

Reference Standard Endotoxins suitable for PyroMAT® System

Introduction

What is a pyrogen?

A pyrogen is, by definition, a substance that produces a rise in temperature in a human or animal. Pyrogens constitute a heterogeneous group of contaminants comprising microbial and non-microbial substances. The most widely known pyrogen is the endotoxin (LPS = Lipo-Polysaccharide), which is produced by gram-negative bacteria. Other microbial substances include those derived from gram-positive bacteria like Lipoteichoic Acid (LTA), particles from viruses and pyrogens originating from yeasts and fungi. Non-microbial pyrogenic substances can be rubber particles, microscopic plastic particles or metal compounds in elastomers.

Why to conduct a pyrogen test?

Pyrogenic substances in pharmaceutical products can induce life-threatening fever reactions after injection into the human body. Therefore, it is a regulatory requirement to test such products for pyrogens to ensure product quality and patient safety.

Purpose of the test is to prove that the amount of pyrogens contained in the product will not exceed a certain threshold, known as the contaminant limit concentration (CLC), that will guarantee the patient safety.

The monocyte activation test (MAT) method has been qualified and validated for the detection of pyrogens by the European Center for the Validation of Alternative Methods (ECVAM) in 2005 and by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in 2008.

It has been among the compendial methods for pyrogen detection in the European Pharmacopeia since 2010 (Chapter 2.6.30). The MAT is also mentioned by the FDA "Guidance For Industry – Pyrogen and Endotoxins testing: Questions and Answers" as an alternative to the rabbit pyrogen test which should be validated according to USP <1225>. Additionally, the USP <151> Pyrogen Test mentions that, "A validated, equivalent *in vitro*

pyrogen or bacterial endotoxin test may be used in place of the *in vivo* rabbit pyrogen test, where appropriate."

Principle of the MAT

The monocyte activation test (MAT) is the human *in vitro* alternative to the rabbit pyrogen test, and allows the detection of the full range of pyrogens, including endotoxins and non-endotoxin pyrogens (NEPs).

By putting the product to be tested in contact with human monocytic cells, it will mimic what happens in the human body: in presence of pyrogens, the monocytes are activated and produce cytokines such as interleukin-6.

The cytokines are then detected using an immunological assay (ELISA) involving specific antibodies and an enzymatic color reaction.

Principle of the PyroMAT® System

The PyroMAT® System uses cryo-preserved Mono-Mac-6 (MM6) human monocytic cells as a source of monocytes.

The response to pyrogenic substances is determined by measurement of interleukin-6 (IL-6) produced by the Mono-Mac-6 cells. For this purpose, the ELISA microplate supplied in the kit is coated with an antibody specific to IL-6.

IL-6 molecules released by MM6 cells during incubation phase are transferred from the cells supernatant to the ELISA plate, and bound by the immobilized primary antibody.

A secondary antibody, linked to an enzyme, is added to form an IL-6 bound complex. After washing any unbound molecules, the IL-6 bound complex is detected in a color reaction started by the addition of an appropriate substrate.

The color development is proportional to the amount of initial IL-6 production in the supernatant and measured with an absorbance reader.

Comparison of Reference Standard Endotoxins (RSE) from different suppliers

Preparation of endotoxin standard solutions is needed to assess the limit of detection (LOD) of the system, to build a standard curve for quantification or to estimate the pyrogen content of a sample, depending on the MAT method used.

The use of a validated Reference Standard Endotoxin is required and such a standard can be supplied by the European Pharmacopoeia (EDQM) or the United States Pharmacopoeia (USP).

Control Standard Endotoxins (CSE) provided by LAL suppliers should not be used for MAT test.

The reference standard endotoxin (RSE) supplied by the USP / EDQM is a reference endotoxin preparation with a certified activity upon reconstitution. Control standard endotoxin preparations (CSEs) are qualified using the RSE, but their activity is certified only in combination with a test system, e.g. a defined preparation of limulus amoebocyte lysate used for the bacterial endotoxin test.

Using these CSEs outside their test system might lead to unexpected results and is not recommended by the respective suppliers. As there is currently no dedicated reference standard for the pyrogen test available, standardization is achieved using a strong pyrogen like the RSE whose production and standardization is not depending on the use of a specific bacterial endotoxin test.

The scope of this application note is to show the suitability of Reference Standard Endotoxins from different suppliers (USP/EDQM) for MAT with the PyroMAT® System.

Materials

Material	Description	Cat. No.
PyroMAT® Kit		Pyr0MATkit
PyroMAT® Cells		Pyr0MATcells
PyroMAT® Endotoxin Standard	European Pharmacopoeia (EP) Reference Standard Endotoxin	1.44161.0001
Sigma-Aldrich RSE	European Pharmacopoeia (EP) Reference Standard Endotoxin	E0150000
ACILA RSE	USP Reference Standard Endotoxin (USP) 10.000 E.U./FI	1220200
NIBSC RSE	3 rd International Standard 10,000 USP Endotoxin units Replacement I.S. for 94/580	10/178

Table 1: Materials used to generate standard curves

Resuspension of RSE

Lyophilized RSE were reconstituted and aliquoted according to supplier guidelines.

