

Detection of pyrogens in albumin 20% solution with the 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) [1].

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 supernatant during incubation phase are transferred in 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.

Quantification of pyrogens with the MAT

For the quantification of the pyrogenic load of a sample, method A in accordance with the European Pharmacopeia [1] can be conducted.

Method A involves a comparison of the preparation being examined with a standard endotoxin dose-response curve. The contaminant concentration of the preparation being examined is to be less than the CLC (Contaminant Limit Concentration) to pass the test.

To ensure both the precision and validity of the test, preparatory tests need to be conducted to assure that:

- The criteria for the endotoxin standard curve are satisfied
- The solution does not interfere with the test
- The test detects endotoxin and non-endotoxin contaminants
- The solution does not interfere with the detection system

Material and Equipment

To perform the MAT and a product specific validation, we recommend using:

- PyroMAT® Cells (Ref: Pyr0MATCELLS)
- PyroMAT® Kit (Ref: Pyr0MATKIT)
- Reference Standard Endotoxin (Ref: 1.44161.0001).
- NEP Control HKSA (Ref: MATHKSA)
- NEP Control Flagellin (Ref: MATFLAGELLIN)
- IL-6 control (Ref: Pyr0MATIL6)

Additional equipment and consumables required:

- Incubator, 37 °C, humidified
- Water bath (37 °C)
- Microplate reader to measure absorbance at 450 nm and 630 nm (reference wavelength)
- Cryo-freezer (< -80 °C)
- Freezer (-20 °C)
- Refrigerator (2-8 °C)
- Vortexer
- 50 mL centrifuge
- Multichannel pipettes with suitable containers
- Adjustable pipettes: (10 µL – 100 µL; 100 µL – 1000 µL) with suitable sterile, pyrogen-free pipette tips
- Pyrogen-free glass tubes
- 2 mL endotoxin-free reaction tubes with caps
- 50 mL endotoxin-free tubes with caps



The PyroMAT® Kit

Quantification of pyrogens with the PyroMAT® system

Preparatory tests – Product Specific Validation (PSV)

European pharmacopeia, chapter 2.6.30

Before routine testing of a pharmaceutical product with MAT, a product specific validation (PSV) must be performed according to the method chosen for routine testing to ensure the validity of the criteria for the endotoxin standard curve, the detectability of endotoxin and non-endotoxin contaminants in the sample and that the sample does not interfere with the test or detection system.

Interferences with the test can be removed by diluting the product up to a certain limit, referred to as the maximum valid dilution (MVD).

The MVD is the maximum dilution factor at which it is still possible to detect the pyrogen limit (i.e., the CLC). It is directly linked to the limit of detection (LOD) of the system.

The more sensitive the system is, the more the product can be diluted to remove interferences.

The MVD of a test solution can be calculated using the following formula:

$$MVD = \frac{CLC \times C}{LOD}$$

CLC = Contaminant Limit Concentration (EU/mg or EU/mL)
C = Concentration of the test solution (mg/mL or mL/mL)
LOD = Limit of Detection (EU/mL).

The CLC is the acceptance criterion for the pass/fail decision, expressed in endotoxin equivalents per milligram or milliliter (EEU/mg or EEU/mL) or per unit of the biological activity of the product.

It is calculated by the following expression:

$$CLC = \frac{K}{M}$$

K = threshold pyrogenic dose per kilogram of body mass (EU/kg)

M = maximum recommended bolus dose of product per kilogram of body mass (mg/kg or mL/kg).

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

When testing for interfering factors, dilutions of the preparation being examined with geometric steps not exceeding the MVD should be performed. The same dilutions spiked with endotoxin at a justified concentration (in case of method A, a concentration near the estimated middle of the endotoxin standard curve) should then be performed.

These dilutions must be tested in parallel in the same experiment and together with an endotoxin standard curve, which shall be used to calculate the concentration of endotoxin-equivalents in each solution.

The mean recovery of the added endotoxin spike is then calculated for each dilution. The test is considered free of interference when recovery of the added endotoxin is within the range of between 50 and 200%.

Where practicable, interference testing should be performed on at least 3 different lots of the preparation being examined to investigate on possible batch-to-batch variation. If the interference cannot be removed by dilution or specific sample preparation of the product within the MVD range, Method C is preferred over Method A and B.

