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Application Note

Detection of pyrogens in a vaccine preparation 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 MonoMac 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

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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 ± 20% of optical density.

Sample specifications: Meningococcal group C conjugate vaccine

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe, its toxins or one of its surface proteins. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of the microorganisms associated with that agent that it later encounters.

Vaccines include a variety of ingredients including antigens, stabilizers, adjuvants, antibiotics, and preservatives. They may also contain residual by-products from the production process. Knowing precisely what is in each vaccine can be helpful when investigating and minimizing adverse events following immunization.

Due to the production process and the composition, vaccines may contain free endotoxin or other pyrogenic substances. Endotoxin may serve as an adjuvant and support the stimulation of the immune system or the antigen may have a pyrogenic effect itself [2,3]. For the release of some vaccines the rabbit pyrogen test (RPT) is still in use while for other products the bacterial endotoxin test (BET) was implemented.

However, both tests have their disadvantages. The RPT is only able to give a qualitative result, while the BET does not detect non-endotoxin pyrogens and is not capable to give any information about interactions and synergetic effects or the pyrogenic activity of the found endotoxin. Additionally, both methods base on animals or animal product and therefore counter the principles of the 3Rs (Replacement, Reduction and Refinement) regarding animal welfare.

The suitability of the MAT test for detection of pyrogens in vaccine products was shown in other studies before. [3,4,5,6,7]

The examined meningococcal group C conjugate vaccine is a liquid preparation of purified capsular polysaccharide derived from Neisseria meningitidis Group C, linked to tetanus toxoid as a carrier and adsorbed to aluminium hydroxide as adjuvants.

For the estimation of the MVD of the product to be tested, the threshold for bacterial endotoxins for the product is 25 IU, corresponding to a pyrogenic load of 25 EU, per single human dose [8].

The single bolus dose of the product is a volume of 0.5 mL. Therefore, the CLC of this product was calculated as

$$CLC = \frac{K}{M} = \frac{25\ EU/dose}{\frac{0.5\ mL}{dose}} = \frac{25\ EU}{0.5\ mL} = 50\ EU/mL$$

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

$$MVD = \frac{50\ EU/mL \times 1}{0.05\ EU/mL} = 1000$$

Product specific validation for testing meningococcal group C conjugate vaccine 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 (NEP) control was not detectable in the undiluted product. From an 1:5 dilution to the MVD all dilutions showed detection of the NEP control.

The undiluted product was found to show interference with the detection of the endotoxin spike, the reaction was fully inhibited. From an 1:30 dilution to the MVD, the product reproducibly showed both detection of the NEP and an endotoxin 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 1:30 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.

Testing of meningococcal group C conjugate vaccine 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.

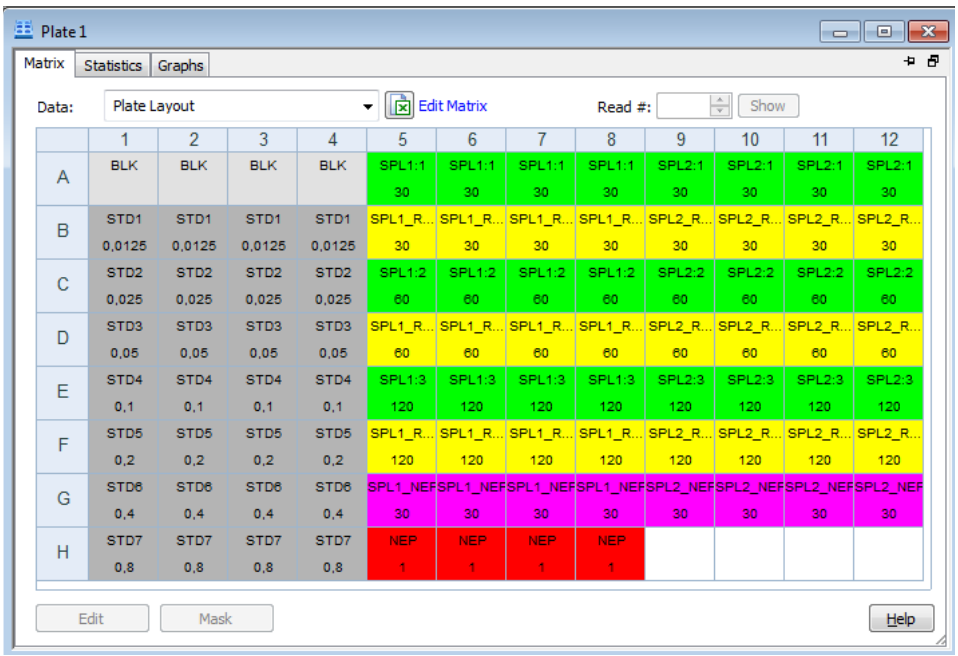
Three dilutions of the product were tested according to method A described in EP: The dilutions 1:30, 1:60 and 1:120 of the sample were all tested with and without endotoxin spike. A quantification of the found endotoxin equivalents for all dilutions of the unspiked and spiked sample was done using the 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.