Dilution of Reference Standard Endotoxin aliquots

The standard endotoxin solutions were prepared from the RSE stock solution at 2000 EU/mL. Seven (7) endotoxin concentrations (0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 EU/mL) were prepared to generate the standard curve according to the following procedure:

- Thaw a 50 µL-aliquot of RSE and vortex at maximum speed during 1 min.
- Perform serial dilutions in endotoxin-free water, using endotoxin-free glass tubes, as described below. Make sure to vortex all the dilutions before using.

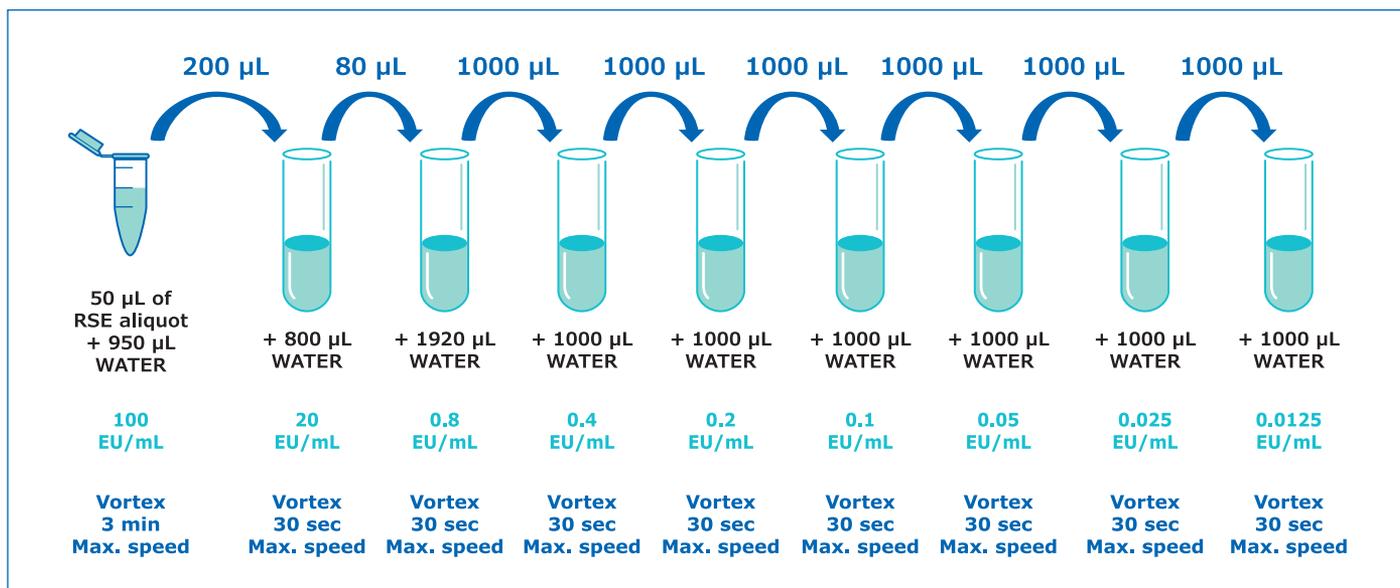


Figure 2: Materials used to generate standard curves

MAT quick procedure with PyroMAT®

Step 1: Preparation and incubation with PyroMAT® cells

- 1**
 - Prepare suitable endotoxin standard dilutions
 - Load the different solutions on the 96-wells cell culture plate
 - Prepare the PyroMAT® cells and dispense in each well
- 2**
 - Incubate the plate for 22 ±2 hours at 37 °C with humidified atmosphere, without CO₂



Step 2: Detection of IL-6 with ELISA

- 3**
 - Transfer the cell supernatants into IL-6 microplate
 - Add the IL-6 conjugate to each well
 - Incubate 2 hours at room temperature
- 4**
 - Remove the liquid and wash the plate 4 times
 - Prepare the substrate solution by mixing color reagent A and B and add the mixture to each well
 - Incubate 30 minutes at room temperature, in the dark
- 5**
 - Add the stop solution
- 6**
 - Read the plate at 450 nm and 630 nm within 30 minutes after adding the stop solution



Figure 3: PyroMAT™ workflow with standard ELISA procedure

Results

Endotoxin standard curves were generated using RSE from different suppliers. To be considered “VALID”, the endotoxin standard curve must fulfill the following acceptance criteria described in the EP Chapter 2.6.30 :

- ➔ **Effect of dose criteria:** a statistical test that confirms a positive dose/effect response.
- ➔ **Goodness of fit:** a statistical test that confirms the suitability of the regression model to describe the raw data. The data are modeled with a 5-parameter logistics regression model.
- ➔ **Blank criteria:** the mean of blank OD value should be below 0.1.
- ➔ **LOD criteria:** the test is valid if an $LOD \leq 0.05$ EU/mL is reached.

