patents, and has given 28 formal presentations related to his research. He has also refereed papers for the Journal of Leukocyte Biology, Immunopharmacology and Immunotoxicology, and Vaccine.

Ken Ishii, M.D., Ph.D.

Dr. Ishii received his M.D. and Ph.D. degrees from the School of Medicine at Yokohama City University in Kanagawa, Japan. He is a Group Leader for the Akira Innate Immunity Project at the Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Osaka University. Dr. Ishii's resume indicates that he has extensive regulatory experience that includes pyrogen testing requirements for pharmaceuticals. Dr. Ishii was formerly a Staff Scientist at the Food and Drug Administration (FDA) Center for Biologics and Evaluation Research (CBER). His work experience includes regulation of Investigational New Drug applications related to DNA-based vaccines and immunotherapy

using DNA vaccine and immunostimulatory DNA (CpG DNA). Dr. Ishii also has regulatory experience related to vaccines and immunotherapies against infectious diseases and allergy. He has authored or coauthored 58 publications in peer-reviewed journals and holds 17 patents.

Jack Levin, M.D.

Dr. Levin received an M.D. from the Yale University School of Medicine in New Haven, CT. He is an Independent Investigator at the Marine Biological Laboratory at Woods Hole, MA. Dr. Levin is also a Professor of Laboratory Medicine and Professor of Medicine at the University of California School of Medicine in San Francisco. He previously held various academic positions (e.g., Professor of Medicine) at Johns Hopkins Hospital in Baltimore and holds additional positions (e.g., Associate Member of the Cancer Research Institute at University of California at Santa Cruz, attending physician and Director of the Anticoagulation Clinic at the Veterans' Administration Medical Center in San Francisco). Dr. Levin is board-certified in Internal Medicine by the American Board of Internal Medicine. Dr. Levin's resume indicates that he has extensive experience studying the pyrogenic response and pyrogen testing (e.g., research in hemoglobin-lipopolysaccharide interactions and pioneered gel-clot LAL technology). Dr. Levin is a former editor-in-chief of the Journal of Endotoxin Research, a member of the American Society of Hematology (serving on various committees), a member of the Corporation, Marine Biological Laboratory, a Fellow of the American College of Physicians, a member of the American Society for Experimental Pathology, American Society for Clinical Investigation, the California Academy of Medicine, the International Endotoxin Society, and numerous other societies. Dr. Levin has co-organized nine international conferences and has 246 publications in peer-reviewed journals, book chapters, or edited series.

Albert Li, Ph.D., MBA

Dr. Li received his Ph.D. in Biomedical Sciences from the University of Tennessee, Oak Ridge and an Executive MBA from the University of Maryland University College in College Park. Dr. Li co-founded three companies to advance drug development. He is Chairman and CSO of ADMET Group, LLC; Founding Chairman, President, and CEO of *In Vitro* ADMET Laboratories in Rockville, MD; and Founding Chairman, President, and CEO of Advanced Pharmaceutical Sciences in Baltimore, MD. Dr. Li's resume indicates that he has a broad level of experience in validation of *in vitro* and alternative methods. Dr. Li has secured multiple research grants to advance a drug candidates from the preclinical laboratory through clinical trials, developed proprietary technology of interest to the pharmaceutical industry, and established a GLP laboratory for *in vitro* efficacy, metabolism, and toxicity testing. Dr. Li has published over 130 scientific papers, numerous books/special journal issues, and is frequently invited to speak in national and international conferences.

David Lovell, Ph.D., FIBiol, CBiol, F.S.S., CStat

Dr. Lovell received a Ph.D. from the Department of Human Genetics and Biometry, University College, London. He is currently Reader in Medical Statistics at the Postgraduate Medical School at the University of Surrey. Previously, he was Associate Director and Head of Biostatistics support to Clinical Pharmacogenomics at Pfizer Global Research and Development in Sandwich, Kent providing data management and statistical support to pharmacogenetics and genomics. He joined Pfizer in 1999 as the Biometrics Head of Clinical

Pharmacogenetics. Before joining Pfizer, Dr. Lovell was the Head of the Science Division at BIBRA International, Carshalton, which included Molecular Biology, Genetic Toxicology, Biostatistics and Computer Services. At BIBRA, Dr. Lovell managed the statistical and computing group providing specialized statistical support to BIBRA's Clinical Unit and contract research work. He conducted and managed research programs on genetics, statistics and quantitative risk assessment for the EU and United Kingdom (U.K.) Government Departments. His research interests at BIBRA were in the use of mathematical and statistical methods together with genetic models in the understanding of toxicological mechanisms and risk assessment problems. Dr. Lovell had previously been a Senior Research Officer with the U.K. Medical Research Council (MRC) Experimental Embryology and Teratology Unit, a visiting Postdoctoral Fellow at the National Institute of Environmental Health Sciences in North Carolina, U.S., a Geneticist at the MRC Laboratories, Carshalton and a Research Assistant in Cytogenetics at Birmingham University. He has acted as a consultant to a number of organizations, has considerable experience of working with Regulatory Authorities, has many publications related to his work and has wide experience of making presentations to a wide range of audiences. He is a member of the U.K. Government's advisory Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment and the Independent Scientific Advisory Committee for Medicines and Healthcare Products Regulatory Agency database research. He served on the NICEATM-ICCVAM Expert Panels that evaluated the Frog Embryo Teratogenesis Assay - Xenopus, *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro* Pyrogen Test Methods.

Melvyn Lynn, M.S., Ph.D.

Dr. Lynn received M.S. and Ph.D. degrees in Microbiology from Rutgers University in New Brunswick, New Jersey. He is currently Senior Director and Global Head, Sepsis and Anti-Infectives Therapeutic Area at Eisai Inc. Dr. Lynn's expertise in the area of pyrogenicity is evidenced from his involvement in the clinical development of TLR4 antagonists and antimicrobials. Dr. Lynn directs global clinical development of a TLR-4 antagonist and antimicrobials and is head of a multifunctional, international project team, for which he regularly interacts with FDA and international regulatory agencies. Dr. Lynn has participated in global Standard Operating Procedure process development teams and served on the Eisai Global Clinical Development Global Development Board to address globalization of clinical development of drugs and clinical processes. Dr. Lynn has authored or coauthored 24 peer-reviewed publications, a review, two book chapters, a research letter, and 28 abstracts. Dr. Lynn has additional drug development experience during his tenure at the Bristol-Myers Squibb Company.

Anthony Mire-Sluis, Ph.D.

Dr. Mire-Sluis received his Ph.D. in Cell Biology and Biochemistry from the Department of Haematology at the Royal Free Hospital Medical School. He is Senior Director – Product Quality and External Affairs at AMGEN, Inc. with former positions as Head of the Cytokine Group at the National Institute for Biological Standards and Control, Director of Bioanalytical Sciences at Genentech, Inc., Head of Analytical Science and Standards in the Center for Biologics Evaluation and Research (CBER) at the FDA and Principal Advisor for Regulatory Science and Review in the Office of Biotechnology Products and Office of Pharmaceutical Sciences in the Center for Drug Evaluation and Research at the FDA. Dr. Mire-Sluis's resume demonstrates his expertise in regulatory science associated with pyrogen

testing with experience in product quality and development of biologicals, in immunology, and prior experience with the FDA. Dr. Mire-Sluis has managerial and product development experience including management of analytical and product quality departments of up to 75 staff (postdoctoral and technical levels). He is involved in strategic planning of development of biotechnology-derived products, including toxicology, assay development, and quality control. Dr. Mire-Sluis has expertise in the detection, measurement, and characterization of biological materials using immunological, molecular biological, and cell biological technology (cytokines, growth factors, enzymes, monoclonal antibodies). He is involved in high throughput screening technology, bioassay and immunoassay designs, risk assessment and process validation. He is a member of the World Health Organization consultative committee for therapeutic drug standardization, Chairman of the International Union of Immunological Societies Standardization Committee and of the human therapeutics committee of the International Association for Biologicals, a board member for the Journal of Immunological Methods, a member of the U.S. Pharmacopeia Biological Assay Statistical Analysis Expert Working Group and the Biological Assay Validation Expert Working Group. Dr. Mire-Sluis has authored almost 100 peer-reviewed publications.

Jonathan Richmond, BSc (Hons) Med.Sci., MB ChB, FRCSEd, FRMS

Dr. Richmond received a Bachelor of Science in Medical Science with Honors (B.Sc. [Hons] Med.Sci.) and Bachelor of Medicine and Bachelor of Surgery (MB ChB) degrees with Distinction in Medicine and Therapeutics from Edinburgh University. Presently, he is head of the Animals Scientific Procedures Division at the Home Office. He is a Fellow of the Royal College of Surgeons of Edinburgh (FRCSEd) and a Fellow of the Royal Society of Medicine (FRMS). Other appointments include convener of the U.K. interdepartmental group on the 3Rs, board member U.K. National Centre for the 3Rs, convener of the International Standards Organization Technical Corrigendum 194/Working Group 3 (*Biocompatibility of Medical Device Materials*), and member of related expert working groups. He is a former member of the EU Committee on Scientific and Technical Progress and past Chairman of the European Commission Technical Expert Working Group on ethical review. He served as chair of the peer review panel for the reduced murine local lymph node assay (LLNA) test protocol and prediction model for ESAC in 2007 and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA. He served on the NICEATM-ICCVAM Expert Panel that evaluated Five *In Vitro* Pyrogen Test Methods. He has a variety of publications in peer-reviewed journals and national and international meetings, on the principles and practice of surgery, regulation of biomedical research, principles of humane research, bioethics, and public policy.

Peter Theran, V.M.D.

Dr. Theran holds a Doctor of Veterinary Medicine degree from the University of Pennsylvania. He has had many years of experience both as a veterinary internal medicine specialist at the Massachusetts Society for Prevention of Cruelty to Animals' Angell Memorial Animal Hospital in Boston, and as the director of Boston University Medical Center's Laboratory Animal Science Center. He presently serves on a number of government committees as an animal welfare member, and is a member of the Board of Directors of the Institute for *In Vitro* Sciences in Gaithersburg, MD and Chimp Haven in Shreveport, Louisiana. He served on the NICEATM-ICCVAM Expert Panels that evaluated the *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro*

Pyrogen Test Methods. He is a former member of the Advisory Committee on Alternative Toxicological Methods and SACATM. He is presently working as a consultant.

Kevin Williams

Mr. Williams received a B.S. degree in Microbiology from Texas A&M University. He is a Microbiologist in the Quality Control Laboratory at Eli Lilly & Company. Mr. Williams' resume indicates that he is a well-noted expert in pyrogen testing (Bacterial Endotoxin Test [BET] and LAL) and validation and he has authored several books on endotoxins. His responsibilities include bacterial endotoxin testing and validation, automation of BET, depyrogenation validation, automated microbial identification system validation, validation of sterility tests, preservative effectiveness testing, microbial purity testing and validation, and bioburden testing and validation. Mr. Williams is a member of the LAL User Steering Committee, the Parenteral Drug Association, and the American Society for Microbiology. He has developed a method to calculate tolerance limits for excipients based on unit formula content of finished drug and developed novel methods of recovering endotoxin from parenteral drug packaging components. Mr. Williams served as editor of the textbook, "Microbial Contamination Control in Parenteral Manufacturing," and contributed a chapter on "Historical and Emerging Themes in Parenteral Manufacturing Control." He also edited the textbook "Endotoxins," and contributed chapters on endotoxin and contamination control.

