2.0 *In Vitro* Pyrogen Test Method Protocol Components

2.1 Overview of How the *In Vitro* Pyrogen Test Methods Are Conducted

Although there are differences among the *in vitro* pyrogen test methods considered in this ICCVAM BRD, the basic procedural steps are consistent across all five methods:

- Interference testing is performed to verify that a test substance does not interfere with either the cell system used or with the specific cytokine-specific enzyme-linked immunosorbent assay (ELISA).
- The test substance is mixed with a suspension of human-derived blood cells.
- The concentration of the specific proinflammatory cytokine (e.g., IL-1β, IL-6) is measured using an ELISA, and is compared to the response curve of an endotoxin standard.
- An internationally accepted endotoxin standard (World Health Organization-LPS [WHO-LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-), or an endotoxin standard that has been calibrated against this standard, is used to generate the standard response curve for the assay. The endotoxin activity of a test substance is calculated by comparing the induced cytokine release with that induced by the endotoxin standard.
- A product "passes" (i.e., is considered negative for endotoxin pyrogen activity) if the cytokine response to the test substance is less than that induced by 0.5 endotoxin units/mL (EU/mL).

2.2 Description and Rationale for the Test Method Components for Proposed Standardized Protocols

The standard operating procedures for each test method assessed in the ECVAM validation studies are provided as Appendix A of each ECVAM BRD. As indicated in **Section 2.1**, there are essential principles of each protocol that are common among the five methods reviewed. These include:

- Isolating and/or culturing human monocytoid cells (either included in WB, separated as a fraction [i.e., PBMCs], or as cell line [i.e., MM6])
- Performing interference testing with each substance
- Treating the cells in suspension with a test substance
- Collecting cytokine release data
- Evaluating the data in relation to the proposed prediction model

Table 2-1 provides a comprehensive comparison of the similarities and differences among the protocols for the five test methods. No rationale was provided for the use of WB in the various test methods; however, Poole et al. (2003) summarized several studies, which indicated that the monocytes present in diluted WB respond to pyrogen/endotoxin by releasing pyrogenic cytokines. The use of the MM6 cell line was justified based on mechanistic considerations and its response to endotoxins.

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Table 2-1 *In Vitro* Pyrogen Test Method Components

	Test Method Component	WB/IL-1β[1]	Cryo WB/IL-1β	WB/IL-6	PBMC/IL-6[1]	MM6/IL-6
	Source of cells	Human WB	Human Cryo WB	Human WB	Human WB	MM6 cell bank (original cell line maintained by Prof. H. Ziegler-Heitbrock, U. Munich)
	Laboratory equipment	• CO_2 cell culture incubator (37°C, 5% CO_2, humidified) • Centrifuge • Consumables as specified in SOP (e.g., heparinized blood tubes for WB methods, 96-well plates or culture tubes, centrifuge tubes, microfuge tubes, pyrogen-free plastic-ware where specified, serological pipets, pyrogen-free hypodermic needles) • Data analysis software • Hemocytometer (e.g., PBMC and MM6 assays) • Laminar Flow Hood (Class II) • Liquid nitrogen, CO_2 freezers, or programmable freezers for cryopreservation methods (Cryo WB/IL-1β or Cryo PBMC) • Microscope, inverted (optional except for PBMC and MM6 assays) • Microtiter Plate Reader (450 nm with 600-690 nm reference filter for IL-1β or 500-590 nm reference filter for IL-6 measurements) • pH meter • Pipettors (8 to 12 multi-channels; 2 to 2000 µL adjustables; pyrogen-free tips (except for ELISA) • Vortex mixer • Water bath				
	Culture medium	None - WB is diluted with PFS	RPMI Complete Medium • RPMI 1640 (part of the Endosafe Kit for cryoblood) • no specific additives needed	None - WB is diluted with PFS	RPMI Complete Medium • RPMI 1640 • HSA • L-Glutamine (2 mM) • Penicillin/streptomycin	RPMI Medium[2] • RPMI 1640 medium • Bovine insulin (0.23 IU/mL) • HEPES (20 mM) • HIFCS (10% or 2%) • L-glutamine (2mM) • MEM non-essential amino acid solution (0.1 mM) • Oxaloacetic acid (1 mM) • Sodium pyruvate (1 mM)
	Other reagents	• Endotoxin standard • PFS • PFW • Validated IL-1β ELISA kit	• DMSO • Endotoxin standard • PFS • PFW • Validated IL-1β ELISA kit	• Endotoxin standard • PFS • PFW • Validated IL-6 ELISA kit	• Endotoxin standard • PFS • PFW • Trypan blue • Validated IL-6 ELISA kit	• DMSO • Endotoxin standard • PFS • PFW • Trypan blue • Validated IL-6 ELISA kit
Dose selection procedures		Interference testing performed to determine the lowest dilution of the test product necessary to achieve an acceptable endotoxin spike recovery (i.e., 50% to 200% recovery)[3]				
Endpoints measured		IL-1β release via ELISA		IL-6 release via ELISA		

