Appendix B5

Draft ICCVAM Recommended Protocol for Future Studies Using the Whole Blood (WB)/Interleukin-6 (IL-6) Test Method

[This Page Intentionally Left Blank]

Draft ICCVAM Recommended Protocol for Future Studies Using the Whole Blood (WB)/Interleukin-6 (IL-6) Test Method

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) WB/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 WB/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 the WB/IL-6 methodology first described by Pool et al. (1998). A table of comparison between the draft ICCVAM recommended protocol and the ECVAM SOPs is provided in **Table 1**. Future studies using the WB/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 any 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.

23
24
25
26
27
28
29
30
31
32
33
34
35
36 ***[This Page Intentionally Left Blank]***
37
38

38 **Table 1 Comparison of Draft ICCVAM Recommended Test Method Protocol with the ECVAM SOP for the Whole**
39 **Blood (WB)/Interleukin-6 (IL-6) Pyrogen Test Method**

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP
Test Substance	Test neat or at minimal dilution that produces no interference	Same as 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 0.5 EU/mL EC <2x Median OD_{450} of 0.5 EU/mL EC
Assay Acceptability Criteria	Mean OD_{450} of 0.5 EU/mL EC ≥1.6X Mean OD_{450} of NSC	Same as ICCVAM protocol
	Mean OD_{450} of PPC is 50% to 200% of 0.5 EU/mL EC	Same as ICCVAM protocol
	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
	Not included	EC SC satisfies ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures Methodology; ICH Q2B, Nov 1996
	EC SC produces OD_{450} values that ascend in a sigmoidal concentration response	Same as ICCVAM protocol
	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded (need at least 3 donors)	High responder blood donors (i.e., >200 pg/mL IL-6) may be excluded (need at least 3 donors)
	Not included	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
Decision Criteria for Pyrogenicity	Mean OD_{450} of TS > Mean corrected OD_{450} of 0.5 EU/mL EC	EC SC data transformed to logit responses by in house program and the linear mean square calculated. TS pyrogen content is compared with the ELC[2] using confidence limits. The preparation being examined must pass the test with blood from three separate donors
	Not included	Limit test is run to determine whether or not a

Protocol Component	Draft ICCVAM Protocol	ECVAM SOP
		TS after correction and dilution contains < 0.5 EU/mL of endotoxin

40 Abbreviations: EC = Endotoxin control; ELC = Endotoxin Limit Concentration; MVD = Maximum Valid Dilution; NSC = Normal saline control; PPC =
41 Positive Product Control; SC = Standard curve; TS = Test substance
42 [1] Median or mean OD_{450} values are corrected (i.e., reference filter reading, if applicable, and NSC are subtracted).
43 [2] 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
44 or 0.5 EU/mL/kg, or an ELC of 0.5 EU/mL).
45
46

47 **1.0 PURPOSE AND APPLICABILITY**

48 The purpose of this protocol is to describe the procedures used to evaluate the presence of a
49 pyrogen (i.e., Gram-negative endotoxin) in a test substance. The presence of Gram-negative
50 endotoxin is detected by its ability to induce cytokine IL-6 release from monocytoid cells in
51 WB. The quantity of IL-6 released is obtained using an enzyme-linked immunosorbent assay
52 (ELISA) that includes monoclonal or polyclonal antibodies specific for IL-6. Release of this
53 cytokine is measured by incubation of WB with test substances or controls (i.e., positive,
54 negative). The amount of pyrogen present is determined by comparing the values of
55 endotoxin equivalents produced by cells exposed to the test substance to those exposed to an
56 internationally-harmonized Reference Standard Endotoxin (RSE)[1] or an equivalent standard
57 expressed in Endotoxin Units (EU)/mL. Based on a rabbit threshold pyrogen dose of 0.5
58 EU/mL, which was established in a retrospective evaluation of rabbit pyrogen test (RPT)
59 data, a test substance is considered pyrogenic if it induces a level of IL-6 release equal to or
60 greater than 0.5 EU/mL.

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

65 **2.0 SAFETY AND OPERATING PRECAUTIONS**

66 All procedures for procurement of eligible blood donors and blood donations should follow
67 the regulations and procedures set forth by institutional guidelines for utilization of human
68 substances, which include but are not limited to blood, tissues, and tissue fluids. Standard
69 laboratory precautions are recommended including the use of laboratory coats, eye
70 protection, and gloves. If necessary, additional precautions required for specific study
71 substances or hazardous chemicals will be identified in the Material Safety Data Sheet
72 (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.

