DRAFT ICCVAM TEST METHOD RECOMMENDATIONS

In Vitro Pyrogenicity Test Methods

December 1, 2006

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1.0 ICCVAM DRAFT RECOMMENDATIONS FOR *IN VITRO* PYROGENICITY TEST METHODS

1.1 Draft Recommended Test Method Uses

ICCVAM has evaluated the validation status of the following *in vitro* test methods proposed as replacements for the *in vivo* rabbit pyrogen test (RPT) only (i.e., not for the bacterial endotoxin test [BET]):

- cryo WB/IL-1 (The Human Whole Blood/IL-1 *In Vitro* Pyrogen Test: Application of cryopreserved human whole blood)
- MM6/IL6 (An Alternative *In Vitro* Pyrogen Test Using the Human Monocytoid Cell Line MONO MAC-6 [MM6])
- PBMC/IL-6 (The Human Peripheral Blood Mononuclear Cell [PBMC]/IL-6 *In Vitro* Pyrogen Test)
- WB/IL-1 (The Human Whole Blood [WB]/IL-1 *In Vitro* Pyrogen Test)
- WB/IL-6 (The Human Whole Blood/IL-6 *In Vitro* Pyrogen Test)

There is sufficient information (see **Table 1**), based on validation studies with a limited number of pharmaceuticals (see **Table 2**), to substantiate the use of these test methods (PBMC/IL-6, cryo WB/IL-1 [96 well plate method], WB/IL-6, and MM6/IL-6) 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[1,2]. While the scientific basis of these test methods suggests that they have the capability to detect pyrogenicity produced by a wider range of pyrogens (i.e., those mediated by non-endotoxin sources), there is insufficient data to support this broader application.

[1] Equivalent methods can be regulated under 21 CFR 610.9 as alternatives to the currently accepted test method(s).

[2] There are substances other than endotoxin that may induce the cellular release of IL-1β and/or IL-6. For this reason, users of these test methods should be aware of the potential for a false positive result, suggesting that endotoxin is present, which actually is due to the presence of another pyrogenic material.

Table 1 Performance Statistics for *In Vitro* Pyrogenicity Test Methods[1]

Test Method	Concordance[2]	Sensitivity	Specificity	False Negative Rate	False Positive Rate
PBMC/IL-6	93.3% (140/150)	92.2% (83/90)	95.0% (57/60)	7.8% (7/90)	5.0% (3/60)
cryo WB/IL-1	91.7% (110/120)	97.4% (75/77)	81.4% (35/43)	2.6% (2/77)	18.6% (8/43)
WB/IL-6	91.9% (136/148)	88.8% (79/89)	96.6% (57/59)	11.2% (10/89)	3.4% (2/59)
MM6/IL-6	93.2% (138/148)	95.5% (85/89)	89.8% (53/59)	4.5% (4/89)	10.2% (6/59)
WB/IL-1 (plate method)	92.0% (129/139)	98.8% (83/84)	83.6% (46/55)	1.2% (1/84)	16.4% (9/55)

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

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

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

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

Users should be aware that the performance characteristics for these *in vitro* pyrogenicity test methods could be revised as additional data become available. Therefore, test method users should consult the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov/) and other sources to obtain the most current information relevant to the current performance and usefulness and limitations of these test methods.

1.2 Draft Recommended Future Studies

To further the use of these five test methods (cryo WB/IL-1, MM6/IL-6, PBMC/IL-6, WB/IL-1 [plate method], and WB/IL-6) as potential replacements for the RPT for detecting non-endotoxin pyrogens, additional studies that include a broader range of pyrogenic materials are recommended. For a direct comparison between the *in vitro* pyrogen test(s) and the RPT, such studies should include parallel RPT testing[3].

The hazards associated with human blood products should be carefully considered and all technical staff must be trained to observe all necessary safety precautions.

