4.0 *In Vivo* Reference Data for the Assessment of Test Method Accuracy

4.1 Description of the Protocol Used to Generate *In Vivo* Data

4.1.1 The Rabbit Pyrogen Test

The RPT protocols most widely accepted by regulatory agencies are outlined in the USP (USP 2007b), the U.S. Code of Federal Regulations (FDA 2005), the European Pharmacopeia ([EP], EP 2005a), and the Japanese Pharmacopeia ([JP], JP 2001), and are summarized in **Table 4-1**. The RPT involves measuring the temperature increase in rabbits following an i.v. injection (via the ear vein) of a test substance in a dose not to exceed 10 mL/kg injected within a period of not more than 10 min. Initially, three rabbits are injected and the increase (or decrease) in temperature relative to the baseline value is measured at 30-min intervals for up to three hr. The resulting data are used to calculate an overall temperature increase by adding the results from all three animals, which is then used to assign a label of pyrogenic or non-pyrogenic.

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Table 4-1 Test Guidelines for the Rabbit Pyrogen Test

RPT Protocol Component	Reference			
	21 CFR 610.13 (FDA 2005)	**EP5.0 2.6.8 (EP 2005a)**	**JP XIV (JP 2001)**	**USP30 NF25 <151> (USP 2007b)**
Number of rabbits	3 or 8[1]	3, 6, 9, or 12[1]	3 or 8[1]	3 or 8[1]
Rabbit species/strain	Not specified	Not specified	Not specified	Not specified
Exclusion criteria for rabbits during the initial selection of rabbits	• Used in a negative pyrogen test in the preceding 2 days • Used in a pyrogen test in which its temperature rose ≥0.6°C in the preceding 2 weeks	• Weight<1.5 kg • Decreased weight in the preceding week • Used in a negative pyrogen test in the preceding 3 days • Used in a positive pyrogen test in the preceding 3 weeks	• Weight<1.5 kg • Decreased weight in the preceding week • Previously used in a positive pyrogen test • Rabbits from negative pyrogen tests may be reused only when a "as a long a resting period as possible is taken"	• Used in a negative pyrogen test in the preceding 2 days • Used in a pyrogen test in which its temperature rose ≥0.6°C in the preceding 2 weeks
Testing room conditions	20 to 23°C	Within 3°C of the housing quarters (temperature not specified)	20 to 27°C and constant humidity	20 to 23°C
Food/water during test	Food withheld during the test, but water available at all times	Food withheld overnight and until end of the test. Water withheld during the test.	Food withheld beginning several hrs. prior to first temperature recording and until the end of the test.	Food withheld during the test period, but water available at all times
Depth of temperature probe in rectum	Not less than 7.5 cm	Approximately 5 cm	6-9 cm	Not less than 7.5 cm
Preliminary test	≤7 days prior to main test, perform all procedures used for the main test except the injection.	• 1-3 days prior to main test, treat test animals with an injection of warmed (38.5°C) pyrogen-free saline • Record temperature at 90 min prior to injection and every 30 min thereafter up to 3 hr. • Exclude any rabbits with an increase of >0.6°C	Not specified	≤7 days prior to main test, perform all procedures used for the main test except the injection.
Baseline temperature	• Record temperature ≤ 30 min prior to injection • For any group of rabbits, use only if baseline temperatures do not vary>1°C among rabbits • Exclude rabbits with baseline temperature>39.8°C	• Mean of two temperature recordings at 40 min and 10 min prior to injection • Exclude rabbits if variation >0.2°C between measurements noted • Exclude rabbits with initial temperature >39.8°C or <38.0°C	• Record temperature three times at one-hr intervals prior to injection • Assuming no appreciable variability among recordings, use the last recording as the baseline value. • Exclude animals if 2nd and 3rd temperature measurements exceed 39.8°C	• Record temperature ≤30 min prior to injection • For any group of rabbits, use only if baseline temperatures do not vary >1°C among rabbits • Exclude rabbits with baseline >39.8°C
Injection volume	≥3 mL/kg *BUT* ≤10mL/kg	≥0.5 mL/kg *BUT* ≤10mL/kg	10 mL/kg, unless otherwise specified	≤10 mL/kg