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

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

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

85 **3.0 MATERIALS, EQUIPMENT, AND SUPPLIES**

86 **3.1 Source of Cells**

87 Leukocytes from fresh WB are the source of cells for cytokine production in the WB/IL-6
88 test method (Hartung and Wendel, 1996; Pool et al., 1998; Schindler et al., 2006). WB is
89 obtained from healthy human volunteers who have provided their consent according to
90 established institutional guidelines. Volunteers are expected not to have taken any drugs
91 (e.g., prescription drugs, recreational drugs, herbal drugs) and to have been free from illness
92 for at least two weeks prior to donation.

93 **3.2 Equipment and Supplies**

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

97

97

98 3.2.1 Blood Incubation

99 *3.2.1.1 Equipment*

100 • Centrifuge

101 • Hood; Bio-safety, laminar flow (recommended)

102 • Incubator; cell culture (37±1°C + 5% CO_2)

103 • Pipetter, multichannel (8- or 12-channel)

104 • Pipetters, single-channel adjustable (20 and 200 μL)

105 • Repeating pipetter

106 • Vortex mixer

107 3.2.1.2 *Consumables*

108 • Centrifuge tubes; nonpyrogenic, polypropylene (15 and 50 mL)

109 • Combitips; repeating pipetter (1.0 and 2.5 mL)

110 • Needle set; Sarstedt multifly, pyrogen-free, 19 mm, 21 gauge for S-Monovette

111 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture

112 • Reaction tubes; polypropylene (1.5 mL)

113 • Reservoirs; fluid

114 • Tips; pipetter, sterile, pyrogen-free (20 and 200 μL)

115 • Tubes; Sarstedt S-Monovette, 7.5 mL, heparinized for blood collection

116 3.2.2 ELISA

117 *3.2.2.1 Equipment*

118 • Microplate mixer

119 • Microplate reader (450 nm with an optional reference filter in the range of

120 540-590 nm)

121 • Microplate washer (optional)

122 • Multichannel pipetter

123 3.2.2.2 *Consumables*

124 • Container; storage, plastic

125 • Deionized water; nonsterile

126 • Plates; microtiter, nonpyrogenic, 96-well, polystyrene, tissue culture

127 • Pyrogen-free water (PFW)

128 • Reservoirs; fluid

129 • Tips; pipetter, nonsterile

130 • Tubes; polypropylene (12 mL)

131 3.2.2.3 *ELISA Kit*

132 An ELISA that measures IL-6 release from WB is used. A variety of IL-6 ELISA kits are
133 commercially available and the IL-6 ELISA procedure outlined in this protocol is intended to
134 serve as an example for using an ELISA kit. If the user prefers to prepare an in-house
135 ELISA, then additional reagents would be required. The IL-6 ELISA should be calibrated
136 using an IL-6 international reference standard (e.g., WHO 89/548) prior to use. The IL-6
137 cytokine assay kits do not provide the RSE or endotoxin equivalent; therefore this reagent
138 must be purchased separately. Results obtained using these products are subject to the assay
139 acceptability and decision criteria described in **Sections 8.0** and **9.0**. IL-6 ELISA kit
140 components may include the following:

141 • ELISA plates coated with anti-human IL-6 capture antibody; monoclonal or
142 polyclonal

143 • Buffered wash solution

144 • Dilution buffer

145 • Enzyme-labeled detection antibody

146 • Human IL-6 reference standard

147 - Pyrogen-free saline (PFS)
148 - Stop solution
149 - TMB/substrate solution

150 **3.3 Chemicals**

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

153 **3.4 Solutions**

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

155 **4.0 ASSAY PREPARATION**

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

157 **4.1 Endotoxin Standard Curve**

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

161 A standard endotoxin curve consisting of a Normal Saline Control (NSC) and five RSE
162 concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) are included in the incubation step
163 (refer to **Table 4-1**) and then transferred to the ELISA plate. To prepare the endotoxin
164 standard curve, first obtain a 2000 EU/mL stock solution by addition of PFW to the
165 lyophilized content of the stock vial by following the instructions provided by the
166 manufacturer (e.g., for a vial containing 10,000 EU, 5 mL of PFW is added). To reconstitute
167 the endotoxin, the stock vial should be vortexed vigorously for at least 30 min or sonicated in
168 a bath sonicator for 5 min. The stock solution is stable for 14 days when stored at 2 to 8°C.
169 An endotoxin standard curve is prepared by making serial dilutions of the stock solution in
170 PFS as described in **Table 4-1**.

