Appendix B3

Draft ICCVAM Recommended Protocol for Future Studies Using the Peripheral Blood Mononuclear Cell (PBMC)/Interleukin-6 (IL-6) Test Method

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PREFACE

This proposed protocol for the detection of pyrogenicity is based on information obtained from 1) The European Centre for the Validation of Alternative Methods (ECVAM) PBMC/IL-6 Background Review Document (BRD) presented in Appendix A of the draft Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Pyrogenicity Test Method BRD, which includes ECVAM Standard Operating Procedures (SOPs) for the PBMC/IL-6 test method, and 2) Information provided to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) by Dr. Thomas Hartung, Head of ECVAM. The ECVAM SOPs are based on various test methods that use human PBMCs to detect cytokine production as a measure of pyrogenicity (Bleeker et al., 1994; Dinarello et al., 1984; Poole et al., 2003). A table of comparison between the draft ICCVAM recommended protocol and the ECVAM SOPs is provided in **Table 1**. Future studies using the PBMC/IL-6 test method may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that the proposed test method protocol might be revised based on additional optimization and/or validation studies. ICCVAM recommends that test method users routinely consult the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov/) to ensure that the most current test method protocol is used.

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Table 1 Comparison of Draft Recommended Test Method Protocol with the ECVAM SOP for the Peripheral Blood Mononuclear Cell (PBMC)/Interleukin-6 (IL-6) Pyrogen Test

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP/ ECVAM Catch-Up Validation SOP
Test Substance	Test neat or at minimal dilution that produces no interference	Test at MVD during validation - otherwise similar to ICCVAM protocol
Incubation Plate (number of control or test groups at n=4 each)	NSC (1)	Same as ICCVAM protocol
	EC (5)	Same as ICCVAM protocol
	TS (14)	Same as ICCVAM protocol
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Same as ICCVAM protocol
Decision Criteria for Interference	0.5 x Median OD_{450}[1] of 1 EU/mL EC <2x Median OD_{450} of 1 EU/mL EC	0.5 x Median OD_{450} of 0.25 EU/mL[3] EC <2x Median OD_{450} of 0.25 EU/mL EC
Assay Acceptability Criteria	Mean OD_{450} of PPC is 50% to 200% of 0.5 EU/mL EC	Mean OD_{450} of PPC is 50% to 200% of 0.25 EU/mL[2] EC
	Mean OD_{450} of NSC ≤0.15	Same as ICCVAM protocol
	Quadratic function of IL-6 SC r^2 ≥0.95	Same as ICCVAM protocol
	EC SC produces OD_{450} values that ascend in a sigmoidal concentration response	Same as ICCVAM protocol
	High responder (i.e., > 200 pg/mL IL-6) or low responder (i.e., Mean OD_{450} for 1 EU/mL EC is signficantly less than that for 1000 pg/mL IL-6) blood donors may be excluded (limited to 1 of 4 donors). One of four donors may be excluded or the test repeated with four additional donors.	Same as ICCVAM protocol
Decision Criteria for Pyrogenicity	Mean corrected OD_{450} of TS > Mean corrected OD_{450} of 0.5 EU/mL EC	EC SC data transformed to 4-parameter logistical model by an in-house program and the linear mean square is calculated. TS pyrogen content is compared with the ELC[3] using confidence limits for significance. The preparation being examined must pass the test with blood from three separate donors. Dixon's test is used to reject outliers.

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP/ ECVAM Catch-Up Validation SOP
	Decision Level 1 Passes if all are negative Fails if 2 or more are positive If one is positive, repeat with 4 more donors *Decision Level 2* Passes if 1/8 are positive Fails if 2 or more of 8 are positive	Same as ICCVAM protocol
	Not included	Limit test is run to determine whether or not a TS after correction and dilution contains < 0.5 EU/mL of endotoxin

Abbreviations: EC = Endotoxin control; ELC = Endotoxin Limit Concentration; MVD = Maximum Valid Dilution; NSC = Normal saline control; PPC = Positive Product Control; SC = Standard curve; TS = Test substance

[1] Median or mean OD_{450} values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

[2] Value of 0.25 EU/mL is arbitrary - should correspond to a concentration in the middle of the SC for the EC.