Appendix B

Relevant Federal Pyrogenicity Regulations and Testing Guidelines

B1 U.S. Code of Federal Regulations (CFR) B-3
B1-1 21 CFR 211.167 - Current Good Manufacturing Practice for Finished Pharmaceuticals: Special Testing Requirements (April 1, 2007) B-5
B1-2 21 CFR 314.50(d)(1)(ii)(a) - Applications: Content and Format of an Application (April 1, 2007) B-6
B1-3 21 CFR 610.9 - General Provisions: Equivalent Methods and Processes (April 1, 2007) B-17
B1-4 21 CFR 610.13 - General Biological Products Standards: Purity (April 1, 2007) B-19
B2 International Organization for Standardization - ISO 10993-11 - Biological Evaluation of Medical Devices - Part 11: Tests for Systemic Toxicity (First Edition 1993-12-15) B-21
B3 U.S. Pharmacopeia (USP) 30-NF25 ..... B-23
B3-1 (85) - Bacterial Endotoxins Test B-23
B3-2 (151) - Pyrogen Test B-23
B3-3 (1041) - Biologics B-23
B4 European Pharmacopeia 5.0 ..... B-25
B4-1 2.6.8 - Pyrogens B-25
B4-2 2.6.14 - Bacterial Endotoxins B-25
B5 Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices (December 1987) B-27

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Appendix B1

U.S. Code of Federal Regulations (CFR)

B1-1 **21 CFR 211.167 - Current Good Manufacturing Practice for Finished Pharmaceuticals: Special Testing Requirements (April 1, 2007)**

B1-2 **21 CFR 314.50 (d)(1)(ii)(a) - Applications: Content and Format of an Application (April 1, 2007)**

B1-3 **21 CFR 610.9 - General Provisions: Equivalent Methods and Processes (April 1, 2007)**

B1-4 **21 CFR 610.13 - General Biological Products Standards: Purity (April 1, 2007)**

These documents are available at:
http://www.access.gpo.gov/cgi-bin/cfrassemble.cgi?title=200442

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Appendix B2

International Organization for Standardization

ISO 10993-11 - Biological Evaluation of Medical Devices - Part 11: Tests for Systemic Toxicity (First Edition 1993-12-15)

This document is available for purchase at:
http://www.iso.org/iso/store.htm

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Appendix B3

U.S. Pharmacopeia (USP) 30-NF25

B3-1 (85) - Bacterial Endotoxins Test
B3-2 (151) - Pyrogen Test
B3-3 (1041) - Biologics

These documents provide a description of the respective biological test and are available for purchase at:
http://www.usp.org/products

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Appendix B4

European Pharmacopeia 5.0

B4-1 2.6.8 - Pyrogens
B4-2 2.6.14 - Bacterial Endotoxins

These documents provide a description of the respective biological test and are available for purchase at:
http://www.edqm.eu/site/Online_Publications-581.html

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Appendix B5

Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices (December 1987)

In July 2011, the U.S. FDA withdrew the Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human Parenteral Drugs, Biological Products, and Medical Devices (1987) because the document no longer reflected the Agency's current thinking on the topic. Current FDA guidance (June 2012) can be found at https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm314718.htm

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Appendix C

ICCVAM Recommended Test Method Protocols

C1 The Human Whole Blood (WB)/Interleukin (IL)-1β *In Vitro* Pyrogen Test.....C-3
C2 The Human WB/IL-1β *In Vitro* Pyrogen Test: Application of Cryopreserved (Cryo) Human WB.....C-25
C3 The Human WB/IL-6 *In Vitro* Pyrogen Test.....C-47
C4 The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test.....C-71
C5 The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 *In Vitro* Pyrogen Test.....C-95

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Appendix C1

The Human Whole Blood (WB)/Interleukin (IL)-1β *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Human Whole Blood (WB)/Interleukin (IL)-1β *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-1β from monocytoid cells in human whole blood (WB). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)[1] WB/IL-1β Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Validation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedures (SOPs) for the WB/IL-1β test (could be referred to as Monocyte Activation Test), which is first described by Hartung and Wendel (1996). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOPs is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the WB/IL-1β pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov) to ensure that the most current protocol is used.

[1]ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOPs for the WB/IL-1β Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM SOP[1]	ECVAM Validation SOP[1]
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test neat or at minimal dilution that produces no interference	Test at MVD
Number of Blood Donors	Minimum of 3 (independent or pooled)	Minimum of 1	Minimum of 1
Decision Criteria for Interference	Mean OD[2] of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1) in triplicate	NSC (1)
	EC (5)	EC (5)in triplicate	EC (2)
	TS (14)	TS (25) in triplicate	TS (3) x EC (5) spikes = 15 TS
	PPC[3] (0)	PPC (0)	PPC (3) = 3 TS
	NPC[3] (0)	NPC (0)	NPC (3) = 3 TS
	LTAC[4] (0)	LTAC (1) in triplicate	LTAC (0)
ELISA Plate	Includes seven point IL-1β SC and blank in duplicate	Not included	Not included
Assay Acceptability Criteria	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test[6]	Outliers rejected using Dixon's test[6]
	Mean OD of NSC ≤0.15	Not included	Not included
	Quadratic function of IL-1β SC r ≥0.95[5]	Not included	Not included
	EC SC produces OD values that ascend in a sigmoidal concentration response	Not included	Not included
	Not included	Mean OD of 0.5 EU/mL EC ≥ 1.6x Mean OD of NSC	Mean OD of 0.5 EU/mL EC ≥ 1.6x Mean OD of NSC
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC[7] TS	OD TS > OD 0.5 EU/mL EC	OD TS > OD 0.5 EU/mL EC

Abbreviations: EC = Endotoxin control; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-1β = Interleukin-1β; LTAC = Lipoteichoic acid (LTA) control; MVD = Maximum valid dilution; NPC = Negative product control; NSC = Negative saline control; OD = Optical density; PPC = Positive product control; SC = Standard curve; SOP = Standard operating procedure; TS = Test substance; WB = Whole blood

[1]ECVAM WB/IL-1β SOPs are presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).
[2]Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).
[3]In the ICCVAM WB/IL-1β protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA. In the ECVAM SOP, PPC and NPC were only included in the ECVAM validation study.
[4]LTAC was only included in the ECVAM SOP.
[5]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.
[6]Included in the ECVAM Trial data report presented in Appendix D of the ICCVAM BRD.
[7]Where unknown, the ELC is calculated (see **Section 12.2**).

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-1β from monocytoid cells in whole blood (WB). The concentration of IL-1β released by incubation of WB cells with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-1β. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by WB cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)[1] or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national /international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at http://www.niehs.nih.gov/odhsb/biosafe/univers.htm. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

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[1]RSEs are internationally harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

Monocytoid cells from WB are the source of cytokine production in the WB/IL-1β test method as described by Hartung and Wendel (1996) and Schindler et al. (2006). In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting monocytes in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640[2]).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (http://www.fda.gov/cber/blood.htm). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at http://www.fda.gov/cber/gdlns/donorhistques.htm#iv. If you have questions regarding the applicability of blood regulations to your specific situation[3], it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be sterile and pyrogen-free.

3.2.1 Blood Incubation

3.2.1.1 *Equipment*

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture (37±1°C + 5% CO_2)

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[2]The requirements for WB can be found at 21 CFR 640.1 *et seq*. In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

[3]The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Pipetter; multichannel (8- or 12-channel)
- Pipetters; single-channel adjustable (20 and 200 μL)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)
- Needle set; multifly, pyrogen-free, 19 mm, 21 gauge
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for blood collection
- Tips; pipetter, sterile, pyrogen-free (20 and 200 μL)

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 600-690 nm[4])
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid

[4]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

- Tips; pipetter, nonsterile
- Tubes; polystyrene (12 mL)

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-1β release is used. A variety of IL-1β ELISA kits are commercially available and the IL-1β ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-1β ELISA should be calibrated using an IL-1β international reference standard (e.g., World Health Organization [WHO] 86/680) prior to use. The IL-1β cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-1β ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-1β capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-1β reference standard
- PFS
- Stop solution
- TMB[5]/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

ELISA solutions are listed in **Section 3.2**.

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the WB/IL-1β pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control

[5]The use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable.

(NSC) and five RSE concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL[1]	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000[2,3]	50	1950	50[4]
50	100	900	5.0
5.0	500	500	2.5
2.5	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

Each stock tube should be vortexed prior to its use to make the subsequent dilution.

[1]To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

[2]A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

[3]The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

[4]This concentration is not used in the assay.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section**

12.3). The calculation of the MVD is dependent on the ELC for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2.** For non-spiked solutions, 200 µL of PFS is added to a well followed by 20 µL of the test substance (i.e., equivalent to the negative product control [NPC]) and 20 µL of WB. Endotoxin-spiked solutions are prepared by adding 180 µL of PFS to each well followed by 20 µL of the test substance, and 20 µL of WB. Then, 20 µL of an endotoxin-spike solution (1.0 EU/mL) (i.e., equivalent to the positive product control [PPC]) is added to each well. The contents of the wells are mixed and incubated as outlined in **Section 6.1.3**, **Steps 6-8**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-1β standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well[1]	
PFS	180	200
Endotoxin-spike solution[2]	20	0
Test substance (neat and each serial dilution)	20	20
WB	20	20
Total[3]	240	240

Abbreviations: PFS = Pyrogen-free saline; WB = Whole blood

[1] n=4 replicates each

[2] Endotoxin concentration is 1.0 EU/mL in PFS.

[3] A total volume of 240 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%. For example, consider the following interference test results in **Table 4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-1β relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not to exceed the MVD, for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., WB incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., WB incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-1β Release

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture[6] from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. WB should be stored at room temperature (RT) and must be used within 4 hr. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

Prior to use in the assay, an equal volume of WB from multiple individual donors should be pooled[7].

6.1.2 Incubation Plate

Test substances are prepared at a level of dilution that did not show interference with the test system, provided that this dilution does not exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

[6]WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

[7]Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

Table 6-1 Overview of Incubation Plate Preparation in the WB/IL-1β Pyrogen Test

Number of Wells	Sample	PFS	EC	Test Sample	WB	Mix the samples; incubate for 10 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO_2.	Mix the samples; immediately transfer to an ELISA plate[3] and run ELISA or store plate in a -20°C or -80°C freezer.
		µL					
20[1]	EC	200	20	0	20		
4	NSC	220	0	0	20		
56[2]	Test samples (1-14)	200	0	20	20		

Abbreviations: EC = Endotoxin control; IL-1β = Interleukin-1β; NSC = Negative saline control; PFS = Pyrogen-free saline; WB = Whole blood

[1]Five EC concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) in quadruplicate

[2]14 test samples (n=4) per plate

[3]An IL-1β standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.3 Incubation Assay for IL-1β Release

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Blood samples are prepared in a microtiter plate using a laminar flow hood. All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer 200 µL of PFS into each well.

Step 3. Transfer 20 µL of test sample or 20 µL of PFS for the NSC into the appropriate wells as indicated in the template.

Step 4. Transfer 20 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer 20 µL of WB into each well and mix by gently swirling the plate.

Step 6. Mix the contents of the wells thoroughly by gently pipetting up and down five times using a multichannel pipetter, changing the tips between each row to avoid cross-contamination.

Step 7. Place the covered plate in a tissue culture incubator for 10 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO_2.

Step 8. Prior to transferring the test samples onto the ELISA plate, mix the contents of the wells by pipetting up and down three times using a multichannel pipetter, changing the tips between each row to avoid cross-contamination.

