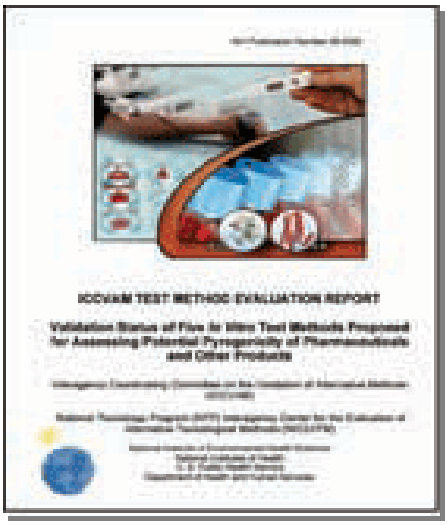
ICCVAM Recommendations on Five *In Vitro* Pyrogen Test Methods

R McFarland[1], M Wind[2], J Kulpa-Eddy[3], R Tice[4], W Stokes[4]

[1]U.S. Federal Drug Administration, Washington, DC, USA, [2]U.S. Consumer Product Safety Commission, Bethesda, MD, [3]U.S. Department of Agriculture, Riverdale, MD, [4]National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), National Institute of Environmental Health Sciences (NIEHS)/National Institutes of Health (NIH) /Department of Health and Human Services (DHHS), Research Triangle Park, North Carolina, USA.

Introduction



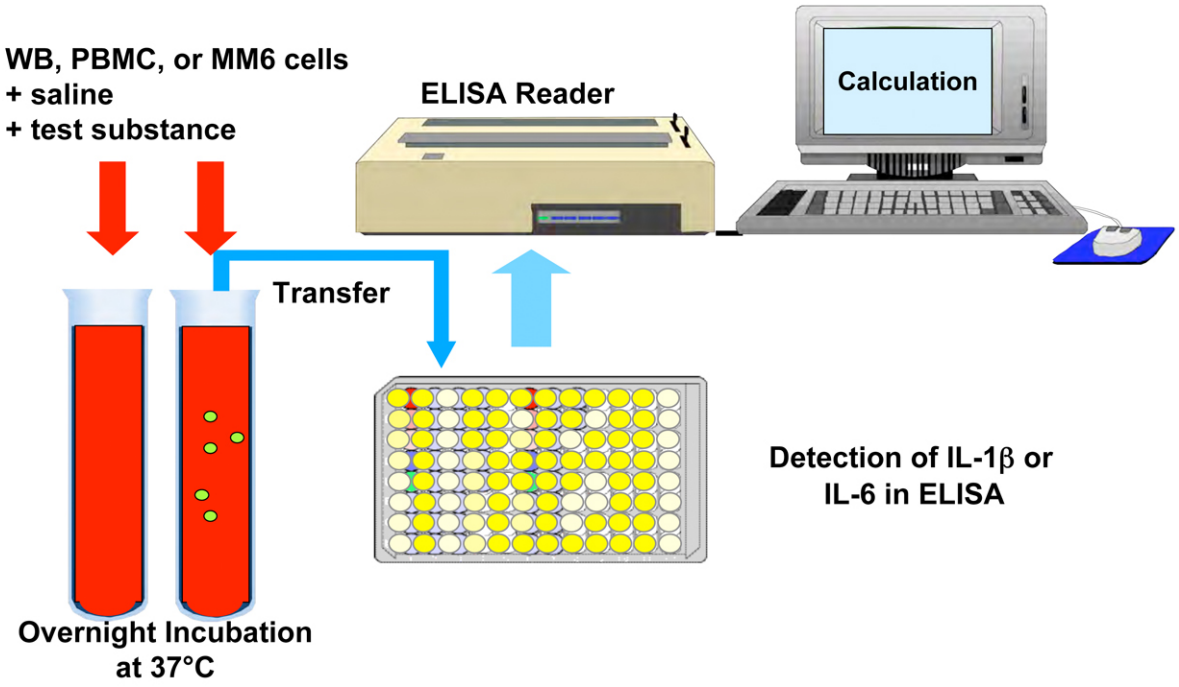
- The U.S., European[1], and Japanese Pharmacopoeias currently recognize two test methods for pyrogen testing: the rabbit pyrogen test (RPT) and the bacterial endotoxin test. Alternative test systems based on the *in vitro* activation of human blood cells have been developed to assess potential pyrogenicity.
- These test methods utilize human whole blood, isolated primary monocytes, or a monocyte cell line (Mono Mac 6) and are based on quantifying cytokine release (IL-1β or IL-6) to identify Gram-negative endotoxin containing substances as potential pyrogens.
- The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), in conjunction with the ICCVAM Pyrogenicity Working Group, evaluated the validation status of five of these test methods as potential replacements for the RPT:
  - 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
- The ICCVAM test method evaluation report (TMER), *Validation Status of Five In Vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products* provides ICCVAM recommendations for these five *in vitro* test methods to assess

potential pyrogenicity of pharmaceuticals and other products in a tiered-testing strategy. These recommendations are based on a comprehensive evaluation of the scientific validation status of the test methods by ICCVAM, and take into consideration the comments and recommendations received from an independent expert peer review panel, ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and the general public.

