ICCVAM-Recommended Test Method Protocol
The Human Whole Blood/Interleukin-6 *In Vitro* Pyrogen Test

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ICCVAM Final Recommended Protocol for Future Studies Using the Human Whole Blood (WB)/Interleukin (IL)-6 *In Vitro* Pyrogen Test

PREFACE

This protocol is for the detection of Gram-negative endotoxin, a pyrogen, in parenteral drugs, as indicated by the release of IL-6 from monocytoid cells in human whole blood (WB). This protocol is based on information obtained from 1) the European Centre for the Validation of Alternative Methods (ECVAM)[1] WB/IL-6 Background Review Document (BRD) presented in Appendix A of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm), 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 ICCVAM BRD includes the ECVAM Standard Operating Procedure (SOP) for the WB/IL-6 test (could be referred to as Monocyte Activation Test), which is based on the WB/IL-6 method first described by Pool et al. (1998). A table of comparison between the ICCVAM recommended protocol and the ECVAM SOP is provided in **Table 1**.

Users should contact the relevant regulatory authority for guidance when using this ICCVAM recommended protocol to demonstrate product specific validation, and any deviations from this protocol should be accompanied by scientifically justified rationale. Future studies using the WB/IL-6 pyrogen test may include further characterization of the usefulness or limitations of the assay for regulatory decision-making. Users should be aware that this 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 protocol is used.

[1]ECVAM is a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre.

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Table 1 Comparison of ICCVAM Recommended Protocol with the ECVAM SOP for the WB/IL-6 Pyrogen Test

Protocol Component	ICCVAM Protocol	ECVAM SOP[1]
Test Substance	Test neat or in serial dilutions that produce no interference, not to exceed the MVD	Test neat or at minimal dilution that produces no interference
Number of Blood Donors	Minimum of 3 (independent or pooled)	Minimum of 3 (independent)
Decision Criteria for Interference	Mean OD[2] of PPC is 50% to 200% of 1.0 EU/mL EC	Mean OD of PPC is 50% to 200% of 1.0 EU/mL EC
Incubation Plate for ELISA (The number of samples or controls measured in quadruplicate)	NSC (1)	NSC (1)
	EC (5)	EC (5)
	TS (14)	TS (14)
	PPC[3] (0)	PPC (0)
	NPC[3] (0)	NPC (0)
ELISA Plate	Includes seven point IL-6 SC and blank in duplicate	Includes seven point IL-6 SC and blank in duplicate
Assay Acceptability Criteria	Mean OD of NSC ≤0.15	NSC < 200 pg/mL IL-6
	Quadratic function of IL-6 SC r ≥0.95[4]	EC SC satisfies ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures Methodology; ICH Q2B, Nov 1996
	EC SC produces OD values that ascend in a sigmoidal concentration response	Wilcoxon rank-sum test used to show that at least 3 of 4 replicates at each increasing EC concentration are higher relative to the next lowest concentration
	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) may be excluded
	Outliers rejected using Dixon's test	Outliers rejected using Dixon's test[5]
Decision Criteria for Pyrogenicity	Endotoxin concentration TS > ELC[6] TS	Endotoxin concentration TS > ELC TS OR 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; ELISA = Enzyme-linked immunosorbent assay; EU = Endotoxin units; IL-6 = Interleukin-6; MVD = Maximum valid dilution; NSC = Negative saline control; OD = Optical density; PPC = Positive product control; SC = Standard curve; TS = SOP = Standard operating procedure; Test substance; WB = Whole blood

[1]ECVAM WB/IL-6 SOP is presented in Appendix A of the ICCVAM BRD (available at http://iccvam.niehs.nih.gov/methods/pyrogen/pyr_brd.htm).

[2]Mean OD values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).

[3]In the ICCVAM WB/IL-6 protocol, PPC and NPC are assessed in the interference test described in **Section 4.2**, which is performed prior to the ELISA. In the ECVAM SOP, PPC and NPC were only included in the ECVAM validation study.