	Test Method Component	WB/IL-1β[1]	Cryo WB/IL-1β	WB/IL-6	PBMC/IL-6[1]	MM6/IL-6
Exposure of the test substance	**Pre-test preparation of cells**	Collect WB, heparinize, and use within 4 hr Plate Method: same collection procedure	• Collect WB, heparinize, and cryopreserve according to the Konstanz or PEI method • Prior to testing, thaw WB at 37°C for 15 min	Collect WB, heparinize, and use within 4 hr	• Collect WB and isolate PBMCs by centrifugation • Resuspend PBMCs in RPMI-C ($1x10^6$ cells/mL) (use PBMCs within 4 hr of initial WB collection)	• Incubate MM6 cells ($4x10^5$ cells/mL media) for 24 hr • Resuspend cells ($2.5x10^6$ cells/mL)[3] prior to testing
	Application of the test substance	Tube method: In a microfuge tube mix 1000µL PFS+100µL sample+100µL WB Plate method: In a 96-well plate mix 200µL PFS+20µL sample+20µL WB	Konstanz method: In a 96-well plate mix 200µL RPMI+20µL sample+20µL WB PEI Method: In a 96-well plate mix 180µL RPMI + 20µL sample+40µL WB	In a 96-well plate: Mix 50 µL standards/samples+100 µL PFS+50 µL WB	In a 96-well plate: Mix 50 µL standards/samples+100 µL RPMI-C+100 µL PBMCs	In a 96-well plate: Mix 50 µL standards/samples+100 µL RPMI-C+100 µL cells in suspension
	Duration of exposure	10-24 hr		16-24 hr		
	Material used for ELISA	Tube method: centrifuge 2 min @ 10,000 x *g*-test supernatant Plate method: mix each well be pipetting and test resuspended mixture	WB/RPMI/sample mixture	WB/saline/sample mixture	Cell supernatant	Cell supernatant
Known limits of use		Intended for parenteral pharmaceuticals, biological products, and medical devices that have been qualified through interference testing				
Nature of the response assessed		Pyrogenic substances induce the release of proinflammatory cytokines (e.g., IL-1β) from monocytoid cells present in human WB		Pyrogenic substances induce the release of proinflammatory cytokines (e.g., IL-6) from monocytoid cells in WB, PBMC, or immortalized MM6 cells		
Appropriate controls	**Positive control (PC)**	0.5 EU/mL WHO-LPS 94/580 [*E. coli* 0113:h10:K-][5]				
	Negative control (NC)	PFS				
	Positive product control (PPC)	Test substance spiked with endotoxin (0.5 EU/mL or a concentration in middle of standard endotoxin curve)				
	Negative product control (NPC)	Test substance spiked with PFS				
Assay acceptability criteria		• PC OD 1.6-fold>NC OD • PPC OD 1.6-fold>NPC OD • PPC OD should be within 50% to 200% of the PC OD • NC OD≤0.100	• PC OD 1.6-fold>NC OD • PPC OD 1.6-fold>NPC OD • PPC OD should be within 50% to 200% of the PC OD • NC OD≤0.100	• PPC OD should be within 50% to 200% of the PC OD • NC OD<200 pg/mL IL-6 standard	• PPC OD should be within 50% to 200% of the PC OD • 1 EU/mL standard OD>1000 pg/mL IL-6 standard • NC OD<0.15 and NC OD<500 pg/mL IL-6 standard	• PC OD±20% of the expected value (i.e., 0.5 EU/mL) • PPC OD should be within 50% to 200% of the PC OD • NC OD<0.200

	Test Method Component	WB/IL-1β[1]	Cryo WB/IL-1β	WB/IL-6	PBMC/IL-6[1]	MM6/IL-6
Nature of data to be collected and methods used for data collection		• The endotoxin content of a test substance is calculated by comparing the induced IL-1β release with that induced by the endotoxin standard curve concentrations		• The endotoxin content of a test substance is calculated by comparing the induced IL-6 release with that induced by the endotoxin standard curve concentrations		
Type of media in which data are stored		Electronic files				
Exclusion criteria		Mean±SD of the OD for each test substance/standard				
Decision criteria for pyrogenicity		OD TS > OD 0.5 EU/mL EC		EC TS > ELC TS	EC TS > ELC TS[6]	EC TS > ELC TS