Appendix A provides Draft Performance Standards for *In Vitro* Pyrogenicity Test Methods that are based on ICCVAM guidelines (ICCVAM 2003[4]). **Appendix B** provides five draft proposed *in vitro* pyrogenicity test method protocols that are based on those used in the ECVAM validation study. **Appendix B1** is the *Proposed Test Method Protocol for the Human Whole Blood/IL-1 In Vitro Pyrogen Test: Application of Cryopreserved Human Whole Blood.* **Appendix B2** is the *Proposed Test Method Protocol for the In Vitro Pyrogen Test Using the Human Monocytoid Cell Line MONO MAC-6 (MM6).* **Appendix B3** is the *Proposed Test Method Protocol for the Human PBMC/IL-6 In Vitro Pyrogen Test*. **Appendix B4** is the *Proposed Test Method Protocol for the Human Whole Blood/IL-1 In Vitro Pyrogen Test*. **Appendix B5** is the *Proposed Test Method Protocol for the Human Whole Blood/IL-6 In Vitro Pyrogen Test*.

[3] In order to demonstrate the utility of these test methods for the detection of non-endotoxin pyrogens, either an international 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*.

[4] ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508. National Institute for Environmental Health Sciences, Research Triangle Park, NC. Available: http://iccvam.niehs.nih.gov/. [accessed 2 June 2005].

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Appendix A

Draft Performance Standards for *In Vitro* Pyrogenicity Test Methods

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1.0 PURPOSE AND BACKGROUND OF PERFORMANCE STANDARDS

1.1 Introduction

Prior to the acceptance of a new test method for regulatory testing applications, validation studies are conducted to assess its reliability (i.e., the extent of intra- and inter-laboratory reproducibility) and its relevance (i.e., the ability of the test method to correctly predict or measure the biological effect of interest) (OECD 1996, 2002; ICCVAM 1997, 2003). The purpose of performance standards is to communicate the basis by which new proprietary (i.e., copyrighted, trademarked, registered) and nonproprietary test methods have been determined to have sufficient relevance and reliability for specific testing purposes. These performance standards, based on test methods accepted by regulatory agencies, can be used to evaluate the reliability and relevance of other test methods that are based on similar scientific principles and measure or predict the same biological or toxic effect. Five *in vitro* pyrogenicity test methods, cryo WB/IL-1 (The Human Whole Blood/IL-1 *In Vitro* Pyrogen Test: Application of cryopreserved human whole blood), MM6/IL6 (An Alternative *In Vitro* Pyrogen Test Using the Human Monocytoid Cell Line MONO MAC-6 [MM6]), PBMC/IL-6 (The Human PBMC/IL-6 *In Vitro* Pyrogen Test, WB/IL-1 (The Human Whole Blood/IL-1 *In Vitro* Pyrogen Test), and WB/IL-6 (The Human Whole Blood/IL-6 *In Vitro* Pyrogen Test) were included in a validation study to evaluate the correlation between *in vitro* cytokine release and the *in vivo* rabbit fever response and the feasibility of using *in vitro* cytokine assays to predict a pyrogenic response.

This section describes the three elements of performance standards identified by ICCVAM (2003) and the ICCVAM process used to develop performance standards during a test method evaluation. These test method performance standards are proposed as standards that can be used to evaluate future *in vitro* pyrogenicity test methods. If other *in vitro* pyrogenicity test methods are adequately validated and demonstrate significantly improved performance, then the test method performance standards may be revised accordingly.

1.2 Elements of ICCVAM Performance Standards

Performance standards are standards based on a validated test method that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar (ICCVAM 2003). The three elements of performance standards are:

- **Essential test method components:** These consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed, mechanistically and functionally similar test method. Essential test method components include unique characteristics of the test method, critical procedural details, and quality control measures.
- **A minimum list of reference substances:** Reference substances are used to assess the accuracy and reliability of a proposed, mechanistically and functionally similar test method. These substances are a representative subset of those used to demonstrate the reliability and the accuracy of the validated test method, and are the minimum number that should be used to evaluate the performance of a proposed, mechanistically and functionally similar test method.
- **Accuracy and reliability values:** These are the accuracy and reliability characteristics that the proposed test method should be comparable to or exceed when evaluated using the minimum list of reference chemicals.