[4]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.
[5]Included in the ECVAM Trial data report presented in Appendix D of the ICCVAM BRD.
[6]Where unknown, the ELC is calculated (see **Section 12.2**)

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the presence of Gram-negative endotoxin, a pyrogen, in parenteral drugs. The presence of Gram-negative endotoxin is detected by its ability to induce the release of interleukin (IL)-6 from monocytoid cells in whole blood (WB). The concentration of IL-6 released by incubation of WB with a test substance or controls (i.e., positive and negative) is quantified using an enzyme-linked immunosorbent assay (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. The amount of pyrogen present is determined by comparing the values of endotoxin equivalents produced by WB cells 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. A test substance is considered pyrogenic if the endotoxin concentration of the test substance exceeds the Endotoxin Limit Concentration (ELC) for the test substance.

The relevance and reliability of this test method to detect non-endotoxin pyrogens have not been demonstrated in a formal validation study, although data are available in the literature to suggest that this assay has the potential to serve this purpose.

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures that use human blood-derived materials should follow national/international procedures for handling blood potentially contaminated with pathogens. An example of such guidelines is the Universal Precautions available at http://www.niehs.nih.gov/odhsb/biosafe/univers.htm. For non-human blood procedures (e.g., ELISAs), standard laboratory precautions are recommended including the use of laboratory coats, eye protection, and gloves. If necessary, additional precautions required for specific chemicals will be identified in the Material Safety Data Sheet (MSDS).

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 minutes (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.

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[1]RSEs are internationally-harmonized reference standards (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6). 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.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Blood Donor Eligibility

Monocytoid cells from fresh WB are the source of cytokine production in the WB/IL-6 test method as described by Hartung and Wendel (1996), Pool et al. (1998), and Schindler et al. (2006). In the United States (U.S.), the collection of blood and blood components for transfusion and further manufacture (including the use of resulting monocytes in a licensed test) is currently regulated under Section 351 of the Public Health Service (PHS) Act (U.S. Code [U.S.C.], Title 42, Chapter 6A) and/or the Federal Food Drug and Cosmetic Act (U.S.C., Title 21, Chapter 9), both of which require compliance with Current Good Manufacturing Practice (cGMP) regulations (21 CFR Parts 210, 211 and 600-640[2]).

These regulations and the associated FDA guidance's provide an important resource for information regarding the currently accepted practice for blood manufacture and collection (including donor screening) (http://www.fda.gov/cber/blood.htm). Specifically, guidance regarding donor screening questionnaires and links to currently acceptable questionnaires can be found at http://fda.gov/cber/gdlns/donorshitques.htm#iv. If you have any questions regarding the application of blood regulations to your specific situation[3], it is recommended that you e-mail the Manufacturers Assistance and Technical Training (MATT) Branch established by FDA at matt@cber.fda.gov for advice.

Any participating blood establishment should address how the unused components of blood donations will be accounted for and ultimately destroyed, and if the establishment will store the blood preparation, describe the storage procedures to be followed.

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 pyrogen-free.

3.2.1 Blood Incubation

3.2.1.1 Equipment

- Centrifuge
- Hood; Bio-safety, laminar flow (recommended)
- Incubator; cell culture (37±1°C + 5% CO_2)

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[2]The requirements for WB can be found at 21 CFR 640.1 *et seq*. In addition, there are specific regulations applicable to red blood cells, platelets, and other blood components. See, for example, 21 CFR 640.10-640.27. Other regulations applicable to the manufacture of blood and blood components include 21 CFR Part 606, the cGMP requirements for blood and blood components, 21 CFR 610.40, the requirements for testing of WB donations, and 21 CFR 640.3, the requirements for determining the suitability of the donor. Blood that enters into U.S. interstate commerce should be tested for antibodies to HIV 1/2, HCV, HTLV I and II, HBc, HBsAg and RPR, WNV and Chagas.

[3]The collection of blood for research and development purposes or as a component of an *in vitro* test (that is not subject to licensure) may potentially not be required to adhere to the FDA regulations outlined above.

- Pipetter, multichannel (8- or 12-channel)
- Pipetters, single-channel adjustable (20 and 200 μL)
- Repeating pipetter
- Vortex mixer

3.2.1.2 *Consumables*

- Centrifuge tubes; polystyrene (15 and 50 mL)
- Combitips; repeating pipetter (1.0 and 2.5 mL)
- Needle set; multifly, pyrogen-free, 19 mm, 21 gauge
- Plates; microtiter, 96-well, polystyrene, tissue culture
- Pyrogen-free saline (PFS)
- Reaction tubes; polystyrene (1.5 mL)
- Reservoirs; for blood collection
- Tips; pipetter, sterile, pyrogen-free (20 and 200 μL)