Abbreviations: Cryo = Cryopreserved; DMSO = Dimethylsulfoxide; EC = Endotoxin concentration; ELC = Endotoxin limit concentration; EU/mL = Endotoxin units/mL; ELISA = Enzyme-linked immunosorbent assay; HIFCS = Heat-inactivated fetal calf serum; HSA = Human serum albumin; IL = Interleukin; IU = International units; LPS = Lipopolysaccharide; MEM = Minimum essential medium; MM6 = Mono Mac 6; NC = Negative control; NPC = Negative product control; OD = Optical density; PBMC = Peripheral blood mononuclear cells; PC = Positive control; PEI = Paul-Ehrlich-Institut; PFS = Pyrogen free saline; PFW = Pyrogen free water; PPC = Positive product control; SD = Standard deviation; SOP = Standard operating procedure; TS = Test substance; WB = Whole blood; WHO = World Health Organization; x *g* = times gravity

[1]As described in **Section 1.1.1**, a catch-up validation studies were also conducted to evaluate the performance of the WB/IL-1β test method using 96-well plates, and the PBMC/IL-6 test method when using cryopreserved PBMCs. The plating procedure (WB/IL-1β) and the cryopreservation procedure (PBMC/IL-6) are the only differences in the test method protocols (see **Appendix A**).

[2]Medium should be qualified for testing by a valid bacterial endotoxin test (i.e., USP30 NF25<85>) indicating that the endotoxin contamination is <0.06 IU/mL); fetal bovine serum concentration for MM6 cells varies based on whether it is for maintenance/propagation (10%) or assay (2%) conditions.

[3]Dilution of the test material should not exceed the maximum valid dilution (MVD), where MVD = (endotoxin limit concentration)/(detection limit of the assay)

[4]Cell numbers represent viable cells based on trypan blue exclusion

[5]Or another endotoxin calibrated against this standard

[6]Includes a sequential decision strategy in which 3 to 4 donors are tested per substance. 1) If all donors show negative - product is non-pyrogenic; 2) If ≥2 donors show a positive - product is pyrogenic; 3) If only one donor shows a positive, an additional 3 to 4 donors are tested and if no more than one donor is positive (out of 6 to 8 donors) - product is non-pyrogenic; otherwise, product is pyrogenic**.**

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2.2.1 Methods Used to Analyze the Data, Including Methods to Analyze for Interference with the Assay

Once a substance has been tested in the requisite number of donor samples (see **Section 2.2.2**), the resulting sample test medium (as indicated in **Table 2-1**) is assayed in quadruplicate in the relevant cytokine ELISA. Outliers are identified using the nonparametric Dixon's test ($p > 0.05$) (Dixon 1950; Barnett and Lewis 1984), the Grubbs' test (Grubbs 1969) for normally distributed samples, or other statistically acceptable methods (Martin and Roberts 2006) and are excluded from the calculations of endotoxin content (see also **Section 5.3** and **Appendix C**). Endotoxin standard curves are included in each assay, from which the endotoxin content of each replicate is estimated using a 4-parameter logistic model.

As indicated in **Table 2-1**, mean optical density (OD) readings are calculated for the positive and negative control samples, as well as for the relevant positive and negative product controls. The acceptable range of the positive product control (50% to 200% of the positive control response) defines the threshold for interference with the test system. If the positive product control response falls outside of this range, the samples are then assayed at the lowest dilution that does not cause interference.

2.2.2 Decision Criteria and the Basis for the Prediction Model Used to Identify a Pyrogenic Substance

As described in **Section 4.2**, historical RPT data were used to establish a threshold pyrogen dose (i.e., the endotoxin dose at which fever was induced in 50% of the rabbits), which was determined to be 5 EU/kg. Based on the largest allowable volume for injection in rabbits (10 mL/kg), the limit of detection that the *in vitro* pyrogen tests must meet was defined as 0.5 EU/mL. Accordingly, the prediction model for each test method was established based on this limit of detection (i.e., a substance is considered pyrogenic if the mean response is greater than or equal to the 0.5 EU/mL standard).