Note: The aliquots may be tested immediately in the ELISA or stored in a -20°C or -80°C freezer for testing at a later time. After transfer to the ELISA plate, freeze the remaining aliquots in a -20°C or -80°C freezer for subsequent experiments, if necessary.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	Void[3]	Void
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1]EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.
[2]TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3]Columns 11 and 12 are reserved for the IL-1β standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-1β Release

6.2.1 IL-1β Standard Curve

An IL-1β standard, supplied with the ELISA kit, is used. IL-1β standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in PFS to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL. Each well on the ELISA plate will receive 100 µL of an IL-1β blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A sample ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-1β standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-1β standard curve is prepared as

described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4**.

Step 1. Add 100 µL of enzyme-labeled detection antibody to each well.

Step 2. After pipetting up and down three times to mix the supernatant, transfer 100 µL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 3. Add 100 µL of each IL-1β standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 90 min on a microplate mixer at 350-400 rpm at RT.

Step 5. Decant and wash each well five to six times with 300 µL Buffered Wash Solution per well and then rinse three times with deionized water. Place the plates upside down and tap to remove the wash solution.

Step 6. Add 200 µL of TMB/Substrate Solution to each well and incubate at RT in the dark for 10 to 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 µL of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD_{450} within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 600-690 nm is recommended.[8]

[8]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-1β[3] 0	IL-1β 0
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	IL-1β 62.5	IL-1β 62.5
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	IL-1β 125	IL-1β 125
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	IL-1β 250	IL-1β 250
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	IL-1β 500	IL-1β 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-1β 1000	IL-1β 1000
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-1β 2000	IL-1β 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-1β 4000	IL-1β 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1]EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.
[2]TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3]IL-1β values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Enzyme-labeled Antibody (µL)	**Material transfer from Incubation Plate (µL)**	**IL-1β standard (0 to 4000 pg/mL) (µL)**	Incubate 90 min on a plate mixer at 350 to 400 rpm at RT.	Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	**TMB/Substrate Solution (µL)**	Incubate for less than 15 min at RT in dark.	**Stop Solution (µL)**	Read each well at OD_{450} with a 600 to 690 nm reference filter.
100	100	100			200		50	

Abbreviations: OD_{450} = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST RESULTS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with a 600 to 690 nm reference filter (recommended)[9]. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-1β standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-1β standard curve produces an $r \geq 0.95$[10] and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett et al. 1994], Grubbs' test [Barnett et al. 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance
- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)

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[9]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

[10]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

Justification of the In Vitro Test Method and Protocol 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 on the procedure used to ensure their integrity from "lot-to-lot" and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)[1]

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)[1,2]

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M, where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)[1,2]

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is[1]:

MVD = (ELC x Product Potency [PP])/λ

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is[1]:

MVD = PP/Minimum Valid Concentration (MVC)

where, MVC = (λ x M)/K

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

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[1]From FDA (1987)
[2]From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)[1,2]

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces ½ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)[1,2]

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)[1,2]

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

Appendix C2

The Human WB/IL-1β *In Vitro* Pyrogen Test: Application of Cryopreserved (Cryo) Human WB

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ICCVAM Final Recommended Protocol for Future Studies Using the Cryopreserved (Cryo) Whole Blood (WB)/Interleukin (IL)-1β *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-1β from monocytoid cells in human whole blood (WB) that have been cryopreserved (Cryo). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)[1] Cryo WB/IL-1β Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedure (SOP) for the Cryo WB/IL-1β test (could be referred to as Monocyte Activation Test), which is based on the Cryo WB/IL-1β method first described by Schindler et al. (2004). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOP is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the Cryo WB/IL-1β pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov) to ensure that the most current protocol is used.

[1]ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOP for the Cryo WB/IL-1β Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM Catch-Up Validation SOP[1]
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test at MVD
Number of Blood Donors	Minimum of 3 (independent or pooled)	5 (pooled)[2]
Decision Criteria for Interference	Mean OD[3] of PPC is 50% to 200% of 0.5 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.5 EU/mL EC
	Not included	Mean OD of PPC ≥ 1.6x Mean OD of NPC
Incubation Plate (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1)
	EC (5)	EC (2)
	TS (14)	TS (3) x EC (5) spikes = 15 TS
	PPC[4] (0)	PPC (3) = 3 TS
	NPC[4] (0)	NPC (3) = 3 TS
ELISA Plate	Includes seven point IL-1β SC and blank in duplicate	Not included
Assay Acceptability Criteria	Mean OD of NSC ≤0.15	Mean OD of NSC ≤100 m OD
	Quadratic function of IL-1β SC r ≥0.95[5]	Not included
	EC SC produces OD values that ascend in a sigmoidal concentration response	Not included
	Not included	Mean OD of 0.5 EU/mL EC ≥ 1.6x Mean OD of NSC
	Not included	If one OD of 1.0 EU/mL EC > Max, ELISA may be repeated using reduced incubation time
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test[6]
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC[7] TS	OD TS > OD 0.5 EU/mL EC[6]

Abbreviations: Cryo = Cryopreserved; EC = Endotoxin control; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-1β = Interleukin-1β; MVD = Maximum valid dilution; NPC = Negative product control; NSC = Negative saline control; OD = Optical density; PPC = Positive product control; SC = Standard curve; SOP = Standard operating procedure; TS = Test substance; WB = Whole blood

[1]ECVAM Cryo WB/IL-1β catch-up validation SOP is presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

[2]Samples are collected from five donors and pooled prior to cryopreservation.

[3]Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

[4]In the ICCVAM Cryo WB/IL-1β protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA.

[5]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

[6]Included in the ECVAM Trial data report presented in Appendix D of the ICCVAM BRD.

[7]Where unknown, the ELC is calculated (see **Section 12.2**).

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-1β from monocytoid cells in whole blood (WB) that have been cryopreserved (Cryo). The concentration of IL-1β released by incubation of Cryo WB cells with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-1β. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by Cryo WB cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)[1] or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national/international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at http://www.niehs.nih.gov/odhsb/biosafe/univers.htm. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as

[1]RSEs are internationally harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

Monocytoid cells from WB are the primary source of cytokine production in the Cryo WB/IL-1β test method as described by Hartung and Wendel (1999) and Schindler et al. (2004, 2006). In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting monocytes in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640[2]).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (http://www.fda.gov/cber/blood.htm). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at http://www.fda.gov/cber/gdlns/donorhistques.htm#iv. If you have questions regarding the applicability of blood regulations to your specific situation[3], it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, and solutions) should be sterile and pyrogen-free.

3.2.1 Blood Incubation

3.2.1.1 *Equipment*

- Centrifuge

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[2]The requirements for WB can be found at 21 CFR 640.1 *et seq*. In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

[3]The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture (37±1°C + 5% CO_2)
- Pipetter; multichannel (8- or 12-channel)
- Pipetters; single-channel adjustable (20 and 200 µL)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)
- Needle set; multifly, pyrogen-free, 19 mm, 21 gauge
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for fluid collection
- RPMI-1640 cell culture medium
- Tips; pipetter, sterile, pyrogen-free (20 and 200 µL)

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 600-690 nm[4])
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, nonsterile

[4]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

- Tubes; polystyrene (12mL)

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-1β release is used. A variety of IL-1β ELISA kits are commercially available and the IL-1β ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-1β ELISA should be calibrated using an international reference standard (e.g., World Health Organization [WHO] 86/680) prior to use. The IL-1β cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-1β ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-1β capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-1β reference standard
- PFS
- Stop solution
- TMB[5]/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

- RPMI-1640 cell culture medium

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB and the procedure for cryopreservation of WB is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the Cryo WB/IL-1β pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.25, 0.50, 1.0, 2.5, and 5.0 EU/mL) are included in the

[5]The use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable.

incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (i.e., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL[1]	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000[2,3]	50	1950	50[4]
50	100	900	5.0
5.0	500	500	2.5
2.5	400	600	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

[1]To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

[2]A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

[3]The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

[4]This concentration is not used in the assay.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section 12.3**). The calculation of the MVD is dependent on the ELC for a test substance. The ELC

can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2.** For non-spiked solutions, 200 µL of RPMI is added to a well followed by 20 µL of the test substance (i.e., equivalent to the negative product control [NPC]) and 20 µL of Cryo WB (**Section 6.1**). Endotoxin-spiked solutions are prepared by adding 180 µL of RPMI to each well followed by 20 µL of the test substance and 20 µL of Cryo WB. Then, 20 µL of an endotoxin-spike solution (1.0 EU/mL) (i.e., equivalent to the positive product control [PPC]) is added to each well. The contents of the wells are mixed and incubated as outlined in **Section 6.1.5**, **Steps 6-9**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-1β standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well[1]	
RPMI	180	200
Endotoxin-spike solution[2]	20	0
Test substance (neat and each serial dilution)	20	20
Cryo WB	20	20
Total[3]	240	240

Abbreviations: Cryo = Cryopreserved; WB = Whole blood

[1]n=4 replicates each

[2]Endotoxin concentration is 1.0 EU/mL in RPMI.

[3]A total volume of 240 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%. For example, consider the following interference test results in **Table 4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-1β relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not to exceed the MVD, for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., WB incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., Cryo WB incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-1β Release

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture[6] from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

6.1.2 Cryopreservation Procedure

The two methods available for cryopreservation of blood are 1) the PEI method developed at the Paul Ehrlich Institute (Langen, Germany) and 2) the Konstanz method developed at the University of Konstanz (Konstanz, Germany).

6.1.3 PEI Method of Cryopreservation

In the PEI method (Schindler et al. 2006), an equal volume of WB from multiple independent donors is pooled[7] and frozen in a cryoprotective phosphate buffer (Sorensen's) containing 20% (v/v) pyrogen-free, clinical-grade DMSO. The tubes can be stored in a -80°C freezer or in the vapor phase of liquid nitrogen until used.

6.1.3.1 *Konstanz Method of Cryopreservation*

In the Konstanz method (Schindler et al. 2004), pyrogen-free, clinical grade DMSO is added to WB of individual donors at a final concentration of 10% (v/v). An equal volume of WB from multiple independent donors is pooled[7] and frozen in a computer-controlled freezer using several cycles of programmed freezing down to -120°C. Tubes of WB are then removed from the instrument and stored in the vapor phase of liquid nitrogen until used.

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[6]WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

[7]Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

6.1.3.2 *Thawing Procedure*

The tubes are thawed in an incubator at 37±1°C for 15 min. Prior to use in the assay, the pooled[7] WB cells should be examined under a microscope to determine that the morphology of the cells is consistent with the appearance of cells that previously yielded acceptable results. The results of this examination should be included in the study report.

6.1.4 Incubation Plate

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

Table 6-1 Overview of Incubation Plate Preparation in the Cryo WB/IL-1β Pyrogen Test (PEI Method)

Number of Wells	Sample	RPMI	EC	Test Sample	Cryo WB[1]	Mix the samples; incubate for 10 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO_2.	Mix the samples; immediately transfer to an ELISA plate[5] and run ELISA or store plate in a -20°C or -80°C freezer.
		µL					
20[2]	EC	180	20	0	40		
4	NSC	180	0	0[3]	40		
56[4]	Test samples (1-14)	180	0	20	40		

Abbreviations: Cryo = Cryopreserved; EC = Endotoxin control; IL-1β = Interleukin-1β; NSC = Negative saline control; PEI = Paul Ehrlich Institute; PFS = Pyrogen-free saline; WB = Whole blood

[1]For the Konstanz method of cryopreservation, 20 µL of Cryo WB is used and the volume of RPMI is adjusted to 200 µL.

