1.0 Introduction And Rationale For The Proposed Use Of In Vitro Pyrogen Test Methods

1.1 Introduction

1.1.1 Historical Background of In Vitro Pyrogen Test Methods and the Rationale for Their Development

A brief summary of the historical development of the five in vitro pyrogen test methods was provided in Section 1.1.1 of each Background Review Document (BRD) provided by the European Centre for the Validation of Alternative Methods (ECVAM), a unit of the Institute for Health and Consumer Protection at the European Commission's Joint Research Centre. These BRDs were provided to the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and are included in Appendix A. This section includes supplementary information and provides a context for United States (U.S.) regulatory considerations.

Pyrogenic substances (i.e., substances that induce fever) may originate from a variety of biological or synthetic/manufacturing sources. They may also be released from microbiological organisms such as bacteria, viruses, and fungi during cell death or following immunological attack (i.e., cell damage or death due to a local or systemic immune response). One of the most potent pyrogenic materials is bacterial endotoxin, which is an outer membrane component of the Gram-negative bacteria cell wall. Pyrogens may also be found in processing and packaging materials, chemicals, raw materials, or equipment used during the manufacturing of parenteral drugs or medical devices. The presence of endotoxins in otherwise sterile biological preparations such as parenteral drugs suggests the presence of past or current bacterial contamination.

The induction of fever by these pyrogenic substances is a complex process and multiple mechanisms are thought to be involved. It is likely that the specific pathway, or combinations of pathways, involved in the production of a fever response depends on a number of variables (e.g., the properties of the pyrogenic substance and the route of administration). In general, pyrogenic substances cause leukocytes (i.e., neutrophils, monocytes/macrophages, and lymphocytes) to release cytokines (e.g., interleukin [IL]-1β, IL-6, and Tumor Necrosis Factor-α [TNF-α]) that act as endogenous pro-inflammatory mediators, often referred to as "endogenous pyrogens" (Dinarello 1999). Once released, these cytokines act on the central nervous system to promote the synthesis of prostaglandins, ultimately producing of a fever response (Dinarello 1999; Netea et al. 2000). These cytokines have been shown to be associated with the fever response induced by pyrogenic substances in both humans and rabbits (Dinarello 1999). Certain bacterial products (e.g., endotoxin) can also stimulate cytokine production directly through the activation of Toll-like receptors (TLRs) (Dinarello 1999; Netea et al. 2000).

References to ECVAM BRD sections are in normal type to distinguish them from references to ICCVAM BRD sections, which are in boldface type.
The translation of released cytokines into a fever response is largely mediated by circumventricular organs. These small neuronal cell groups allow neurons to come in contact with a variety of circulating substances directly from the bloodstream, which are thought to control the febrile response through projections to sites in the hypothalamus and brain stem (Saper and Breder 1994; Dinarello 1999; Beutler and Rietschel 2003).

The U.S., European, and Japanese Pharmacopeias currently recognize two test methods for pyrogen testing, the *in vivo* rabbit pyrogen test (RPT) and the *in vitro* bacterial endotoxin test (BET), also referred to as the *Limulus* amebocyte lysate (LAL) test. The BET is accepted because of its sensitivity to the presence of Gram-negative endotoxins. However, the test method has well documented limitations, including its inability to respond to non-endotoxin pyrogens, as well as its susceptibility to interference from certain types of materials (e.g., high protein and lipid levels, glucans). In contrast, the RPT is capable of detecting both endotoxin and non-endotoxin pyrogens. However, disadvantages of the RPT include the need for interspecies extrapolation from rabbits to humans.

In 2002, a total of 243,838 rabbits were used in the U.S. for all research and testing purposes, of which 6,324 rabbits were reported as experiencing more than slight or momentary pain and/or distress where anesthetics, analgesics, or tranquilizers could not be administered for scientific reasons (U.S. Department of Agriculture [USDA] 2002). Eight of these cases were specifically attributed to pyrogenicity testing, presumably based on induction of a fever response (USDA 2002). Thus, although the potential for more than slight or momentary pain and/or distress exists for pyrogenicity testing when a fever response is induced, it does not appear that a fever response is common. In the European Union (EU), approximately 313,000 total rabbits were used for all scientific purposes in 2005 (CEC 2007). Of these, approximately 276,000 rabbits were used for pharmaceutical products and medical device testing (i.e., either research and development, production and quality control, or toxicological and other safety evaluations). Although the number of rabbits specifically used for pyrogenicity testing was not reported, it is likely that this number is significantly less than the total of 276,000. Additional animal use numbers, including data reported from Canada and the United Kingdom (U.K.) are summarized in Section 10.1.

