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Application Note

Detection of pyrogens in hormone-based drugs with the PyroMAT® System

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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 = Lipopolysaccharide), which is produced by Gram-negative bacteria. Other microbial substances include those derived from Gram-positive bacteria such as lipoteichoic acid (LTA) or, 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 carry out a pyrogen test?

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

For health and safety reasons, health authority agencies are required to ensure the absence of pyrogenic substances in injectable drugs. Currently, the most frequently used tests are the rabbit pyrogen test (RPT) and/or the bacterial endotoxin test (BET) 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 of giving any information about interactions and synergetic effects or the pyrogenic activity of the found endotoxin. Additionally, both methods are based on animals or animal products and therefore counter the principles of the 3Rs (Replacement, Reduction and Refinement) regarding animal welfare.

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

The monocyte activation test (MAT) method was qualified and validated for the detection of pyrogens by the European Center for the Validation of Alternative Methods (ECVAM) in 2005 and also by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in 2008.[1]

It has been among the compendial methods for pyrogen detection in the European Pharmacopeia since 2010 (Chapter 2.6.30).[2]

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>[3,4] 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.”[5,6]

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).[7,8]

By putting the product to be tested in contact with human monocytic cells, it mimics what happens in the human body: in presence of pyrogens, the monocytes are activated and produce cytokines such as Interleukin-1 and Interleukin-6.[9]

The cytokines are then detected using an immunological assay (ELISA) involving specific antibodies and an enzymatic color reaction.[10]

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 the MM6 cell supernatant during the incubation phase are transferred in the ELISA plate, and are 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 molecule, 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 is 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 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 should be less than the CLC (Contaminant Limit Concentration) to pass the test.

To ensure both the accuracy 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

Product Specific Validation (PSV) for hormone drugs

Prerequisites – 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.

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

The MVD of a test solution is 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 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 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 is 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 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 contaminant causing positive responses in the rabbit pyrogens test or adverse drug reactions in man can be used. Where those batches are not available, validation should be done including at least 2 non-endotoxin ligands such as Flagellin or Heat Killed *Staphylococcus aureus* (HKSA) 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 for the preparation being examined is to be within ± 20% of optical density.

Sample specifications: hormone-based drugs

For definition a hormone is any signaling molecule produced by glands in multicellular organisms that are transported by the circulatory system that target distant organs to regulate physiology and behavior[11]. Hormones are used to communicate between organs and tissues for physiological regulation and behavioral activities, such as digestion, metabolism, respiration, tissue function, sensory perception, sleep, excretion, lactation, stress, growth and development, movement, reproduction, and mood.[12]

Selected hormones, their function and the effect of their deficiency in the human body are listed below. There are several pathologies characterized by hormone deficiency and consequently, related hormone drug therapies (HT) have been developed to treat these gland malfunctions.[13]

Human Growth hormone (hGH)

Human Growth hormone (hGH) stimulates growth and cell reproduction and regeneration. hGH is naturally released by the anterior pituitary gland, a pea-sized gland located at the base of the skull. Adequate level of hGH, is one of the essential factors for growth in children. Children with Growth Hormone Deficiency (GHD) do not produce enough of hGH, which has a high impact on the natural physical development. Symptoms include failure to meet height and weight growth standards. Consequently, hGH and its synthetic version, called somatropin, are used to treat this kind of disorder. Injections of somatropin can help to increase the growth rates.[14]

Human Chorionic Gonadotropin (hCG)

Human chorionic gonadotropin (hCG) hormone supports the normal development of an egg in a woman's ovary to stimulate the egg release during ovulation.[15] This hormone is produced by the placenta after implantation. The presence of hCG is detected in some pregnancy tests. hCG interacts with the LHCG receptor of the ovary and promotes the maintenance of the corpus luteum during the beginning of pregnancy. This allows the corpus luteum to secrete the hormone progesterone during the first trimester. Progesterone enriches the uterus with a thick lining of blood vessels and capillaries so that it can sustain the growing fetus. Recombinant Human Chorionic Gonadotropin (r-hCG) is the synthetic version of hCG hormone administered to stimulate ovulation, treat infertility in women and increase sperm count in men. Moreover, in case of pituitary gland disorder, r-hCG is also used in young boys when their testicles have not dropped down into the scrotum normally.