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)[4]
- Microplate washer (optional)
- Multichannel pipetter

3.2.2.2 *Consumables*

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

[4]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

3.2.2.3 *ELISA Kit*

An ELISA that measures IL-6 release 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. The IL-6 ELISA should be calibrated using an IL-6 international reference standard (e.g., World Health Organization [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
- PFS
- Stop solution
- TMB[5]/substrate solution

3.3 Chemicals

- Endotoxin (e.g., WHO-lipopolysaccharide [LPS] 94/580 *Escherichia coli* [*E. coli*] O113:H10:K-; United States Pharmacopeia [USP] RSE *E. coli* LPS Lot G3E069; USP RSE *E. coli* Lot G; FDA *E. coli* Lot EC6)

3.4 Solutions

ELISA solutions are listed in **Section 3.2**.

4.0 ASSAY PREPARATION

All test substances, endotoxin, and endotoxin-spiked solutions should be stored as specified in the manufacturer's instructions. The collection of WB is outlined in **Section 6.1**.

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 WB/IL-6 pyrogen test. A standard endotoxin curve consisting of a Negative Saline Control (NSC) and five RSE concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 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

[5]The use of an IL-6 ELISA kit with a chromagen other than TMB is acceptable.

the stock vial by following the instructions provided by the manufacturer (e.g., 5 mL of PFW is added to a vial containing 10,000 EU). To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use. The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer. An endotoxin standard curve is prepared as described in **Table 4-1** by making serial dilutions of the stock solution in PFS with vigorous vortexing at each dilution step. Dilutions should not be stored, because dilute endotoxin solutions are not as stable as concentrated solutions due to loss of activity by adsorption, in the absence of supporting data to the contrary.

Table 4-1 Preparation of Endotoxin Standard Curve

Stock Endotoxin EU/mL[1]	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration in Tube EU/mL
2000[2,3]	20	1980	20[4]
20	100	900	2.0
2.0	500	500	1.0
1.0	500	500	0.50
0.50	500	500	0.25
0.25	500	500	0.125
0	0	1000	0

Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline

Each stock tube should be vortexed prior to its use to make the subsequent dilution.

[1]To reconstitute the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min. Subsequent dilutions should be vortexed vigorously immediately prior to use.

[2]A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.

[3] The stock solution is stable for not more 14 days when stored at 2 to 8°C or for up to 6 months when kept in a -20°C freezer.

[4]This concentration is not used in the assay.

4.2 Interference Test

For every test substance lot, interference testing must be performed to check for interference between the test substance and the cell system and/or ELISA. The purpose of the interference test is to determine whether the test substance (or specific lot of test substance) has an effect on cytokine release.

4.2.1 Interference with the Cell System

All test substances must be labeled as pyrogen-free (i.e., endotoxin levels at an acceptable level prior to release by the manufacturer) to ensure that exogenous levels of endotoxin do not affect the experimental outcome. Liquid test substances should be diluted in PFS. Solid test substances should be prepared as solutions in PFS or, if insoluble in saline, dissolved in dimethyl sulfoxide (DMSO) and then diluted up to 0.5% (v/v) with PFS, provided that this concentration of DMSO does not interfere with the assay. To ensure a valid test, a test substance cannot be diluted beyond its Maximum Valid Dilution (MVD) (refer **to Section 12.3**). The calculation of the MVD

is dependent on the ELC for a test substance. The ELC can be calculated by by dividing the threshold human pyrogenic dose by the maximum recommended human dose in a single hour period (see **Section 12.2**) (USP 2007; FDA 1987). Furthermore, test substances should not be tested at concentrations that are cytotoxic to blood cells.

4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

The WHO-LPS 94/580 [*E. coli* O113:H10:K-] or equivalent internationally harmonized RSE is recommended for preparation of the endotoxin-spike solution and the endotoxin standard curve (see **Section 4.1**).