For validation of the detection of non-endotoxin contaminants, historical batches that have been found to be contaminated with non-endotoxin contaminants causing positive responses in the rabbit pyrogens test or adverse drug reaction in man can be used. Where those batches are not available, validation should be done including at least 2 non-endotoxin ligands for toll-like receptors, one of which is to be spiked into the preparation being examined.

Once the optimum dilution of the preparation being examined has been identified, this dilution needs to be tested for interference in the detection system. The agreement between a dilution series in presence and absence of the preparation being examined is to be within $\pm 20\%$ of optical density.

Sample specifications: Human Serum Albumin

Human serum albumin (HSA) 20% solution for infusion is indicated for the restoration and maintenance of circulating blood volume where volume deficiency has been demonstrated and use of a colloid is appropriate. [2].

Due to production process, albumin is known to be often contaminated with (1,3)- β - glucans origination from filter material. These (1,3)- β -glucans are known to be pro-inflammatory molecules activating monocytes and show synergetic effects with other pyrogens like endotoxin, leading to more intensive pyrogenic reactions in man [3,4].

The suitability of the MAT test for detection of pyrogens in albumin products was shown in various studies before [5,6,7]

The administration dosage for the tested product is variable and personalized based on specific indication, patient's clinical status and response. Depending on the indication, doses of 20 – 100 g albumin 20% may be applied.

HSA 20% is a hyperoncotic solution and rapid administration can lead to rapid volume expansion and cardiac failure. It should be infused slowly to avoid this, therefore larger doses are typically given within several hours.

In the absence of acute hemorrhage, total daily albumin dosage should not exceed the theoretical amount present in total normal plasma volume (about 2 g/kg body weight), nevertheless, the product to be examined was designed to allow also higher dosage with a maximum dose of 6g/kg/day.

For the estimation of the MVD of the product to be tested, the threshold pyrogenic dose for parenteral applied solutions is 5 EU/kg (K).

The single bolus dose of the product was considered as a volume of 100 mL, corresponding to a dose of 20 g albumin. For an average adult, a body weight of 70 kg can be assumed. Therefore, the CLC of this product was calculated as

$$CLC = \frac{K}{M} = \frac{5 \text{ EU/kg}}{\frac{100 \text{ mL}}{70 \text{ kg}}} = \frac{350 \text{ EU}}{100 \text{ mL}} = 3.5 \text{ EU/mL}$$

For the PyroMAT® system, the LOD is 0.05 EU/mL, so

$$MVD = \frac{3.5 \text{ EU/mL} \times 1}{0.05 \text{ EU/mL}} = 70$$

Product specific validation for testing albumin 20% for infusion with the PyroMAT® system

Assurance of the criteria for the endotoxin standard curve:

A standard curve using Reference Standard Endotoxin was performed to verify that the criteria for endotoxin standard curve were valid.

- The regression of response on log dose was statistically significant ($p < 0.01$)
- The regression of response on log dose did not deviate significantly from linearity ($p > 0.05$)

Test for interfering factors and method validation for detection of non-endotoxin contaminants:

A dilution series from undiluted product up to the MVD was prepared and a test for interfering factors and method validation for non-endotoxin monocyte-activating contaminants (NEPs) according to EP was performed, comparing endotoxin-spiked sample dilutions as well as NEPs-spiked sample dilutions with the same unspiked sample dilutions.

Tests results showed non-endotoxin pyrogen (NEPs) control was detectable in all dilutions of the product from undiluted sample to MVD.

The undiluted product was found to show some interference with the detection of the endotoxin spike (in average around 200% spike recovery) so this dilution may occasionally fail validity criteria. From an 1:10 dilution to the MVD, the product reproducibly showed

both detection of the NEP and a spike recovery within the range 50-200%, allowing to rule out interference with the test.

Test for interference in the detection system:

The so found optimum dilution was forwarded to a test for interference in the detection system. A dilution series of IL-6 control was tested in absence and presence of the sample dilution. All IL-6 control dilutions showed less than 20% difference between dilution in absence and presence of the product, therefore no interference with the detection system could be found.

Conclusion

Assurance of criteria for the standard curve	Valid
Test for interfering factors	Valid
Detection of NEP contaminants	Valid
Interference in the detection system	Valid

All criteria of the product specific validation were fulfilled and the dilution 1:10 was chosen as the first valid dilution for the Method A.