Human Follicle-Stimulating hormone (hFSH)

Follicle-stimulating hormone (FSH), also known as follitropin, is a glycoprotein gonadotropin secreted by the anterior pituitary in response to gonadotropin-releasing hormone released by the hypothalamus. hFSH regulates ovulation, the growth and development of eggs in a woman's ovaries.

In males hFSH stimulates primary spermatocytes to form secondary spermatocytes.[16] Low level of FSH secretion can result in failure of gonadal function (hypogonadism). This condition is typically manifested in males as failure in production of normal numbers of spermatozoon, and in females as cessation of reproductive cycles. The recombinant and synthetic form of hFSH (r-hFSH) is used to treat infertility in women who cannot ovulate and to stimulate sperm production in men. r-hFHS is often used in combination with another hormone, called human chorionic gonadotropin (hCG).[17]

Human Luteinizing hormone (hLH)

Luteinizing hormone (LH), also known as lutropin, is a hormone produced by gonadotropic cells in the anterior pituitary gland.[18] In females, an acute rise of LH triggers ovulation and development of the corpus luteum. In males LH stimulates Leydig cell production of testosterone.

LH acts synergistically with FSH. LH deficiency frequently occurs in conjunction with follicle-stimulating hormone (FSH) deficiency because LH and FSH are secreted by the same pituitary gonadotrope cells. LH deficiency can manifest in females or males as delayed puberty, hypogonadism at any age, or reproductive abnormalities that can be dramatic or subtle. Lutropin alpha (hLH) is used as fertility medication to help follicles (eggs) in the ovaries to develop and mature. It is used in combination with follitropin alpha (hFSH) when a women's pituitary gland does not produce enough hLH.[19]

In the present study all the suitability tests for pyrogen detection performed with the PyroMAT® System on a selection of injectable hormone drugs are described. The hormone drugs selected are summarized below:

Name	Abbreviation	Tissue	Effect
Growth hormone	hGH	Anterior pituitary gland	Stimulates growth and cell reproduction
Human chorionic gonadotropin	hCG	Placenta	Promotes maintenance of corpus luteum during beginning of pregnancy
Follicle-stimulating hormone	hFSH	Anterior pituitary gland	In female: stimulates maturation of Graafian follicles in the ovary
Luteinizing hormone	hLH	Anterior pituitary gland	In female: ovulation In male: stimulates Leydig cell

Low Endotoxin Recovery (LER) phenomenon is well known in the Bacterial Endotoxin test (BET) as the inability of the assay to detect lipopolysaccharide (LPS), due to a “masking effect” caused by chelators or detergents commonly used in buffer formulations for medical products and recombinant proteins.[20] In the presence of LER effect, the masked LPS is considered a potential danger, as it may pose a health threat in pharmaceutical products or compromise experimental results.[21,22, 23]

Consequently, with the aim to verify that in the final drug formulation, in addition to the active ingredient, the excipients (such as proteins, surfactants, essential amino acids, salts and preservatives) do not interfere with the pyrogen detection giving the LER effect[24] endotoxin, a group of drug substances (active ingredients) and drug products (final formulation containing excipients) were tested using the PyroMAT® System.

The drug substances and drug products tested in the present study were:

- hGH drug substance
- hCG drug substance
- hCG drug product
- hFSH drug substance
- hFSH drug product
- hLH drug substance
- hLH drug product

PSV plate layout

All the Product Specific Validation tests have been processed using the following plate layout scheme. The data analysis was performed with the Gen5™ ver3.03 software commercialized by BioTek, and the PyroMAT_PSV_I_II_III_A protocol that can be downloaded for free on our website.