171

171

172 **Table 4-1 Preparation of Endotoxin Standard Curve**

Stock Endotoxin EU/mL	µL of Stock Endotoxin	µL of PFS	Endotoxin Concentration in Tube EU/mL
2000[1,2]	20	1980	20[3]
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

173 Abbreviations: EU = Endotoxin units; PFS = Pyrogen-free saline
174 Each stock tube should be vortexed prior to its use to make the subsequent dilution.
175 [1] A 2000 EU/mL stock solution of endotoxin is prepared according to the manufacturer's instructions.
176 [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
177 the endotoxin at -80°C.
178 [3] This concentration is not used in the assay.
179

180 **4.2 Test Substances**

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

187 4.2.1 Interference Testing

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

193 For many marketed products, values for the MVD and the Endotoxin Limit Concentration
194 (ELC) are published in the U.S. Pharmacopeia, the European Pharmacopoeia, and/or Food
195 and Drug Administration (FDA) guidelines. However if one or both of these values are not

196 available, then calculation of the MVD is dependent on the ELC (see **Section 12.3**). If
197 unknown, the ELC can be approximated by dividing the maximum hourly dose of the
198 product by the hourly dose received per patient. For example, if a product were used at an
199 hourly dose of 100 mg per patient, then the ELC would be 350 EU/100 mg or 3.5 EU/mg.

200 4.2.1.1 *Reference Endotoxin for Spiking Test Substances*

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

206 4.2.1.2 *Spiking Test Substances with Endotoxin*

207 For interference testing, non-spiked and endotoxin-spiked test substances are prepared in
208 microplate wells (n=4 replicates) and an *in vitro* pyrogen test is performed. Either PFS or a
209 fixed concentration (a concentration selected from the middle of the EC standard curve) of
210 the RSE (i.e., 1 EU/mL) in PFS is added to the test substance in serial two-fold dilutions. An
211 illustrative example of endotoxin spiking dilutions is shown in **Table 4-2.** For non-spiked
212 solutions, 50 μL of PFS is added to a well followed by 50 μL of WB and mixed by inversion.
213 Then, 50 μL of the test substance (neat or at serial dilution) is added followed by 100 μL of
214 PFS and the well contents are mixed. Endotoxin-spiked solutions are prepared by adding 50
215 μL of PFS to each well followed by 50 μL of WB and mixed by inversion. Then, 50 μL of
216 the test substance (neat or at serial dilution), 50 μL of an endotoxin spike solution (1.0
217 EU/mL), and 50 μL of PFS are added and the well contents are mixed (see example
218 presented in **Table 4-2)**.

219

219

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

Sample Addition	Spiked	Unspiked
	µL/well[1]	
Pyrogen-free saline (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

223 Abbreviations: WB = Whole blood
224 [1] n=4 replicates each
225 [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
226 remaining components and volume of PFS.
227 [3] Endotoxin concentration is 1.0 EU/mL in PFS.
228 [4]A total volume of 250 µL per well is used for the incubation.
229
230
231

232 The lowest dilution of the test substance that yields an endotoxin spike recovery of 50% to
233 200% in the pyrogen test is determined. The optical density (OD) values of the endotoxin-
234 spiked and non-spiked test substances are calibrated against the endotoxin calibration curve.
235 The resulting EU value of the non-spiked test substance is subtracted from the corresponding
236 EU value of the endotoxin-spiked test substance at each dilution. The % recovery for each
237 sample dilution is then determined from the endotoxin spike solution concentration set to
238 100%. For example, consider the following interference test results in **Table 4-3**:

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

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

241
242
243 Based on these results, the dilution of the test substance used in the *in vitro* pyrogen test
244 would be 1:4 (i.e., the lowest dilution between 50% and 200% of the 1 EU/mL EC).

245 4.2.2 Interference with ELISA

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

250 5.0 CONTROLS

251 5.1 Negative Control

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

254 5.2 Solvent Control

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

257 5.3 Positive Control

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

260 5.4 Benchmark Controls

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

266 • consistent and reliable source(s) for the chemicals (e.g., parenteral
267 pharmaceuticals, medical device eluates)

268 • structural and functional similarities to the class of the substance being tested

269 • known physical/chemical characteristics

270 • supporting data on known effects in animal models

271 • known potency in the range of response

272 **5.5 Positive Product Control (PPC)**

273 The PPC is a test substance diluted to a level that does not interfere with the test method and
274 does not exceed the MVD. The PPC is obtained by spiking a test substance with a known EC
275 (e.g., 1 EU/mL) and demonstrating that 50% to 200% of the EC is recovered.