[3] Where unknown, the ELC is calculated (e.g., Based on a rabbit sensitivity of 5 EU/kg, for a product injected at 10 mL/kg, the detection limit is 5 EU/10 mL/kg or 0.5 EU/mL/kg, or an ELC of 0.5 EU/mL).

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of a pyrogen (i.e., Gram-negative endotoxin) in a test substance. The presence of Gram-negative endotoxin is detected by its ability to induce cytokine IL-6 release from human PBMCs. The quantity of IL-6 released is obtained using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. Release of this cytokine is measured by incubation of PBMCs with test substances or controls (i.e., positive, negative). The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by PBMCs exposed to the test substance to those exposed to an internationally-harmonized Reference Standard Endotoxin (RSE)[1] or an equivalent standard expressed in Endotoxin Units (EU)/mL.

The focus of this protocol is on the use of the PBMC/IL-6 test method specifically for the detection of Gram-negative endotoxin in parenteral pharmaceuticals. The relevance and reliability for non-endotoxin pyrogens (e.g., lipoteichoic acid) has not been demonstrated in a formal validation study.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures for procurement of eligible blood donors and blood donations should follow the regulations and procedures set forth by institutional guidelines for utilization of human substances, which include but are not limited to blood, tissues, and tissue fluids. Standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific study substances or hazardous chemicals will be identified in the Material Safety Data Sheet (MSDS).

[1] RSEs are internationally-harmonized reference standards (e.g., WHO *E. coli* Lipopolysaccharide [LPS] 94/580 [0113:H10:K-]; USP RSE Lot G3E069; FDA Lot EC-6). Equivalent endotoxins include commercially available *E. coli*-derived LPS Control Standard Endotoxin (CSE) or other *E. coli* LPS preparations that have been calibrated with an appropriate RSE.

The stop solution used in the ELISA kit is acidic and corrosive and should be handled with the proper personal protective devices. If this reagent comes into contact with skin or eyes, wash thoroughly with water. Seek medical attention, if necessary.

Tetramethylbenzidine (TMB) solution contains a hydrogen peroxide substrate and 3, 3', 5, 5'- TMB. This reagent is a strong oxidizing agent and a suspected mutagen. Appropriate personal protection should be used to prevent bodily contact.

Bacterial endotoxin is a toxic agent (i.e., can induce sepsis, shock, vascular damage, antigenic response) and should be handled with care. Skin cuts should be covered and appropriate personal protective devices should be worn. In case of contact with endotoxin, immediately flush eyes or skin with water for at least 15 min. If inhaled, remove the affected individual from the area and provide oxygen and/or artificial respiration as needed. Skin absorption, ingestion, or inhalation may produce fever, headache, and hypotension.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Source of Cells

PBMCs from fresh WB are the source of cells for cytokine production in the PBMC/IL-6 test method (Poole et al., 2003). WB is obtained from healthy human volunteers who have provided their consent according to established institutional guidelines. Volunteers are expected not to have taken any drugs (e.g., prescription drugs, recreational drugs, herbal drugs) and to have been free from illness for at least two weeks prior to donation.

PBMCs are isolated from WB using density gradient centrifugation (refer to **Section 6.1.1.1**). The isolated PBMC suspension may be used directly in the PBMC/IL-6 test assay (**Section 6.1.3**) or frozen for later use (**Section 6.1.1.3**).

3.2 Equipment and Supplies

For all steps in the protocol, excluding the ELISA procedure, the materials that will be in close contact with samples and/or blood cells (e.g., pipet tips, containers, solutions) should be sterile and free from detectable pyrogens.

3.2.1 Preparation of PBMCs

3.2.1.1 *Equipment*

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture (37±1°C + 5% CO_2)
- Lymphoprep™
- Pipetter; multichannel (8- or 12-channel)
- Pipetters; single-channel adjustable (20, 200, and 1000 µL)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; nonpyrogenic, polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (2.5 and 5.0 mL)
- Cryotubes; screw-cap, 2 mL
- Filters; sterile, 0.22 µm
- Needle set; Sarstedt multifly, pyrogen-free, 19 mm, 21 gauge for S-Monovette
- Phosphate buffered saline (PBS); sterile
- Pipets; serological, sterile (5, 10, and 25 mL)
- Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; fluid
- RPMI-1640 cell culture medium; supplemented with the following reagents to yield RPMI-Complete (RPMI-C)
    - FCS; heat-inactivated (5 mL or a 1% final concentration)
    - L–Glutamine; 2 mM

