2.0 ICCVAM Recommendations for *In Vitro* Pyrogen Test Methods

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

2.1 ICCVAM Recommendations: Test Method Uses and Limitations

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

2.1.1 Independent Peer Review Panel Conclusions and Recommendations

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

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

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

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

2.1.2 ECVAM Scientific Advisory Committee (ESAC) Statement of Validity

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

However, ESAC concluded that these tests have been scientifically validated for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of this pyrogen, in materials currently evaluated and characterized by rabbit pyrogen tests. In contrast, as described in **Section 2.1**, ICCVAM has concluded that the current validation database for these test methods is inadequate to support such a definitive statement based on the ECVAM validation study design, which did not include biologics or medical devices and evaluated only a limited range and number of pharmaceutical products and additionally did not include parallel testing with the RPT.

2.2 ICCVAM Recommendations: Test Method Protocols

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

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

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

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

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

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

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

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

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

[4]Dixon 1950.

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

2.3 ICCVAM Recommendations: Future Studies

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

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

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

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

The Panel also recommended other studies for consideration:

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

2.4 ICCVAM Recommendations: Performance Standards

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

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