276 **5.6 Negative Product Control (NPC)**

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

279 **6.0 EXPERIMENTAL DESIGN**

280 **6.1 Incubation with Test Samples and Measurement of IL-6 Release**

281 6.1.1 <u>Collection of Human Blood</u>
282 WB is obtained from healthy human volunteers who have provided their consent according
283 to established institutional guidelines. Volunteers are expected not to have taken any drugs
284 and to have been free from illness for at least two weeks prior to donation. The criteria for
285 rejection of data from donors that are low responders or that are suspect due to veracity of
286 health information is addressed in **Section 8.0**.

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

292 6.1.2 <u>Incubation Plate</u>
293 Test substances are prepared at a level of dilution that did not show interference with the test
294 system or for which it is known that interference does not occur. Each incubation plate can
295 accommodate an endotoxin standard curve, a NSC, and 14 test substances (see Table **6-1**).

296 **Table 6.1 Overview of Incubation Plate Preparation in the WB/IL-6 Test Method**

Number of Wells	Sample	PFS	EC	Test Sample	WB	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	200	20	0	20		
4	NSC	220	0	0	20		
56[2]	Test samples (1-14)	200	0	20	20		

297 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; PFS = Pyrogen-free saline WB =
298 Whole blood
299 [1] Five EC concentrations (0.125, 0.25, 0.50, 1.0, and 2.0 EU/mL) in quadruplicate.
300 [2] 14 test samples (n=4 each) per plate.
301 [3] An IL-6 standard curve is prepared in Columns 11 and 12 on the ELISA plate. Therefore, 80 wells are
302 available for test samples and controls on the incubation plate.
303

304 6.1.3 Incubation Assay for IL-6 Release

305 Blood samples are prepared in a microtiter plate using a laminar flow hood. All consumables
306 and solutions must be sterile and pyrogen-free. Each plate should be labeled appropriately
307 with a permanent marker. An overview of the incubation plate preparation is shown in **Table**
308 **6-1**. The incubation procedure is outlined below:

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

310 • **Step 2.** Using a pipetter, transfer 150 μL of PFS into each well.

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

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

315 • **Step 5.** Transfer 50 μL of WB into each well and mix by gently swirling the
316 plate.

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

320 • **Step 7.** Place the covered plate in a tissue culture incubator for 10 to 24 hr at
321 37±1°C in a humidified atmosphere containing 5% CO_2.

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

325 *Note: The aliquots may be tested immediately in the ELISA or stored at -20°C or*
326 *-80°C for testing at a later time. After transfer to the ELISA plate, freeze the*
327 *remaining aliquots at -20°C or -80°C for subsequent experiments, if necessary (see*
328 *Assay Acceptability and Decision Criteria in **Sections 8.0** and **9.0**).*

329

329

330 **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

331 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
332 [1] EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.
333 [2] TS number (e.g., TS 1) represents an arbitrary sequence for individual test substances.
334 [3] Columns 11 and 12 are reserved for the IL-6 standard curve on the ELISA plate (see **Table 6-3**).
335
336

337 **6.2 ELISA to Measure IL-6 Release**

338 6.2.1 IL-6 Standard Curve

339 An IL-6 standard, supplied with the ELISA kit, is used. IL-6 standards are typically supplied
340 in lyophilized form and should be reconstituted according to the manufacturer's instructions.
341 The stock solution should be diluted in PFS to the following concentrations: 0, 62.5, 125,
342 250, 500, 1000, 2000, and 4000 pg/mL in volumes of at least 500 µL. Each well on the
343 ELISA plate will receive 100 µL of an IL-6 blank or standard.