1.3 ICCVAM Process for the Development of Performance Standards

The process followed by ICCVAM for developing performance standards for new test methods is as follows:

- NICEATM and the ICCVAM Pyrogenicity Working Group (PWG) develop proposed performance standards for consideration during the ICCVAM evaluation process. If performance standards are proposed by a test method sponsor, they will be considered by ICCVAM at this stage. Generally, the

proposed performance standards are based on the information and data provided in the test method submission or on other available applicable data.

- The ICCVAM/NICEATM Peer Review Panel evaluates the proposed performance standards for completeness and appropriateness during its evaluation of the validation status of the proposed test method. The proposed performance standards, as well as the test method submission, are made available to the public for comment prior to and during the Peer Review Panel meeting.
- The PWG, with the assistance of NICEATM, prepares the final performance standards for ICCVAM approval, taking into consideration the recommendations of the Peer Review Panel and public comments.

Performance standards recommended by ICCVAM are incorporated into ICCVAM test method evaluation reports, which are then provided to U.S. Federal agencies and made available to the public. Regulatory authorities can then reference the performance standards in the ICCVAM report when they communicate their acceptance of a new test method. In addition, performance standards adopted by U.S. Federal regulatory authorities can be provided in guidelines issued for new test methods. Availability of ICCVAM test method evaluation reports are announced routinely in the *Federal Register*, in NTP Newsletters, and by e-mail to ICCVAM/NICEATM listserve groups.

1.4 ICCVAM Development of Performance Standards for *In Vitro* Pyrogenicity Test Methods

1.4.1 Current Regulatory Testing Requirements for Pyrogenicity

The major regulatory requirement for pyrogenicity testing is for end product release of human and animal parenteral drugs, medical devices, and human biological products. Results from pyrogenicity testing are used to limit to an acceptable level the risks of febrile reaction in the patient to injection and/or implantation of the product of concern. The current U.S. legislation requiring the use of pyrogenicity testing is stated in the Federal Food, Drug, and Cosmetic Act (U.S.C., Title 21, Chapter 9). In addition, the U.S.

Pharmacopeia (USP) maintains sterility requirements for pharmaceuticals that include pyrogenicity testing. As detailed in **Table 1-1**, the U.S. Food and Drug Administration (FDA) is the principal U.S. regulatory agency that requires pyrogenicity testing, with different Centers within the FDA regulating the affected products (i.e., human and animal parenteral drugs, biological products, and medical devices). **Table 1-1** also shows the statutory protocol requirements used by each FDA Center, along with the comparable international standards presently required by European Union member nations.

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

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

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

1.4.2 Test Methods for Assessing Pyrogenicity

The currently recognized test methods for evaluating pyrogenicity are the *in vivo* rabbit pyrogen test (RPT) and the bacterial endotoxin test (BET). The RPT (USP28 NF23<151>, EP5.0 2.6.8) involves measuring the rise in body temperature evoked in rabbits by the intravenous injection of a test solution. The RPT is a sequential test, using the response of the first three rabbits tested to determine the need for additional testing. The BET (USP28 NF23<85>, EP5.0 2.6.14) is used to detect or quantify the presence of

gram-negative bacterial endotoxins using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus*). There are three different BET techniques: the gel-clot technique (based on gel formation due to clotting of *Limulus* amoebocyte lysate [LAL] in the presence of endotoxin); the turbidometric technique (based on the development of turbidity after cleavage of an endogenous substrate); and the chromogenic technique (based on color development resulting from cleavage of a synthetic peptide-chromagen complex).

1.4.3 Intended Regulatory Uses for *In Vitro* Pyrogenicity Test Methods

The *in vitro* pyrogenicity test methods are not intended as replacements for the BET. However, five of these methods (cryo WB/IL-1, MM6/IL-6, PBMC/IL-6, WB/IL-1[plate method], and WB/IL-6) may be considered for the detection of the presence of Gram-negative endotoxin in materials that are currently tested in the RPT, subject to product-specific validation to demonstrate equivalency.