Injection time	≤10 min	≤4 min, unless otherwise indicated	Not specified, but injection should occur within 15 min of the third pretest temperature recording	≤10 min
Injection site	Marginal ear vein	Marginal ear vein	Marginal ear vein	Marginal ear vein
Pre-warming of test material	37°C±2°C	38.5°C	37°C	37°C±2°C
Temperature recording intervals after injection	30 min intervals for 1 to 3 hr	≤30 min intervals for 3 hr	1 hr intervals for 3 hr	30 min intervals for 1 to 3 hr

Abbreviations: CFR = U.S. Code of Federal Regulations; EP = European Pharmacopeia; FDA = U.S. Food and Drug Administration; JP = Japanese Pharmacopeia; RPT = Rabbit pyrogen test; USP = United States Pharmacopeia

[1]Each test is initially conducted with three animals and additional animals are tested to resolve equivocal results in the first three animals

4.1.2 Current In Vivo Pyrogen Test Method Protocols

As indicated in **Table 4-1**, U.S. and international regulatory agencies have tailored the RPT protocol to suit their specific needs and goals in protecting human health. The current test method protocols (i.e., FDA 2005; EP 2005a; JP 2001; USP 2007b) recommend using healthy, adult rabbits with no specific breed/strain requirements. Rabbits are to be adequately acclimated to their surroundings and housed in an environment free from excessive external stimuli. Each rabbit is conditioned prior to the test with a sham test that includes all of the procedural steps except the injection (see also **Section 1.2**). Reuse of test rabbits is permitted only after an appropriate withdrawal period has been completed (see also **Section 1.2**).

The test is conducted in a room that is designated solely for pyrogen testing, in which the temperature is within 3°C of the uniform temperature of the housing room (i.e., 20°C±3°C). Food is withheld during the test, but access to water is continuous. The baseline temperature, which is used to calculate the increase in temperature during the test, is measured 30-40 min prior to injection of the test substance. In each group of rabbits tested, the variation in baseline temperature among the rabbits should not vary more than 1°C, and rabbits with an initial temperature greater than 39.8°C are excluded from testing.

The test substance is pre-warmed to approximately 37°C and injected (≤10 mL/kg) into the marginal ear vein, completing each injection within 10 min. The rectal temperature is recorded at 30-min intervals for up to three hr after the injection. The decision criteria outlined in **Table 4-2** are then used to determine a pyrogenic response. As shown in **Table 4-2**, the decision criteria by which labels of pyrogenic or non-pyrogenic are assigned vary among the USP, FDA, EP, and JP test guidelines.

Table 4-2 Decision Criteria for Determining a Pyrogenic Response in the Rabbit Pyrogen Test

RPT Protocol	No. Rabbits	Product passes if:	Product fails if:
USP30 NF25<151> (USP 2007b)	3	0/3 rabbits show an increase of ≥0.5°C	NA[1]
	5[1]	≤3/8 rabbits show an increase of ≥0.5°C *AND* the summed responses ≤3.3°C	>3/8 rabbits show an increase of ≥0.5°C *AND/OR* the sum of all responses >3.3°C
21 CFR 610.13 (FDA 2005)	3	0/3 rabbits show an increase of ≥0.5°C	NA[1]
	5[1]	≤3/8 rabbits show an increase of ≥0.6°C *AND* the summed responses ≤3.7°C	>3/8 rabbits show an increase of ≥0.6°C *AND/OR* the summed responses >3.7°C
EP5.0 2.6.8 (EP 2005a)	3	Summed responses ≤1.15°C	Summed responses >2.65°C
	6[2]	Summed responses ≤2.80°C	Summed responses >4.30°C
	9[2]	Summed responses ≤4.45°C	Summed responses >5.95°C
	12	Summed responses ≤6.60°C	Summed responses >6.60°C
JP XIV (JP 2001)	3	3/3 rabbits show an increase of <0.6°C *AND* the summed responses ≤1.4°C	≥2/3 rabbits show an increase ≥0.6°C
	5[3]	≥4/5 rabbits show an increase <0.6°C	≥2/5 rabbits show an increase ≥0.6°C

CFR = U.S. Code of Federal Regulations; EP = European Pharmacopeia; FDA = U.S. Food and Drug Administration; JP = Japanese Pharmacopeia; NA = Not applicable; USP = United States Pharmacopeia; RPT = Rabbit pyrogen test

[1]If ≥1/3 rabbits show an increase of ≥0.5°C, continue test with an additional five rabbits.

[2]Three additional animals are tested when the summed responses falls in between the previous range.

[3]Five additional animals are tested when neither criterion is met, and results are based on these five animals only.

