ICCVAM Evaluation of Five *In Vitro* Test Methods for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products

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Introduction

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

More recent efforts have focused on the development of an *in vitro* test system that combines the sensitivity of the BET with the wide range of pyrogens (i.e., both endotoxin and non-endotoxin pyrogens) detectable by the RPT. With this intention, test systems based on the activation of human monocytes *in vitro* have been developed that take advantage of the role of these cells in the fever response.

ECVAM *In Vitro* Pyrogen Test Method Submissions

In June 2005, Background Review Documents (BRDs) for five of these *in vitro* pyrogen test methods were submitted to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for consideration as replacements for the RPT. The submitted test methods were:

- **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**

The proposed *in vitro* pyrogen test methods are based on the measurement of proinflammatory cytokines (i.e., IL-1 or IL-6), released from human monocytoid cells, or from a monocytoid cell line (MM6), in response to exposure to a pyrogenic substance (**Figure 1**).

Each ECVAM BRD summarizes the validation studies conducted with one of the five *in vitro* pyrogen test methods, which is in turn compiled into a single document, the ICCVAM BRD, which compares and contrasts these five test methods.

ICCVAM *In Vitro* Pyrogen BRD

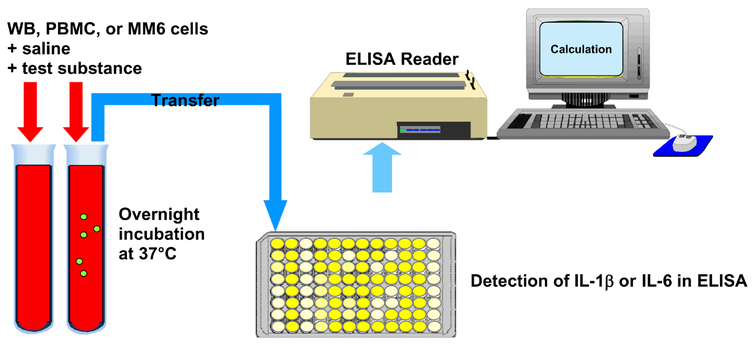
The ICCVAM BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of five *in vitro* pyrogen test methods, including what is known about their relevance and reliability, the scope of the substances tested, and the availability of a standardized test method protocol for each test method.

The information summarized is based on data contained in the five individual BRDs submitted by ECVAM, as well as the available information obtained from published studies and any additional unpublished data provided by ECVAM.

The test methods were reviewed for their ability to detect the presence of Gram-negative endotoxin that has been spiked into a variety of parenteral pharmaceuticals.

The draft ICCVAM BRD is publicly available on the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov)

Figure 1 Overview of the *In Vitro* Pyrogen Test Methods[1]



[1]Modified from figure provided by Dr. Thomas Montag-Lessing (Paul Ehrlich Institute)

Decision Criteria for Determining a Positive Response

A sample is considered pyrogen-free if it induces cytokine release significantly lower than the one induced by 0.5 EU LPS ($p < 0.01$, one-sided)

In order to qualify, the positive product control spiked with 0.5 EU LPS must be positive.

The assays using multiple donors (WBT/IL-6 and PBMC/IL-6) require all donor samples test negative

- Retesting is allowed if a single positive donor tests positive.
- Two or more positive donors is considered a positive result.

Test Method Accuracy

Ten parenteral pharmaceutical products were used to determine test method accuracy (**Table 1**). Each drug was spiked with four concentrations of a World Health Organization (WHO) *Escherichia coli* Gram-negative endotoxin standard and tested once in three different laboratories. Accuracy was determined against a threshold value of 0.5 EU/mL obtained from a regression plot of historical RPT data (n=171 Chinchilla bastard rabbits).

The accuracy of the five test methods ranged from 81% to 93%, sensitivity ranged from 73% to 99%, specificity ranged from 81% to 97%, false negative rates ranged from 3% to 27%, and false positive rates ranged from 3% to 19% (**Table 3**).