[2]Five EC concentrations (0.25, 0.50, 1.0, 2.5, 5.0 EU/mL) in quadruplicate

[3]20 µL of PFS is added instead of the test sample.

[4]14 test samples (n=4) per plate

[5]An IL-1β standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.5 Incubation Assay for IL-1β Release

Cryo WB is prepared in a microtiter plate using a laminar flow hood (refer to **Section 6.1.1**). All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer either 180 or 200 µL of RPMI into each well (for the PEI or Konstanz method of cryopreservation, respectively – refer to **Step 5** below).

Step 3. Transfer 20 µL of test sample or 20 µL of PFS for the NSC into the appropriate wells as indicated in the template.

Step 4. Transfer 20 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer either 40 or 20 µL of Cryo WB (for the PEI or Konstanz method of cryopreservation, respectively) into each well and mix by gently swirling the plate.

Step 6. Mix the contents of the wells thoroughly by gently pipetting up and down five times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Step 7. Place the covered plate in a tissue culture incubator for 10 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO_2.

Step 8. If using the Konstanz method, freeze the plate in a -20°C or -80°C freezer until the contents of the well are completely frozen and then, thaw the plate at RT or in a water bath not exceeding 37±1°C.

Step 9. Prior to transferring the test samples onto the ELISA plate, mix the contents of the wells by pipetting up and down three times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Note: The aliquots may be tested immediately in the ELISA or stored in a -20°C or -80°C freezer for testing at a later time. After transfer to the ELISA plate, freeze the remaining aliquots in a -20°C or -80°C freezer for subsequent experiments, if necessary.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	Void[3]	Void
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance

[1]EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.

[2]TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.

[3]Columns 11 and 12 are reserved for the IL-1β standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-1β Release

6.2.1 IL-1β Standard Curve

An IL-1β standard, supplied with the ELISA kit, is used. IL-1β standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in RPMI to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL. Each well on the ELISA plate will receive 100 µL of an IL-1β blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-1β standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-1β standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4.**

Step 1. Add 100 µL of enzyme-labeled detection antibody to each well.

Step 2. After pipetting up and down three times to mix the supernatant, transfer 100 µL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 3. Add 100 µL of each IL-1β standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 90 min on a microplate mixer at 350-400 rpm at RT.

Step 5. Decant and wash each well three times with 300 µL Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 µL of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 µL of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD_{450} within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 600-690 nm is recommended.[8]

[8]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 5.0	EC 5.0	EC 5.0	EC 5.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-1β[3] 0	IL-1β 0
B	EC 2.5	EC 2.5	EC 2.5	EC 2.5	TS4	TS4	TS4	TS4	TS11	TS11	IL-1β 62.5	IL-1β 62.5
C	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS5	TS5	TS5	TS5	TS12	TS12	IL-1β 125	IL-1β 125
D	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS6	TS6	TS6	TS6	TS12	TS12	IL-1β 250	IL-1β 250
E	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS7	TS7	TS7	TS7	TS13	TS13	IL-1β 500	IL-1β 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-1β 1000	IL-1β 1000
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-1β 2000	IL-1β 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-1β 4000	IL-1β 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1]EC value (e.g., EC 5.0) represents the endotoxin concentration in EU/mL.
[2]TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3]IL-1β values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Enzyme-labeled Antibody (µL)	**Material transfer from Incubation Plate (µL)**	**IL-1β standard (0 to 4000 pg/mL) (µL)**	Incubate 90 min on a plate mixer at 350 to 400 rpm at RT.	Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	**TMB/Substrate Solution (µL)**	Incubate for less than 15 min at RT in dark.	**Stop Solution (µL)**	Read each well at OD_{450} with a 600 to 690 nm reference filter.
100	100	100			200		50	

Abbreviations: OD_{450} = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST RESULTS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with

a 600 to 690 nm reference filter (recommended)[9]. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-1β standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-1β standard curve produces an $r \geq 0.95$[10] and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett et al. 1984], Grubbs' test [Barnett et al. 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance
- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, and resuspension solvent)

Justification of the In Vitro Test Method and Protocol Used

Test Method Integrity

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[9]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

[10]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

- 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 on the procedure used to ensure their integrity from "lot-to-lot" and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)[11]

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)[11,12]

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M, where:

[11]From FDA (1987)
[12]From USP (2007)

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)[11,12]

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is[11]:

MVD = (ELC x Product Potency [PP])/λ

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is[11]:

MVD = PP/Minimum Valid Concentration (MVC)
where, MVC = (λ x M)/K
where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

12.5 Parenteral Threshold Pyrogen Dose (K)[11,12]

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces ½ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)[11,12]

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)[11,12]

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

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Appendix C3

The Human WB/IL-6 *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Human Whole Blood (WB)/Interleukin (IL)-6 *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-6 from monocytoid cells in human whole blood (WB). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)[1] WB/IL-6 Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedure (SOP) for the WB/IL-6 test (could be referred to as Monocyte Activation Test), which is based on the WB/IL-6 method first described by Pool et al. (1998). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOP is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the WB/IL-6 pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov) to ensure that the most current protocol is used.

[1]ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

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Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOP for the WB/IL-6 Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM SOP[1]
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test neat or at minimal dilution that produces no interference
Number of Blood Donors	Minimum of 3 (independent or pooled)	Minimum of 3 (independent)
Decision Criteria for Interference	Mean OD[2] of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate for ELISA (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1)
	EC (5)	EC (5)
	TS (14)	TS (14)
	PPC[3] (0)	PPC (0)
	NPC[3] (0)	NPC (0)
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate
Assay Acceptability Criteria	Mean OD of NSC ≤0.15	NSC < 200 pg/mL IL-6
	Quadratic function of IL-6 SC r ≥0.95[4]	EC SC satisfies ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures Methodology; ICH Q2B, Nov 1996
	EC SC produces OD values that ascend in a sigmoidal concentration response	Wilcoxon rank-sum test used to show that at least 3 of 4 replicates at each increasing EC concentration are higher relative to the next lowest concentration
	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test[5]
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC[6] TS	Endotoxin concentration TS > ELC TS OR Limit test is run to determine whether or not a TS after correction and dilution contains < 0.5 EU/mL of endotoxin

Abbreviations: EC = Endotoxin control; ELC = Endotoxin limit concentration; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-6 = Interleukin-6; MVD = Maximum valid dilution; NSC = Negative saline control; OD = Optical density; PPC = Positive product control; SC = Standard curve; TS = SOP = Standard operating procedure; Test substance; WB = Whole blood

[1]ECVAM WB/IL-6 SOP is presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

[2]Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

[3]In the ICCVAM WB/IL-6 protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA. In the ECVAM SOP, PPC and NPC were only included in the ECVAM validation study.

[4]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.
[5]Included in the ECVAM Trial data report presented in Appendix D of the ICCVAM BRD.
[6]Where unknown, the ELC is calculated (see **Section 12.2**)

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-6 from monocytoid cells in whole blood (WB). The concentration of IL-6 released by incubation of WB with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by WB cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)[1] or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national/international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at http://www.niehs.nih.gov/odhsb/biosafe/univers.htm. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

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[1]RSEs are internationally-harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

Monocytoid cells from fresh WB are the source of cytokine production in the WB/IL-6 test method as described by Hartung and Wendel (1996), Pool et al. (1998), and Schindler et al. (2006). In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting monocytes in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640[2]).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (http://www.fda.gov/cber/blood.htm). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at http://fda.gov/cber/gdlns/donorshitques.htm#iv. If you have any questions regarding the application of blood regulations to your specific situation[3], it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how the unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be sterile and pyrogen-free.

3.2.1 Blood Incubation

3.2.1.1 Equipment

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture (37±1°C + 5% CO_2)

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[2]The requirements for WB can be found at 21 CFR 640.1 *et seq*. In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

[3]The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Pipetter, multichannel (8- or 12-channel)
- Pipetters, single-channel adjustable (20 and 200 μL)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)
- Needle set; multifly, pyrogen-free, 19 mm, 21 gauge
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for blood collection
- Tips; pipetter, sterile, pyrogen-free (20 and 200 μL)

3.2.2 ELISA

3.2.2.1 Equipment

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 540-590 nm)[4]
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, nonsterile
- Tubes; polystyrene (12 mL)

[4]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-6 release is used. A variety of IL-6 ELISA kits are commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-6 ELISA should be calibrated using an IL-6 international reference standard (e.g., World Health Organization [WHO] 89/548) prior to use. The IL-6 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-6 reference standard
- PFS
- Stop solution
- TMB[5]/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

ELISA solutions are listed in **Section 3.2**.

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the WB/IL-6 pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of

[5]The use of an IL-6 ELISA kit with a chromagen other than TMB is acceptable.

the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL[1]	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration in Tube EU/mL
2000[2,3]	20	1980	20[4]
20	100	900	2.0
2.0	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

Each stock tube should be vortexed prior to its use to make the subsequent dilution.

[1]To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

[2]A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

[3] The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

[4]This concentration is not used in the assay.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer **to Section 12.3**). The calculation of the MVD

is dependent on the ELC for a test substance. The ELC can be calculated by by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2.** For non-spiked solutions, 50 µL of PFS is added to a well followed by 50 µL of WB and mixed by inversion. Then, 50 µL of the test substance (i.e., equivalent to the negative product control [NPC]) is added followed by 100 µL of PFS and the well contents are mixed. Endotoxin-spiked solutions are prepared by adding 50 µL of PFS to each well followed by 50 µL of WB and mixed by inversion. Then, 50 µL of the test substance, 50 µL of an endotoxin-spike solution (1.0 EU/mL), and 50 µL of PFS (i.e., equivalent to the positive product control [PPC]) are added to each well. The contents of the wells are mixed and incubated as outlined in **Section 6.1.3**, **Steps 6-8**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-6 standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well[1]	
PFS (total volume added)	100[2]	150[2]
Endotoxin-spike solution[3]	50	0
Test substance (neat and each serial dilution)	50	50
WB	50	50
Total[4]	250	250

Abbreviations: PFS = Pyrogen-free saline; WB = Whole blood

[1]n=4 replicates each

[2]50 µL of WB and 50 µL of PFS are added to each well and mixed by inversion prior to the addition of the remaining components and volume of PFS.

[3]Endotoxin concentration is 1.0 EU/mL in PFS.

[4]A total volume of 250 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by

setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%. For example, consider the following interference test results in **Table 4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-6 relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not exceed the MVD for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., WB incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., WB incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-6 Release

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture[6] from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. WB should be stored at room temperature (RT) and must be used within 4 hr[7]. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

Prior to use in the assay, an equal volume of WB from multiple individual donors should be pooled[8].

6.1.2 Incubation Plate

Test substances are prepared at a level of dilution that did not show interference with the test system, provided that this dilution does not exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test substances (see Table **6-1**).

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[6]WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

[7]Although the ECVAM SOP did not describe the use of cryopreserved WB for the WB/IL-6 test method, the use of cryopreserved WB with the WB/IL-1 test method was outlined and this methodology may also be appropriate for the WB/IL-6 test method, but this has yet to be demonstrated.