1.4.4 Similarities and Differences in the Endpoints of In Vitro Pyrogenicity Test Methods and Currently Recognized Pyrogenicity Test Methods

The endpoint measured in the *in vitro* pyrogenicity test methods is cytokine release, either IL-1β or IL-6, depending on the test method employed. The RPT involves measuring the rise in body temperature evoked in rabbits by the intravenous injection of a test solution. While there is not a direct association between the endpoints measured in these assays, cytokine release is involved in the development of an inflammatory response, which can result in an increase in temperature. Therefore, the *in vitro* release of pro-inflammatory cytokines, such as IL-1β and IL-6, is intended to predict the onset of such a response. The cell types used for the various *in vitro* methods presumably are those that would be directly associated with an inflammatory response. Both the *in vitro* and *in vivo* tests provide quantitative endpoints.

There are different endpoints for the BET, depending on the technique used. The gel-clot technique is based on the observation of gel formation due to clotting of LAL in the presence of endotoxin. The turbidometric technique evaluates the development of turbity

after cleavage of an endogenous substrate. Finally, the chromogenic technique measures color development resulting from cleavage of a synthetic peptide-chromogen complex. Clearly, there are no biological similarities between the endpoints measured in the *in vitro* test methods and the various BET techniques. However, like the *in vitro* test methods, the turbidometric and chromogenic techniques provide quantitative measurements, while the gel-clot technique is qualitative.

2.0 *IN VITRO* PYROGENICITY TEST METHODS

2.1 Background

Pre-validation and validation studies have been completed to evaluate the ability of the five *in vitro* pyrogenicity test methods to be used as alternatives to the RPT. This section briefly describes the principles of *in vitro* pyrogenicity test methods followed by the recommended performance standards that would be used to evaluate test methods that are functionally and mechanistically similar to these methods. The performance standards consist of 1) essential test method components, 2) reference substances, and 3) the comparable accuracy and reliability that should be achieved.

2.2 Principles of *In Vitro* Pyrogenicity Test Methods

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

- The test substance is applied to the specific human-derived cells used in the *in vitro* test method (i.e., mixed with a suspension of cells).
- The test substance is incubated with the cells for a specified period of time.
- The concentration of pro-inflammatory cytokines (e.g., IL-1β, IL-6) is quantified via a cytokine-specific enzyme-linked immunosorbent assay (ELISA) by comparison to a standard curve.

- Using an endotoxin standard curve, the endotoxin content of the product is calculated.
- A product "passes" (i.e., is considered negative for endotoxin) if the endotoxin content is < 0.5 endotoxin units (EU)/mL.

While it is possible that comparable cell types derived from other species would also be capable of detecting the presence of Gram-negative endotoxin, one of the strengths of these test methods is that they are derived from human tissues, and thus avoid the potential uncertainty associated with cross-species interpretation. Therefore, test method developers are encouraged to focus on human cell-based systems.

Investigators using an *in vitro* pyrogenicity test method for detecting the presence of Gram-negative endotoxin must be able to demonstrate that the assay is valid for its intended use. This includes demonstrating that any modification to the existing validated reference test method does not adversely affect its performance characteristics. *In vitro* pyrogenicity test methods may be used to test pharmaceuticals, biological products, and medical devices. Interference testing must be included to demonstrate that the properties of the test substance do not impede the release and detection of proinflammatory cytokines.

2.3 Essential Test Method Components for *In Vitro* Pyrogenicity Test Methods

Essential test method components consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a mechanistically and functionally similar proposed test method. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to these components will help assure that a proposed test method is based on the same concepts as the corresponding validated test method.

The following is a description of the essential test method components for *in vitro* pyrogenicity test methods.