The tested dilutions are specific for each drug and are described in the following sections.

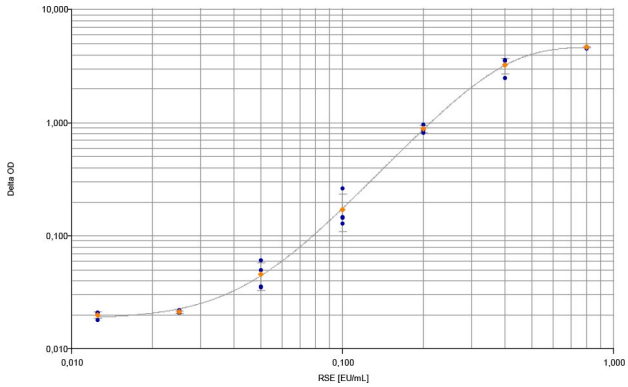
	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK	BLK	BLK	BLK	SPL1:1	SPL1:1	SPL1:1	SPL1:1	SPL1:3	SPL1:3	SPL1:3	SPL1:3	Well ID
					100	100	100	100	400	400	400	400	Conc/Dil
B	STD1	STD1	STD1	STD1	SPL1_RSE:1	SPL1_RSE:1	SPL1_RSE:1	SPL1_RSE:1	SPL1_RSE:3	SPL1_RSE:3	SPL1_RSE:3	SPL1_RSE:3	Well ID
	0,0125	0,0125	0,0125	0,0125	100	100	100	100	400	400	400	400	Conc/Dil
C	STD2	STD2	STD2	STD2	SPL1_NEP1:1	SPL1_NEP1:1	SPL1_NEP1:1	SPL1_NEP1:1	SPL1_NEP1:3	SPL1_NEP1:3	SPL1_NEP1:3	SPL1_NEP1:3	Well ID
	0,025	0,025	0,025	0,025	100	100	100	100	400	400	400	400	Conc/Dil
D	STD3	STD3	STD3	STD3	SPL1:2	SPL1:2	SPL1:2	SPL1:2	SPL1:4	SPL1:4	SPL1:4	SPL1:4	Well ID
	0,05	0,05	0,05	0,05	200	200	200	200	800	800	800	800	Conc/Dil
E	STD4	STD4	STD4	STD4	SPL1_RSE:2	SPL1_RSE:2	SPL1_RSE:2	SPL1_RSE:2	SPL1_RSE:4	SPL1_RSE:4	SPL1_RSE:4	SPL1_RSE:4	Well ID
	0,1	0,1	0,1	0,1	200	200	200	200	800	800	800	800	Conc/Dil
F	STD5	STD5	STD5	STD5	SPL1_NEP1:2	SPL1_NEP1:2	SPL1_NEP1:2	SPL1_NEP1:2	SPL1_NEP1:4	SPL1_NEP1:4	SPL1_NEP1:4	SPL1_NEP1:4	Well ID
	0,2	0,2	0,2	0,2	200	200	200	200	800	800	800	800	Conc/Dil
G	STD6	STD6	STD6	STD6	NEP1	NEP1	NEP1	NEP1					Well ID
	0,4	0,4	0,4	0,4	1	1	1	1					Conc/Dil
H	STD7	STD7	STD7	STD7	NEP2	NEP2	NEP2	NEP2					Well ID
	0,8	0,8	0,8	0,8	1	1	1	1					Conc/Dil

Figure 1. Example of plate layout for PSV Method A

Assurance of the criteria for the endotoxin standard curve:

For all the following PSV tests a standard curve using Reference Standard Endotoxin (RSE) was performed to verify that the criteria for the 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 ($p > 0.05$)



RSE standard curve validity crieria	Effect of Dose	Goodness of fit
	Valid	Valid
	Blank delta OD	**LOD**
	Valid	Valid

Figure 2. Example of Endotoxin Standard Curve obtained with the PyroMAT® System

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

A test for interfering factors and method validation for non-endotoxin pyrogens (NEPs) according to EP was performed, comparing endotoxin-spiked sample dilutions as well as NEP-spiked sample dilutions with the same unspiked sample dilutions.