For three of the test methods, results from multiple donors (Cryo WB/IL-1β [n=5], WB/IL-6 [n=3], and PBMC/IL-6 [n=3 to 4]) are required to determine the potential pyrogenicity of a test substance. In contrast, a single donor sample is used for the WB/IL-1β test method, as is a single cell sample for the MM6/IL-6 test method. As outlined in **Table 2-2**, unlike the Cryo WB/IL-1β test method, the WB/IL-6 and PBMC/IL-6 test methods employ a decision strategy that takes into account the individual responses of each donor sample.

Table 2-2 Prediction Model Used for *In Vitro* Pyrogen Test Methods

Test Method	No. Donors	No. Positive	No. Negative	Decision
PBMC/IL-6	4[1]	4	0	Pyrogenic
		3	1	Pyrogenic
		2	2	Pyrogenic
		1	3	Non-pyrogenic
		0	4	Non-pyrogenic
	3[1]	3	0	Pyrogenic
		2	1	Pyrogenic
		1	2	Non-pyrogenic
		0	3	Non-pyrogenic
WB/IL-6	3	3	3	Pyrogenic
		2	1	Pyrogenic
		1	2	Non-pyrogenic
		0	3	Non-pyrogenic
Cryo WB/IL-1β	5 (pooled)[2]	1	0	Pyrogenic
		0	1	Non-pyrogenic
WB/IL-1β	1	1	0	Pyrogenic
		0	1	Non-pyrogenic
MM6/IL-6	NA[3]	1	0	Pyrogenic
		0	1	Non-pyrogenic

Abbreviations: Cryo = Cryopreserved; IL = Interleukin; MM6 = Mono Mac 6; NA = Not applicable; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

[1]Samples are collected from four donors for the PBMC/IL-6 test method. One donor sample may be excluded based on quality criteria, in which case the prediction model may be applied to results from three donors.

[2]Samples are collected from five donors for the Cryo WB/IL-1β test method and pooled prior to cryopreservation.

[3]Not applicable, because source material is obtained from an immortalized cell line.

2.2.3 Information and Data to be Included in the Study Report and Availability of Standard Forms for Data Collection and Submission

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

Test Substances and Control Substances

- Name and type (e.g., pharmaceutical, biological product, medical device eluate, etc.) of test product
- Purity and composition of the test substance or preparation
- Physicochemical properties, such as physical state, volatility, pH, stability, chemical class, water solubility, relevant to the conduct of the study
- Quality assurance (QA) data and known biological properties
- Treatment of the test/control substances prior to testing, if applicable (e.g., vortexing, sonication, warming; solvent used)
- Stability, if known

Justification of the Specific Protocol(s) 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 of the procedure used to ensure their integrity from lot-to-lot and over time

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data from the testing laboratory (which should be included in the report)
- Acceptable negative control data, including historical control ranges from the testing laboratory (which should be included in the report)

Test Conditions

- Cell system used; donor information, if relevant
- Calibration information for the equipment used for measuring cytokine release (e.g., spectrophotometer)
- Details of test procedure used
- Description of modifications of the test procedure made by the testing laboratory for the substance being tested
- Reference to the laboratory's historical data for the cell system and protocol
- Description of data and QA evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Good Laboratory Practice (GLP) QA Statement

- This statement addresses all GLP inspections and audits made during the study, and the dates the results were reported to the Study Director. This statement also serves to confirm that the final report reflects the raw data.

Reporting requirements for GLP-compliant studies are provided in the relevant guidelines (e.g., Organisation for Economic Co-operation and Development [OECD] 1998; U.S. Environmental Protection Agency [EPA] 2003a, 2003b; FDA 2003).

2.3 Basis for Selection of the Test Method Systems

One of the difficulties associated with the currently required pyrogen test methods (i.e., BET and RPT) is that both require extrapolation of the response from a non-human system to the human. In contrast, and as discussed in **Section 1.1.1**, all five of these test methods employ human cells in an attempt to mimic the human fever response *in vitro*.

The WB test methods (i.e., Cryo WB/IL-1β, WB/IL-1β, WB/IL-6) offer the convenience of performing the assay directly on a human blood sample, with minimal pretest preparation. The Cryo WB/IL-1β test method was developed to offer the convenience of an increased time interval between the time of blood collection and the time a test is initiated (since the fresh blood methods require testing within four hr of collection), as well as increased standardization through the pooling of five donor samples to produce a larger sample bank of cells to use in the test. The MM6/IL-6 test method provides increased standardization by using an immortalized cell line that may be maintained in the laboratory indefinitely, and transferred among laboratories. Finally, the PBMC/IL-6 test method was developed in an attempt to improve pyrogen detection sensitivity by using the monocyte fraction of WB, which is considered to be the most sensitive human blood cell type to the presence of endotoxin.