Test Method Reliability

Intralaboratory repeatability was evaluated by comparing the optical density (OD) readings for cytokine measurements at each spike concentration (0.06 to 0.5 EU/mL) against saline. Although variability (based on up to 20 replicates per concentration) increased with endotoxin spike concentration, variability did not affect the ability to detect the threshold endotoxin level

Three parenteral pharmaceutical products were used to determine test method reproducibility (**Table 2**). Intralaboratory reproducibility was evaluated with mean correlations expressed as a percentage of agreement among pairs of runs (i.e., run 1 vs. run 2; run 1 vs. run 3; run 2 vs. run 3) were calculated. Agreement across 3 runs within a single laboratory ranged from 75% to 100% (data not shown). One run per lab using the Cryo WB/IL-1 96 well plate method resulted in a range of 83 to 92% agreement (**Table 4**)

Interlaboratory reproducibility was evaluated in two different studies (Study A and Study B) in which each run from one laboratory was compared to all other runs of another laboratory and the proportion of equally qualified samples determined. The agreement across three laboratories for each test method ranged from 58% to 86% (**Table 5**). In the second study, the results from the 10 substances used in the accuracy analysis (**Table 1**) were compared. Agreement across laboratories ranged from 57% to 88%, depending on the test method used (**Table 6**).

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

Test Substance[2]	Source	Lot Number(s)	Active Ingredient	Indication	MVD (-fold)
Beloc®	Astra Zeneca	DA419A1	Metoprolol tartrate	Heart dysfunction	140
Binotal®	Grünenthal	117EL2	Ampicillin	Antibiotic	140
Ethanol 95%	B. Braun	2465Z01	Ethanol	Diluent	35
Fenistil®	Novartis	21402 26803[3]	Dimetindenmaleat	Antiallergic	175
Glucose 5%	Eifelfango	1162 3132[3]	Glucose	Nutrition	70
MCP®	Hexal	21JX22	Metoclopramid	Antiemetic	350
Orasthin®	Hoechst	W015	Oxytocin	Initiation of delivery	700
Sostril®	Glaxo Wellcome	1L585B 3H01N[3]	Ranitidine	Antiacidic	140
Syntocinon®	Novartis	S00400	Oxytocin	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, 0.5, or 1.0 EU/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Each sample contained the appropriate spike concentration when tested at its Maximum Valid Dilution (MVD).
[3]Indicates the lot number used in the catch-up validation study.

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

Test Substance[2]	Source	Agent	Indication
Gelafundin®	Braun Melsungen	Gelatin	Transfusion
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, 0.5, or 1.0 EU/mL of endotoxin (WHO-LPS 94/580 [*E. coli* O113: H10:K-]). Each sample contained the appropriate spike concentration when tested at its Maximum Valid Dilution (MVD).

Table 3 Performance Characteristics for *In Vitro* Pyrogen Test Methods[1]

Test Method	Accuracy[2]	Sensitivity	Specificity	False Negative Rate	False Positive Rate
Cryo WB/IL-1	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)[3]	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-1 (tube)	81% (119/147)	73% (64/88)	93% (55/59)	27% (24/88)	7% (4/59)
WB/IL-1 (96-well plate)[4]	93% (129/139)	99% (83/84)	84% (46/55)	1% (1/84)	16% (9/55)

Abbreviations: Cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin-6; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood
[1]Based on results of 10 parenteral drugs tested in each of three different laboratories, samples of each drug were tested with or without being spiked with a Gram-negative endotoxin standard (0, 0.25, 0.5, 0.5, or 1.0 EU/mL).
[2]Percentage (Number of correct runs/total number of runs)
[3]A modification of the PBMC/IL-6 test method using cryopreserved PBMCs.
[4]A modification of the WB/IL-1 test method using 96-well plates instead of tubes for the test substance incubation.