An *in vitro* test system that combines the sensitivity of the BET with the wide range of pyrogens detectable by the RPT would be an obvious improvement for pyrogen testing. With this intention, test systems based on the activation of human monocytes *in vitro* were developed that take advantage of an increased understanding of the biological mechanisms responsible for the human fever reaction (Dinarello 1999). Initial efforts focused on peripheral blood mononuclear cells (PBMC), which release proinflammatory cytokines when exposed to endotoxin (Duff and Atkins 1982; Dinarello et al. 1984). A number of similar test systems, using either whole blood (WB), PBMCs, or monocytoid cell lines (e.g., Mono Mac 6 [MM6], THP-1) were subsequently developed (Tsuchiya et al. 1980; Poole et al. 1988; Ziegler-Heitbrock et al. 1988; Hartung and Wendel 1996; Hartung et al. 2001; Poole et al. 2003; Gaines Das et al. 2004). Five test systems developed from human monocytoid cells were selected by ECVAM for prevalidation and validation studies with the intent of comparing their effectiveness for replacing the RPT and thereby eliminating the use of rabbits for pyrogen testing. The results of these studies have been published (Hoffmann et al. 2005a; Schindler et al. 2006). The five tests selected were:

- The Human WB/IL-1β *In Vitro* Pyrogen Test²
• The Human WB/IL-1β In Vitro Pyrogen Test: Application of Cryopreserved (Cryo) Human WB
• The Human WB/IL-6 In Vitro Pyrogen Test
• The Human PBMC/IL-6 In Vitro Pyrogen Test
• The MM6/IL-6 In Vitro Pyrogen Test

1.1.2 Peer Reviews of In Vitro Pyrogen Test Method Validation Studies

The ECVAM-sponsored validation studies of each of these in vitro test methods have been the subject of a recent formal peer review convened by the ECVAM Scientific Advisory Committee (ESAC). Two members of the ESAC served as co-chairpersons for the review Panel, which consisted of five additional U.S. and European reviewers. These reviewers assessed the ability of each test method to serve as a complete replacement for the RPT. Based on this review, the ESAC declared that, “these tests have been scientifically validated for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of this pyrogen, in materials currently evaluated and characterized by RPTs.” Although the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) requested the ESAC peer review report, this document is not currently permitted by ECVAM to be publicly disseminated.

This BRD was prepared for an ICCVAM independent peer review panel (Panel) to evaluate these in vitro pyrogen test methods and to consider the ICCVAM draft recommendations for each in vitro test method. Because individual BRDs for each test method were provided by ECVAM, the ICCVAM BRD provides information that was common to all five in vitro test methods and references the appropriate sections of the ECVAM BRDs for specifics related to individual test methods. The recommendations of the ICCVAM Panel, combined with the information and analyses presented in the ICCVAM and ECVAM BRDs and any comments by the public or the Scientific Advisory Committee on Alternative Toxicological Methods were considered by ICCVAM prior to making its final recommendations on the usefulness and limitations of each test method, the proposed standardized test method protocols, performance standards, and any additional studies considered necessary to further develop or characterize any or all of these in vitro test methods.

1.2 Regulatory Rationale and Applicability

1.2.1 Current Regulatory Testing Requirements and ICCVAM Prioritization Criteria

This section reviews and summarizes the extent to which the five ICCVAM prioritization criteria (ICCVAM 2003) apply to the in vitro pyrogen test methods under consideration.

Criteria 1. The extents to which the proposed test methods are (a) applicable to regulatory testing needs and (b) applicable to multiple agencies/programs.

