

**1. The submitted test method should relate to regulations or guidelines in Japan.**

Subject to all applicable provisions of the 15th edition of the Japanese Pharmacopeia (JP 15), published in 2006, as well as Test Methods for Biosafety Monitoring of Medical Devices (Pharmaceutical Bureau, Evaluation Licensing Division Administrative Notice, Medical Devices Evaluation No. 36).

Methods for detection of pyrogenic substances contained in JP 15 include a Pyrogen Test and a Bacterial Endotoxins (limulus) Test.

According to General Rules for Preparations and other sections on Injections in JP 15, the Bacterial Endotoxin Test is preferred, but it also stipulates that the Pyrogen Test can be used when application of the Bacterial Endotoxin Test is impracticable.

Test for Rubber Closure for Aqueous Infusions Methods (JP 15) as well as Test Methods for Biosafety Monitoring of Medical Devices both require implementation of the Pyrogen Test.

**2. The submitted test method and supporting validation data should have been subjected to a transparent and independent peer review process.**

ICCVAM has performed a third-party review of both the ECVAM EC Statement on the Validity of In-Vitro Tests and the ECVAM Background Review Document. In May of 2008, it published both an ICCVAM Test Method Evaluation Report and ICCVAM Background Review Document. The JaCVAM Pyrogen Test Review Committee reviewed these reports.

ICCVAM validations practice due diligence using a modular approach as stipulated by OECD guideline GD34.

The reports include validations of five test protocols and test substances.

A comparative study of correlativity with rabbit pyrogen tests, including reference documentation, was given for reports that were not accepted as documentation for overall evaluation.

The documentation did contain some problematic handling of data, but nothing that affected its conclusions to any significant degree, and we accept ICCVAM's overall evaluation.

3. The data generated by the test method should adequately measure or predict the endpoint of interest. For replacement test methods, the data should show a linkage between the proposed test method and an existing test method, and/or the proposed test method and effects in the target or model species.

In vitro pyrogen test methods (in vitro PBMC assays) assess the existence of pyrogens by measuring inflammatory cytokine (interleukin IL-1 $\beta$  or IL-6) secreted from human peripheral blood mononuclear cells or human monocyte-like cell lines. This is a scientifically valid approach, based on an endotoxin-induced pyrogenic mechanism.

We have confirmed that results were largely in agreement with those from the Pyrogen Test, which is used as a standard reference and which measures changes in body temperature of rabbits, and those from the Bacterial Endotoxins Test.

Since the test uses human cells, it is potentially a more accurate predictor of pyrogenic potency in humans than either the Pyrogen Test or the Bacterial Endotoxins Test, which use animal cells, but this has not been verified.

Only endotoxins from gram-negative bacteria were included in the validation report, and non-endotoxins have yet to be fully verified.

4. The test method should generate data useful for hazard/risk assessment purposes.

This test method is capable of assessing hazards, as it provides either a positive or negative result, based on whether or not a particular dose of endotoxins results in the quantity of cytokines crossing a certain threshold. Just like test methods for detection of pyrogenic substances that use rabbits, this test method was not designed for risk assessment.

5. The submitted test method and supporting validation data should adequately cover a spectrum of chemicals and products representative of those administered by the regulatory program or agency for which the test method is proposed, and the applicability and limitations of the test method should be clearly described.

The accuracy and replicability of the submitted test method has been validated with a total of 13 substances. Each of these substances is a drug to which exotoxins had been added, and no validations of substances, biological products, or medical devices that exhibit pyrogenic potency without the addition of endotoxins were

performed. Therefore, the capacity of this test method to detect non-endotoxins is unclear. This test method is capable of assessing the pyrogenic potency of drugs suitable for parenteral administration, but is considered insufficient with drugs for medical devices or oral exposure.

Reference documentation includes a comparative study of in vitro PBMC tests, endotoxin tests, and rabbit pyrogen tests using test specimens comprising human serum albumin preparations reported to have induced fever as a side effect during clinical practice, recombinant factor VIII preparations, and plasma preparations to which endotoxins had been added. Results have also been reported for various glucan, lipoteichoic acid, fungal spores, medical equipment, and lipids, indicating the potential of this test method to detect pyrogenic potency in substances for which other test methods are not applicable. We do not yet have, however, sufficient data to validate these claims.

6. The test method should be sufficiently robust (relatively insensitive to minor changes in protocol) and transferable among properly-equipped laboratories with adequately-trained staff.

Whole human blood within four hours of collection, frozen blood, and peripheral blood mononuclear cells or cell lines taken from whole blood are used in the measurement of IL-1 $\beta$  or IL-6. A battery of five test methods (Whole Blood(WB)/IL-1, stored blood CryoWB/IL-1, WB/IL-6, PBMC/IL-6, and monocyte cell line MM6/IL-6) exists, for which ICCVAM has proposed recommended protocols. As predictors of pyrogenic potency, each of these test methods yields roughly identical rates of agreement.

Modified versions of PBMC/IL-6 and WB/IL-1 test methods have also been validated. Lack of a clear description of the process under which the modifications were made, however, precludes assessment of their robustness.

An ELISA kit is used to measure cytokines, so transferability was not reviewed, since we consider any experienced technician capable of acquiring those skills. Although inter-laboratory reproducibility for WB/IL-1 is problematic, the other test methods all exhibit good intra- and inter-laboratory reproducibility.

7. The test method should be both time and cost effective as well as likely to be used in a regulatory context.

We infer that these test methods are more cost effective than the Pyrogen Test, which uses rabbits and requires significant time to implement when multiple animals are used.

Time and cost effectiveness of these tests relative to the Bacterial Endotoxins Test is not clear.

The use of human blood is subject to regulatory restrictions.

8. Justification should be provided (scientific, ethical, economical) for the new or updated test method in light of existing test methods.

Compared with pyrogen tests that use rabbits, these new test methods fulfill the principles of the 3Rs and provide economic justification.

Compared with endotoxin tests, these new test methods have different modes of action, but ethical and economic justification remains unclear.

Based on the above, the JaCVAM Regulatory Acceptance Board has determined the following for these in vitro pyrogen test methods.

These test methods are based on an endotoxin-induced pyrogenic mechanism that is scientifically valid in principle.

The validation studies present results that exhibit a high correlation with the rabbit pyrogen test, but it remains necessary to increase the number of compounds and implement validation at multiple laboratories before these test methods can be adopted as an alternative test method.

At present, these test methods are not suitable as a replacement for endotoxin tests. Given that JP 15 makes allowances for the move from test methods that use rabbits for detection of pyrogenic substances to those that test for endotoxins, these test methods afford only limited value in Japan.

We recognize potential for these test methods, but do not find them to be immediately valid alternatives at this point in time.