- Penicillin/streptomycin (10,000 IU/mL penicillin, 10 mg/mL streptomycin)
- Syringes; sterile (100 µL and 30 mL)
- Tips; pipetter, sterile, pyrogen-free (20, 200, and 1000 µL)
- Tubes; Sarstedt S-Monovette, 7.5 mL, heparinized for blood collection

3.2.2 ELISA

3.2.2.1 *Equipment*

- Microplate mixer
- Microplate reader (450 nm with an optional reference filter in the range of 540-590 nm)
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

- Container; storage, plastic
- Deionized water; nonsterile
- Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture
- Pyrogen-free water (PFW)
- Reservoirs; fluid
- Tips; pipetter, nonsterile
- Tubes; polystyrene (12 mL)

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-6 release from PBMCs is used. A variety of IL-6 ELISA kits are commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to serve as an example for using an ELISA kit. If the user prefers to prepare an in-house ELISA, then additional reagents would be required. The IL-6 ELISA should be calibrated using an IL-6 international reference standard (e.g., WHO 89/548) prior to use. The IL-6

cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore, this reagent must be purchased separately. Results obtained using these products are subject to the assay acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit components may include the following:

- ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or polyclonal
- Buffered wash solution
- Dilution buffer
- Enzyme-labeled detection antibody
- Human IL-6 reference standard
- Pyrogen-free saline (PFS)
- Stop solution
- TMB/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO *E. coli* LPS 2nd International Standard 94/580; USP RSE *E. coli* LPS Lot G3E069; USP RSE *E. Coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

- RPMI-C cell culture medium

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored at 4°C.

4.1 Endotoxin Standard Curve

An internationally harmonized RSE or equivalent is used to generate the endotoxin standard curve. The use of any other *E. coli* LPS requires calibration against a RSE using the PBMC/IL-6 test method.

A standard endotoxin curve consisting of a Normal Saline Control (NSC) and five RSE concentrations (0.063, 0.125, 0.25, 0.50, and 1.0 EU/mL) are included in the incubation step (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the lyophilized content of the stock vial by following the instructions provided by the manufacturer (e.g., for a vial containing 10,000 EU, 5 mL of PFW is added). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for 5 min. The stock solution is stable for 14 days when stored at 2 to 8°C. An endotoxin standard curve is prepared by making serial dilutions of the stock solution in PFS as described in **Table 4-1**.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration EU/mL
2000[1,2]	20	1980	20[3]
20	100	1900	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0.125	500	500	0.063
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline
Each stock tube should be vortexed vigorously prior to its use to make the subsequent dilution.
[1] A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.
[2] The stock solution of USP RSE may be stored in aliquots and kept at -20°C for up to 6 months. Do not store the endotoxin at -80°C.
[3] This concentration is not used in the assay.

4.2 Test Substances

Liquid test substances should be tested neat or, if interference is detected (see **Section 4.2.1**), diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethylsulfoxide (DMSO) then diluted up to 0.5% (v/v) with PFS provided that this concentration does not interfere with the assay. The test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for 5 min.

4.2.1 Interference Testing

Interference testing must be carried out on any test sample for which no interference information is available. The purpose of the interference test is to determine the lowest dilution (i.e., highest concentration) of a test substance from which an endotoxin spike can be detected (i.e., based on the decision criteria described in **Section 4.2.1.2**). However, to ensure a valid test, a test substance should not be diluted beyond its Maximum Valid Dilution (MVD).

For many marketed products, values for the MVD and the Endotoxin Limit Concentration (ELC) are published in the U.S. Pharmacopeia, the European Pharmacopoeia, and/or Food and Drug Administration (FDA) guidelines. However if one or both of these values are not available, then calculation of the MVD is dependent on the ELC (see **Section 12.3**). If unknown, the ELC can be approximated by dividing the maximum hourly dose of the product by the hourly dose received per patient. For example, if a product is used at an hourly dose of 100 mg per patient, then the ELC would be 350 EU/100 mg or 3.5 EU/mg.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent international RSE (e.g., USP G3E069, FDA EC-6) is recommended for preparation of the endotoxin control (EC). If a different *E. coli* LPS is used and the potency relative to the RSE is not provided, then each lot must be calibrated against the RSE in the PBMC/IL-6 test method. For interference testing, an endotoxin standard curve (see **Section 4.1**) should be included on each plate.