[8]Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

Table 6-1 Overview of Incubation Plate Preparation in the WB/IL-6 Pyrogen Test

Number of Wells	Sample	PFS	EC	Test Sample	WB	Mix the samples; incubate for 16 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO_2.	Mix the samples; immediately transfer to an ELISA plate[3] and run ELISA.
		µL					
20[1]	EC	100	50	0	50		
4	NSC	150	0	0	50		
56[2]	Test samples (1-14)	100	0	50	50		

Abbreviations: EC = Endotoxin control; IL-6 = Interleukin-6; NSC = Negative saline control; PFS = Pyrogen-free saline WB = Whole blood

[1]Five EC concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) in quadruplicate

[2]14 test samples (n=4 each) per plate

[3]An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.3 Incubation Assay for IL-6 Release

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Blood samples are prepared in a microtiter plate using a laminar flow hood. All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

- **Step 1.** Refer to the incubation plate template presented in **Table 6-2.**
- **Step 2.** Using a pipetter, transfer 100 µL of PFS into each well.
- **Step 3.** Transfer 50 µL of test sample or 50 µL of PFS for the NSC into the appropriate wells as indicated in the template.
- **Step 4.** Transfer 50 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.
- **Step 5.** Transfer 50 µL of WB into each well and mix by gently swirling the plate.
- **Step 6.** Mix the contents of the wells thoroughly by gently pipetting up and down several times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.
- **Step 7.** Place the covered plate in a tissue culture incubator for 16 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO_2.

- **Step 8.** Prior to transferring the test samples to the ELISA plate, mix the contents of the wells by pipetting up and down using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	Void[3]	Void
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1] EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.
[2] TS number (e.g., TS 1) represents an arbitrary sequence for individual test substances.
[3] Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-6 Release

6.2.1 IL-6 Standard Curve

An IL-6 standard, supplied with the ELISA kit, is used. IL-6 standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in PFS to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 µL. Each well on the ELISA plate will receive 50 µL of an IL-6 blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-6 standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-6 standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4.**

Step 1. After pipetting up and down three times to mix the supernatant, transfer 50 µL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 2. Add 50 µL of each IL-6 standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 3. Add 200 µL of the enzyme-labeled detection antibody (neat as supplied, or diluted, if necessary) to each of the wells.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr at RT.

Step 5. Decant and wash each well three times with 300 µL Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 µL of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 µL of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD_{450} within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 540 to 590 nm is recommended.[9]

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6[3] 0	IL-6 0
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1]EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.
[2]TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3]IL-6 values in columns 11 and 12 are in pg/mL.

[9]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-4 Overview of ELISA Procedure

Material transfer from Incubation Plate (µL)	**IL-6 standard (0 to 4000 pg/mL) (µL)**	**Enzyme-labeled Antibody (µL)**	Cover the Incubation Plate and incubate for 2 to 3 hr at RT.	Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	**TMB/Substrate Solution (µL)**	Incubate for less than 15 min at RT in the dark.	**Stop Solution (µL)**	Read each well at OD_{450} with a 540 to 590 nm reference filter.
50	50	200			200		50	

Abbreviations: OD_{450} = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST METHODS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with a 540 to 590 nm reference filter (recommended)[10]. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6 standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-6 standard curve produces an $r \geq 0.95$[11] and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

Blood donors (or a pool of blood donors) are considered to be high responders if their concentration of IL-6 is greater than 200 pg/mL. High responders should be excluded from analysis. The preparation being examined is required to pass the test with blood donations from at least three different donors (i.e., either as a pool of three individual donors or as three individual donors tested independently).

[10]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.
[11]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett and Lewis 1994], Grubbs' test [Barnett and Lewis 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance
- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

Justification of the Test Method and the Protocol 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 on the procedure used to ensure their integrity from “lot-to-lot” and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure used

- Description of any modification to the test procedure
- Reference to historical data of the model
- Description of the evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)[1]

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)[1,2]

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M, where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)[1,2]

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is[1]:

MVD = (ELC x Product Potency [PP])/λ

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is[1]:

MVD = PP/Minimum Valid Concentration (MVC)

where, MVC = (λ x M)/K

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

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[1]From FDA (1987)
[2]From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)[1,2]

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces ½ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)[1,2]

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)[1,2]

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

Appendix C4

The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Human Peripheral Blood Mononuclear Cell (PBMC)/Interleukin (IL)-6 *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-6 from human peripheral blood mononuclear cells (PBMCs). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)[1] PBMC/IL-6 Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedures (SOPs) for the PBMC/IL-6 test (could be referred to as Monocyte Activation Test), which is based on various methods that use human PBMCs to detect cytokine production as a measure of pyrogen presence (Bleeker et al. 1994; Dinarello et al. 1984; Poole et al. 2003). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOPs is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the PBMC/IL-6 pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov) to ensure that the most current protocol is used.

[1]ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

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Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOPs for the PBMC/IL-6 Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM Catch-Up Validation SOP[1]	ECVAM Validation SOP[1]
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test at MVD	Test at MVD
Number of Blood Donors	Minimum of 3 (independent or pooled)	Minimum of 3[2] (independent)	Minimum of 4 (independent)
Decision Criteria for Interference	Mean OD[3] of PPC is 50% to 200% of 0.25 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.25 EU/mL EC	Mean OD of PPC is 50% to 200% of 0.25 EU/mL EC
Incubation Plate for ELISA (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1)	NSC (1)
	EC (5)	EC (5)	EC (5)
	TS (14)	TS (2) x EC (5) spikes = 10 TS	TS (2) x EC (5) spikes = 10 TS
	PPC[4] (0)	PPC (2) = 2 TS	PPC (2) = 2 TS
	NPC[4] (0)	NPC (2) = 2TS	NPC (2) = 2TS
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate
Assay Acceptability Criteria	Mean OD of NSC ≤0.15	Mean OD of NSC ≤0.15	Mean OD of NSC ≤0.15
	Quadratic function of IL-6 SC r ≥0.95[5]	Quadratic function of IL-6 SC r ≥0.95	Quadratic function of IL-6 SC r ≥0.95
	EC SC produces OD values that ascend in a sigmoidal concentration response	EC SC produces OD values that ascend in a sigmoidal concentration response	EC SC produces OD values that ascend in a sigmoidal concentration response
	High responder blood donors (i.e., > 200 pg/mL IL-6) or low responder blood donors (i.e., Mean OD of 1EU/mL EC is significantly less than that of 1000 pg/mL IL-6) may be excluded	High responder blood donors (i.e., > 200 pg/mL IL-6) or low responder blood donors (i.e., Mean OD of 1EU/mL EC is significantly less than that of 1000 pg/mL IL-6) may be excluded	High responder blood donors (i.e., > 200 pg/mL IL-6) or low responder blood donors (i.e., Mean OD of 1EU/mL EC is significantly less than that of 1000 pg/mL IL-6) may be excluded
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC[6] TS	Endotoxin concentration TS > ELC TS	Endotoxin concentration TS > ELC TS[7]

Abbreviations: EC = Endotoxin control; ELC = Endotoxin limit concentration; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-6 = Interleukin-6; MVD = Maximum valid dilution; NSC = Negative saline control; OD = Optical density; PBMC = Peripheral blood mononuclear cell; PPC = Positive product control; SC = Standard curve; SOP = Standard operating procedure; TS = Test substance

[1]ECVAM PBMC/IL-6 SOPs are presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

[2]Sample are cryopreserved prior to use in the assay.

[3]Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

[4]In the ICCVAM PBMC/IL-6 protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA.

[5]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

[6]Where unknown, the ELC is calculated (see **Section 12.2**).

[7]Decision criteria for individual donors were defined in the ECVAM Validation SOP for the PBMC/IL-6 test method. Test method users should refer to these criteria if multiple donors are tested independently.

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-6 from human peripheral blood mononuclear cells (PBMCs). The concentration of IL-6 released by incubation of PBMCs with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by PBMCs exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)[1] or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national/international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at http://www.niehs.nih.gov/odhsb/biosafe/univers.htm. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'- TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

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[1]RSEs are internationally-harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

PBMCs from fresh whole blood (WB) are the source of cells for cytokine production in the PBMC/IL-6 test method as reported by Poole et al. (2003)[2]. In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting PBMCs in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640[3]).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (http://www.fda.gov/cber/blood.htm). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at http://www.fda.gov/cber/gdlns/donorhistques.htm#iv. If you have questions regarding the applicability of blood regulations to your specific situation[4], it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be sterile and pyrogen-free.

3.2.1 Preparation of PBMCs

3.2.1.1 *Equipment*

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)

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[2]As indicated by the ECVAM Catch-Up Validation SOP for the PBMC/IL-6 test method, PBMCs that have been cryopreserved can also be used as the source of cells in the PBMC/IL-6 test method.

[3]The requirements for WB can be found at 21 CFR 640.1 *et seq*. In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

[4]The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Incubator; cell culture (37±1°C + 5% CO_2)
- Lymphoprep™
- Pipetter; multichannel (8- or 12-channel)
- Pipetters; single-channel adjustable (20, 200, and 1000 µL)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (2.5 and 5.0 mL)
- Cryotubes; screw-cap, 2 mL
- Filters; sterile, 0.22 µm
- Needle set; multifly, pyrogen-free, 19 mm, 21 gauge
- Phosphate buffered saline (PBS); sterile
- Pipettes; serological, sterile (5, 10, and 25 mL)
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for blood collection
- RPMI-1640 cell culture medium (500 mL); supplemented with the following reagents to yield RPMI-C
  - Human serum albumin (HSA); 5 mL or a 1% final concentration)
  - L–Glutamine; 200 mM
  - Penicillin/streptomycin (10,000 IU/mL penicillin, 10 mg/mL streptomycin)
- Syringes; sterile (100 µL and 30 mL)
- Tips; pipetter, sterile, pyrogen-free (20, 200, and 1000 µL)

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer

- Microplate reader (450 nm with an optional reference filter in the range of 540-590 nm)[5]
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, nonsterile
- Tubes; polystyrene (12 mL)

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-6 release is used. A variety of IL-6 ELISA kits are commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-6 ELISA should be calibrated using an IL-6 international reference standard (e.g., World Health Organization [WHO] 89/548) prior to use. The IL-6 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-6 reference standard
- PFS
- Stop solution
- TMB[6]/substrate solution

[5]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

[6]The use of an IL-6 ELISA kit with a chromagen other than TMB is acceptable.

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

- RPMI-C cell culture medium; supplemented as described in **Section 3.2.1.2**

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB, the isolation of PBMCs from WB, and the procedure for cryopreservation of PBMCs is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the PBMC/IL-6 pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.063, 0.125, 0.25, 0.50, and 1.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL[1]	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000[2,3]	40	3960	20[4]
20	100	1900	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0.125	500	500	0.063
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

Each stock tube should be vortexed vigorously prior to its use to make the subsequent dilution.

[1]To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

[2]A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

[3]The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

[4]This concentration is not used in the assay.

To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when

stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section 12.3**). The calculation of the MVD is dependent on the ELC for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 0.25 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2.** For non-spiked solutions**,** 150 µl of RPMI-C is added to a well followed by 50 µl of the test substance (i.e., equivalent to the negative product control [NPC]) and 50 µL of PBMCs and the well contents are mixed. Endotoxin-spiked solutions are prepared by adding 100 µL of RPMI-C to each well followed by 50 µL of the test substance, and 50 µL of an endotoxin-spike solution (0.25 EU/mL) (i.e., equivalent to the positive product control [PPC]). Finally, 50 µL of PBMCs are added to each well and the wells are mixed and incubated as outlined in **Section 6.1.3, Steps 6-8**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-6 standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well[1]	
RPMI-C	100	150
Endotoxin-spike solution[2]	50	0
Test substance (neat and each serial dilution)	50	50
PBMCs[3]	50	50
Total[4]	250	250

Abbreviations: PBMC = Peripheral blood mononuclear cells
[1]n=4 replicates each
[2]Endotoxin concentration is 0.25 EU/mL in RPMI-C.
[3]PBMCs are resuspended in RPMI-C (1 x 10^6 cells/mL).
[4]A total volume of 250 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 0.25 EU/mL) at 100%. For example, consider the following interference test results in **Table 4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-6 relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not to exceed the MVD, for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance to be used in the *in vitro* pyrogen test would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 0.25 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., PBMCs incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., PBMCs incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-6 Release

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture[7] from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of

[7]WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

6.1.1.1 *Isolation of PBMCs from WB*

PBMCs are isolated from WB using density gradient centrifugation. The PBMC suspension must be isolated within 2 hr of WB collection using Lymphoprep™. The isolated PBMC suspension may be used immediately (**Section 6.1.2**) or frozen for later use (**Section 6.1.1.3**). The isolation procedure described below is a modification of the manufacturer's instructions as outlined in the ECVAM SOP for the PBMC/IL-6 pyrogen test.