2.3.1 *In Vitro* Cell Culture Conditions

- A mammalian cell line, primary cells, or heparinized whole blood (as described above, either would preferably be of human origin) is used. Cryopreserved cells/whole blood may be used, where it has been demonstrated that cryopreservation is not detrimental to the test method.
- Fresh whole blood may be stored at room temperature, but should be used within four hours of collection. Blood donors should be in good health (i.e., not suffering from bacterial or viral infections for at least one week prior to donation), and not taking any medications known to influence cytokine production (e.g., immunosuppressant or anti-inflammatory drugs). As an additional measure, assay acceptance criteria allow for the identification of low or high responders such that results with Blood/PBMCs from a compromised donor are omitted.
- Where necessary, cells are propagated in sterile tissue culture flasks and then subcultured to sterile 96-well plates for use in testing. Initial cell seeding should be done at a density that allows rapid growth throughout the exposure period. However, cell density should not reach confluency by the end of the test exposure period.
- Appropriate cell culture growth conditions (e.g., 37°C ± 1°C, 90% ± 10% humidity, 5.0% ± 1% CO_2 in ambient air) should be maintained throughout the testing period. The cell cultures should be free of contamination with bacteria, mycoplasma, or fungi.

Cell culture media should be prequalified by the testing laboratory via a standardized protocol before initiating the test to guarantee that the media provide cells with appropriate nutrients to meet the growth criteria needed for the test method.

2.3.2. Application of the Test Substances

2.3.2.1 *Test Substance Preparation*

- All disposables (e.g., pipette tips, pipettes, culture ware, etc.) should be labeled sterile, pyrogen free.
- Test substances (i.e., pharmaceuticals, biological products) should be diluted to their respective maximum valid dilution (MVD) in sterile, pyrogen-free 0.9% NaCl.
- Medical devices can be directly incubated with to the cells in suspension. Alternatively, and if necessary, eluates/extracts from medical devices may be prepared with a volume of pyrogen-free water appropriate to their use and, where applicable, to the surface area that comes in contact with body tissues or fluids.
- Each test should contain a range of concentrations of either the international reference standard endotoxin (i.e., WHO LPS 94/580), or an LPS standard that has been calibrated against this standard, with which to generate a standard curve. *NOTE: In the ECVAM validation study, concentrations of 0, 0.25, 0.5, and 1.0 endotoxin units (EU)/mL were used to establish the decision criteria for a pyrogenic response based on the in vivo rabbit threshold fever concentration (i.e., the concentration at which an increase in temperature was recorded in 50% animals tested) (see* ***Section 2.3.5****).*
- Test substances should be fully solubilized (i.e., no visual observation of test substance in the dosing solution).

2.3.2.2 *Test Substance Application and Sample Collection*

- Whole blood samples may be dosed in either 96-well plates or microcentrifuge tubes.
- The cells should be exposed for from 16 to 24 hours
- Each substance should be tested in a minimum of three replicates.

- At the end of the exposure period, supernatants may be collected either directly from each well, or following centrifugation for microcentrifuge tubes.

2.3.3 Control Substances

2.3.3.1 Negative Control

To ensure that the test system is functioning properly and that the specific test is valid, the negative control (i.e., 0.9% NaCl) should not induce a significant increase in IL-1β or IL-6 release.

2.3.3.2 Positive Control

The purpose of a positive control chemical is to demonstrate that the cell system is responding with adequate sensitivity to a pyrogenic substance for which the magnitude of the pyrogenic response is well characterized. Each test should generate a response that is comparable to the historical range generated by the laboratory. Therefore, the positive control should be the international reference standard endotoxin (i.e., WHO-LPS 94/580 [*E. coli* 0113:h10:K-]), or an endotoxin standard that has been calibrated against this standard. A laboratory should perform a minimum of 10 *in vitro* pyrogenicity tests using the positive control over a number of days to develop a minimum historical database of cyokine data. Typically, for biologically based test methods, suggested acceptable ranges for the positive control response are within two to three standard deviations of the historical mean response, but developers of proprietary test methods may establish tighter ranges. The positive control chemical should be tested concurrently with (and independent of) the test substance. Test substances spiked with known quantities of the positive control should be used for interference testing.