Additional information on standardization of the cellular components required for the test methods is presented in the ECVAM response to ICCVAM PWG questions (see question #5 in **Appendix B**).

2.4 Proprietary Components

Data from the test methods that use the IL-6 endpoint (i.e., WB/IL-6, PBMC/IL-6, MM6/IL-6) were obtained using either an in-house IL-6 ELISA developed by Novartis Pharma AG (Basel, Switzerland) or the Central Laboratory for the Blood Transfusion Service (CLB) Human IL-6 ELISA kit (Amsterdam, The Netherlands). In the ECVAM response to ICCVAM PWG questions (**Appendix B**), it was stated that both IL-6 ELISAs use the same monoclonal anti-IL-6 antibody for detection. At the present time, the Novartis IL-6 ELISA is not available for purchase; however, the CLB IL-6 ELISA kit is commercially available. Importantly, other commercially available IL-6 ELISAs may be individually validated and used in these procedures.

The MM6 cell line was generated by Professor Ziegler-Heitbrock at the University of Munich (refer to **Appendix A**, as well as Section 2.4 of the ECVAM MM6/IL-6 BRD). These cells are currently available from The German Collection of Microorganisms and Cell Cultures (DSMZ). However, a legal agreement with Professor Ziegler-Heitbrock stating that the MM6 cell line will be used for research purposes only is required prior to purchasing the cells. At the present time, any organization (e.g., pharmaceutical company) wishing to use the cells for product testing has to negotiate a fee for provision of the cells and a royalty payment per batch of product tested.

According to the U.S. Patent and Trademark Office (USPTO), patents are held for "Test for determining pyrogenic effect of a material" (U.S. 5,891,728, April 6, 1999), and "Pyrogenicity test for use with automated immunoassay systems" (U.S. 6,696,261 B2, February 24, 2004). These patents cover the WB/IL-1β and WB/IL-6 test methods, respectively. In addition, and related to the WB/IL-1β test method, there is a patent application pending for "Test procedure with biological system - Preparations containing deep-frozen blood are used for determining blood response" (USPTO 436518000).

There are several measures in the study validity criteria that may be used to verify the integrity of proprietary components. As outlined in **Table 2-1**, an endotoxin standard curve is established for each assay, which is in turn used to define the endotoxin activity of the test

substances. In addition, positive and negative controls, along with positive and negative product controls, are used for interference testing, and serve as internal controls for each assay.

2.5 Number of Replicates

2.5.1 Number of Donors

There is no rationale provided for the number of donors included for each test method. As described in **Section 2.2.2**, samples from multiple donors are required for three of the test methods. The Cryo WB/IL-1β test method uses pooled blood from five different donors and the WB/IL-6 and PBMC/IL-6 test methods use blood from at least three donors, which are tested individually. In contrast, a single donor sample is used for the WB/IL-1β test method.

2.5.2 Number of Assay Replicates

Once each substance has been tested in the requisite number of donor samples (see **Section 2.2.2**), the resulting sample test medium is assayed in quadruplicate in the relevant cytokine ELISA. As indicated in **Section 2.2.1**, the nonparametric Dixon's test (Dixon 1950; Barnett and Lewis 1984) or Grubbs' test (Grubbs 1969) for normally distributed samples is used to detect outliers among the replicates. Section 2.5 of the ECVAM BRDs states that four replicates were chosen, as it is considered the minimum number for inclusion in Dixon's test.

2.6 Modifications to the Test Method Protocols Based on ECVAM Validation Study Results

In the MM6/IL-6 test method, prevalidation studies demonstrated that pre-incubation of the cells at a defined initial concentration of 2 x 10^7 cells/50 mL RPMI-C for 24 hr greatly improved test method performance. Therefore, this modification was included in the validation study, and subsequently carried forward to the recommended MM6/IL-6 test method protocol.

For the PBMC/IL-6 test method, a single blood donor was initially used as a source of PBMCs. However, the use of PBMCs from four separate donors (assayed individually) was shown to reduce variability, and this modification was carried forward in the recommended PBMC/IL-6 test method protocol.

No modifications were made to the WB/IL-1β, Cryo WB/IL-1β, and WB/IL-6 test method protocols as a result of the prevalidation or validation testing experiences.

2.7 Differences Between Comparable Validated Test Methods with Established Performance Standards

The differences between the *in vitro* pyrogen test methods and the currently accepted pyrogen test methods (i.e., BET and RPT) are described in **Sections 1.2.3** and **1.3.2**.