PSV for hGH drug product

The hGH drug product Maximum Valid Dilution (MVD) calculation is reported in the table below.

Sample	Molecular Name	CLC (EU/mL)	PyroMAT® Limit of Detection (EU/mL)	MVD
hGH Drug Product	Somatropin	≤40	0.05	800

The experiment on hGH drug product was carried out using the dilutions 1:100, 1:200, 1:400 and 1:800. In order to demonstrate the method robustness, tests were carried out using two different PyroMAT® Cell lots.

The tested dilution range showed valid NEP control detection and RSE spike recoveries.

		Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve Assay 1 – 2 – 3		Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system Assay 1 - 2 -3		Valid			
		Dil 1:100	**Dil 1:200**	**Dil 1:400**	**Dil 1:800**
Results EEU* / mL Assay 1 – 2 – 3		< 5.00	<10.00	<20.00	<40.00
RSE Spike Recovery %	Assay 1	92.3%	68.5%	77.0%	92.2%
	Assay 2	83.2%	72.5%	78.5%	87.5%
	Assay 3	106.0%	88.9%	93.0%	93.4%
HKSA Detection in the sample Assay 1 – 2 – 3		Valid	Valid	Valid	Valid

*Endotoxin Equivalent Units

In summary, all the results coming from the three PSV experiments were consistent and valid. To verify if the pure sample could give interference with the PyroMAT® test, a fourth PSV experiment was carried out using a series of lower dilutions (1:1, 1:25, 1:50 and 1:100).

Data results are showed below:

Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Dil. Factor <= MVD	Spike Rec. (%)	Nep Detection	Conclusion
hGH -DP	1	800	0.009	0.009	6.8	40	<	0.05	Valid	0	Invalid	Invalid
	1		0.009									
	1		0.008									
	1		0.008									
	25	800	0.016	0.017	9	40	<	1.25	Valid	73.6	Valid	Valid
	25		0.018									
	25		0.018									
	25		0.015									
	50	800	0.016	0.016	6.5	40	<	2.5	Valid	97.3	Valid	Valid
	50		0.016									
	50		0.016									
	50		0.014									
	100	800	0.015	0.021	53.7	40	<	5	Valid	86.6	Valid	Valid
	100		0.015									
	100		0.037									
	100		0.015									

The undiluted product (1:1) showed interference with the endotoxin spike (RSE) detection:

- Spike Recovery % = 0

Moreover, the non-endotoxin pyrogen (NEP) control was not detectable in the undiluted product:

- NEP Detection = INVALID

From 1:25 dilution to the MVD, all dilutions showed again normal NEP and RSE controls detection.

In the end, to confirm the lowest working dilution, a fifth experiment was carried out using the following dilution series: 1:10, 1:25, 1:50 and 1:100.

Data and results are shown below:

Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Dil. Factor <= MVD	Spike Rec. (%)	Nep Detection	Conclusion
hGH -DP	10	800	0.015	0.015	3.4	40	<	0.5	Valid	74.5	Valid	Valid
	10		0.015									
	10		0.015									
	10		0.014									
	25	800	0.016	0.015	6.9	40	<	1.25	Valid	97.1	Valid	Valid
	25		0.014									
	25		0.014									
	25		0.014									
	50	800	0.014	0.014	0	40	<	2.5	Valid	77.7	Valid	Valid
	50		0.014									
	50		0.014									
	50		0.014									
	100	800	0.015	0.015	6.9	40	<	5	Valid	74.5	Valid	Valid
	100		0.015									
	100		0.013									
	100		0.015									

In the last experiment, the 1:10 dilution gave valid results for both endotoxin spike recovery and non-endotoxin pyrogen (NEP) control detection.