4.2.1.2 *Spiking Test Substances with Endotoxin*

For interference testing, non-spiked and endotoxin-spiked test substances are prepared in microplate wells (n=4 replicates) and an *in vitro* pyrogen test is performed. Either RPMI-C or a fixed concentration (a concentration selected from the middle of the EC standard curve) of the RSE (i.e., 0.25 EU/mL) in RPMI-C is added to the test substance in serial two-fold dilutions. An illustrative example of endotoxin spiking solutions is shown in **Table 4-2.** For non-spiked solutions**,** 150 µl of RPMI-C is added to a well followed by 50 µl of the test substance (neat or at serial dilution) and 50 µL of PBMCs and the well contents are mixed. Endotoxin-spiked solutions are prepared by adding 100 µL of RPMI-C to each well followed

by 50 µL of the test substance (neat or at serial dilution) and 50 µL of an endotoxin spike solution (0.25 EU/mL). Finally, 50 µL of PBMCs are added and the well contents are mixed (see example presented in **Table 4-2**).

Table 4-2 Preparation of Endotoxin-Spiked and Non-spiked Solutions for Determination of Test Substance Interference in the Incubation and ELISA Test Systems

Sample Addition	Spiked	Non-spiked
	µL/well[1]	
RPMI-C	100	150
Endotoxin spike solution[2]	50	0
Test substance (neat and each serial dilution)	50	50
PBMCs[3]	50	50
Total[4]	250	250

Abbreviations: PBMC = Peripheral blood mononuclear cells
[1] n=4 replicates each
[2] Endotoxin concentration is 0.25 EU/mL in RPMI-C.
[3] PBMCs are resuspended in RPMI-C (1 x 10^6 cells/mL).
[4] A total volume of 250 µL per well is used for the incubation.

The lowest dilution of the test substance that yields an endotoxin spike recovery of 50% to 200% in the pyrogen test is determined. The optical density (OD) values of the endotoxin-spiked and non-spiked test substances are calibrated against the endotoxin calibration curve. The resulting EU value of the non-spiked test substance is subtracted from the corresponding EU value of the endotoxin-spiked test substance at each dilution. The % recovery for each sample dilution is then determined from the endotoxin spike solution concentration set to 100%. For example, consider the following interference test results in Table **4-3**:

Table 4-3 Example of Interference Data Used to Determine Sample Dilution for Assay

Sample Dilution	% Recovery of Endotoxin Control
None	25
1:2	49
1:4	90
1:8	110

Based on these results, the dilution of the test substance used in the *in vitro* pyrogen test would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 0.25 EU/mL EC).

4.2.2 Interference with ELISA

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference, then a subsequent experiment similar to that described in **Section 4.2.1** would need to be performed to confirm that the test substance(s) does not directly interfere with the ELISA. For this experiment, an ELISA would be performed in the absence of PBMCs.

5.0 CONTROLS

5.1 Negative Control

A negative control (e.g., RPMI-C) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.2 Solvent Control

Solvent controls are recommended to demonstrate that the solvent is not interfering with the test system when solvents other than PFS are used to dissolve test substances.

5.3 Positive Control

An internationally standardized EC (e.g., WHO 94/580; USP G3E069; 0.5 EU/mL) is included in each experiment to verify that an appropriate response is induced.

5.4 Benchmark Control

Benchmark controls may be used to demonstrate that the test method is functioning properly, or to evaluate the relative pyrogenic potential of chemicals (e.g., parenteral pharmaceuticals, medical device eluates) of a specific class or a specific range of responses, or for evaluating the relative pyrogenic potential of a test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemicals (e.g., parenteral pharmaceuticals, medical device eluates)
- structural and functional similarities to the class of the substance being tested
- known physical/chemical characteristics

- supporting data on known effects in animal models
- known potency in the range of response

5.5 Positive Product Control (PPC)

The PPC is a test substance diluted to a level that does not interfere with the test method and does not exceed the MVD. The PPC is obtained by spiking a test substance with a known EC (e.g., 0.25 EU/mL) and demonstrating that 50% to 200% of the EC is recovered.