Data interpretation

The data analysis was performed with Gen5 software and the PyroMAT™ Method A protocol 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.



	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	BLK	SPL1:1 30	SPL1:1 30	SPL1:1 30	SPL1:1 30	SPL2:1 30	SPL2:1 30	SPL2:1 30	SPL2:1 30
B	STD1 0,0125	STD1 0,0125	STD1 0,0125	STD1 0,0125	SPL1_R... 30	SPL1_R... 30	SPL1_R... 30	SPL1_R... 30	SPL2_R... 30	SPL2_R... 30	SPL2_R... 30	SPL2_R... 30
C	STD2 0,025	STD2 0,025	STD2 0,025	STD2 0,025	SPL1:2 60	SPL1:2 60	SPL1:2 60	SPL1:2 60	SPL2:2 60	SPL2:2 60	SPL2:2 60	SPL2:2 60
D	STD3 0,05	STD3 0,05	STD3 0,05	STD3 0,05	SPL1_R... 60	SPL1_R... 60	SPL1_R... 60	SPL1_R... 60	SPL2_R... 60	SPL2_R... 60	SPL2_R... 60	SPL2_R... 60
E	STD4 0,1	STD4 0,1	STD4 0,1	STD4 0,1	SPL1:3 120	SPL1:3 120	SPL1:3 120	SPL1:3 120	SPL2:3 120	SPL2:3 120	SPL2:3 120	SPL2:3 120
F	STD5 0,2	STD5 0,2	STD5 0,2	STD5 0,2	SPL1_R... 120	SPL1_R... 120	SPL1_R... 120	SPL1_R... 120	SPL2_R... 120	SPL2_R... 120	SPL2_R... 120	SPL2_R... 120
G	STD6 0,4	STD6 0,4	STD6 0,4	STD6 0,4	SPL1_NEF 30	SPL1_NEF 30	SPL1_NEF 30	SPL1_NEF 30	SPL2_NEF 30	SPL2_NEF 30	SPL2_NEF 30	SPL2_NEF 30
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.

Plate 1

Matrix | Statistics | Graphs

Data: Raw Data - Delta OD Summary (1/2) | Edit Matrix | Read #: | Show

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	BLK	SPL1:1	SPL1:1	SPL1:1	SPL1:1	SPL2:1	SPL2:1	SPL2:1	SPL2:1
	0,014	0,014	0,014	0,014	0,017	0,014	0,013	0,015	0,000	0,000	0,000	0,000
B	STD1	STD1	STD1	STD1	SPL1_R...	SPL1_R...	SPL1_R...	SPL1_R...	SPL2_R...	SPL2_R...	SPL2_R...	SPL2_R...
	0,015	0,018	0,015	0,014	0,715	0,707	0,810	0,531	0,000	0,000	0,000	0,000
C	STD2	STD2	STD2	STD2	SPL1:2	SPL1:2	SPL1:2	SPL1:2	SPL2:2	SPL2:2	SPL2:2	SPL2:2
	0,018	0,017	0,018	0,026	0,012	0,012	0,013	0,012	0,000	0,000	0,000	0,000
D	STD3	STD3	STD3	STD3	SPL1_R...	SPL1_R...	SPL1_R...	SPL1_R...	SPL2_R...	SPL2_R...	SPL2_R...	SPL2_R...
	0,038	0,047	0,038	0,042	1,716	1,112	1,585	0,759	0,000	0,000	0,000	0,000
E	STD4	STD4	STD4	STD4	SPL1:3	SPL1:3	SPL1:3	SPL1:3	SPL2:3	SPL2:3	SPL2:3	SPL2:3
	0,161	0,141	0,195	0,124	0,013	0,012	0,013	0,015	0,000	0,000	0,000	0,000
F	STD5	STD5	STD5	STD5	SPL1_R...	SPL1_R...	SPL1_R...	SPL1_R...	SPL2_R...	SPL2_R...	SPL2_R...	SPL2_R...
	0,934	1,439	1,541	0,662	1,430	2,130	1,850	2,139	0,000	0,000	0,000	0,000
G	STD6	STD6	STD6	STD6	SPL1_NEF	SPL1_NEF	SPL1_NEF	SPL1_NEF	SPL2_NEF	SPL2_NEF	SPL2_NEF	SPL2_NEF
	3,820	3,669	3,761	3,799	3,351	3,469	3,501	3,429	0,000	0,000	0,000	0,000
H	STD7	STD7	STD7	STD7	NEP	NEP	NEP	NEP				
	4,355	4,499	4,314	4,470	2,099	2,959	3,299	2,715				

Edit | Mask | Help

The standard curve was valid for all the criteria.