344 6.2.2 ELISA

345 The manufacturer's instructions provided with the ELISA kit should be followed and a
346 typical experimental design is outlined below. If the user prefers to prepare an in-house
347 ELISA, then appropriate modifications and validation of these changes would be necessary.
348 The ELISA should be carried out at RT and therefore all components must be at RT prior to
349 use. Do *not* thaw frozen specimens by heating them in a water bath. A suggested ELISA
350 plate template is shown in **Table 6-3**, which includes a five-point EC standard curve, a NSC,

351 an eight-point IL-6 standard curve (0 to 4000 pg/mL), and 14 test substances in
352 quadruplicate. The EC standard curve, the NSC, and the test sample supernatants are
353 transferred directly from the incubation plate. The IL-6 standard curve is prepared as
354 described in **Section 6.2.1**. An overview of the ELISA plate preparation is shown in **Table 6-**
355 **4.**

356 Immediately prior to the ELISA procedure, dilute or mix any assay components according to
357 the manufacturer's instructions.

358 **Step 1.** After pipetting up and down three times to mix the supernatant, transfer 50
359 μL from each well of the Incubation Plate (A1-10; H1-10) to the ELISA plate.

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

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

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

366 **Step 5.** Decant and wash each well five to six times with 300 mL Buffered Wash
367 Solution and then rinse three times with deionized water. Place the plates upside
368 down and tap to remove water.

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

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

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

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

375

375 **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

376 Abbreviations: EC = Endotoxin control; NSC = Negative saline control; TS = Test substance
377 [1] EC value (e.g., EC 2.0) represents the endotoxin concentration in EU/mL.
378 [2] TS number (e.g., TS1) represents an arbitrary sequence for individual test substances.
379 [3] IL-6 values in columns 11 and 12 are in pg/mL.
380
381

382 **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 to 25°C.	**TMB/Substrate Solution (µL)**	Incubate 15 min at RT in the dark.	**Stop Solution (µL)**	Read optical density at 450 nm with a 540-590 nm wavelength reference filter.
50	200		200		50	

383 Abbreviations: RT = Room Temperature
384
385

386 7.0 EVALUATION OF TEST METHODS

387 7.1 OD Measurements

388 The OD of each well is obtained by reading the samples in a standard microplate
389 spectrophotometer (i.e., plate reader) using a visible light wavelength of 450 nm (OD_{450}) with

390 a 540 to 590 nm reference filter (recommended). OD_{450} values are used to determine assay
391 acceptability and in the decision criteria for detection of endotoxin in a test sample (see
392 **Sections 8.0** and **9.0**).

393 8.0 CRITERIA FOR AN ACCEPTABLE TEST

394 Obtain the PPC and the corresponding NPC by interference testing of a test substance in the
395 presence and absence of a fixed quantity of endotoxin (i.e., 1 EU/mL) in quadruplicate. An
396 EC (five-point standard curve) and a NSC should be included in each experiment. An IL-6
397 standard curve should be included in each ELISA plate as shown in the template presented in
398 **Table 6-3**. An assay is considered acceptable only if the following minimum criteria are met:

399 • The quadratic function of the IL-6 standard curve produces an $r^2 \geq 0.95$ and the
400 OD_{450} of the blank control is below 0.15.

401 • The endotoxin standard curve produces OD_{450} values that ascend in a
402 sigmoidal concentration response.

403 Blood donors are considered to be high responders if their concentration of IL-6 is greater
404 than 200 pg/mL. High responders should be excluded from analysis. From a set of four
405 donors, a maximum of one donor may be excluded from the test. Furthermore, the
406 preparation being examined is required to pass the test with blood donations from at least
407 three different donors.

408 9.0 DATA INTERPRETATION/DECEISION CRITERIA

409 9.1 Decision Criteria for Determination of Pyrogenicity

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

412 Calculate the mean OD_{450} values of all of the replicates in each experimental group. Calibrate
413 the mean OD_{450} value for each test substance using the endotoxin standard curve and
414 document the estimated endotoxin concentration. Multiply the estimated endotoxin
415 concentration by the dilution factor, if necessary. This value represents the pyrogenicity of
416 the sample in terms of endotoxin equivalents for that particular donor. The *t*-test is used to
417 compare the data of a test sample against the data of the EC (0.5 EU/mL) that is performed in

418 parallel. If this test results in a significant *p*-value (i.e., smaller than 1%), then the sample is
419 considered to be non-pyrogenic, and as pyrogenic if otherwise (Hoffmann et al., 2005), as
420 long as the assay acceptability criteria in **Section 8.0** has been met.