2.3.3.3 Benchmark Controls

Benchmark controls may be useful to demonstrate that the test method is functioning properly for detecting the pyrogenic potential of chemicals (e.g., parenterals or medical device eluates) of a specific chemical 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:

- structural and functional similarity to the class of the substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

2.3.4 Cytokine Measurements

Only standardized, quantitative methods should be used to measure cytokine release (e.g., an enzyme immunoassay with a species-specific antibody for the relevant proinflammatory cytokine). Each assay should contain a range of concentrations of the relevant cytokine standard (e.g., IL-1β, IL-6) in order to generate a standard curve for the analytical assay. The protocol should be compatible with analytical laboratory equipment (e.g., spectrophotometer) that allows a quick and precise measurement of the endpoint. Colorimetric, fluorometric, or luminometric endpoints should have the optical density (OD) measured at the appropriate wavelength, and OD values for blanks should be subtracted from all measurements. Each supernatant should be assayed with a minimum of three replicates.

2.3.5 Interpretation of Results

The endpoint values obtained for each test sample can be used to calculate the level of cytokine release relative to the positive control samples (i.e., the endotoxin standard curve). A sample is considered positive for a pyrogenic response if the level of cytokine release is greater than or equal to that induced by the 0.5 EU/mL endotoxin standard, the reported threshold fever concentration for the *in vivo* rabbit test (see **Section 2.3.2.1**).

2.3.6 Test Report

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

Test Substances and Control Substances

- Name of pharmaceutical, biological product, medical device eluate, etc.

- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility) relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., vortexing, sonication, warming; 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.

2.4 Reference Substances for *In Vitro* Pyrogenicity Test Methods

Reference substances are used to assess the accuracy and reliability of a proposed, mechanistically and functionally similar test method and are a representative subset of those used to demonstrate the reliability and the accuracy of the validated test method. These substances are:

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

To demonstrate technical proficiency with the validated test method, the user should evaluate his/her ability to identify Gram-negative endotoxin that has been spiked into each of the reference substances listed in **Table 2-1.** These eight substances are marketed pharmaceuticals that were tested in the ECVAM *in vitro* pyrogenicity test methods validation study. Only released clinical lots that have been labeled as having no detectable pyrogens should be used as reference substances to be spiked with Gram-negative endotoxin. As indicated in **Section 2.3.3**, the spike should be either the international reference standard endotoxin (WHO-LPS 94/580 [*E. coli* 0113:h10:K-]) or an endotoxin standard that has been calibrated against this standard. Each reference substance should be tested clean (i.e., unspiked) and spiked with endotoxin (0.5 EU/mL).

Table 2-1 Recommended Reference Substances for *In Vitro* Pyrogenicity Test Methods

Test Substance[1]	Source	Active Ingredient	MVD (-fold)
Beloc®	Astra Zeneca	Metoprolol tartrate	140
Binotal®	Aventis	Ampicillin	140
Ethanol 13% (w/w)	B. Braun	Ethanol	35
Fenistil®	Novartis	Dimetindenmaleat	175
Glucose 5% (w/v)	Eifel	Glucose	70
MCP®	Hexal	Metoclopramid	350
Orasthin®	Aventis	Oxytocin	700
Sostril®	GSK	Ranitidine	140

[1]Each reference substance should be spiked with 0.5 EU/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Each sample should contain the appropriate spike concentration when tested at its Maximum Valid Dilution (MVD). MVD = (endotoxin limit concentration)/(detection limit of the assay)
Positive Control: 0.9% NaCl spiked with 0.5 EU/mL
Negative Control: 0.9% NaCl

2.5 Accuracy and Reliability

The third element of the performance standards is the determination of accuracy (also known as relevance) and reliability values. A proposed test method, functionally and mechanistically similar to the *in vitro* pyrogenicity test methods described above, will use selected reference substances to assess accuracy and reliability.