- The TMER contains ICCVAM recommendations for:
  - Test method uses
  - Standardized test method protocols
  - Future studies
  - Proposed reference substances

[1] Subsequent to release of the TMER, the European Directorate for the Quality of Medicine (EDQM) reported March 21, 2009 (available at: http://www.edqm.eu/medias/fichiers/133rd_Session_of_the_Eu.pdf) that the European Pharmacopeia Commission formally adopted monograph 2.6.30. "Monocyte Activation Test" (formerly alternative pyrogen test) during its 133rd session in March 2009 for implementation into the European Pharmacopeia in 2010.

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



Test Method Accuracy

- Ten parenteral pharmaceutical products (**Table 1**) were used to determine test method accuracy (**Table 2**).
- Each drug was spiked with four concentrations of a World Health Organization (WHO) *Escherichia coli* Gram-negative endotoxin standard (WHO-LPS 94/580 [*E. Coli* 0113:H10K-]) and tested once in three different laboratories.
- Accuracy was determined against a threshold value of 0.5 EU/mL, which was established based on a regression analysis of historical RPT data (n = 171 Chinchilla bastard rabbits).
- Results (**Table 2**):
  - Accuracy of the five 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%
  - False positive rates ranged from 3% to 23%

A complete description of all databases and the resulting accuracy and reliability analyses conducted for each of these test methods can be obtained at http://iccvam.niehs.nih.gov/methods/pyrogen/pyrogen.htm.

ICCVAM Pyrogenicity Working Group

Department of Agriculture

Jodie Kulpa-Eddy, D.V.M

Environmental Protection Agency

Karen Hamernik, Ph.D.

National Institute of Environmental Health Sciences

William Stokes, D.V.M., D.A.C.L.A.M. (Director, NICEATM)
Raymond Tice, Ph.D.

European Centre for the Validation of Alternative Methods

Marlies Halder, Ph.D.[1]

Food and Drug Administration

Center for Biologics Evaluation and Research

Mustafa Akkoyunlu, M.D., Ph.D.
Pankaj Amin
Christine Anderson
Kimberly Benton, Ph.D.
Joseph George, Ph.D.
Christopher Joneckis, Ph.D.
Richard McFarland, M.D. (Chair)

Center for Drug Evaluation and Research

David Hussong, Ph.D.
Abigail Jacobs, Ph.D.
Robert Mello, Ph.D.
Raju Kammula, D.V.M., Ph.D., D.A.B.T.
Daniela Verthelyi, M.D., Ph.D.
Jiaqin Yao, Ph.D.

Center for Devices and Radiological Health

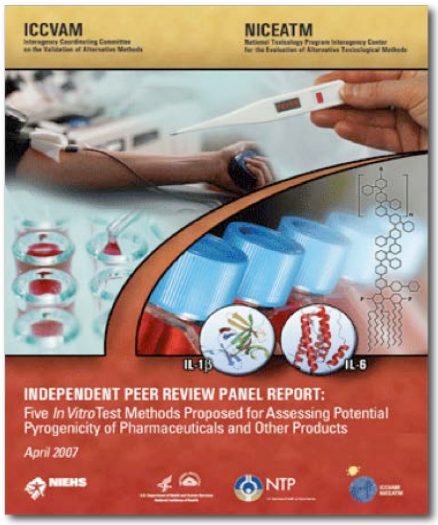
Ramesh Panguluri

Center for Food Safety and Applied Nutrition

Penelope Rice, Ph.D.

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

ICCVAM International Pyrogenicity Peer Review Panel



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

Brian Crowe, Ph.D., Director of Immunology Vaccines, Baxter Vaccine AG, Orth an der Donau, Austria

Nancy Flournoy, Ph.D., Professor and Chair, Department of Statistics, University of Missouri-Columbia, Columbia, Missouri

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

Ken Ishii, M.D., Ph.D., Group Leader, Akira Innate Immunity Project, ERATO, Japan Science and Technology Agency, Osaka University, Osaka, Japan

Jack Levin, M.D., Professor of Laboratory Medicine, VA Hospital, University of California-San Francisco, San Francisco, California

Albert Li, Ph.D., Chairman and Chief Scientific Officer, *In Vitro* ADMET Laboratories, Rockville, Maryland

David Lovell, Ph.D., Reader in Medical Statistics, University of Surrey, Guilford, United Kingdom

Melvyn Lynn, Ph.D., Senior Director and Global Head, Sepsis and Anti-infectives Therapeutic Area, Eisai Medical Research, Inc., Ridgefield Park, New Jersey

Anthony Mire-Sluis, Ph.D., Senior Director, Product Quality and External Afffairs, AMGEN, Inc, Thousand Oaks, California

Jon Richmond, M.D., Head of Division, Animals Scientific Procedures Division, Home Office, Tayside, United Kingdom

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

Kevin Williams, Microbiologist, Eli Lilly, Indianapolis, Indiana

Table 1 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, or 1.0 Endotoxin Units (EU)/mL of endotoxin (World Health Organization [WHO]-Lipopolysaccharide 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 Cryo WB/IL-1β test method.