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2As indicated in the ECVAM BRDs for the WB/IL-1 and PBMC/IL-6 test methods, catch-up validation studies were conducted to evaluate the performance of the WB/IL-1 test method when using 96-well plates, and the PBMC/IL-6 test method when using cryopreserved PBMCs. The plating procedure (WB/IL-1) and the cryopreservation procedure (PBMC/IL-6) are the only differences in the test method protocols (see Appendix A). These modifications were not submitted by ECVAM as separate test methods, and are therefore not being considered as separate test methods in this BRD. However, where relevant, comparative information is provided (e.g., see Table 2-1 and Sections 6.1 and 7.2).
Pyrogenicity testing is primarily used by regulatory authorities for end-product release of human and animal parenteral drugs, biological products, and medical devices. The results from these assays are used to limit, to an acceptable level, the risks of febrile reaction in the patient exposed to the product of concern by injection and/or implantation. As detailed in Table 1-1, the U.S. Food and Drug Administration (FDA) is the principal U.S. regulatory agency that requires pyrogenicity testing, with different Centers within the FDA regulating the affected products. The Center for Biologics Evaluation and Research, the Center for Drug Evaluation and Research, the Center for Devices and Radiological Health, and the Center for Veterinary Medicine require that human injectable drugs (including biological products), animal injectable drugs, and medical devices be tested for the presence of pyrogenic substances. The current U.S. legislation requiring the use of pyrogenicity testing is set forth in the Federal Food, Drug, and Cosmetic Act (U.S. Code [U.S.C.], Title 21, Chapter 9). In addition, the U.S. Pharmacopeia (USP) maintains sterility requirements for pharmaceuticals that include pyrogenicity testing. Table 1-1 also shows the statutory protocol requirements used by each FDA Center, along with the comparable enabling legislation and statutory protocol requirements of the EU member nations.

Additionally, the prediction model described in the ECVAM BRDs is based on a pyrogen threshold dose of 0.5 EU/mL. While this level of detection would be sufficient for many parenteral drugs and medical devices, the endotoxin limit set by the FDA for intrathecal drugs and devices that contact cerebrospinal fluid is 0.06 EU/ml. In response to an ICCVAM Pyrogenicity Working Group (PWG) request for more data to support the use of these test methods for discriminating an endotoxin threshold dose lower than 0.5 EU/ml, ECVAM provided supplemental data (see question #6 in Appendix B).
### Table 1-1  Summary of U.S. and European Legislation and Statutory Protocol Requirements for Pyrogenicity Testing

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<tr>
<th>Agency</th>
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<th>Legislation</th>
<th>Statutory Protocol Requirements</th>
<th>Non-Governmental Standards</th>
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<td>FDA-CDER</td>
<td>Human parenteral pharmaceuticals</td>
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<td>USP30 NF25&lt;151&gt; (USP 2007b)</td>
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<td>FDA-CDRH</td>
<td>Medical devices</td>
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<td>ISO 10993-11 (ISO 2006)</td>
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<td>FDA-CVM</td>
<td>Veterinary pharmaceuticals</td>
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<td>Europe</td>
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Criteria 2. Warranted, based on the extent of expected use or application and impact on human, animal, or ecological health.

The proposed test methods are intended to replace a method that is used extensively in pharmaceutical, biological product, and medical device development and registration (i.e., the RPT).

Criteria 3: The potential for the proposed test methods, compared to current test methods accepted by regulatory agencies, to (a) refine animal use (decrease or eliminate pain and distress), (b) reduce animal use, or (c) replace animal use.  

The two most common pyrogen tests presently used (i.e., RPT and BET) require the use of animals. The RPT is performed in rabbits that can be maintained and reused (under certain circumstances) for multiple tests. According to USP30 NF25<151> (USP 2007b), rabbits may not be reused more than once every 48 hours (hr) after a negative test, not less than two

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3Refinement alternative is defined as a new or revised test method that refines procedures to lessen or eliminate pain or distress to animals, or enhances animal well-being. Reduction alternative is defined as a new or revised test method that reduces the number of animals required. Replacement alternative is defined as a new or revised test method that replaces animals with nonanimal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate) (ICCVAM 1997).
weeks following either (1) a maximum rise of 0.6°C or more, or (2) an animal is included in a test with a substance that is classified as pyrogenic. The BET is performed using hemolymph (the equivalent of blood, which requires drawing approximately 20% of the animal’s total blood volume) obtained from *Limulus polyphemus* (horseshoe crabs). Although the donor horseshoe crabs are returned to the wild, some mortality (up to 15%) is associated with the procedure (Walls et al. 2002).