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

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

Run Comparison[1]	PBMC/IL-6			MM6/IL-6		
	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3
1 vs 2	92% (11/12)	100% (12/12)	100% (12/12)	100% (12/12)	92% (11/12)	100% (12/12)
1 vs 3	100% (12/12)	100% (12/12)	92% (11/12)	100% (12/12)	92% (11/12)	92% (11/12)
2 vs 3	92% (11/12)	100% (12/12)	92% (11/12)	100% (12/12)	100% (12/12)	92% (11/12)
Mean	95%	100%	95%	100%	95%	95%
Agreement[2] across 3 runs	92%	100%	94%	100%	92%	92%

Abbreviations: Cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin-6; MM6 = Mono Mac 6; ND = Not done; PBMC = Peripheral blood mononuclear cells; WB = Whole blood
[1]Comparison between 3 individual runs within each laboratory
[2]All possible combinations of runs among the 3 laboratories were compared
[3]Not done. The ECVAM Cryo WB/IL-1 BRD states that an assessment of intralaboratory reproducibility was performed using the WB IL-1 (fresh blood) test method, and it was assumed that intralaboratory variability would not be affected by the change to cryopreserved blood assayed in 96-well plates.
[4]Not assessed due to lack of sufficient data. The sensitivity criteria were not met for 1/3 substance in run 2, and 1/3 substance in run 3.

Table 5 Interlaboratory Reproducibility of *In Vitro* Pyrogen Test Methods (Study A)

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

Abbreviations: Cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin-6; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood
[1]Data from three substances (see **Table 2**) spiked with endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]) at 0, 0, 0.5 and 1.0 EU/mL tested three times in three different laboratories, with the exception of Cryo WB/IL-1 (only the preliminary run from each laboratory used for analysis)
[2]Some of the runs did not meet the assay acceptance criteria and therefore were excluded from the analysis.
[3]For the Cryo WB/IL-1 test method, each substance tested only once in each laboratory.
[4]All possible combinations of runs among the 3 laboratories were compared (with the exception of Cryo WB/IL-1, which was only tested once in each laboratory, resulting in only one possible combination per substance).

Table 6 Interlaboratory Reproducibility of *In Vitro* Pyrogen Test Methods (Study B)

Lab	Agreement Between Laboratories[1]						
	WB/IL-1 (Tube)	WB/IL-1 (Plate)	Cryo WB/IL-1	WB/IL-6	PBMC/IL-6	PBMC/IL-6 (Cryo)	MM6/IL-6
1 vs 2	73% (35/48)	92% (11/12)	84% (38/45)	85% (41/48)	84% (42/50)	96% (48/50)	90% (45/50)
1 vs 3	82% (40/49)	83% (10/12)	88% (21/24)	85% (41/48)	86% (43/50)	76% (38/50)	90% (43/48)
2 vs 3	70% (33/47)	92% (11/12)	100% (25/25)	88% (44/50)	90% (45/50)	80% (40/50)	83% (40/48)
Mean	75%	89%	91%	86%	87%	84%	88%
Agreement across 3 labs	57% (27/47)	83% (10/12)	88% (21/24)	79% (38/48)	80% (40/50)	76% (38/50)	81% (39/48)

Abbreviations: Cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin-6; 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, 0.5, and 1.0 EU/mL tested once in three different laboratories

Figure 2 Timeline for ICCVAM Evaluation

June 2005
ECVAM Submission of Five *In Vitro* Pyrogen Test Methods to ICCVAM/NICEATM

December 2006
Publication of Draft ICCVAM Background Review Document (BRD) on Five *In Vitro* Pyrogen Test Methods

February 6, 2007
Independent Scientific Peer Review Panel Public Meeting Held at the Natcher Center on the National Institutes of Health (NIH) Campus

April 2007
Publication of the Independent Peer Review Panel Report

June 2007
Public Comments Due on Peer Review Panel Report
SACATM Comments on Peer Review Panel Report

Fall 2007
Finalize ICCVAM BRD and Test Method Evaluation Report
Finalize ICCVAM Test Method Recommendations and Forward to Federal Agencies for Consideration

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Pyrogenicity Working Group (PWG)

U.S. Environmental Protection Agency (EPA)
Ayaad Assad, D.V.M., Ph.D.
Karen Hamernik, Ph.D.
Louis (Gino) Scarano

U.S. Food and Drug Administration (FDA)
Mustafa Akkoyunlu, M.D., Ph.D.
Pankaj Amin
Christine Anderson
Kimberly Benton, Ph.D.
Joseph George, Ph.D.
David Hussong, Ph.D.
Abby Jacobs, Ph.D.
Christopher Joneckis, Ph.D.
Raju Kammula, D.V.M., Ph.D., D.A.B.T.
Jodie Kulpa-Eddy, D.V.M.
Richard McFarland, M.D. (Chair)
Ramesh Panguluri

U.S. Food and Drug Administration (FDA) (Con't)
Robert Mello, Ph.D.
Penelope Rice, Ph.D.
Leonard Schechtman, Ph.D. (retired 2006)
Amy Rosenberg, M.D.
Daniela Verthelyi, M.D., Ph.D.
Jiaqin Yao, Ph.D.