421 9.1.1 Decision Level 1

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

426 9.1.2 Decision Level 2

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

430 **10.0 STUDY REPORT**

431 The test report should include the following information:

432 *Test Substances and Control Substances*

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

438 *Justification of the Test Method and the Protocol Used*

439 *Test Method Integrity*

440 • The procedure used to ensure the integrity (i.e., accuracy and reliability) of the
441 test method over time
442 • If the test method employs proprietary components, documentation on the
443 procedure used to ensure their integrity from "lot-to-lot" and over time

444 - The procedures that the user may employ to verify the integrity of the
445 proprietary components

446 *Criteria for an Acceptable Test*

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

449 *Test Conditions*

450 - Cell system used
451 - Calibration information for the spectrophotometer used to read the ELISA
452 - Details of test procedure used
453 - Description of any modification to the test procedure
454 - Reference to historical data of the model
455 - Description of the evaluation criteria used

456 *Results*

457 - Tabulation of data from individual test samples

458 *Description of Other Effects Observed*

459 *Discussion of the Results*

460 *Conclusion*

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

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

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

468

468 **11.0 REFERENCES**

469 EPA. 2003a. Good Laboratory Practice Standards. Toxic Substances Control Act. 40 CFR
470 792.

471 EPA. 2003b. Good Laboratory Practice Standards. Federal Insecticide, Fungicide, and
472 Rodenticide Act. 40 CFR 160.

473 FDA. 2003. Good Laboratory Practices for Nonclinical Laboratory Studies. 21 CFR 58.

474 Hartung T, Wendel A. 1996. Detection of pyrogens using human whole blood. *In vitro*
475 Toxicol 9(4):353-359.

476 Hoffmann S, Peterbauer A, Schindler S, Fennrich S, Poole S, Mistry Y, Montag-Lessing T,
477 Spreitzer I, Löschner B, van Aalderen M, Bos R, Gommer M, Nibbeling R, Werner-
478 Felmayer G, Loitzl P, Jungi T, Brcic M, Brügger P, Frey E, Bowe G, Casado J, Coecke S, de
479 Lange J, Mogster B, Næss LM, Aaberge IS, Wendel A, Hartung T. 2005. International
480 validation of novel pyrogen tests based on human monocytoid cells. J Immunol Methods
481 298:161-173.

482 Organisation for Economic Co-operation and Development (OECD). 1998. OECD Series on
483 Principle of Good Laboratory Practice and Compliance Monitoring. No. 1. OECD Principles
484 of Good Laboratory Practice (as revised in 1997). OECD, ENV/MC/CHEM (98)17. Paris:
485 OECD.

486 Pool EJ, Johaar G, James S, Petersen I, Bouic P. 1998. The detection of pyrogens in blood
487 products using an *ex vivo* whole blood culture assay. J Immunoassay 19:95-111.

488 Schindler S, Spreitzer I, Löschner B, Hoffmann S, Hennes K, Halder M, Brügger P, Frey E,
489 Hartung T, Montag T. 2006. International validation of pyrogen tests based on cryopreserved
490 human primary blood cells. J Immunol Methods 316:42-51.

491

492

492 **12.0 TERMINOLOGY AND FORMULA**

493 **12.1 Assay Sensitivity (λ)**

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

497 **12.2 Endotoxin Control**

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

501 **12.3 Endotoxin Limit Concentration (ELC)**

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

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

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

509 **12.4 Maximum Valid Dilution (MVD)**

510 The MVD is the maximum dilution of a test substance that can be tolerated in a test system
511 without exceeding the ELC, if the test substance must be diluted as a result of assay
512 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).

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

515 MVD = (ELC x Product Potency [PP])/λ

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

519 If the ELC is not known, the MVD is:

520 MVD = PP/Minimum Valid Concentration (MVC)

521 where, MVC = (λ x M)/K

522 where, M is the maximum human dose

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

526 **12.5 Negative Product Control (PPC)**

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

529 **12.6 Negative Saline Control (NSC)**

530 The NSC is WB incubated with PFS (used for dilution of test substance) and is used as the
531 blank.

532 **12.7 Non-intrathecal Threshold Pyrogen Dose (K)**

533 The value K represents the threshold pyrogen dose for parenteral products for rabbits and
534 humans. Based on experimental data, K is fixed at 5.0 EU/kg. For intrathecal products, K is
535 0.2 EU/kg.

536 **12.8 Positive Product Control (PPC)**

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

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

541 **12.9 Product Potency (PP)**

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

543 **12.10 Rabbit Pyrogen Test Dose of Maximum Human Dose (M)**

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

550

551

552

553

554