2.5.1 Accuracy

When evaluated using the minimum list of recommended reference substances (**Table 2-1**), the proposed test method should have performance characteristics that are comparable to the performance of the validated *in vitro* pyrogenicity test methods. Accuracy is defined as the closeness of agreement between a test method result and an accepted reference value (ICCVAM 2003). The substances tested in the ECVAM validation studies are included so that the performance of the proposed test method can be determined and compared to that of the validated reference test methods.

The accuracy of these assays to identify a pyrogenic concentration of Gram-negative endotoxin was evaluated. This accuracy evaluation characterizes the extent that additional test methods will be necessary to achieve accurate *in vitro* predictions of

contamination by Gram-negative endotoxin for labeling and lot release purposes. **Table 2-2** shows that overall accuracy among the test methods is comparable (91.7% to 93.3%), with false negative rates ranging from 1.2% to 11.2%, and false positive rates ranging from 3.4% to 18.6%.

Table 2-2 Performance Statistics for *In Vitro* Pyrogenicity Test Methods[1]

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

[1]Based on combined results of 10 different substances tested in three different laboratories
[2]Percentage (Number of correct runs/total number of runs)

2.5.2 Reliability

Test method reliability (intralaboratory repeatability and intra- and inter-laboratory reproducibility) is the degree to which a test method can be performed reproducibly within and among laboratories over time (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period. Intralaboratory reproducibility refers to the determination of the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the determination of the extent to which different laboratories can replicate results using the same protocol and test chemicals, and indicates the extent to which a test method can be transferred successfully among laboratories.

659 The reliability of the proposed test method for the reference substances should be
660 comparable to or better than that of the validated *in vitro* pyrogenicity test methods. The
661 following sections provide these reference statistics.
662
663 2.5.2.1 *Intralaboratory Repeatability*
664 In the ECVAM validation study, intralaboratory repeatability was evaluated in each test
665 method by testing saline and various endotoxin spikes (0.06 to 0.5 EU/mL) in saline and
666 evaluating the closeness of agreement between optical density readings for cytokine
667 measurements for each concentration. Up to 20 replicates per concentration were tested
668 and results indicated that variability in measurements increased with endotoxin
669 concentration, but that the 0.5 EU/mL spike concentration (i.e., the threshold for
670 pyrogenicity for each test method) was clearly distinguishable from lower concentrations.
671
672 2.5.2.2 *Intralaboratory Reproducibility*
673 Intralaboratory reproducibility was evaluated with three marketed pharmaceuticals spiked
674 with various concentrations of endotoxin. Reproducibility was assessed from three
675 identical, independent runs conducted in each of the three testing laboratories (with the
676 exception of the cryo WB/IL-1 test method[1]). From these results, agreement between
677 different runs was determined for each substance in three laboratories. As shown in
678 **Table 2-3**, the agreement across three runs in an individual lab ranged from 75% to
679 100%.
680

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

Table 2-3 Intralaboratory Reproducibility of *In Vitro* Pyrogenicity Test Methods

Run Comparison[1]	WB/IL-1			Cryo WB/IL-1			WB/IL-6			PBMC/IL-6			MM6/IL-6		
	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3
1 vs 2	92%	100%	100%	ND	ND	ND	75%	92%	100%	92%	100%	100%	100%	92%	100%
1 vs 3	83%	88%	92%	ND	ND	ND	100%	92%	100%	100%	100%	92%	100%	92%	92%
2 vs 3	92%	NA	92%	ND	ND	ND	75%	100%	100%	92%	100%	92%	100%	100%	92%
Mean	89%	-	94%	ND	ND	ND	83%	94%	100%	94%	100%	94%	100%	94%	94%
Agreement across 3 runs	83%	-	92%	ND	ND	ND	75%	92%	100%	92%	100%	94%	100%	92%	92%

NA: Not assessed due to lack of sufficient data. The sensitivity criteria were not met for 1/3 substances in run 2, and 1/3 substances in run 3; **ND**: Not done. The cryo WB/IL-1 test method BRD states that an assessment of intralaboratory reproducibility was performed in the WB IL-1 (fresh blood) test method, and it is assumed that variability is not affected by the change to cryopreserved blood assayed in 96-well plates.