Quantitative method A according to EP

Planning Test Execution

Once a valid dilution has been identified through the Product Specific Validation, Method A (quantitative test) can be performed to assess the pyrogenicity of samples from this product.

The lowest dilution / highest concentration of the sample, that was found free of interference in the PSV is to be chosen as initial dilution and 2-fold serial dilutions are tested.

For quantification, the middle section of the dose-response curve of the endotoxin standard is to prefer, giving the most exact results, while quantification within the upper plateau of the standard curve can lead to inaccuracy due to reaching the endpoint of the reaction and therefore is not recommended.

For most exact results, we therefore recommend to analyze sample dilutions which do not exceed the measuring range of 0,05 to 0,4 EU/mL.

Data interpretation

The data analysis was performed with Gen5 software and the PyroMAT® Method A available on our website. Information related to the sample were completed directly on the software: sample name, CLC.

For interpretation, the layout for method A was modified with the appropriate dilution factors for this sample matrix.

Testing of albumin 20% for infusion with method A

The test setup was performed according to the user guide of the PyroMAT® system.

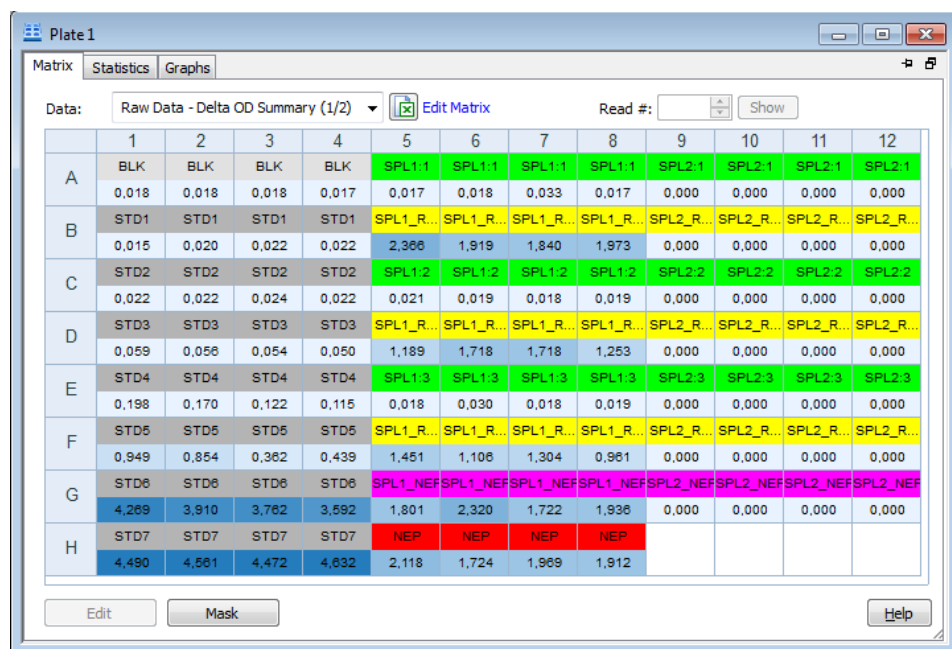
An endotoxin standard curve was performed for the test.

Three dilutions of the product were tested according to method A described in EP: The dilutions 1:10, 1:20 and 1:40 of the sample were all tested with and without endotoxin spike. A quantification of the found endotoxin equivalents for all dilutions of the unspiked and spiked sample was done using the endotoxin standard curve. The endotoxin spike recovery for all three sample dilutions was calculated.