4.2.1.2 *Spiking Test Substances with Endotoxin*

Non-spiked and endotoxin-spiked test substances are prepared in quadruplicate and an *in vitro* pyrogen test is performed. A fixed concentration of the RSE (i.e., 1.0 EU/mL or a concentration equal to or near the middle of the endotoxin standard curve) is added to the undiluted test substance (or in serial two-fold dilutions, not to exceed the MVD). An illustrative example of endotoxin-spiking solutions is shown in **Table 4-2.** For non-spiked solutions, 50 µL of PFS is added to a well followed by 50 µL of WB and mixed by inversion. Then, 50 µL of the test substance (i.e., equivalent to the negative product control [NPC]) is added followed by 100 µL of PFS and the well contents are mixed. Endotoxin-spiked solutions are prepared by adding 50 µL of PFS to each well followed by 50 µL of WB and mixed by inversion. Then, 50 µL of the test substance, 50 µL of an endotoxin-spike solution (1.0 EU/mL), and 50 µL of PFS (i.e., equivalent to the positive product control [PPC]) are added to each well. The contents of the wells are mixed and incubated as outlined in **Section 6.1.3**, **Steps 6-8**. An ELISA is then performed as outlined in **Section 6.2**, without the IL-6 standard curve.

Table 4-2 Preparation of Endotoxin-Spiked and Non-Spiked Solutions for Determination of Test Substance Interference

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

Abbreviations: PFS = Pyrogen-free saline; WB = Whole blood

[1]n=4 replicates each

[2]50 µL of WB and 50 µL of PFS are added to each well and mixed by inversion prior to the addition of the remaining components and volume of PFS.

[3]Endotoxin concentration is 1.0 EU/mL in PFS.

[4]A total volume of 250 µL per well is used for the incubation.

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 spike recovery for each sample dilution is calculated as a percentage by

setting the theoretical value (i.e., endotoxin-spike concentration of 1.0 EU/mL) at 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

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

If a spike recovery between 50% and 200% is obtained, then no interference of the test substance with either the cell system or the ELISA is demonstrated (i.e., the test substance does not increase or decrease the concentration of IL-6 relative to the endotoxin spike). The lowest dilution (i.e., highest concentration) of a test substance that yields an endotoxin-spike recovery between 50% and 200% is determined. The test substance is then diluted in serial two-fold dilutions beginning at this dilution, not exceed the MVD for use in the assay. Based on the results illustrated in **Table 4-3**, the initial dilution of the test substance would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1.0 EU/mL EC).

4.2.2 Interference at the MVD

If the data obtained from the experiment in **Section 4.2.1** suggests the presence of interference at the MVD, then consideration should be given for using another validated pyrogen test method.

5.0 CONTROLS

5.1 Benchmark Controls

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 substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response

5.2 Endotoxin Control

The EC (i.e., WB incubated with an internationally harmonized RSE) serves as the positive control in each experiment. The results should be compared to historical values to insure that it provides a known level of cytokine release relative to the NSC.

5.3 Negative Saline Control

The NSC (i.e., WB incubated with PFS instead of the test substance) 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.

5.4 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.

6.0 EXPERIMENTAL DESIGN

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

6.1.1 Collection of Human Blood

Human volunteers that have met the donor eligibility criteria described in **Section 3.1** are used as the source of WB. All components of the blood collection system (e.g., syringes, tubes, connecting lines) must be sterile and pyrogen-free. WB is drawn by venipuncture[6] from the medial cubital or cephalic vein of either the right or left arm and collected in a sterile container that contains anticoagulant solution (e.g., heparin). The total volume of blood collected per donor (i.e., up to 500 mL) will be dictated by experimental design and determined by the test method user. WB should be stored at room temperature (RT) and must be used within 4 hr[7]. All subsequent handling of WB should be performed in a laminar flow hood using sterile technique to prevent contamination.

Prior to use in the assay, an equal volume of WB from multiple individual donors should be pooled[8].

6.1.2 Incubation Plate

Test substances are prepared at a level of dilution that did not show interference with the test system, provided that this dilution does not exceed the MVD. Each incubation plate can accommodate an endotoxin standard curve, a NSC, and 14 test substances (see Table **6-1**).

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[6]WB is obtained using Universal Precautions (e.g., latex gloves, labcoats, safety glasses) and sterile equipment (e.g., syringes, needles, collection tubes) within a hospital or clinical setting by qualified and adequately trained personnel (i.e., registered nurse, licensed phlebotomist, or medical doctor).

[7]Although the ECVAM SOP did not describe the use of cryopreserved WB for the WB/IL-6 test method, the use of cryopreserved WB with the WB/IL-1 test method was outlined and this methodology may also be appropriate for the WB/IL-6 test method, but this has yet to be demonstrated.

[8]Multiple donors (i.e., a minimum of three) should meet the acceptability criteria as outlined in **Section 8.0** either as a pool of multiple individual donors or as multiple individual donors tested independently.