To form a lower, denser layer, 15 mL of PBS and 20 mL of Lymphoprep™ should be added to each tube containing 15 mL of WB. The tubes are then centrifuged at 340 x g for 45 min at RT. After centrifugation, a white band of PBMCs should be visible at approximately the 25 mL graduation mark on the tube. If cryopreservation of PBMCs is to be performed (see **Section 6.1.1.3**), carefully remove 18 mL of supernatant above the PBMC band and transfer it to a new tube for preparing a cryoprotective solution. The remaining supernatant above the PBMC band should be aspirated and discarded. Using a 10 mL pipet, transfer the PBMC layer to a new centrifuge tube.

6.1.1.2 *Washing PBMCs*

The PBMCs are resuspended in a total volume of 50 mL of PBS and centrifuged at 340 x g for 15 min. The supernatant is poured off and the cellular sediment resuspended in 10 mL of PBS by pipetting up and down several times with a serological pipet. The total volume in each tube is adjusted to 50 mL with PBS and centrifuged at 340 x g for 10 min. After centrifugation, the PBMCs should be resuspended in RPMI-C and an equal volume of cell suspension from multiple individual donors should be pooled[8].

Prior to use in the assay, the pooled PBMCs should be examined under a microscope to determine that the morphology of the cells is consistent with the appearance of cells that previously yielded acceptable results. It is advisable that cell number and cell viability be determined using appropriate methods (e.g., hemocytometer and vital dye or flow cytometer and fluorescent marker). The cell count of the PBMC suspension should be adjusted to 1 x 10^6 cells/mL in RPMI-C. The percentage of viable PBMCs should exceed 80% for their inclusion in the test. The results of these examinations should be included in the study report. If PBMCs are prepared from fresh WB, then the cell suspension must be used in the assay within 4 hr from the time of WB collection.

6.1.1.3 *Procedure for Cryopreservation and Thawing of PBMCs*

To freeze the PBMCs, prepare a cryoprotective solution by adding 2 mL of pyrogen-free DMSO to the supernatant (18 mL) collected in the centrifugation procedure outlined in **Section 6.1.1.1**. Cool the cryoprotective solution to between 2 and 8°C. Centrifuge the isolated PBMCs as instructed in **Section 6.1.1.2** and then add 6 mL of the chilled cryoprotective solution to the cell sediment and prepare aliquots in cryotubes. The cryotubes

[8]Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

are placed in a Styrofoam box for thermal insulation and slowly frozen in a -80°C freezer. After 72 hr, the tubes can be transferred to liquid nitrogen for prolonged storage.

To thaw the cryopreserved PBMCs, submerge the tubes in a water bath at 37±1°C. After thawing, the cell suspensions are pooled in a single 50 mL centrifuge tube and RPMI-C is added to give a total volume of 40 mL. The PBMCs are centrifuged at 340 x g for 10 min, the supernatant removed, and the cells resuspended in 10 mL of RPMI-C.

Prior to use in the assay, it is advisable that cell number and cell viability be examined as described in **Section 6.1.1.2**. The cell count of the PBMC suspension should be adjusted to 1 x 10^6 cells/mL in RPMI-C. The percentage of viable PBMCs should exceed 80% for their inclusion in the test. The results of this examination should be included in the study report.

6.1.2 Incubation Plate

Test substances are prepared at a level of dilution that did not show interference with the test system, provided that this dilution does not exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

Table 6-1 Overview of Incubation Plate Preparation in the PBMC/IL-6 Pyrogen Test

Number of Wells	Sample	RPMI-C	EC	Test Sample	PBMCs	Mix the samples; incubate for 16 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO_2.	Mix the samples; immediately transfer to an ELISA plate[4] and run ELISA.
		µL					
20[1]	EC	100	50	0	100		
4	NSC	100	0	0[2]	100		
56[3]	Test samples (1-14)	100	0	50	100		

Abbreviations: EC = Endotoxin control; IL-6 = Interleukin-6; NSC = Negative saline control; PBMC = Peripheral blood mononuclear cell

[1] Five EC concentrations (0.063, 0.125, 0.25, 0.50, and 1.0 EU/mL) in quadruplicate

[2] 50 µL of PFS is added instead of the test sample.

[3] 14 test samples (n=4 each) per plate

[4] An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.3 Incubation Assay for IL-6 Release

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. PBMC samples are prepared in a microtiter plate using a laminar flow hood (refer to **Section 6.1.1**). All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled

appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the suggested incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer 100 µL of RPMI-C into each well.

Step 3. Transfer 50 µL of test sample or 50 µL of PFS for the NSC into the appropriate wells as indicated in the template.

Step 4. Transfer 50 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer 100 µL of a well-mixed PBMC suspension into each well and mix by gently swirling the plate.

Step 6. Mix the contents of the wells thoroughly by pipetting up and down several times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Step 7. Place the covered plate in a tissue culture incubator for 16 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO_2.

Step 8. Prior to transferring the test samples to the ELISA plate, mix the contents of the wells by pipetting up and down using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 1.0	EC 1.0	EC 1.0	EC 1.0	TS3	TS3	TS3	TS3	TS11	TS11	Void[3]	Void
B	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.063	EC 0.063	EC 0.063	EC 0.063	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1]EC value (e.g., EC 1.0) represents the endotoxin concentration in EU/mL.
[2]TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3]Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-6 Release

6.2.1 IL-6 Standard Curve

An IL-6 standard, supplied with the ELISA kit, is used. IL-6 standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in RPMI-C to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 µL. Each well on the ELISA plate will receive 50 µL of an IL-6 blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-6 standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-6 standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4.**

Step 1. After pipetting up and down very carefully three times (avoid detachment of the adherent PBMCs) to mix the supernatant, transfer 50 µL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 2. Add 50 µL of each IL-6 standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 3. Add 200 µL of the enzyme-labeled detection antibody (neat as supplied, or diluted, if necessary) to each of the wells.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr at RT.

Step 5. Decant and wash each well three times with 300 µL Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 µL of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 µL of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD_{450} within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 540 to 590 nm is recommended.[9]

[9]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 1.0	EC 1.0	EC 1.0	EC 1.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6[3] 0	IL-6 0
B	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.063	EC 0.063	EC 0.063	EC 0.063	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1]EC value (e.g., EC 1.0) represents the endotoxin concentration in EU/mL.
[2]TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3]IL-6 values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Material transfer from Incubation Plate (µL)	**IL-6 standard (0 to 4000 pg/mL) (µL)**	**Enzyme-labeled Antibody (µL)**	Cover the Incubation Plate and incubate for 2 to 3 hr at RT.	Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	**TMB/Substrate Solution (µL)**	Incubate for less than 15 min at RT in dark.	**Stop Solution (µL)**	Read each well at OD_{450} with a 540 to 590 nm reference filter.
50	50	200			200		50	

Abbreviations: OD_{450} = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST METHODS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with

a 540 to 590 nm reference filter (recommended)[10]. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6 standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-6 standard curve produces an r ≥0.95[11] and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

Blood donors (or a pool of blood donors) are considered to be low responders if their OD_{450} value obtained for 1.0 EU/mL EC is below the OD_{450} value obtained for 1000 pg/mL IL-6. Blood donors (or a pool of blood donors) who produce an OD_{450} value for the NSC that is above the OD_{450} value at 500 pg/mL IL-6 are considered to be high responders. Low and high responders should be excluded from analysis. The preparation being examined is required to pass the test with blood donations from at least three different donors (i.e., either as a pool of three individual donors or as three individual donors tested independently).

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett and Lewis 1994], Grubbs' test [Barnett and Lewis 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection[12]

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance

[10]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

[11]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

[12]Decision criteria for individual donors were defined in the ECVAM SOP for the PBMC/IL-6 test method. Test method users should refer to these criteria if multiple donors are tested independently.

- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

Justification of the In Vitro Test Method and Protocol 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 on the procedure used to ensure their integrity from "lot-to-lot" and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure used
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

11.0 REFERENCES

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)[1]

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)[1,2]

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M, where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product is used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)[1,2]

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is[1]:

MVD = (ELC x Product Potency [PP])/λ

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is[1]:

MVD = PP/Minimum Valid Concentration (MVC)

where, MVC = (λ x M)/K

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

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[1]From FDA (1987)
[2]From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)[1,2]

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces ½ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)[1,2]

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)[1,2]

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

Appendix C5

The Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6 *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Monocytoid Cell Line Mono Mac 6 (MM6)/Interleukin (IL)-6 *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-6 from the monocytoid cell line Mono Mac 6 (MM6). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)[1], MM6/IL-6 Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), and 2) information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ICCVAM BRD includes the ECVAM Standard Operating Procedures (SOPs) for the MM6/IL-6 test (could be referred to as Monocyte Activation Test), which are based on the methodology published by Taktak et al. (1991). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOPs is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the MM6/IL-6 pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov) to ensure that the most current protocol is used.

[1]ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

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Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOPs for the MM6/IL-6 Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM SOP[1]	ECVAM Validation SOP[1]
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test neat or at minimal dilution that produces no interference	Test at MVD
Decision Criteria for Interference	Mean OD[2] of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate for ELISA (The number of samples or controls in quadruplicate)	NSC (1)	NSC (1)	NSC (1)
	EC (5)	EC (5)	EC (5)
	TS (14)	TS (14)	TS (2) x EC (5) spikes = 10 TS
	PPC[3] (0)	PPC (0)	PPC (2) = 2 TS
	NPC[3] (0)	NPC (0)	NPC (2) = 2 TS
	PC[4] (0)	PC (0)	PC (1) = 1 TS
	NC[4] (0)	NC (0)	NC (1) = 1 TS
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate	Not included
Assay Acceptability Criteria	Quadratic function of IL-6 SC r ≥0.95[5]	Not included	Not included
	Mean OD of NSC ≤0.15	Not included	Not included
	EC SC produces OD values that ascend in a sigmoidal concentration response	Endotoxin concentration (0.5 IU/mL) > background (defined as the mean +2SD (n-1)	Mean OD of each EC > Mean OD of next lower EC concentration (minimum of 4 data points needed for valid SC)
	Not included	Not included	PC = ±20% of the theoretical value
	Not included	Not included	OD NC < 0.200
	Not included	Not included	OD PC > LOQ[6]
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC[7] TS	Endotoxin concentration TS .> ELC TS	OD TS > OD 0.5 EU/mL EC

Abbreviations: EC = Endotoxin control; ELC = Endotoxin limit concentration; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-6 = Interleukin-6; IU = International units; LOQ = Limit of quantification; MM6 = Mono Mac 6; MVD = Maximum valid dilution;
NC = Negative control; NPC = Negative product control; NSC = Negative saline control; OD = Optical density; PC = Positive control;

PPC = Positive product control; SC = Standard curve; SD = Standard deviation; SOP = Standard operating procedure; TS = Test substance

[1]ECVAM MM6/IL-6 SOP and ECVAM MM6/IL-6 Validation SOP are presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

[2]Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

[3]In the ICCVAM MM6/IL-6 protocol, PPC and NPC are assessed in the interference test described in **Section 4.3**, which is performed prior to the ELISA. In the ECVAM SOPs, PPC and NPC were only included in the ECVAM validation study.