4.2 Reference Data Used to Assess *In Vitro* Test Method Accuracy

The ECVAM BRDs state that due to ethical and legal reasons, the RPT was not conducted in parallel to the *in vitro* test methods. Instead, historical RPT data produced over a 5-year period at the Paul-Ehrlich Institut (PEI), which is the German Federal Agency of Sera and Vaccines, were used (Hoffmann et al. 2005a). These data were generated for internal quality control studies from 171 rabbits (Chinchilla Bastards). Chinchilla Bastards are reported to be a more sensitive strain than the New Zealand White rabbit strain for pyrogenicity testing (Hoffmann et al. 2005b). However, neither the USP (USP 2007b) nor the EP (EP 2005a) prescribes a specific rabbit strain for the RPT.

4.3 Availability of Original Records for the *In Vivo* Reference Data

Section 4.1 of each ECVAM BRD indicates that the PEI provided the historical RPT data.

4.4 *In Vivo* Data Quality

The historical RPT studies were conducted at the PEI, which supports regional German regulatory authorities, provides marketing approval of certain marketed biological products (e.g., sera, vaccines, test allergens), and functions as a WHO collaborating center for QA of blood products and *in vitro* diagnostics. The unit for pyrogen and endotoxin testing of the PEI is accredited following ISO/IEC 17025 (International Standards Organization [ISO]

2005). In a request for additional information from ECVAM, it was stated that the RPT data was generated according to the EP monograph, but the detailed protocol used by this laboratory was not provided.

4.5 Availability and Use of Toxicity Information from the Species of Interest

A number of studies have concluded that humans and rabbits have approximately the same threshold to pyrogenic stimulation, although higher doses are more pyrogenic and more toxic in humans (Co Tui and Schrift 1942; Westphal 1956; Keene et al. 1961). Moreover, Greisman and Hornick (1969) compared three purified endotoxin preparations in rabbits and in male volunteers and showed that the threshold pyrogenic dose was similar in both species. However, the dose-response relationships for humans were considerably steeper than those for the rabbit at each dose tested.

As stated in **Section 1.2.1**, the major regulatory requirement for pyrogenicity testing is for end-product release of human and animal parenteral drugs, medical devices, and human biological products. The results from such testing are used to limit, to an acceptable level, the risks of febrile reactions from injection and/or implantation of the product of concern.

Endotoxin can produce a number of acute effects on human health. McKinney et al. (2006) reported increased cytokine expression patterns in a cohort of subjects experiencing systemic adverse events (i.e., fever, rash, lymphadenopathy) after smallpox vaccine administration. Martich et al. (1993) studied systemic, cardiovascular, pulmonary, cytokine release, and the inflammatory response resulting from i.v. injection of small doses of endotoxin in humans to understand mechanisms of sepsis and septic shock. Burrell (1994) later reviewed the available literature on the adverse human responses to bacterial endotoxin. In addition, environmental or chronic exposure to inhaled bacterial endotoxin (present in soil, in water, and on vegetation) may lead to an inflammation in the airways and/or gastrointestinal disturbances (Rylander 2002). Therefore, for protection of both human and animal health, it is vital that the test method employed provide an accurate estimation of the potential for a pyrogenic reaction.

4.6 Information on the Accuracy and Reliability of the *In Vivo* Test Method

Hoffmann et al. (2005a) modeled the sensitivity and specificity of the RPT using historical data (summarized in **Section 4.2**) to establish a threshold pyrogen dose (i.e., the endotoxin dose at which fever was induced in 50% of the rabbits). A threshold value of 0.5 EU/mL was defined by regression analysis of the data. The performance characteristics of the RPT (i.e., sensitivity and specificity) were then determined using a 2 x 2 contingency table, incorporating the parameters obtained from the regression analysis. The authors considered the prevalence of the endotoxin spikes included in the ECVAM accuracy evaluations in the validation studies (i.e., 0 EU/mL: 20%; 0.25 EU/mL: 20%; 0.5 EU/mL: 40%; 1.0 EU/mL: 20%) and applied the threshold pyrogen dose of 0.5 EU/mL to calculate theoretical values for sensitivity (58%) and specificity (88%) of the RPT.

The accuracy and reliability of the RPT for endotoxin testing has been considered adequate for U.S. and international regulatory needs for many years. Since its inclusion in the USP in 1941, the RPT has been used extensively and is the preferred method for detection of pyrogenicity for product development, because of the inability of the BET to detect non-endotoxin pyrogens.