Table 6-1 Overview of Incubation Plate Preparation in the WB/IL-6 Pyrogen Test

Number of Wells	Sample	PFS	EC	Test Sample	WB	Mix the samples; incubate for 16 to 24 hr 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.
		µL					
20[1]	EC	100	50	0	50		
4	NSC	150	0	0	50		
56[2]	Test samples (1-14)	100	0	50	50		

Abbreviations: EC = Endotoxin control; IL-6 = Interleukin-6; NSC = Negative saline control; PFS = Pyrogen-free saline WB = Whole blood

[1]Five EC concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 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 (see **Table 6-3**). Therefore, 80 wells are available for test samples and controls on the incubation plate.

6.1.3 Incubation Assay for IL-6 Release

Test substances should be vortexed vigorously for at least 30 min or sonicated in a bath sonicator for at least 5 min prior to use in the assay. Test substances should be prepared in serial two-fold dilutions beginning at a level of dilution that did not show interference with the test system (see **Section 4.2**) in as many subsequent dilutions that are necessary to be within the linear range of the endotoxin standard curve, not to exceed the MVD. Blood 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 incubation plate template presented in **Table 6-2.**
- **Step 2.** Using a pipetter, transfer 100 µL of PFS into each well.
- **Step 3.** Transfer 50 µL of test sample or 50 µL of PFS for the NSC into the appropriate wells as indicated in the template.
- **Step 4.** Transfer 50 µL of the EC (standard curve) in quadruplicate into the appropriate wells according to the template.
- **Step 5.** Transfer 50 µL of WB into each well and mix by gently swirling the plate.
- **Step 6.** Mix the contents of the wells thoroughly by gently 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 to 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] 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	Void[3]	Void
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	Void	Void
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	Void	Void
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	Void	Void
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	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 2.0) represents the endotoxin concentration in EU/mL.
[2] TS number (e.g., TS 1) 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 PFS 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. The ELISA should be carried out at RT and therefore all components must be at RT prior to use. Frozen specimens should not be thawed 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, an eight-point IL-6 standard curve (0 to 4000 pg/mL), and available wells for up to 14 test substances and a NSC each 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 three times 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 RT.

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.[9]

Table 6-3 ELISA Plate - Sample and Control Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	EC[1] 2.0	EC 2.0	EC 2.0	EC 2.0	TS3	TS3	TS3	TS3	TS11	TS11	IL-6[3] 0	IL-6 0
B	EC 1.0	EC 1.0	EC 1.0	EC 1.0	TS4	TS4	TS4	TS4	TS11	TS11	IL-6 62.5	IL-6 62.5
C	EC 0.50	EC 0.50	EC 0.50	EC 0.50	TS5	TS5	TS5	TS5	TS12	TS12	IL-6 125	IL-6 125
D	EC 0.25	EC 0.25	EC 0.25	EC 0.25	TS6	TS6	TS6	TS6	TS12	TS12	IL-6 250	IL-6 250
E	EC 0.125	EC 0.125	EC 0.125	EC 0.125	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 2.0) 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.

[9]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.

Table 6-4 Overview of ELISA Procedure

Material transfer from Incubation Plate (µL)	**IL-6 standard (0 to 4000 pg/mL) (µL)**	**Enzyme-labeled Antibody (µL)**	Cover the Incubation Plate and incubate for 2 to 3 hr at RT.	Decant and wash each well three times with 300 µL Buffered Wash Solution and three times with deionized water.	**TMB/Substrate Solution (µL)**	Incubate for less than 15 min at RT in the dark.	**Stop Solution (µL)**	Read each well at OD_{450} with a 540 to 590 nm reference filter.
50	50	200			200		50	

Abbreviations: OD_{450} = Optical density at 450 nm; 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)[10]. OD values are used to determine assay acceptability and in the decision criteria for pyrogen detection (see **Sections 8.0** and **9.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

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 $r \geq 0.95$[11] and the OD of the blank control is below 0.15.
- The endotoxin standard curve produces OD values that ascend in a sigmoidal concentration response.

Blood donors (or a pool of blood donors) are considered to be high responders if their concentration of IL-6 is greater than 200 pg/mL. High responders should be excluded from analysis. The preparation being examined is required to pass the test with blood donations from at least three different donors (i.e., either as a pool of three individual donors or as three individual donors tested independently).