[4]PC and NC were only included in the ECVAM validation study. PC is 50 pg/mL endotoxin in saline. NC is 0.9% saline.

[5]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

[6]LOQ is the mean OD of the NSC + 10x the SD of the mean OD for the NSC.

[7]Where unknown, the ELC is calculated (See **Section 12.2**).

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of IL-6 from Mono Mac 6 (MM6) cells, a human cell line derived from a patient with acute monocytic leukemia (Zeigler-Heitbrock et al. 1988). The concentration of IL-6 released by incubation of MM6 cells with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by MM6 cells exposed to the test substance to those exposed to an internationally harmonized Reference Standard Endotoxin (RSE)[1] or an equivalent standard expressed in Endotoxin Units (EU)/mL. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PROCEDURES

All procedures should be performed following standard laboratory precautions, including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'-TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 minutes (min). If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

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[1]RSEs are internationally harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

3.0 MATERIALS, EQUIPMENT AND SUPPLIES

3.1 Source of Cells

The MM6 cell line is a human monocytic cell line originally described by Professor H.W.L. Ziegler-Heitbrock at the Institute for Immunology, University of Munich, Germany (Ziegler-Heitbrock et al. 1988). The MM6 cell line may be purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, http://www.dsmz.de) by individuals working at non-profit organizations. Prior to transaction, a legal agreement must be reached with Professor H.W.L. Ziegler-Heitbrock stating that the cells will be used for research purposes only. Any contract research organization or pharmaceutical company wanting to obtain the MM6 cell line must contact Professor H.W.L. Ziegler-Heitbrock to negotiate a fee for provision and a royalty payment per batch of product tested. Contact information for Professor H.W.L. Ziegler-Heitbrock is as follows: Professor Dr. H.W.L. Ziegler-Heitbrock, University of Leicester, Dept. of Microbiology, University Road, Leicester LE1 9HN, United Kingdom, e-mail: ziehei@gmx.de.

MM6 cells should be maintained according to the instructions provided by the DSMZ and Professor Dr. H.W.L. Ziegler-Heitbrock, which should stipulate the permissible limit to the passage number for these cells.

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples (e.g., pipet tips, containers, solutions) should be sterile and pyrogen-free.

3.2.1 Utilization of MM6 cells

3.2.1.1 Equipment

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture (37±1°C + 5% CO_2)
- Inverted Microscope
- pH meter
- Pipetter; multichannel (8- or 12-channel)
- Pipetters; single-channel adjustable (20, 200, and 1000 µL)
- Repeating pipetter
- Vortex mixer
- Water bath

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)

- Cryotubes; screw-cap (2 mL)
- Filters; sterile, 0.22 μm
- Flasks; tissue culture
- Phosphate buffered saline (PBS); sterile
- Pipettes; sterile
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- RPMI-1640 cell culture medium supplemented as described in **Section 4.3** to yield either RPMI-C or RPMI-M
- Tips; pipetter, sterile, pyrogen-free (20 and 200 μL)
- Tubes; polystyrene

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 540-590 nm)[2]
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, 96-well, polystyrene
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, sterile and nonsterile
- Tubes; polystyrene (12 mL)

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-6 release is used. A variety of IL-6 ELISA kits are commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. The IL-6 ELISA should be calibrated using an IL-6

[2] The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

international reference standard (e.g., World Health Organization [WHO] 89/548) prior to use. The IL-6 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-6 reference standard
- PFS
- Stop solution
- TMB[3]/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; U.S. Food and Drug Administration [FDA] *E. coli* Lot EC6)

3.4 Solutions

- RPMI-1640 cell culture medium; supplemented as described in **Section 4.3**

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The preparation of MM6 cells for use in the assay is outlined in **Section 6.1**.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the MM6/IL-6 pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in

[3]The use of an IL-6 ELISA kit with a chromagen other than TMB is acceptable.

a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL[1]	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000[2,3]	40	3960	20[4]
20	100	900	2.0
2.0	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline
Each stock tube should be resonicated and vortexed vigorously before the subsequent dilution.
[1]To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.
[2]A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.
[3]The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.
[4]This concentration is not used in the assay.

4.2 Cell Culture Medium

MM6 cells are maintained in RPMI containing 10% FBS, denoted as RPMI-M. For use in the ELISA procedure, the concentration of FBS is reduced to 2% and referred to as RPMI-C. Each medium is prepared and stored as described by the manufacturer.

4.2.1 RPMI-M

- Bovine insulin; 0.23 IU/mL
- FBS; heat-inactivated at 55±1°C (50 mL or a 10% final concentration)
- HEPES buffer; 20 mM
- L–Glutamine; 2 mM
- MEM non-essential amino acids; 0.1 mM
- Oxaloacetic acid; 1 mM
- Penicillin/streptomycin (10,000 IU/mL penicillin, 10 mg/mL streptomycin)
- RPMI-1640 medium (500 mL)
- Sodium pyruvate; 1 mM

4.2.2 Starting a Culture of MM6 Cells

To initiate a culture of MM6 cells, remove a vial of the primary stock from liquid nitrogen. Thaw the vial on ice. Gently mix and transfer the cells to a 50 mL centrifuge tube and add 10 mL of RPMI-M. Centrifuge at 100 x g for 5 min at room temperature (RT). Remove the supernatant and resuspend the cells in ice-cold RPMI-M. Centrifuge at 100 x g for 5 min at RT. Remove the supernatant and resuspend the MM6 cells in 2 mL of RPMI-M. Add 8 mL of RPMI-M to a tissue culture flask and transfer the cell suspension to the flask. Cells should be examined microscopically to ensure that the cells are not clumped together. Place the flasks in a cell culture incubator and maintain the cells at 37±1°C + 5% CO_2.

4.2.3 Propagation of MM6 Cells

Remove the cell culture flask from the incubator and examine the cells under a microscope to to determine that the morphology of the cells is consistent with the appearance of MM6 cells that previously yielded acceptable results. Centrifuge at 100 x g for 8 min at RT. Remove the supernatant, resuspend the cell pellet in 4 mL of RPMI-M, and gently pipet up and down to mix. It is advisable that cell number and cell viability be determined using appropriate methods (e.g., hemocytometer and vital dye or flow cytometer and fluorescent marker). The percentage of cell viability should exceed 80% for further propagation. The results of these examinations should be included in the study report. Transfer the cells (2 x 10^5 cells/mL) to new tissue culture flasks and add RPMI-M. Place the flasks in a cell culture incubator and maintain the cells at 37±1°C + 5% CO_2.

4.2.4 Preparation of a MM6 Cell Bank

To initiate a bank of MM6 cells, centrifuge the cell culture(s) at 100 x g for 8 min at 2 to 8°C. Remove the supernatant and resuspend the cells in FBS at 2 to 8°C. It is advisable to determine cell number and cell viability as outlined in **Section 4.2.3** and adjust the cell concentration to 4 x 10^6 cells/mL and store on ice for 10 min. Add an equal volume of ice-cold FBS containing 10% dimethyl sulfoxide (DMSO) drop-wise to the cell suspension (final concentration is 2 x 10^6 cells/mL with 5% DMSO). Transfer the cell suspension to sterile, pyrogen-free cryotubes (1 mL/tube). Place the tubes in a well-insulated polystyrene box and store in a -80°C freezer for greater than 48 hours (hr) and then transfer to a liquid nitrogen container.

4.3 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.3.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in DMSO and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be

diluted beyond its Maximum Valid Dilution (MVD) (refer to **Section 12.3**). The calculation of the MVD is dependent on the ELC for a test substance. The ELC can be calculated by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to MM6 cells.

4.3.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.3.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin spiking solutions is shown in **Table 4-2.** For non-spiked solutions, 150 µL of RPMI-C and 50 µL of the test substance (i.e., equivalent to the negative product control [NPC]) are added to a well. Endotoxin-spiked solutions are prepared by adding 100 µL of RPMI-C, 50 µL of the test substance, and 50 µL of an endotoxin-spike solution (1.0 EU/mL) (i.e., equivalent to the positive product control [PPC]). Finally, MM6 cells (50 µL) are added to each well and the wells are mixed and incubated as outlined in **Section 6.1.3**, **Steps 6-7**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-6 standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

Sample Addition	Spiked	Non-spiked
	µL/well[1]	
RPMI-C	100	150
Endotoxin-spike solution[2]	50	0
Test substance (neat and each serial dilution)	50	50
MM6 cells[3]	50	50
Total[4]	250	250

Abbreviations: MM6 cells = Mono Mac 6

[1]n=4 replicates each

[2]Endotoxin concentration is 1.0 EU/mL in RPMI-C.

[3]MM6 cells are resuspended in RPMI-C (2.5 x 10^6 cells/mL).

[4]A total volume of 250 µL per well is used for the incubation.

The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The spike recovery for each sample dilution is calculated as a percentage by setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 100%. For example, consider the following interference test results in Table 4-3:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-6 relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not to exceed the MVD, for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance to be used in the *in vitro* pyrogen test would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.3.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., MM6 cells incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., MM6 cells incubated with PFS instead of the test substance) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.4 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-6 Release

6.1.1 Preincubation of MM6 Cells

To perform an ELISA on the following day, obtain an MM6 cell suspension (30 to 50 mL) from propagation flasks and centrifuge at 100 x g for 8 min at RT. Remove the supernatant, resuspend the cell pellet in 2 mL of RPMI-C and gently pipet up and down to mix. It is advisable to determine cell number and cell viability as outlined in **Section 4.2.3**. The percentage of viable MM6 cells should exceed 80% to be suitable for use in the test. The results of these examinations should be included in the study report. Transfer the cells (4 x 10^5 cells/mL) to new tissue culture flasks and add RPMI-C. Place the flasks in a cell culture incubator and maintain the cells at 37±1°C + 5% CO_2 for 16 to 24 hr. In general, the preincubation of 2.0 x 10^7 cells in 50 mL RPMI-C will provide enough cells for one 96-well assay plate

6.1.2 Preparation of MM6 Cells for the Incubation Assay

Prepare the MM6 cells just prior to addition to the incubation plate (**Section 6.1.3, Step 5**). Centrifuge 30 to 50 ml of cell suspension at 100 x g for 8 min at RT. Pour off the supernatant and resuspend the cells in approximately 2 ml of RPMI-C. It is advisable that cell number and cell viability be determined as outlined in **Section 4.2.3**. The percentage of viable MM6 cells should exceed 80% to be suitable for use in the test. The results of these examinations should be included in the study report. Dilute the cells with RPMI–C to a volume that gives a concentration of 2.5 x 10^6 cells/ml.

6.1.3 Incubation Plate

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test substances (see **Table 6-1**).