Heat killed *Staphylococcus aureus* (HKSA) was used as additional control for detection of non-endotoxin pyrogens within the sample, tested with the highest concentration of the product to be examined.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	BLK	SPL1:1 10	SPL1:1 10	SPL1:1 10	SPL1:1 10	SPL2:1 10	SPL2:1 10	SPL2:1 10	SPL2:1 10
B	STD1 0,0125	STD1 0,0125	STD1 0,0125	STD1 0,0125	SPL1_R... 10	SPL1_R... 10	SPL1_R... 10	SPL1_R... 10	SPL2_R... 10	SPL2_R... 10	SPL2_R... 10	SPL2_R... 10
C	STD2 0,025	STD2 0,025	STD2 0,025	STD2 0,025	SPL1:2 20	SPL1:2 20	SPL1:2 20	SPL1:2 20	SPL2:2 20	SPL2:2 20	SPL2:2 20	SPL2:2 20
D	STD3 0,05	STD3 0,05	STD3 0,05	STD3 0,05	SPL1_R... 20	SPL1_R... 20	SPL1_R... 20	SPL1_R... 20	SPL2_R... 20	SPL2_R... 20	SPL2_R... 20	SPL2_R... 20
E	STD4 0,1	STD4 0,1	STD4 0,1	STD4 0,1	SPL1:3 40	SPL1:3 40	SPL1:3 40	SPL1:3 40	SPL2:3 40	SPL2:3 40	SPL2:3 40	SPL2:3 40
F	STD5 0,2	STD5 0,2	STD5 0,2	STD5 0,2	SPL1_R... 40	SPL1_R... 40	SPL1_R... 40	SPL1_R... 40	SPL2_R... 40	SPL2_R... 40	SPL2_R... 40	SPL2_R... 40
G	STD6 0,4	STD6 0,4	STD6 0,4	STD6 0,4	SPL1_NEF... 10	SPL1_NEF... 10	SPL1_NEF... 10	SPL1_NEF... 10	SPL2_NEF... 10	SPL2_NEF... 10	SPL2_NEF... 10	SPL2_NEF... 10
H	STD7 0,8	STD7 0,8	STD7 0,8	STD7 0,8	NEP 1	NEP 1	NEP 1	NEP 1				

After reading the plate, the data interpretation was performed with the software.



The standard curve was valid for all the criteria.

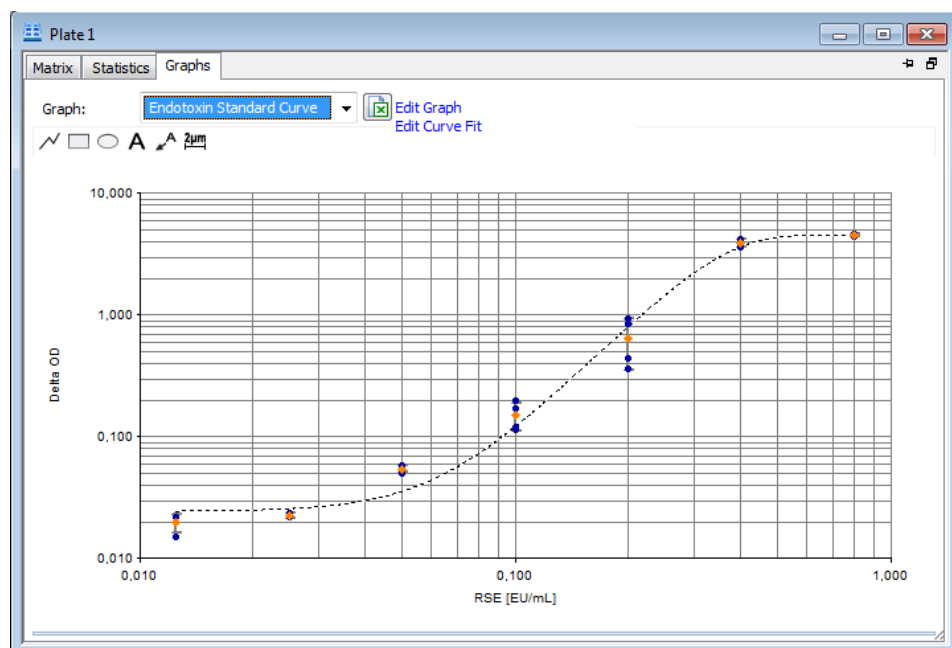


Plate 1

Matrix Statistics Graphs

Data: Assurance of criteria for the Endotox Edit Table Well Type: Blank

Effect of Dose Criteria	Goodness of Fit Criteria	BLK Delta OD Criteria	LOD Criteria
VALID	VALID	VALID	VALID