5.6 Negative Product Control (NPC)

The NPC is the test substance diluted to the MVD and then spiked with PFS. It is the negative control for the PPC.

6.0 EXPERIMENTAL DESIGN

6.1 Incubation with Test Samples and Measurement of IL-6 Release

6.1.1 Collection of Human Blood

WB is obtained from healthy human volunteers who have provided their consent according to established institutional guidelines. Volunteers are expected not to have taken any drugs and to have been free from illness for at least two weeks prior to donation. The criteria for rejection of data from donors that are low responders or that are suspect due to veracity of health information is addressed in **Section 8.0**.

Fresh WB is drawn by venipuncture using a multifly needle set and collected in heparinized tubes (15 IU/mL lithium heparin). All components of the blood collection system (i.e., syringes, tubes, connecting lines) must be sterile and pyrogen-free. Blood should be stored at room temperature (RT) and must be used within 4 hr. Prior to use in the assay, the collection tubes should be gently inverted 1 to 2 times. **Do Not Vortex**.

6.1.1.1 *Isolation of PBMCs from WB*

PBMCs must be isolated within two hours of WB collection using Lymphoprep™. The isolation procedure described below is a modification of the manufacturer's instructions as outlined in the ECVAM SOPs for the PBMC/IL-6 test method.

To each 15 mL of heparinized WB, add 15 mL of PBS and a sterile 10 mL pipet into the tube. Add 20 mL of Lymphoprep™ to each of the two tubes, through the inserted pipet, to form a lower, denser, layer. The tubes are then centrifuged at 340 x g for 45 min at room RT. After centrifugation, a white band of PBMCs should be visible at approximately the 25 mL graduation mark on the tube. Carefully remove the uppermost 9 mL from each tube and discard. If cryopreservation of PBMCs is to be performed (see **Section 6.1.1.3**), then transfer the layer to a new 50 mL tube for preparing a cryoprotective solution. The remaining supernatant above the PBMC band should be aspirated and discarded. Using a 10 mL pipet, transfer the PBMC layer to a new 50 mL centrifuge tube.

6.1.1.2 *Washing PBMCs*

The PBMCs are resuspended in a total volume of 50 mL of PBS and centrifuged at 340 x g for 15 min. The supernatant is poured off and the cellular sediment resuspended in 10 mL of PBS by pipetting up and down several times with a serological pipet. Adjust the total volume in each tube to 50 mL with PBS and centrifuge at 340 x g for 10 min for a second time. After centrifugation, the PBMCs are resuspended with 15 mL of RPMI-C and the two aliquots of PBMCs are pooled into a single tube. Cell counts (expressed per mL) are determined using a hemacytometer. If the cell count is above 1.2 x 10^6/mL, the cell suspension should be diluted to 1 x 10^6 cell/mL in RPMI-C. This suspension must be used in the PBMC/IL-6 assay within 4 hr from the time of WB collection.

6.1.1.3 *Procedure for Cryopreservation and Thawing of PBMCs*

To freeze the PBMCs, prepare a cryoprotective solution by adding 2 mL of pyrogen-free DMSO to the 18 mL of supernatant collected in the centrifugation procedure outlined in **Section 6.1.1.1**. Cool the cryoprotective solution to between 2 and 8°C. Centrifuge the isolated PBMCs as instructed in **Section 6.1.1.2** and then add 6 mL of the chilled cryoprotective solution to the cell sediment. Pool the cell suspensions from the same donor and transfer 1.0 mL aliquots to appropriately labeled screw-cap cryotubes. The cryotubes are placed in a styrofoam box for thermal insulation and slowly frozen to -80°C. After 72 hr, the tubes can be transferred to liquid nitrogen for prolonged storage.

To thaw the cryopreserved PBMCs, take two tubes (each at 1.0 mL) from a single donor and submerge in a water bath at 37±1°C. After thawing, the cell suspensions are pooled in a

single 50 mL centrifuge tube and RPMI-C is added to give a total volume of 40 mL. The PMBCs are centrifuged at 340 x g for 10 min, the supernatant removed, and the cells resuspended in 10 mL of RPMI-C. Viability may be tested and should yield greater than 95% cell survival.

6.1.2 Incubation Plate

Test substances are prepared at a level of dilution that did not show interference with the test system or for which it is known that interference does not occur. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test samples (see **Table 6-1**).