Test for interference in the detection system:

The defined optimum dilution (Dil 1:10) was forwarded to a test for interference in the detection system (ELISA). 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:

In conclusion, a total of six different experiments were carried out on hGH drug product. The results were valid and in accordance to the defined acceptance criteria. Moreover the tests results showed that the PyroMAT® system is applicable for detection of pyrogens in hGH drug product.

All the tested dilutions are reported in the table below:

Dilution Tested								
DP	1:1	1:10	1:25	1:50	1:100	1:200	1:400	1:800
	INVALID	VALID	VALID	VALID	VALID	VALID	VALID	VALID

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 acceptance criteria for the product specific validation were fulfilled and the dilution 1:10 of the drug product was chosen as the first valid dilution to be tested in routine with Method A.

PSV for hCG drug substance and drug product

The hCG drug substance and drug product Maximum Valid Dilutions (MVD) calculation to be tested are reported in the table below.

Sample	Molecular Name	CLC (EU/mL)	PyroMAT® Limit of Detection (EU/mL)	MVD
hCG drug substance	Choriogonadotropin alfa	≤ 28	0.05	560
hCG drug products	Choriogonadotropin alfa	≤ 30	0.05	600

For hCG drug substance, the experiment was carried out using the dilutions 1:10, 1:20, 1:40 and 1:400.

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:10	**Dil 1:20**	**Dil 1:40**	**Dil 1:400**
Results EEU / mL	<0.50	<1.00	<2.00	<20.00
RSE Spike Recovery %	106.0%	103.4%	87.2%	81.0%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

All tested dilutions were valid with detection of NEP controls and RSE spike recoveries within the range of 50-200%, as defined in the acceptance criteria.

With the aim to verify that the excipients present in the final drug formulation do not interfere with the pyrogen detection, the related hCG drug product was also tested, using dilutions 1:8, 1:16, 1:32 and 1:600.

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:8	**Dil 1:16**	**Dil 1:32**	**Dil 1:600**
Results EEU / mL	<0.40	<0.80	<1.60	<30.00
RSE Spike Recovery %	93.7%	98.3%	82.7%	80.0%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

The experiment performed on the drug product confirmed that all tested dilutions, from 1:8 dilution to 1:600, were valid and NEP and RSE controls were correctly detected.

Conclusion:

In conclusion, the tests performed on the hCG drug substance and drug product were valid and in accordance with the defined acceptance criteria. Moreover the test results showed that the PyroMAT® system is applicable to detect pyrogens in hCG drug substance (DS) and drug product (DP).

Dilution Tested				
DP	1:8	1:16	1:32	1:600
	VALID	VALID	VALID	VALID
DS	1:10	1:20	1:40	1:400
	VALID	VALID	VALID	VALID

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 acceptance criteria for the product specific validation were fulfilled and the dilution 1:8 of the drug product was chosen as the first valid dilution to be tested in routine with Method A.

PSV for hFSH drug substance and drug product

The hFSH drug substance and drug product maximum valid dilution (MVD) calculation is reported in the table below.

Sample	Molecular Name	CLC (EU/mL)	PyroMAT® Limit of Detection (EU/mL)	MVD
hFSH drug substance	Follitropin alpha	≤1	0.05	20
hFSH drug products	Follitropin alpha	≤12	0.05	240

The experiment on hFSH drug substance was carried out using the dilutions 1:1, 1:2, 1:10 and 1:20. In order to demonstrate the method robustness, tests were carried out using two different PyroMAT® Cell lots.

The tested dilution range showed valid NEP control detection and RSE spike recoveries.