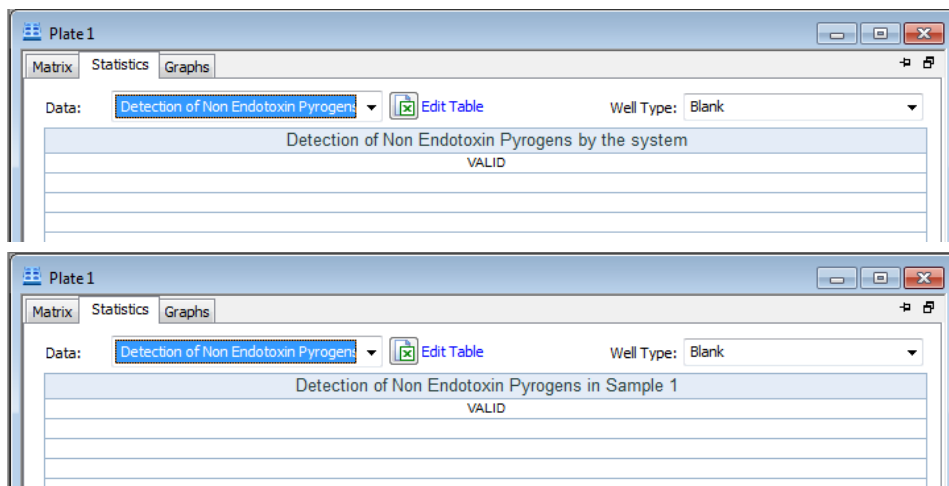
Plate 1

Matrix Statistics Graphs

Data: Additional Criteria (Not required by E) Edit Table Well Type: Blank

Minimum of reactivity
CONFORM

The NEP-control confirmed detection of non-endotoxin pyrogens in the system and in the sample.



The chosen sample dilutions were appropriate for the sample with all tested dilutions being within the MVD and spike recovery within 50-200%.

The data analysis showed validity of the test being fulfilled for all sample dilutions.

The sample itself showed a pyrogenic load of <0.5 EEU*/mL which is below the CLC (3.5 EU/mL) and therefore being considered "not pyrogenic".

* EEU: Endotoxin Equivalent Unit

Well ID	Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]	[EEU/mL] x DIL	Spike Rec. (%)	CONCLU...
SPL1	albumin	10	70,0	0,017	0,021	36,9	3,5	<0,500	143,6	PASS
		10		0,018						
		10		0,033						
		10		0,017						
		20	70,0	0,021	0,019	6,5	3,5	<1,000	125,4	PASS
		20		0,019						
		20		0,018						
		20		0,019						
		40	70,0	0,018	0,021	27,5	3,5	<2,000	116,1	PASS
		40		0,030						
		40		0,018						
		40		0,019						

Results

The capability of the MAT for the detection of Pyrogens in albumin preparations is described in several studies [5,6,7].

The examined albumin preparation showed an enhancement of the reaction of the Monocytes in the undiluted sample that could be overcome by dilution within the authorized dilution range (not exceeding the MVD).

The data show that PyroMAT® system is suitable for detecting both endotoxin and non-endotoxin pyrogenic contaminations in preparations of albumin.

References

- [1] European Pharmacopoeia, chapter Monocyte activation test (2.6.30)
- [2] Tullis JL: Albumin 2. Guidelines for Clinical Use. JAMA 1977; 237:460-463
- [3] Sandle T "Pharmaceutical Product Impurities: Considering Beta Glucans" (2013) americanpharmaceuticalreview
- [4] Pardo-Ruiz Z1, Menéndez-Sardiñas DE2, Pacios-Michelena A1, Gabilondo-Ramírez T2, Montero-Alejo V1, Perdomo-Morales R3. "Soluble β -(1,3)-glucans enhance LPS-induced response in the monocyte activation test, but inhibit LPS-mediated febrile response in rabbits: Implications for pyrogenicity tests" Eur J Pharm Sci. 2016 Jan 1;81:18-26
- [5] Spreizer I. "Advanced Pyrogen Testing" (2017) americanpharmaceuticalreview
- [6] Spreizer I., Fischer M., Hartzsch K., Luderitz-Puchel U., Montag T. „Comparative study of rabbit pyrogen test and human whole blood assay on human serum albumin." Altex. 19 Suppl 1/02, 73-75
- [7] Perdomo-Morales R., Pardo-Ruiz Z., Spreitzer I, Lagarto A, Montag T. "Monocyte Activation Test (MAT) Reliably Detects Pyrogens in Parenteral Formulations of Human Serum Albumin" Altex 28, 3/11, 227-235.

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