Plate 1

Matrix | Statistics | Graphs

Graph: Endotoxin Standard Curve | Edit Graph | Edit Curve Fit

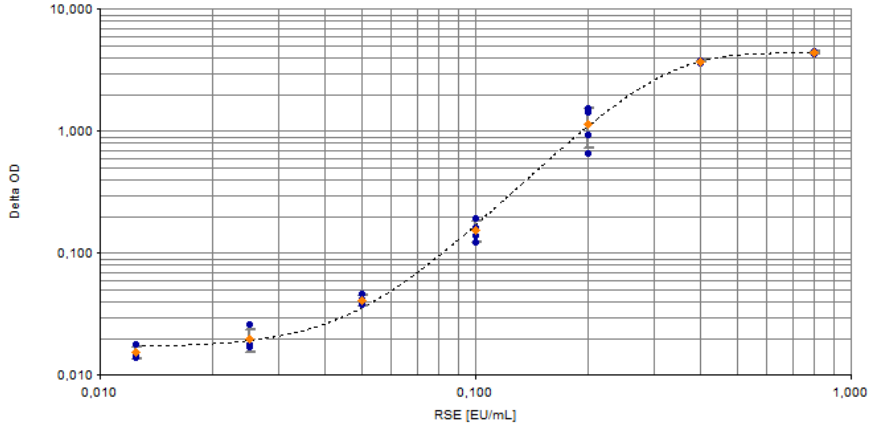


Plate 1

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

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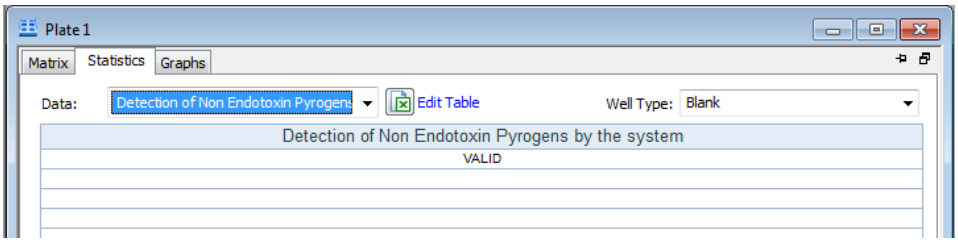
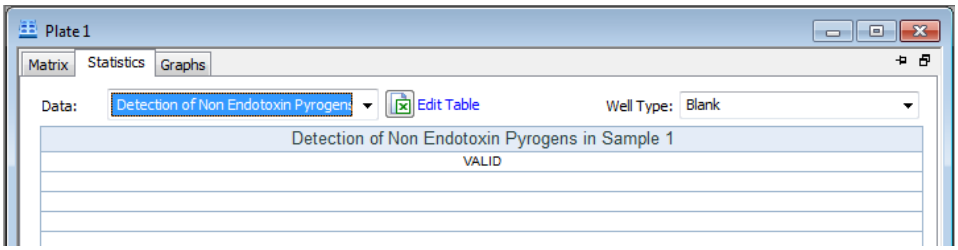
The NEP-control confirmed detection of non-endotoxin pyrogens in the system and in the sample.



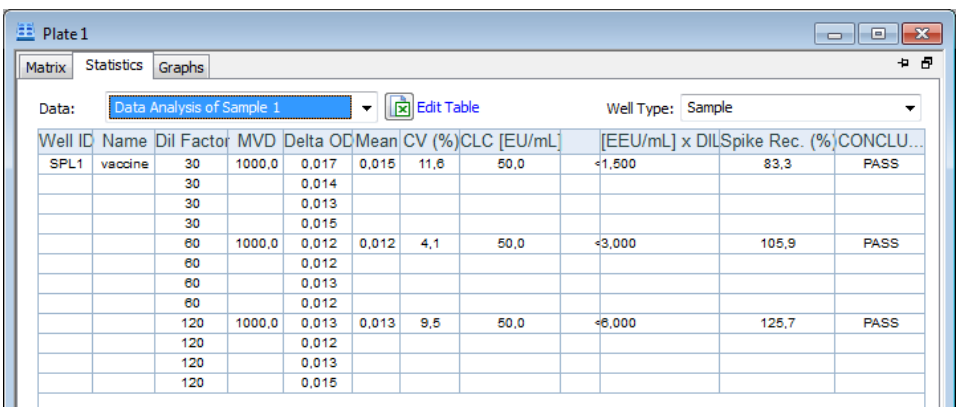


The chosen sample dilutions were appropriate for the sample with all tested dilutions being within the MVD and spike recovery within 50-200%.

