ICCVAM Recommendations on the Use of Five *In Vitro* Test Methods for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products

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Abstract

Pyrogen testing is a critical step for ensuring the safety of parenteral pharmaceuticals. The U.S., European, and Japanese Pharmacopoeias currently recognize two pyrogen tests, both of which require animals (i.e., the rabbit pyrogen test [RPT] and the bacterial endotoxin test). Concerns for animal welfare have led researchers to develop alternative cell-based test methods that use human cells. ICCVAM recently evaluated the validation status of five *in vitro* test methods for assessing potential pyrogenicity of pharmaceuticals and other products as potential replacements for the RPT. These methods use IL-1 or IL-6 ELISAs to measure an increase in cytokines when human monocytoid cells (i.e., whole blood, isolated monocytes, or a Mono Mac 6 cell line) are exposed to Gram-negative endotoxin. The accuracy evaluation was based on 10 parenteral pharmaceuticals, each spiked with four concentrations of endotoxin. Accuracy ranged from 81% to 93%, false negative rates ranged from 1% to 27%, and false positive rates ranged from 3% to 23%. Quantitative and qualitative reliability analyses indicated that the test methods were generally reproducible within and among testing laboratories. Based on the results of these analyses, ICCVAM recommends that, while none of these five *in vitro* test methods should be considered as a complete replacement for the RPT, they could be considered to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis, subject to product-specific validation by the appropriate regulatory agency. When used in this manner, these methods should further reduce the number of animals needed for pyrogenicity testing. ICCVAM recommends, consistent with U.S. Animal Welfare Regulations, that *in vitro* pyrogen tests must be considered prior to testing in animals and that an alternative test method be used when deemed appropriate.

Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is charged by the ICCVAM Authorization Act of 2000[1] with evaluating the scientific validity of new, revised, and alternative toxicological test methods with potential applicability to U.S. Federal agency safety testing requirements. ICCVAM is also required to provide recommendations to U.S. Federal agencies regarding the usefulness and limitations of such test methods.

ICCVAM recently evaluated the validation status of five *in vitro* test methods for assessing the potential pyrogenicity of pharmaceuticals and other products proposed as potential replacements for the *in vivo* rabbit pyrogen test (RPT). The ICCVAM evaluation is summarized in the test method evaluation report (TMER) *In Vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products*.

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

Each of these methods is based on the measurement of pro-inflammatory cytokines (i.e., IL-1β or IL-6) that are released from human monocytoid cells or a monocytoid cell line in response to exposure to a pyrogenic substance (**Figure 1**).

The ICCVAM recommendations are based on a comprehensive evaluation (see the timeline in **Figure 2**) of the scientific validation status of the test methods, and take into consideration the comments and recommendations received from:

- An independent scientific peer review panel (Panel)
- ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)
- The general public

The TMER includes ICCVAM recommendations for test method uses and limitations, test method protocols, additional studies and development of performance standards.

[1]42 U.S.C. § 2851-2, 2851-5 (2000)

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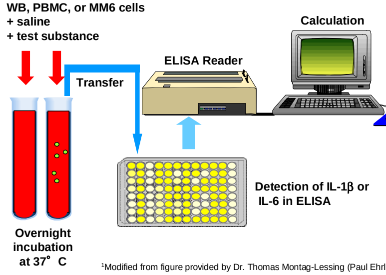
Figure 1

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



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

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Figure 2

Timeline for Development of ICCVAM Test Method Evaluation Report

Jun 2005	European Centre for the Validation of Alternative Methods (ECVAM) submission of five *in vitro* pyrogen test methods
Dec 2006	ICCVAM draft comprehensive Background Review Document (BRD) on five *in vitro* pyrogen test methods published
Feb 2007	Independent Peer Panel meeting convened to assess the current validation status of the five *in vitro* pyrogen test methods
May 2007	Peer Panel Report published
Jun 2007	SACATM comments on the ICCVAM draft BRD and draft recommendations, the Peer Panel report, and all public comments
Oct 2007	ICCVAM endorses final BRD and final test method recommendations
Spring 2008	ICCVAM BRD and TMER to be forwarded to U.S. Federal agencies for consideration
Fall 2008	Responses of relevant U.S. Federal agencies to the ICCVAM recommendations due in writing within 180 days

References

Dixon WJ. 1950. Analysis of extreme values. Ann Math Stat 21:488-506.
ICCVAM. 2000. ICCVAM Authorization Act of 2000. Public Law 106-545. http://iccvam.niehs.nih.gov/about/about_ICCVAM.htm.

Data and Analyses

Test Method Accuracy

- Ten parenteral pharmaceutical products (**Table 1**) were used to determine test method accuracy (**Table 3**).
- 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, which was established based on a regression analysis of historical RPT data (n = 171 Chinchilla bastard rabbits).
- Results (**Table 3**):
  - 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%.

Table 1

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

Test Substance[2]	Source	Lot Numbers	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.

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.

Test Method Reliability

- Intralaboratory repeatability was evaluated by comparing the closeness of agreement among optical density readings for cytokine measurements at each spike concentration within the range of 0.06 to 0.5 EU/mL against saline. Although the variability (based on up to 20 replicates per concentration) increased with endotoxin spike concentration, it was low enough that the 0.05 EU/mL spike concentration could still be distinguished from the lower concentrations.
- Three marketed parenteral pharmaceutical products spiked with various concentrations of endotoxin were used to determine test method reproducibility (**Table 2**).
- 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 **Tables 5 and 6**) in which each run from one laboratory was compared to all other runs of another laboratory.
  - The first study used the three parenteral products (**Table 2**) spiked with endotoxin or a negative saline control in triplicate (except for the Cryo WB/IL-1β, which used only one run per laboratory). The agreement across three laboratories for each test method ranged from 58% to 86% (**Table 5**).
  - The second study compared the results from the 10 substances used in the accuracy analysis (**Table 1**). Agreement across laboratories ranged from 57% to 88% (**Table 6**).

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-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)				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)				
	TC (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 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-1 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).

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ICCVAM Test Method Recommendations

Test Method Uses and Limitations

Based on this evaluation, ICCVAM recommendations are as follows:

- 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 Bacterial Endotoxins Test (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[1])[2].
- Potential users should consider the false negative/false positive rates as well as ease of use in selecting any test method for possible use.
- 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.

[1]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)).
[2]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.

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**.
- These recommended protocols, which are based primarily on ECVAM Standard Operating Procedures for each test method, have been updated by NICEATM and ICCVAM to address inadequacies identified by the Panel, including modifications made to standardize essential test method components across the five *in vitro* test methods. These modifications are not expected to reduce test method performance.
- 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.

Additional Studies

- It is recognized that 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.
- 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 vivo/in vitro* studies should be retrospectively evaluated, or *in vitro* testing should be performed in parallel with RPT and/or BET that are conducted in the future for regulatory purposes[1].
- Future validation studies should include the following considerations:
  - Both endotoxin-spiked and non-endotoxin spiked samples should be included. In this regard, non-endotoxin standards should be characterized prior to their use in any study, if possible.
  - All aspects of the studies should be compliant with national and international Good Laboratory Practice guidelines.
  - Additional studies should include products that have intrinsic pro-inflammatory properties in order to determine if such substances are amenable to these tests.
  - 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 parallel testing should be limited only to those studies that will fill existing data gaps.
  - 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.
  - 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.
  - 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.

[1]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., World Health Organization[WHO]-Lipopolysaccharide 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*.

Development of Performance Standards

- As indicated above, these five *in vitro* 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.
- For this reason, it is not 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.

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