Table 6-1 Overview of Incubation Plate Preparation in the PBMC/IL-6 Test Method

Number of Wells	Sample	RPMI-C	EC	Test Sample	PBMCs	Mix the samples; incubate overnight at 37±1°C in a humidified atmosphere with 5% CO_2.	Mix the samples; immediately transfer to an ELISA plate[3] and run ELISA or store plate at -20°C or -80°C.
		µL					
20[1]	EC	100	50	0	100		
4	NSC	150	0	0	100		
56[2]	Test samples (1-14)	100	0	50	100		

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; PBMC = Peripheral blood mononuclear cell

[1] Five EC concentrations (0.063, 0.125, 0.25, 0.50, and 1.0 EU/mL) in quadruplicate.

[2] 14 test samples (n=4 each) per plate.

[3] An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate. Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.3 Incubation Assay for IL-6 Release

PBMC samples are prepared in a microtiter plate using a laminar flow hood. All consumables and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately with a permanent marker. An overview of the incubation plate preparation is shown in **Table 6-1**. The incubation procedure is outlined below:

Step 1. Refer to the suggested incubation plate template presented in **Table 6-2**.

Step 2. Using a pipetter, transfer 100 µL of RPMI-C into each well.

Step 3. Transfer 50 μL of test sample into the appropriate wells as indicated in the template.

Step 4. Transfer 50 μL of the EC (standard curve) and the NSC controls in quadruplicate into the appropriate wells according to the template.

Step 5. Transfer 100 μL of a well-mixed PBMC suspension into each well and mix by gently swirling the plate.

Step 6. Mix the contents of the wells thoroughly by pipetting up and down several times using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Step 7. Place the covered plate in a tissue culture incubator for 16 to 24 hr at 37±1°C in a humidified atmosphere containing 5% CO_2.

Step 8. Prior to transferring the test samples onto the ELISA plate, mix the contents of the wells by pipetting up and down using a multichannel pipetter, changing the tips between each row in order to avoid cross-contamination.

Table 6-2 Incubation Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 1.0	EC 1.0	EC 1.0	EC 1.0	TS3	TS3	TS3	TS3	TS11	TS11	Void[3]	Void
B	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.063	EC 0.063	EC 0.063	EC 0.063	TS7	TS7	TS7	TS7	TS13	TS13	Void	Void
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	Void	Void
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	Void	Void
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	Void	Void

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1] EC value (e.g., EC 1.0) represents the endotoxin concentration in EU/mL.
[2] TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3] Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).

6.2 ELISA to Measure IL-6 Release

6.2.1 IL-6 Standard Curve

An IL-6 standard supplied with the ELISA kit is used. IL-6 standards are typically supplied in lyophilized form and should be reconstituted according to the manufacturer's instructions. The stock solution should be diluted in RPMI-C to the following concentrations: 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 µL. Each well on the ELISA plate will receive 50 µL of an IL-6 blank or standard.

6.2.2 ELISA

The manufacturer's instructions provided with the ELISA kit should be followed and a typical experimental design is outlined below. If the user prefers to prepare an in-house ELISA, then appropriate modifications and validation of these changes would be necessary. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Do *not* thaw frozen specimens by heating them in a water bath. A suggested ELISA plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, a NSC, an eight-point IL-6 standard curve (0 to 4000 pg/mL), and 14 test substances in quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are transferred directly from the incubation plate. The IL-6 standard curve is prepared as described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-4.**

Step 1. After pipetting up and down very carefully three times (avoid detachment of the adherent PBMCs) to mix the supernatant, transfer 50 µL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

Step 2. Add 50 µL of each IL-6 standard (0 to 4000 pg/mL) into the respective wells on the ELISA plate.

Step 3. Add 200 µL of the enzyme-labeled detection antibody (neat as supplied, or diluted, if necessary) to each of the wells.

Step 4. Cover the microtiter plate(s) with adhesive film and incubate for 2 to 3 hr at 20 to 25°C.

Step 5. Decant and wash each well three times with 300 µL Buffered Wash Solution and then rinse three times with deionized water. Place the plates upside down and tap to remove water.

Step 6. Add 200 µL of TMB/Substrate Solution to each well and incubate at RT in the dark for 15 min. If necessary, decrease the incubation time.