Table 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 substance was spiked with 0, 0.5, or 1.0 Endotoxin Units (EU)/mL of endotoxin (World Health Organization [WHO]-Lipopolysaccharide 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.

Table 3 Performance Characteristics for *In Vitro* Pyrogen Test Methods[1,2]

Test Method	Accuracy	Sensitivity	Specificity	False Negative Rate	False Positive Rate
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)[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-1β (Tube)	81% (119/147)	73% (64/88)	93% (55/59)	27% (24/88)	7% (4/59)
WB/IL-1β (96-well plate)[4]	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 is the proportion of correct outcomes (positive and negative) of a test method. Sensitivity is the proportion of true positive substances that are correctly classified as positive. Specificity is the proportion of true negative substances that are correctly classified as negative. False positive rate is the proportion of true negative substances that are falsely identified as positive. False negative rate is the proportion of true positive substances that are falsely identified as negative.

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

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

Test Method Reliability

Intralaboratory Repeatability

- Closeness of agreement among optical density readings was compared for cytokine measurements at each spike concentration within the range of 0.06 to 0.5 EU/mL against saline.
  - Variability (based on up to 20 replicates per concentration) was low enough that the 0.5 EU/mL spike concentration could repeatedly be distinguished from the lower concentrations.

Intra- and Interlaboratory Reproducibility

- Three marketed parenteral pharmaceutical products spiked with various concentrations of endotoxin were used to determine test method reproducibility (**Table 3**).
  - **Intralaboratory reproducibility** was evaluated with mean correlations expressed as a percentage of agreement between pairs of the independent runs (**Table 4**). Agreement across 3 runs within a single laboratory ranged from 75% to 100%.
  - **Interlaboratory reproducibility** was evaluated in two different studies (see **Table 5** and **Table 6**) in which each run from one laboratory was compared to all other runs of another laboratory.
    - The agreement across the laboratories for each test method in each study ranged from 57% to 88%.

Table 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			Lab			Lab			Lab			Lab		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	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.

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

Lab Comparison	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 2**) spiked with endotoxin (World Health Organization-[WHO] Lipopolysaccharide 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).

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

Lab Comparison	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 (World Health Organization [WHO]-Lipopolysaccharide 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.

Table 7 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-1**
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)				Not applicable
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 1 EU/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; MVD = Maximum valid dilution;

MM6 = Mono Mac 6; 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; SOP = Standard operating procedure; 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 recommended protocols, PPC and NPC are assessed in the interference test, 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] The ELC is expressed as the ratio of the threshold pyrogen dose and the maximum human dose for pyrogenicity administered on a weight basis (kg) in 1 hr, or the RPT dose (whichever is larger).

ICCVAM Test Method Recommendations

Test Method Uses and Limitations

- Based on the ICCVAM evaluation, 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.
- 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 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][2]).[3]
- 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.

Test Method Protocols

- When testing is conducted, the *in vitro* pyrogen test method protocols should be based on the ICCVAM recommended protocols, which are summarized in **Table 7**.
- Using these 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.
- 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.

Future Studies

These test methods could be applicable to a wider range of pyrogens (i.e., endotoxin and non-endotoxin) and test materials, provided that they are adequately validated for such uses. Therefore, future validation studies should include the following considerations:

- Both endotoxin-spiked and non-endotoxin spiked samples should be included. Non-endotoxin standards should be characterized prior to their use in any study.

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[2] 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)).

[3] Substances other than endotoxin may induce 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.

- Test substances that better represent all categories of sample types intended for testing by the methods should be included (e.g., pharmaceuticals, biologicals, and medical devices).
- Wherever possible, historical data generated with the same test samples in both *in vivo/in vitro* studies should be retrospectively evaluated, or *in vitro* studies should be performed in parallel with RPT and/or BET that are conducted in the future for regulatory purposes.
- Test materials that have been identified clinically as pyrogenic would be invaluable for use in future validation studies and might preclude the need to use of animals.
- 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.
    - Any parallel testing should be limited only to those studies that will fill existing data gaps.

Regulatory Acceptance

- In May 2009, all U.S. Federal regulatory agencies endorsed the ICCVAM pyrogen test method recommendations.
- In March 2006, the ECVAM Scientific Advisory Committee (ESAC) endorsed a statement of validity for these five *in vitro* test methods 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.
- The European Pharmacopeia also adopted these test methods in March 2009.

Conclusions

- 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 deemed appropriate for a specific testing situation.
- When used in this manner, these methods should reduce the number of animals needed for pyrogenicity testing.
- 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.

References

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ICCVAM. 2000. ICCVAM Authorization Act of 2000. Public Law 106-545 (available at: http://iccvam.niehs.nih.gov/about/about_ICCVAM.htm).

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