[10]The TMB chromagen is measured at OD_{450}. However, the use of an IL-1β ELISA kit with a chromagen other than TMB is acceptable. The ELISA should be measured at a wavelength appropriate for the specific chromagen used.
[11]Correlation coefficient (r), an estimate of the correlation of x and y values in a series of n measurements.

An outlying observation that represents either a pool of multiple independent donors or a single individual donor may be excluded if there is confirmation that the accuracy of the medical information provided by an individual donor is suspect, or if the aberrant response is identified using acceptable statistical methodology (e.g., Dixon's test [Dixon 1950; Barnett and Lewis 1994], Grubbs' test [Barnett and Lewis 1994; Grubbs 1969; Iglewicz and Houghlin 1993]).

9.0 DATA INTERPRETATION/DECISION CRITERIA

9.1 Decision Criteria for Pyrogen Detection

A test substance is considered pyrogenic when the endotoxin concentration of the test substance exceeds the ELC for the test sample. The ELC can be calculated as shown in **Section 12.2**.

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)
- Quality assurance data
- Treatment of the test/control substances prior to testing (e.g., vortexing, sonication, warming, resuspension solvent)

Justification of the Test Method and the 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 modification to the test procedure
- Reference to historical data of the model
- Description of the 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)[1]

The variable λ is defined as the labeled sensitivity (in EU/mL) of the LAL Reagent in endpoint assays (e.g., the BET gel-clot technique). For kinetic BET assays, λ is the lowest point used in the endotoxin standard curve.

12.2 Endotoxin Limit Concentration (ELC)[1,2]

The ELC for parenteral drugs is expressed in Endotoxin Units (EU) per volume (mL) or weight (mg). The ELC is equal to K/M, where:

K is the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for intravenous administration. For intrathecal administration, K is equal to 0.2 EU/kg (see also **Section 12.5**).

M is the rabbit test dose or the maximum recommended human dose of product (mL or mg) per body weight (kg) in a single hour period (see also **Section 12.8**).

For example, if a non-intrathecal product were used at an hourly dose of 10 mL per patient, then the ELC would be 0.50 EU/mL.

12.3 Maximum Valid Dilution (MVD)[1,2]

The MVD is the maximum allowable dilution of a test substance at which the endotoxin limit can be determined. The calculation of the MVD is dependent on the ELC for a test substance. When the ELC is known, the MVD is[1]:

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 0.065 EU/mL. The calculated MVD would be 1:52.3 or 1:52. The test substance can be diluted no more than 1:52 prior to testing.

If the ELC is not known, the MVD is[1]:

MVD = PP/Minimum Valid Concentration (MVC)

where, MVC = (λ x M)/K

where, M is the maximum human dose

As an example, for Cyclophosphamide Injection, the PP is 20 mg/mL, M is 30 mg/kg, and assay sensitivity is 0.065 EU/mL. The calculated MVC is 0.390 mg/mL and the MVD is 1:51.2 or 1:51. The test substance can be diluted no more than 1:51 in the assay prior to testing.

12.4 Negative Product Control (NPC)

For interference testing, the NPC is a test sample to which pyrogen-free saline (PFS) is added. The NPC is the baseline for determination of cytokine release relative to the endotoxin-spiked PPC.

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[1]From FDA (1987)
[2]From USP (2007)

12.5 Parenteral Threshold Pyrogen Dose (K)[1,2]

The value K is defined as the threshold human pyrogenic dose of endotoxin (EU) per body weight (kg). K is equal to 5.0 EU/kg for parenteral drugs except those administered intrathecally; 0.2 EU/kg for intrathecal drugs.

12.6 Positive Product Control (PPC)

For interference testing, 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 optical density (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.

12.7 Product Potency (PP)[1,2]

The test sample concentration expressed as mg/mL or mL/mL.

12.8 Rabbit Pyrogen Test (RPT) Dose or Maximum Human Dose (M)[1,2]

The variable M is equal to the rabbit test dose or the maximum recommended human dose of product per kg of body weight in a single hour period. M is expressed in mg/kg or mL/kg and varies with the test substance. For radiopharmaceuticals, M equals the rabbit dose or maximum human dose/kg at the product expiration date or time. Use 70 kg as the weight of the average human when calculating the maximum human dose per kg. If the pediatric dose/kg is higher than the adult dose, then it shall be the dose used in the formula.