Step 7. Add 50 µL of Stop Solution to each well.

Step 8. Tap the plate gently after the addition of Stop Solution to aid in mixing.

Step 9. Read the OD_{450} within 15 min of adding the Stop Solution. Measurement with a reference wavelength of 540 to 590 nm is recommended.

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 1.0	EC 1.0	EC 1.0	EC 1.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6[3] 0	IL-6 0
B	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.125	EC 0.125	EC 0.125	EC 0.125	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.063	EC 0.063	EC 0.063	EC 0.063	TS7	TS7	TS7	TS7	TS13	TS13	IL-6 500	IL-6 500
F	NSC	NSC	NSC	NSC	TS8	TS8	TS8	TS8	TS13	TS13	IL-6 1000	IL-6 1000
G	TS1[2]	TS1	TS1	TS1	TS9	TS9	TS9	TS9	TS14	TS14	IL-6 2000	IL-6 2000
H	TS2	TS2	TS2	TS2	TS10	TS10	TS10	TS10	TS14	TS14	IL-6 4000	IL-6 4000

Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
[1] EC value (e.g., EC 1.0) represents the endotoxin concentration in EU/mL.
[2] TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
[3] IL-6 values in columns 11 and 12 are in pg/mL.

Table 6-4 Overview of ELISA Procedure

Material transfer from Incubation Plate (µL)	**Enzyme-labeled Antibody (µL)**	Cover the Incubation Plate and incubate for 2 to 3 hr at 20-25°C.	**TMB/Substrate Solution (µL)**	Incubate 15 min at RT in dark.	**Stop Solution (µL)**	Read optical density at 450 nm with a 540-590 nm wavelength reference filter.
50	200		200		50	

Abbreviations: RT = Room temperature

7.0 EVALUATION OF TEST METHODS

7.1 OD Measurements

The OD of each well is obtained by reading the samples in a standard microplate spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with a 540 to 590 nm reference filter (recommended). OD_{450} values are used to determine assay acceptability and in the decision criteria for the detection of endotoxin in a test substance (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

Obtain the PPC and the corresponding NPC by interference testing of a test substance in the presence and absence of a fixed quantity of endotoxin (i.e., 0.25 EU/mL) in quadruplicate. An EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6 standard curve should be included in each ELISA as shown in the template presented in **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

- The quadratic function of the IL-6 standard curve produces an $r \geq 0.95$ and the OD_{450} of the blank control is below 0.15.
- The endotoxin standard curve produces OD_{450} values that ascend in a sigmoidal concentration response.

Blood donors are considered to be low responders if their mean OD_{450} value obtained for 1 EU/mL EC is significantly below the mean OD_{450} value obtained for 1000 pg/mL IL-6.

Blood donors who produce a mean OD_{450} value for the NSC that is significantly above the mean OD_{450} value at 500 pg/mL IL-6 are considered to be high responders. Low and high responders should be excluded from analysis. Each test must be performed with PBMCs from four different donors. From a set of four donors, a maximum of one donor may be excluded from the assessment; otherwise, the test must be repeated with four different donors.

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Determination of Pyrogenicity

The validity of the endotoxin standard curve should be calculated using a four-parameter logistic model. If necessary to satisfy the model, endotoxin concentrations may be modified.

Calculate the mean OD_{450} values of all of the replicates in each experimental group. Calibrate the mean OD_{450} value for each test substance using the endotoxin standard curve and document the estimated endotoxin concentration. Multiply the estimated endotoxin concentration by the dilution factor, if necessary. This value represents the pyrogenicity of the sample in terms of endotoxin equivalents for that particular donor. The *t*-test is used to compare the data of a test sample against the data of the EC (0.5 EU/mL) that is performed in parallel. If this test results in a significant *p*-value (i.e., smaller than 1%), then the sample is considered to be non-pyrogenic, and as pyrogenic if otherwise (Hoffmann et al., 2005), as long as the assay acceptability criteria in **Section 8.0** has been met.

9.1.1 Decision Level 1

- If all donors show a negative reaction, then the product passes.
- If two or more donors show a positive reaction, then the product fails.
- If one donor shows a positive reaction, then an additional test with four donors has to be performed (go to Decision Level 2).

9.1.2 Decision Level 2

- If out of 6 to 8 donors, only one donor shows a positive reaction, then the product passes.
- In any other case, the product fails.