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

The sample itself showed a pyrogenic load of <1.5 EEU*/mL which is below the CLC (50 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	vaccine	30	1000,0	0,017	0,015	11,6	50,0	<	1,500	83,3	PASS
		30		0,014							
		30		0,013							
		30		0,015							
		60	1000,0	0,012	0,012	4,1	50,0	<	3,000	105,9	PASS
		60		0,012							
		60		0,013							
		60		0,012							
		120	1000,0	0,013	0,013	9,5	50,0	<	6,000	125,7	PASS
		120		0,012							
		120		0,013							
		120		0,015							

Results

The capability of the MAT for the detection of pyrogens in the tested meningococcal group C conjugate vaccine preparation was shown and is comparable to the evaluation on the use of the MAT for testing vaccines described in other studies [4,5,6,7].

The examined vaccine preparation showed inhibition 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 the vaccine.

SPEAK TO A SPECIALIST

References

[1] European Pharmacopoeia, chapter Monocyte activation test (2.6.30)

[2] Williams K. "The Emerging View of Endotoxin as an IIRMI" (2016) BioPharm International Volume 28, Issue 2, pg 24–31

[3] Carlin G., Viitanen E. "*In vitro* pyrogenicity of the diphtheria, tetanus and acellular pertussis components of a trivalent vaccine". Vaccine 23 (2005), 3709–3715

[4] Kaaijk P, van Straaten I. van de Waterbeemd D et al. „Preclinical safety and immunogenicity evaluation of a nonavalent PorA native outer membrane vesicle vaccine against serogroup B meningococcal disease." Vaccine 31 (2013), 1065-1071

[5] Stoddard, M. B., Pinto, V., Keiser, P. B. et al. "Evaluation of a whole-blood cytokine release assay for use in measuring endotoxin activity of group B Neisseria meningitidis vaccines made from lipid A acylation mutants." (2010) Clin Vaccine Immunol 17, 98-107. http://dx.doi.org/10.1128/CVI.00342-09

[6] Vipond C, Findlay L, Feavers I, Care R. "Limitations of the Rabbit Pyrogen Test for Assessing Meningococcal OMV Based Vaccines" Altex 33(1), 2016, 47-53

[7] Nordgren K. "Evaluation of the MAT for the safety testing of meningococcal B vaccine Bexsero: a collaborative study" PharmaLab Congress 2017

[8] European Pharmacopoeia, Meningococcal group C conjugate vaccine (2112)

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Now is the TIME!

Pyrogen Detection:
detect endotoxins and non-endotoxin pyrogens in one non animal-based method

SigmaAldrich.com/PyroMAT

Used to detect a broad range of pyrogens in parenteral products including pharmaceuticals, biopharmaceuticals or medical devices, the monocyte activation test (MAT) provides an in vitro alternative to conventional animal testing methods in accordance with regulatory guidelines.

With **our new Mono-Mac-6 cell line-based MAT,** we provide a robust, reliable and standardized method for pyrogen detection.

PyroMAT™ System
All the advantages of the monocyte activation test combined with the benefits of using a cell line.

The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.

Pyrogen Detection:
Standardize your method, meet regulations & save rabbits

Technical library and more information on **SigmaAldrich.com/pyromat**

Webinar:
in vitro pyrogen testing, the new trend

Want to know more about in vitro pyrogen testing? Discover the benefits of the ready-to-use Monocyte Activation Test kit, learn how to use it and go through a **real-life case study**.

Watch it on:
SigmaAldrich.com/pyromat-webinar1

Webinar:
Monocyte Activation Test: a powerful tool to assess pyrogenic risk in pharmaceutical process

Microbial risk in pharmaceutical process cannot be limited to viable microorganisms. Learn how the MAT can help you to **assess the pyrogenic risk** resulting from your production process.

Watch it on:
SigmaAldrich.com/pyromat-webinar2

Video:
PyroMAT™ step by step process

How to use the PyroMAT™ system to test for endotoxin and non-endotoxin pyrogens.

Watch it on:
SigmaAldrich.com/video-pyromat

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MilliporeSigma
400 Summit Dr.
Burlington, MA 01803

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