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:1	**Dil 1:2**	**Dil 1:10**	**Dil 1:20**
Results EEU / mL	<0.05	<0.10	<0.50	<1.00
RSE Spike Recovery %	90.3%	93.1%	89.5%	95.3%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

From the pure drug substance (1:1) to the MVD, all dilutions showed valid NEP and RSE controls detection.

With the aim to verify that the excipients present in the final drug formulation do not interfere with the pyrogen detection, the related hFSH drug product was tested using dilutions 1:8, 1:16, 1:32 and 1:240.

		Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve Assay 1 – 2 – 3		Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system Assay 1 - 2 -3		Valid			
		Dil 1:8	**Dil 1:16**	**Dil 1:32**	**Dil 1:240**
Results EEU* / mL Assay 1 – 2 – 3		<0.40	<0.80	<1.60	<12.0
RSE Spike Recovery %	**Assay 1**	68.2%	73.5%	83.0%	87.5%
	Assay 2	73.8%	80.0%	63.2%	69.5%
	Assay 3	75.3%	85.2%	73.3%	76.8%
HKSA Detection in the sample Assay 1 – 2 – 3		Valid	Valid	Valid	Valid

Summarizing, all the results coming from the three PSV experiments were consistent and valid. The experiment performed on the pure hFSH drug substance (1:1) showed valid results for the endotoxin spike recovery and for the non-endotoxin pyrogen (NEP) controls detection.

For hFSH drug product experiments, starting from 1:8 dilution up to the MVD, all conditions showed valid NEP and RSE controls results.

Test for interference in the detection system:

The defined optimum dilution (Dil 1:8) 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:

In conclusion, a total of four different PSV tests were performed on hFSH drug substance and drug product. The results were valid and in accordance with the defined acceptance criteria. Moreover the test results show that the PyroMAT® System is applicable to detect pyrogens in hFSH drug substance (DS) and drug product (DP).

The tested dilutions are reported in the table below:

Dilution Tested				
DP	1:8	1:16	1:32	1:240
	VALID	VALID	VALID	VALID
DS	1:1	1:2	1:10	1:20
	VALID	VALID	VALID	VALID

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 acceptance criteria for the product specific validation were fulfilled and the dilution 1:8 of the drug product was chosen as the first valid dilution to be tested in routine with Method A.

PSV for hLH drug substance and drug product

The hLH drug substance and drug product maximum valid dilutions (MVD) calculation are reported in the table below.

Sample	Molecular Name	CLC (EU/mL)	PyroMAT® Limit of Detection (EU/mL)	MVD
hLH drug substance	Lutropin Alfa	≤ 100	0.05	2000
hLH drug products	Lutropin Alfa	≤ 8	0.05	160

The experiment on hLH drug substance was carried out using the dilutions 1:10, 1:20, 1:40 and 1:160.

The tested dilution range showed valid NEP control detection and RSE spike recoveries.

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:10	**Dil 1:20**	**Dil 1:40**	**Dil 1:160**
Results EEU / mL	<0.50	<1.00	<2.00	<8.00
RSE Spike Recovery %	85.4%	96.8%	101.3%	105.5%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

All tested dilutions were valid and able to detect NEP and RSE controls within the range of 50-200%, as defined in the acceptance criteria.

With the aim to verify that the excipients present in the final drug formulation, do not interfere with the pyrogen detection the related hLH drug product was tested, using the same experimental conditions (Dilutions 1:10, 1:20, 1:40 and 1:160).

The hLH drug product results are showed below:

	Effect of Dose	Goodness of fit	BLK delta OD	LOD
RSE standard curve	Valid	Valid	Valid	Valid
	Dil 1:10	**Dil 1:20**	**Dil 1:40**	**Dil 1:160**
Results EEU / mL	<0.50	<1.00	<2.00	<8.00
RSE Spike Recovery %	100.3%	95.2%	111.4%	101.0%
HKSA Detection in the sample	Valid	Valid	Valid	Valid
HKSA 1X & Flagellin 1X detection by the system	Valid			

All tested dilutions (1:10, 1:20, 1:40 and 1:160) were valid and showed normal NEP control detection and RSE spike recoveries.