10.0 STUDY REPORT

The test report should include the following information:

Test Substances and Control Substances

- Name of test substance

- Purity and composition of the substance or preparation
- Physicochemical properties (e.g., physical state, water solubility)
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

Justification of the In Vitro Test Method and Protocol 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 on the procedure used to ensure their integrity from "lot-to-lot" and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative control data

Test Conditions

- Cell system used
- Calibration information for the spectrophotometer used to read the ELISA
- Details of test procedure used
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003) should be followed.

11.0 REFERENCES

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12.0 TERMINOLOGY AND FORMULA

12.1 Assay Sensitivity (λ)

For an *in vitro* cell-based assay, the variable λ is defined as the lowest statistically significant point on the standard endotoxin concentration-response curve and represents the relative sensitivity of the test method for the detection of endotoxin (i.e., level of detection).

12.2 Endotoxin Control (EC)

The EC is incubated with PBMCs and serves as the positive control for the experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

12.3 Endotoxin Limit Concentration (ELC)

The ELC is the maximum allowable concentration of endotoxin for a particular product and is expressed in EU per volume (mL) or weight (mg). The ELC is defined by the FDA or specified in the USP[2]. It is calculated as the product of K/M, where:

K is the threshold pyrogen dose for parenteral use in rabbits or humans (5.0 EU/kg). At an injection volume of 10 mL/kg, K is equal to 0.5 EU/mL.

M is the larger of the rabbit dose or the maximum human dose administered in one hour as defined below and varies with test substance[3].

12.4 Maximum Valid Dilution (MVD)

The MVD is the maximum dilution of a test substance that can be tolerated in a test system without exceeding the ELC, if the test substance must be diluted as a result of assay interference. Dilutions beyond the MVD would not be valid for endotoxin detection in the

[2] ELC values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, Pharmacopoeial guidelines, FDA publications).

[3] Values for most marketed pharmaceutical products are provided in the USP or in other pharmacopoeial or regulatory publications (e.g., European Pharmacopoeia, Japanese Pharmacopoeia, pharmacopoeial guidelines, FDA publications).

test system. Calculation of the MVD is dependent on whether or not the ELC for a test substance is published. When the ELC is known, the MVD is:

MVD = (ELC x Product Potency [PP])/λ

As an example, for "Cyclophosphamide Injection," the ELC is 0.17 EU/mg, PP is 20 mg/mL, and the assay sensitivity is 0.1 EU/mL. The calculated MVD would be 34. The test substance can be diluted no more than 1:34 prior to testing.

If the ELC is not known, the MVD is:

MVD = PP/Minimum Valid Concentration (MVC)

where, MVC = (λ x M)/K

where, M is the maximum human dose

As an example, for "Cylophosphamide Injection," the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.1 EU/mL. The calculated MVC is 0.6 mg/mL and the MVD is 33.3. The test substance can be diluted no more than 1:33 in the assay prior to testing.

12.5 Negative Product Control (NPC)

The NPC is a test sample to which PFS is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

12.6 Negative Saline Control (NSC)

The NSC is PBMCs (in RPMI-C) incubated with PFS (used for dilution of test substance) and is used as the blank.

12.7 Parenteral Threshold Pyrogen Dose (K)

The value K represents the threshold pyrogen dose for parenteral products for rabbits and humans. Based on experimental data, K is fixed at 5.0 EU/kg. For intrathecal products, K is 0.2 EU/kg.

12.8 Positive Product Control (PPC)

The PPC is a test substance spiked with the control standard endotoxin (i.e., 0.5 EU/mL or an amount of endotoxin equal to that which produces ½ the maximal increase in OD from the

endotoxin standard curve) to insure that the test system is capable of endotoxin detection in the product as diluted in the assay.

12.9 Product Potency (PP)

The concentration for a test substance is the PP typically expressed as μg/mL or mg/mL.

12.10 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)

The variable M represents the rabbit test dose or the maximum human dose in 1 hr. The variable M is expressed in mg/kg and varies with the test substance. For radiopharmaceuticals, M should be adjusted to account for product activity (radioactive decay) at time administration. An average human standard weight of 70 kg is used for the calculation. If a pediatric dose should be used and it is higher than the adult dose, then it should be used in the formula.