National Institute of Environmental Health Sciences (NIEHS)
William Stokes, D.V.M., D.A.C.L.A.M. (Director, NICEATM)
Raymond Tice, Ph.D. (Deputy Director, NICEATM)

U.S. Department of Agriculture (USDA)
Jodie Kulpa-Eddy, D.V.M.

ECVAM Liaison
Marlies Halder, Ph.D.[1]

[1]Dr. Halder was responsible for the trial plan of the catch-up validation study, but she was not involved in the evaluation of the data.

Independent Scientific Peer Review Panel Meeting

A public meeting of the Pyrogenicity Peer Review Panel ("Panel") organized by ICCVAM/NICEATM was held at the National Institutes of Health (NIH) campus on February 6, 2007

Charges to the "Panel"

- Review the draft Pyrogenicity Background Review Document (BRD) for completeness and for any errors and omissions
- Evaluate the extent to which each of the applicable criteria for validation and acceptance criteria (ICCVAM 2003) have been adequately addressed for the test methods and their specific proposed use
- Comment on the extent to which the draft ICCVAM test method recommendations are supported by information provided in the draft BRD for proposed:
  - Test method uses
  - Recommended standardized protocols
  - Test method performance standards
  - Additional studies

In Vitro Pyrogenicity Independent Peer Review Panel

Karen Brown, Ph.D., Panel Chair, DRL Pharma and Pair O' Doc's Enterprises, Parkville, Missouri

Brian Crowe, Ph.D., Baxter Vaccine AG, Orth an der Donau, Austria

Nancy Flournoy, Ph.D., University of Missouri-Columbia, Columbia, Missouri

Ihsan Gursel, Ph.D., Bilkent University, Bilkent, Ankara, Turkey

Ken Ishii, MD, Ph.D., ERATO, Japan Science and Technology Agency, Osaka University, Osaka, Japan

Jack Levin, MD, University of California-San Francisco, San Francisco, California

Albert Li, Ph.D., *In Vitro* ADMET Laboratories, Rockville, MD

David Lovell, Ph.D., University of Surrey, Guilford, United Kingdom

Melvyn Lynn, Ph.D., Eisai Medical Research, Inc., Ridgefield Park, New Jersey

Anthony Mire-Sluis, Ph.D., AMGEN, Inc, Thousand Oaks, California

Jon Richmond, MD, Home Office, Tayside, United Kingdom

Peter Theran, V.M.D., Massachusetts Society for the Prevention of Cruelty to Animals, Novato, California

Kevin Williams, Eli Lilly, Indianapolis, Indiana

Overall Peer Review Outcomes

- 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)".

Conclusions

A final ICCVAM BRD and Test Method Evaluation Report, which will include the final ICCVAM test method recommendations, will be available by Fall 2007. In finalizing their test method recommendations, ICCVAM will consider the information contained in the BRD, recommendations from the independent peer review panel, public comments, and SACATM comments. ICCVAM will forward its final test method recommendations to U.S. regulatory agencies for their consideration (**Figure 2**).

References

ICCVAM. 2003. NIH publication No: 03-4508. http://iccvam.niehs.nih.gov/SuppDocs/SubGuidelines/SD_subg034508.pdf

ICCVAM Authorization Act of 2000. 2000. 42 U.S.C. § 2851-2. 2851-5. http://iccvam.niehs.nih.gov/about/about_ICCVAM.htm

Acknowledgments

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NIEHS National Institute of Environmental Health Sciences

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

NICEATM The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

More information on ICCVAM and NICEATM can be accessed at http://iccvam.niehs.nih.gov/