Conclusion:

In conclusion, the performed tests on hLH drug substance and drug product were valid and in accordance to the defined acceptance criteria. Moreover the test results showed that the PyroMAT® System is applicable for detection of pyrogens in hLH drug substance (DS) and drug product (DP).

Dilution Tested				
DS/DP	1:10	1:20	1:40	1:160
	VALID	VALID	VALID	VALID

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 acceptance criteria for the product specific validation were fulfilled and the dilution 1:10 of the drug product was chosen as the first valid dilution to be tested with Method A.

Quantitative Pyrogen Test with 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 the same drug 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 preferred, giving the most exact results, while quantification within the upper plateau of the standard curve can lead to inaccuracy, this is due to reaching the endpoint of the reaction and is therefore not recommended.

For the most exact results, we therefore recommend analyzing sample dilutions which do not exceed the measuring range of 0.05 to 0.4 EU/mL.

Testing of r-hFSH and r-hGH drug products 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 r-hFSH and r-hGH drug products were tested according to method A described in Eu. Ph. 2.6.30.

The dilutions 1:8, 1:16 and 1:32 of r-hFSH drug product and dilutions 1:10, 1:20 and 1:40 of r-hGH drug product 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 carried out 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, spiked in the highest concentration of the product to be examined.

Data interpretation

The data analysis was performed with Gen5™ software version 3.03 and the PyroMAT® Software 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 filled with the appropriate dilution factors for this sample matrix.

					r-hFSH DP				r-hGH DP				
	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK	BLK	BLK	BLK	SPL1:1 8	SPL1:1 8	SPL1:1 8	SPL1:1 8	SPL2:1 10	SPL2:1 10	SPL2:1 10	SPL2:1 10	Well ID Conc/Dil
B	STD1 0,0125	STD1 0,0125	STD1 0,0125	STD1 0,0125	SPL1_RSE:1 8	SPL1_RSE:1 8	SPL1_RSE:1 8	SPL1_RSE:1 8	SPL2_RSE:1 10	SPL2_RSE:1 10	SPL2_RSE:1 10	SPL2_RSE:1 10	Well ID Conc/Dil
C	STD2 0,025	STD2 0,025	STD2 0,025	STD2 0,025	SPL1:2 16	SPL1:2 16	SPL1:2 16	SPL1:2 16	SPL2:2 20	SPL2:2 20	SPL2:2 20	SPL2:2 20	Well ID Conc/Dil
D	STD3 0,05	STD3 0,05	STD3 0,05	STD3 0,05	SPL1_RSE:2 16	SPL1_RSE:2 16	SPL1_RSE:2 16	SPL1_RSE:2 16	SPL2_RSE:2 20	SPL2_RSE:2 20	SPL2_RSE:2 20	SPL2_RSE:2 20	Well ID Conc/Dil
E	STD4 0,1	STD4 0,1	STD4 0,1	STD4 0,1	SPL1:3 32	SPL1:3 32	SPL1:3 32	SPL1:3 32	SPL2:3 40	SPL2:3 40	SPL2:3 40	SPL2:3 40	Well ID Conc/Dil
F	STD5 0,2	STD5 0,2	STD5 0,2	STD5 0,2	SPL1_RSE:3 32	SPL1_RSE:3 32	SPL1_RSE:3 32	SPL1_RSE:3 32	SPL2_RSE:3 40	SPL2_RSE:3 40	SPL2_RSE:3 40	SPL2_RSE:3 40	Well ID Conc/Dil
G	STD6 0,4	STD6 0,4	STD6 0,4	STD6 0,4	SPL1_NEP 8	SPL1_NEP 8	SPL1_NEP 8	SPL1_NEP 8	SPL2_NEP 10	SPL2_NEP 10	SPL2_NEP 10	SPL2_NEP 10	Well ID Conc/Dil
H	STD7 0,8	STD7 0,8	STD7 0,8	STD7 0,8	NEP 1	NEP 1	NEP 1	NEP 1					Well ID Conc/Dil

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

The standard curve was valid for all the criteria.