The need for horseshoe crab hemolymph has potentially been reduced with the development of recombinant Factor C, the endotoxin sensitive protein that initiates clotting in the traditional BET. This commercial product, which was originally cloned from the horseshoe crab (Ding et al. 1997; Ding and Ho 1998, 2001), is currently being compared to the BET for submission and inclusion in the USP.

It should also be noted that the FDA has accepted data from the PBMC test developed by Novartis and Baxter Healthcare, which in conjunction with RPT and BET results, were used to support the safety testing of a specific single drug product (New Drug Application Number 16-267/S-037).

**Criteria 4: The potential for the proposed test methods to provide improved prediction of adverse health or environmental effects, compared to current test methods accepted by regulatory agencies.**

Sufficient data are presented to allow an assessment of the performance of the proposed test methods relative to the RPT (see Section 6.0). Because these methods are conducted using cells of human origin, it is postulated that they may reflect the human physiological response better than the currently employed, non-human based methods (i.e., RPT and BET).

These *in vitro* test methods have also been found to be useful for detecting test substances that, in the absence of endotoxin contamination, have evoked an adverse response in patients (Marth and Kleinhappl 2002; Martis et al. 2005). For example, numerous cases of aseptic peritonitis in dialysis patients that were not febrile have been attributed to peptidoglycan contamination of the dialysate (Martis et al. 2005). This dialysate solution met all European and USP standards prior to product release, but the PBMC/IL-6 test method detected increased levels of IL-6 when the dialysate was tested following product recall (Martis et al. 2005). In Marth and Kleinhappl (2002), a case study of a vaccine that was approved for release by the Austrian health authorities, but later produced a fever response in humans, has been described. When this vaccine was subsequently tested in the WB/IL-1β test method, it produced a positive result (Marth and Kleinhappl 2002).

**Criteria 5: The extent to which the test method provides other advantages (e.g., reduced cost and time to perform) compared to current methods.**

As outlined in Table 11-1, cost estimates obtained from various contract laboratories that perform the RPT and from the only contract laboratory known to perform an *in vitro* pyrogen test indicate that the *in vitro* test methods are considerably more cost effective to perform than the RPT. With respect to time considerations, the *in vitro* test methods require two half-days (i.e., one before and one after the overnight incubation) to complete, provided that cryopreserved blood is available and that interference testing is not required. The RPT can be performed within one day. However, before using a rabbit for the first time in a RPT, it must
be conditioned by a sham test that includes all steps of pyrogenicity testing except for injection, according to USP30 NF25<151> (USP 2007b).

1.2.2 Intended Uses of the Proposed In Vitro Pyrogen Test Methods

The proposed test methods are intended as an end-product release test for the identification of Gram-negative endotoxin in human and animal parenteral drugs, biological products, and medical devices. The results from pyrogen testing are used to limit, to an acceptable level, the risks of febrile reaction to the injection and/or implantation of the product of concern.

1.2.3 Similarities and Differences in the Endpoints Measured by the Proposed Test Methods and the In Vivo Reference Test Method

The endpoint measured in the in vitro pyrogen test methods is release of proinflammatory cytokines, either IL-1β or IL-6, in response to a test substance challenge, depending on the specific cell type employed. As described in Section 1.1.1, the pathogenesis of fever is induced by bacterial products that stimulate the production of IL-1α, IL-1β, and TNF-α, which leads to secondary synthesis of IL-6 and subsequent induction of prostaglandin synthesis (Netea et al. 2000). Direct injection of either IL-1 or IL-6 in several species causes fever, but much higher concentrations of IL-6 are needed. For example, in the rabbit, up to 100-fold more IL-6 is needed to produce a fever compared to IL-1 (Dinarello 2004).

The RPT involves measuring the rise in body temperature evoked in rabbits by the intravenous (i.v.) injection of a test solution. Although there is no direct association between the endpoints measured in the in vitro test methods and the RPT, fever is mediated by proinflammatory cytokines and therefore, it is reasonable to postulate that the cytokine-inducing potential of a pyrogen should correlate with its pyrogenic potential (Nakagawa et al. 2002). Moreover, Nakagawa et al. (2002) evaluated the utility of in vitro pyrogen test methods for detecting and quantifying various pyrogens. For example, the authors demonstrated that the responsiveness of human WB cells to pyrogens was very similar to that of a subline of MM6 cells, where endotoxin treatment (1 ng/mL) resulted in the production of IL-6 (~1 ng/mL) and IL-1 (~0.1 ng/mL).