An additional criterion was implemented in the protocol to assess the reactivity of the standard curve:

- ➔ **Minimum of reactivity:** OD of the 4 replicates of the highest standard (0.8 EU/mL) should be above 3.

It is not required by the European pharmacopeia and is given as an additional indication for the customer.

Data analysis was performed using the PyroMAT® data analysis tool, which consists of a specific protocol developed for PyroMAT® using Gen5 Software (Biotek).

The **Figure 4** presents the curves that were obtained with the PyroMAT® system using Reference Standard Endotoxins (RSE) from four different suppliers as described in **table 1**.

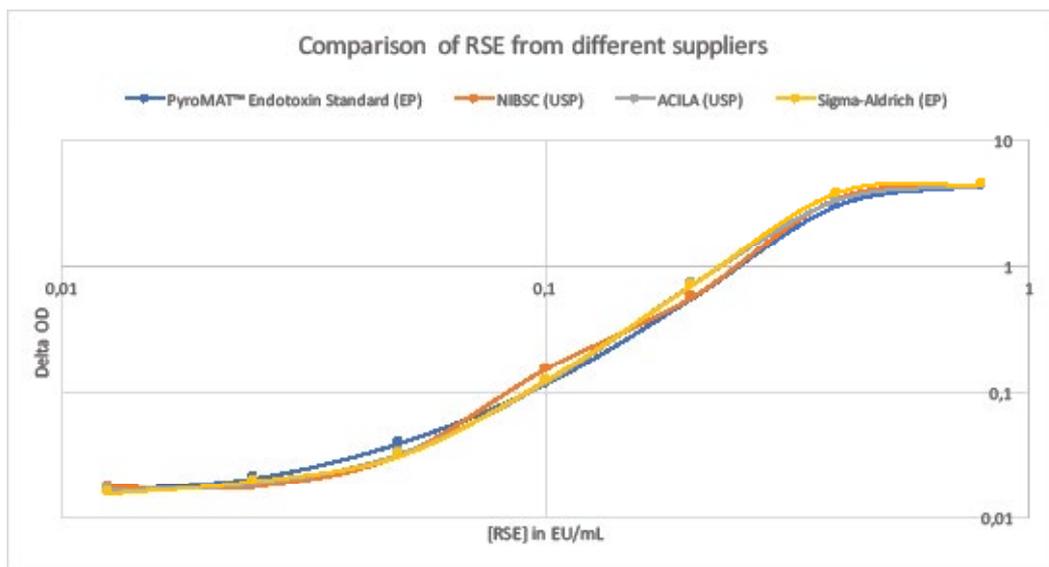


Figure 4: Comparison of standard curves generated with various Reference Standard Endotoxins

The validity of the acceptance criteria for the endotoxin standard curve was determined using the PyroMAT® data analysis tool (protocol for Gen5 Software).

The **Figure 5** shows the results obtained and the legend used in the software for data interpretation:

Material	Effect of close	Goodness of Fit	Blank Delta OD	LOD criteria	Minimum of reactivity
PyroMAT® Endotoxin Standard	VALID	VALID	VALID	VALID	CONFORM
Sigma-Aldrich RSE	VALID	VALID	VALID	VALID	CONFORM
ACILA RSE	VALID	VALID	VALID	VALID	CONFORM
NIBSC RSE	VALID	VALID	VALID	VALID	CONFORM
Legend					
Effect of Dose Criteria:	VALID: $p < 0.01$ INVALID: $p \geq 0.01$	LOD Criteria	VALID: $LOD \leq 0.05$ EU/mL INVALID: $LOD > 0.05$ EU/mL		
Goodness of Fit Criteria:	VALID: $p > 0.05$ INVALID: $p \leq 0.05$	Additional Criteria – Minimum of reactivity	CONFORM: All replicates of Delta OD at STD7 are above 3 NOT REACHED: At least one replicate of Delta OD at STD7 is below 3		
BLK Delta OD Criteria:	VALID: $MEAN(BLK) < 0.1$ INVALID: $MEAN(BLK) \geq 0.1$?????: Unable to Evaluate		

Figure 5: Acceptance criteria for the endotoxin standard curves and legend for data interpretation

All standard curves generated with RSE from different suppliers passed the acceptance criteria of a valid standard curve according to the EP chapter 2.6.30.

Conclusion

All four different Reference Standard Endotoxins (RSE) tested led to the generation of a valid standard curve and can be used to perform MAT with PyroMAT® system.

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