Table 6-1 Overview of Incubation Plate Preparation in the MM6/IL-6 Pyrogen Test

Number of Wells	Sample	RPMI-C	EC	Test Sample	MM6[1]	Mix the samples; incubate for 16 to 24 hr at 37±1°C in a humidified atmosphere with 5% CO_2.	Mix the samples; immediately transfer to an ELISA plate[5] and run ELISA.
		µL					
20[2]	EC	100	50	0	100		
4	NSC	100	0	0[3]	100		
56[4]	Test samples (1-14)	100	0	50	100		

Abbreviations: EC = Endotoxin control; IL-6 = Interleukin-6; NSC = Negative saline control; MM6 = Mono Mac 6

[1]MM6 cell concentration is 2.5 x 10^6 cells/mL.

[2]Five EC concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) in quadruplicate

[3]50 µl of PFS is added instead of the test sample.

[4]14 test samples (n=4 each) per plate

[5]An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.4 Incubation Assay for IL-6 Release

MM6 cells are prepared in a microtiter plate using a laminar flow hood (refer to **Section 6.1.2**). All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the suggested incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer 100 µL of RPMI-C into each well.

Step 3. Transfer 50 µL of test sample or 50 µL of PFS for the NSC into the appropriate wells as indicated in the template.

Step 4. Transfer 50 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer 100 µL of a well-mixed MM6 cell suspension into each well.

Step 6. Place the covered plate in a tissue culture incubator for 16 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO_2.

Step 7. Remove 150 µL of the supernatant from each well, without disrupting the cells, and transfer to the IL-6 ELISA plate.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	Void[3]	Void
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1]EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.
[2]TS number (e.g., TS 1) represents an arbitrary sequence for individual test substances.
[3]Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-6 Release

6.2.1 IL-6 Standard Curve

An IL-6 standard supplied with the ELISA kit is used. IL-6 standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in RPMI-C to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 µL. Each well on the ELISA plate will receive 50 µL of an IL-6 blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, an eight-point IL-6 standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-6 standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4.**

Step 1. After pipetting up and down very carefully three times (avoid detachment of the adherent MM6 cells) to mix the supernatant, transfer 50 µL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 2. Add 50 µL of each IL-6 standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 3. Add 200 µL of the enzyme-labeled detection antibody (neat as supplied, or diluted, if necessary) to each of the wells.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr at RT.

Step 5. Decant and wash each well three times with 300 µL Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 µL of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 µL of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD_{450} within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 540 to 590 nm is recommended[4].

[4]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6[3] 0	IL-6 0
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1]EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.
[2]TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3]IL-6 values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Material transfer from Incubation Plate (µL)	**IL-6 standard (0 to 4000 pg/mL) (µL)**	**Enzyme-labeled Antibody (µL)**	Cover the Incubation Plate and incubate for 2 to 3 hr at RT.	Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	**TMB/Substrate Solution (µL)**	Incubate for less than15 min at RT in dark.	**Stop Solution (µL)**	Read each well at OD_{450} with a 540 to 590 nm reference filter.
50	50	200			200		50	

Abbreviations: OD_{450} = Optical density at 450 nm; RT = Room temperature

7.0 EVALUATION OF TEST RESULTS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with a reference filter of 540 to 590 nm (recommended)[5]. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6 standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-6 standard curve produces an $r \geq 0.95$[6] and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

An outlying observation may be excluded if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett and Lewis 1994] or Grubbs' test [Barnett and Lewis 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance
- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

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[5]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

[6]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

Justification of the In Vitro Test Method and Protocol 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 on the procedure used to ensure their integrity from "lot-to-lot" and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure used
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

11.0 REFERENCES

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)[1]

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)[1,2]

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M, where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)[1,2]

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is[1]:

MVD = (ELC x Product Potency [PP])/λ

As an example, for Cyclophosphamide Injection, the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is[1]:

MVD = PP/Minimum Valid Concentration (MVC)

where, MVC = (λ x M)/K

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

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[1]From FDA (1987)
[2]From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)[1,2]

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, the PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces ½ the maximal increase in optical density (OD) from the endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.7 Product Potency (PP)[1,2]

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)[1,2]

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.

Appendix D

Federal Register Notices, Public Comments, and Relevant SACATM Meeting Minutes

D1 *Federal Register* Notices

Federal Register notices are available at https://www.federalregister.gov/

D1-1 Vol. 70, No. 241, pp. 74833-74834, December 16, 2005: Peer Panel Evaluation of *In Vitro* Pyrogenicity Testing Methods: Request for Comments, Nominations of Experts, and Submission of *In Vivo* and *In Vitro* Data

D1-2 Vol. 71, No. 238, pp. 74533-74534, December 12, 2006: Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments

D1-3 Vol. 72, No. 89, pp. 26395-26396, May 9, 2007: Peer Review Panel Report on Five *In Vitro* Pyrogen Test Methods: Availability and Request for Public Comments

D2 Public Comments Received in Response to *Federal Register* Notices

Public comments are available on request from NICEATM

D3 ICCVAM Evaluation of *In Vitro* Pyrogen Test Methods: Excerpt from SACATM Draft Meeting Minutes

Past SACATM meeting minutes are available at https://ntp.niehs.nih.gov/events/past/index.html?type=SACATM

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Appendix E

ESAC Statement on the Validity of *In Vitro* Pyrogen Tests

ESAC Statement on the Validity of *In Vitro* Pyrogen Tests E-3

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EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
European Centre for the Validation of Alternative Methods (ECVAM)

STATEMENT ON THE VALIDITY OF IN-VITRO PYROGEN TESTS

At its 24th meeting, held on 20-21 March 2006 at the European Centre for the validation of alternative methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

Following a review of scientific reports and peer reviewed publications on the following range of in-vitro pyrogen tests:

1. Human Whole Blood IL-1,
2. Human Whole Blood IL-6,
3. PBMC IL-6,
4. MM6 IL-6, and
5. Human Cryopreserved Whole Blood IL-1,

it is concluded 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.

These methods have the potential to satisfy regulatory requirements for the detection and quantification of these pyrogens in these materials subject to product-specific validation.

The test methods have the capacity of detecting pyrogenicity produced by a wider range of pyrogens, but the evidence compiled for, and considered within this peer review and validation process, is not sufficient to state that full scientific validation of this wider domain of applicability has been demonstrated and confirmed.

Thus, the above test methods can currently be considered as full replacements for the evaluation of materials or products where the objective is to identify and evaluate pyrogenicity produced by Gram-negative endotoxins, but not for other pyrogens.

This endorsement takes account of the dossiers prepared for peer review; the views of independent experts who evaluated the dossiers against defined validation criteria; supplementary submissions made by the Management Team; and the considered view of the Peer Review Panel appointed to oversee the process.

Thomas Hartung
Head of Unit
ECVAM
Institute for Health & Consumer Protection
Joint Research Centre
European Commission
Ispra

21 March 2006

1. The ESAC was established by the European Commission, and is composed of nominees from the EU Members States, industry, academia and animal welfare, together with representatives of the relevant Commission services.

   This statement was endorsed by the following Members of the ESAC:

   Prof Helmut Tritthart (Austria)
   Dr Dagmar Jírová (Czech Republic)
   Prof Elisabeth Knudsen (Denmark)
   Dr Timo Ylikomi (Finland)
   Prof André Guillouzo (France)
   Dr Manfred Liebsch (Germany)
   Dr Efstathios Nikolaidis (Greece)
   Dr Katalin Horvath (Hungary)
   Prof Michael Ryan (Ireland)
   Dr Annalaura Stammati (Italy)
   Dr Mykolas Maurica (Lithuania)
   Prof Eric Tschirhart (Luxembourg)
   Dr Jan van der Valk (The Netherlands)
   Dr Dariusz Sladowski (Poland)
   Prof Milan Pogačnik (Slovenia)
   Dr Argelia Castaño (Spain)
   Dr Patric Amcoff (Sweden)
   Dr Jon Richmond (UK)
   Dr Odile de Silva (COLIPA)
   Dr Julia Fentem (ECETOC)
   Dr Nathalie Alépée (EFPIA)
   Prof Robert Combes (ESTIV)
   Dr Maggy Jennings (Eurogroup for Animal Welfare)
   Mr Roman Kolar (Eurogroup for Animal Welfare)

   The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself.

   Mr Thomas Hartung (ECVAM; chairman)
   Mr Jens Linge (ECVAM; ESAC secretary)
   Mr Juan Riego Sintes (ECB)
   Ms Beatrice Lucaroni (DG Research, Unit F.5)
   Mr Sylvain Bintein (DG Environment, Unit C.3)
   Mr Sigfried Breier (DG Enterprise, Unit F.3)
   Prof Dr Constantin Mircioiu (Romania)
   Dr William Stokes (NICEATM, USA)
   Prof Dr Vera Rogiers (ECOPA)

Annex

The novel pyrogen tests are based on the human fever reaction. Monocytoid cells, either primary from human blood or as propagated cell lines, detect pyrogens of different chemical nature and respond by the release of inflammatory mediators such as cytokines. Since lipopolysaccharides from Gram-negative bacteria are the only type of proven pyrogen, for which an International reference material is available, the tests were standardised to detect the presence of significantly less than 0.5 Endotoxin Units of this preparation, which is considered to be the threshold level for fever induction in the most sensitive rabbit species according to pharmacopoeia test procedures.
The five tests which were sufficiently reproducible and exceeded the rabbit test with regard to sensitivity and specificity for the detection of lipopolysaccharide spiked samples, differ with regard to cell source and preparation, cryopreservation and cytokine measured. The tests have been described elsewhere (1-4). The concept of the validation study (5) and the international validation studies are available (6-7).

1. Poole, S., Thorpe, R., Meager, A., Hubbard, A.R., Gearing, A.J. (1988) Detection of pyrogen by cytokine release. Lancet 8577, 130.

2. Taktak, Y.S., Selkirk, S., Bristow, A.F., Carpenter, A., Ball, C., Rafferty, B., Poole, S. (1991) Assay of pyrogens by interleukin-6 release from monocytic cell lines. J. Pharm. Pharmacol. 43, 578.

3. Hartung, T., Wendel, A. (1996) Detection of pyrogens using human whole blood. In Vitro Toxicol. 9, 353.

4. Schindler S, Asmus S, von Aulock S, Wendel A, Hartung T and Fennrich S. (2004) Cryopreservation of human whole blood for pyrogenicity testing. J. Immunol. Meth. 294, 89-100.

5. Hartung, T., Aaberge, I., Berthold, S., Carlin, G., Charton, E., Coecke, S., Fennrich, S., Fischer, M., Gommer, M., Halder, M., Haslov, K., Jahnke, M., Montag-Lessing, T., Poole, S., Schechtman, L., Wendel, A., Werner-Felmayer, G. (2001) Novel pyrogen tests based on the human fever reaction. The report and recommendations of ECVAM Workshop 43. European Centre for the Validation of Alternative Methods. Altern. Lab. Anim. 29, 99.

6. Hoffmann S, Peterbauer A, Schindler S, Fennrich S, Poole S. Mistry Y, Montag-Lessing T, Spreitzer I, Loschner B, vam Aalderen M, Bos R, Gommer M, Nibbeling R, Werner-Felmayer G, Loitzl P, Jungi T, Brcic M, Brugger P, Frey E, Bowe G, Casado J, Coecke S, de Lange J, Mogster B, Naess LM, Aaberge IS, Wendel A and Hartung T. (2005) International validation of novel pyrogen tests based on the human fever reaction. J. Immunol. Meth. 298, 161-173.

7. Schindler S, Spreitzer I, Loschner, Hoffmann S, Hennes K, Halder M, Brügger P, Frey E, Hartung T and Montag T. (2006) International validation of pyrogen tests based on cryopreserved human primary blood cells. J. Immunol. Meth. 316, 42-51.