Because the RPT is based solely on a rise in body temperature, no data were found on proinflammatory cytokine levels in rabbits following injection with endotoxin to permit a direct comparison with the in vitro test methods.

1.2.4 Use of the Proposed Test Methods in an Overall Strategy of Hazard or Safety Assessment

As detailed in Table 1-1, current U.S. and European regulatory requirements exist to test pharmaceutical products, biological products, and medical devices for pyrogenicity. The pyrogen tests that are currently acceptable to regulatory authorities require the use of rabbits or horseshoe crab hemolymph. According to ECVAM, the in vitro test methods are intended to replace the RPT for the identification of pyrogens where: (a) the test material is incompatible with the BET or (b) the test material contains a non-endotoxin mediated pyrogen. However, as detailed in Section 3.0, only Gram-negative endotoxin was included in the validation study. Therefore, other types of pyrogens have not been adequately validated.
The extent to which the RPT is performed only for detecting the presence of endotoxin is not clear.

1.3 Scientific Basis for the In Vitro Pyrogen Test Methods

1.3.1 Purpose and Mechanistic Basis of the In Vitro Pyrogen Test Methods

The proposed methods are intended to detect pyrogens in parenteral pharmaceuticals, medical devices, and human biological products. These methods provide an in vitro model of the initiation of the human fever response by measuring proinflammatory cytokine release (i.e., IL-1β or IL-6) from human monocytes/monocytoid cells exposed to pyrogens. These proinflammatory cytokines are associated with the initiation of the in vivo fever response.

1.3.2 Similarities and Differences of Modes of Action Between the In Vitro Pyrogen Test Methods and the Fever Response in Humans and/or Rabbits

As detailed in Section 1.2.3, each of the five proposed in vitro test methods measure proinflammatory cytokine release (i.e., IL-1β or IL-6) from human monocytoid cells as an indicator of the presence of a pyrogenic substance. By comparison, the RPT measures a change in body temperature in rabbits over a specified time period following an i.v. injection of a test substance. Although the relative sensitivities of each species to Gram-negative endotoxins vary, the responses of humans, horseshoe crabs (via hemolymph gelatin), and rabbits to these pyrogens have been studied extensively, and test methods based on blood products or blood cells from each of these species appear to be capable of responding to pyrogens (Greisman and Hornick 1969; Cooper et al. 1971; Brunson and Watson 1974; Hoffman et al. 2005a). Several studies directly comparing the in vitro pyrogen test methods with either the RPT and/or BET are summarized in Section 9.1, Moseby et al. (2000), and in the ECVAM response to ICCVAM PWG questions (see question #1 in Appendix B).

The recent discovery and characterization of the TLR family, which recognizes a diverse range of molecules such as lipids, proteins, and nucleic acids derived from pathogens, has led to an enhanced understanding of the signaling pathways activated by endotoxin. More specifically, TLR-4 has been identified as the receptor directly utilized by endotoxin to elicit an immune response. Upon recognition of endotoxin, TLR-4 initiates a rapid and complex signaling cascade, which activates transcription factors (i.e., NF-κB, AP-1, and interferon regulatory factors) to produce proinflammatory cytokines and other immune modulators, thereby leading to a protective immune response (Ishii et al. 2005; Ishii and Akira 2006). It is important to recognize that this TLR-dependent production of proinflammatory cytokines is distinct from the endotoxin-induced synthesis of IL-1 that then converges on the same signaling pathway via the IL-1 receptor (Conti et al. 2004). In addition to endotoxin, TLR-4 recognizes numerous other microbial components such as respiratory syncytial virus proteins and anthrolysin O (Ishii et al. 2005, Ishii and Akira 2006). When proinflammatory cytokine mRNA levels (i.e., IL-1, IL-6, and TNF-α) were compared in response to various TLR-4 agonists, endotoxin induced the highest level of expression (Park et al. 2004).