[1]Comparison between 3 individual runs within each laboratory

688 2.5.2.3 *Interlaboratory Reproducibility*

689 Interlaboratory reproducibility was evaluated in two different studies. In both studies,
690 each run from one laboratory was compared with all other runs of another laboratory. The
691 proportion of equally classified samples provides a measure of reproducibility. In the first
692 study, like the intralaboratory reproducibility evaluation, interlaboratory reproducibility
693 was evaluated with the three marketed pharmaceuticals spiked with endotoxin, and tested
694 three times in three different laboratories. As shown in **Table 2-4**, the agreement across
695 three laboratories for each test method (where three runs per laboratory were conducted)
696 ranged from 72% to 86%, depending on the test method considered. In comparison, the
697 agreement across three laboratories for the WB/IL-1 (96-well plate method) and cryo
698 WB/IL-1 test methods, for which only one run per laboratory was conducted, was 83%
699 and 92%, respectively.
700
701 **Table 2-4 Interlaboratory Reproducibility of *In Vitro* Pyrogenicity Test**
702 **Methods**

Lab Comparison[1]	Agreement Between Laboratories[1]				
	WB/IL-1	Cryo WB/IL-1	WB/IL-6	PBMC/IL-6	MM6/IL-6
1 vs 2	92% (11/12)	92% (11/12)	72% (78/108)[2]	81% (87/108)	97% (105/108)
1 vs 3	83% (10/12)	92% (11/12)	75% (81/108)[2]	86% (93/108)	89% (96/108)
2 vs 3	92% (11/12)	92% (11/12)	97% (105/108)[2]	89% (96/108)	86% (93/108)
Mean	89%	92%	81%	85%	90%
Agreement across 3 labs[3]	83% (10/12)	92% (11/12)	72% (234/324)[2]	78% (252/324)	86% (279/324)

703 [1]Data from three substances (see **Table 2-3**) spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]) at 0, 0, 0.5 and 1.0
704 EU/mL tested three times in three different laboratories, with the exception of the WB/IL-1 (96-well plate method) and the cryo
705 WB/IL-1 (only the preliminary run from each laboratory used for analysis)
706 [2]Some of the runs did not meet the assay acceptance criteria and therefore were excluded from the analysis.
707 [3]All possible combinations of runs among the 3 laboratories were compared (with the exception of cryo WB/IL-1, which was only
708 tested once in each laboratory, resulting in only one possible combination per substance).
709

710 In a second study, reproducibility was evaluated with the same ten substances used for
711 evaluating accuracy. In this study, the ten substances were spiked with five
712 concentrations of endotoxin and tested once in each of three laboratories. As indicated in
713 **Table 2-5**, the agreement across three laboratories for each test method ranged from 79%
714 to 88%, depending on the test method considered.
715
716

716 **Table 2-5 Interlaboratory Reproducibility of *In Vitro* Pyrogenicity Test**
717 **Methods**

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

718 [1]Data from 10 substances spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]) at 0, 0.25, 0.5, 0.5, and 1.0 EU/mL
719 tested once in three different laboratories

3.0 REFERENCES

ICCVAM. 1997. Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication No. 97-3981. Research Triangle Park, NC. National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf [accessed 20 July 2006].

ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508. National Institute for Environmental Health Sciences, Research Triangle Park, NC. Available: http://iccvam.niehs.nih.gov/. [accessed 20 July 2006].

OECD. 1996. Final Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Methods. (Solna Report) Paris: Organisation for Economic Co-operation and Development. Available: http://www.oecd.org [accessed 20 July 2006].

OECD. 2002. Report of the Stockholm Conference on Validation and Regulatory Acceptance of New and Updated Methods in Hazard Assessment. Paris: Organisation for Economic Co-operation and Development. Available: http://www.oecd.org [accessed 20 July 2006]

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Appendix B

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

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