Additional information on testing of other types of pyrogens was also provided by ECVAM and is included in Appendix B.
It has been recognized for many years that humans are responsive to relatively low doses of endotoxin, whereas rodents require much higher doses to elicit a response. In recent years, these species differences have been attributed, in part, to structural differences in TLR-4. For this reason, caution should be used when extrapolating findings from other mammals to humans with respect to endotoxin and TLR-4 signaling (Stoll et al. 2006). Furthermore, TLR-4 mutations have been identified in mice and humans, and it is likely that such defects are associated with altered gene expression and increased susceptibility to infection (Norata et al. 2005; van Deventer 2000; von Aulock et al. 2003).

1.3.3 Range of Substances Amenable to the In Vitro Pyrogen Test Methods and Limits of These Methods

The proposed methods are intended for the identification of pyrogenic substances in parenteral pharmaceuticals, biological products, and medical devices. Because they are based on cultured human monocytes/monocytoid cells, they are considered capable of detecting both Gram-negative endotoxin and non-endotoxin-based pyrogens. While Section 9.0, Moesby et al. (2005), and the ECVAM response to ICCVAM PWG questions (see question #2 in Appendix B) provide a number of published studies demonstrating that the in vitro pyrogen test methods are able to detect non-endotoxin pyrogens, the ECVAM validation studies focused specifically on Gram-negative endotoxin due to the unavailability of standardized, non-endotoxin pyrogens (see Section 3.0).

Because these test methods measure the release of proinflammatory cytokines, drugs that are cytotoxic to blood cells or that induce a substantial proinflammatory response (e.g., IL-1 receptor antagonists, interferon [IFN]-γ, and rheumatic factors) are not amenable to testing by these methods (Hartung et al. 2001; Ishii et al. 2005; Ishii and Akira 2006). As described in Section 2.0, each test method includes an interference test to identify problematic test samples.

1.4 Validation of the In Vitro Pyrogen Test Methods

The ICCVAM Authorization Act of 2000 mandates that “[each] Federal Agency … shall ensure that any new or revised … test method … is determined to be valid for its proposed use prior to requiring, recommending, or encouraging [its use].” Validation is the process by which the reliability and relevance of an assay for a specific purpose are established (ICCVAM 1997). Relevance is defined as the extent to which an assay will correctly predict or measure the biological effect of interest (ICCVAM 1997). For the in vitro pyrogen test methods described in this ICCVAM BRD, relevance is restricted to how well the assays detect the presence of Gram-negative endotoxin. Reliability is defined as the reproducibility of a test method within and among laboratories and should be based on performance with a diverse set of substances that are representative of the types of chemical and product classes that are to be tested and the range of responses that needs to be identified. The validation process is designed to provide data and information that will allow ICCVAM to make recommendations on the applicability of a test method and U.S. Federal agencies to consider those recommendations in light of their regulatory mandates.

The first stage in the evaluation of a new test procedure is the preparation of a BRD that presents and evaluates the relevant data and information about the test method, including its
mechanistic basis, proposed uses, reliability, and performance characteristics (ICCVAM 1997). This ICCVAM BRD summarizes the available information on each of the five in vitro pyrogen test methods listed in Section 1.1.1.

Where adequate data are available, the qualitative and quantitative performance of the proposed alternative test method is evaluated, and its reliability is compared with the reliability of the currently accepted test method. This ICCVAM BRD will aid in identifying essential test method components that should be considered during the identification of a standardized protocol for use of the test method.

1.5 Search Strategies and Selection of Citations for the ICCVAM In Vitro Pyrogen Test Methods BRD

NICEATM conducted an online literature search for relevant information on the five in vitro pyrogen test methods using multiple internet databases (i.e., PubMed, SCOPUS, TOXLINE, Web of Science). Specifically, records were sought using various combinations of the terms: in vitro, WB, WB cells, PBMC, Mono Mac 6, MM6, endotoxin, lipopolysaccharide (LPS), pyrogen, LAL, BET, IL-1, and IL-6. This search was conducted to supplement and update the list of peer-reviewed publications related to in vitro pyrogen testing that was provided in the ECVAM BRDs. U.S., EU, and Japanese pyrogenicity test guidelines were obtained from relevant regulatory agencies via the internet or through direct requests. The resulting database of 370 references confirmed that the lists of references included in the ECVAM BRDs were complete and up-to-date.