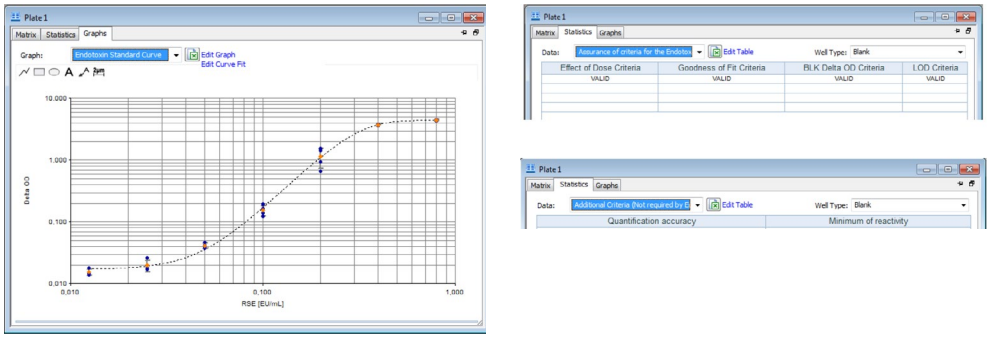


Figure 3. Standard curve obtained with PyroMAT® Software - Method A

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

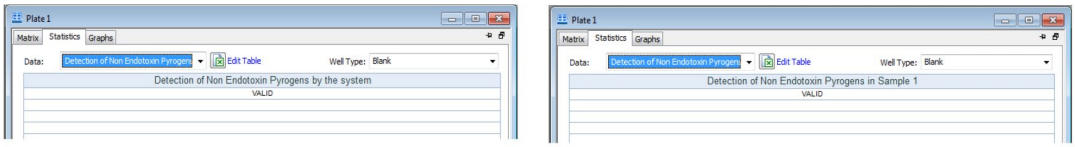


Figure 4. Result of NEP control detection in water and spike in the sample obtained with PyroMAT® Software - Method A

The chosen sample dilutions were appropriate for both samples 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 r-hFSH sample itself showed a pyrogenicity <0.4 EU/mL that is below the CLC (12 EU/mL) and therefore being considered “not pyrogenic”.

Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Spike Rec. (%)	Conclusion
r-hFSH DP	8	240	0.014	0.013	30.1	12	<	0.4	64.2	PASS
	8		0.018							
	8		0.009							
	8		0.011							
	16	240	0.013	0.015	15	12	<	0.8	66.3	PASS
	16		0.018							
	16		0.014							
	16		0.014							
	32	240	0.013	0.013	4.6	12	<	1.6	70.3	PASS
	32		0.013							
	32		0.012							
	32		0.012							

The r-hGH sample itself showed a pyrogenicity <0.5 EU/mL that is below the CLC (40 EU/mL) and therefore being considered “not pyrogenic”.

Name	Dil Factor	MVD	Delta OD	Mean	CV (%)	CLC [EU/mL]		[EEU/mL] x DIL	Spike Rec. (%)	Conclusion
r-hGH DP	10	800	0.014	0.014	11.2	40	<	0.5	70.5	PASS
	10		0.012							
	10		*0.159*							
	10		0.015							
	20	800	*0.047*	0.014	7.1	40	<	1	82.5	PASS
	16		0.014							
	16		0.013							
	16		0.015							
	32	800	0.015	0.023	49.8	40	<	2	75	PASS
	32		0.03							
	32		0.012							
	32		0.036							

NOTE: The numeric values reported among asterisks *nnn* were considered outlier and not taken into account for the final calculation.

Conclusion

The capability of PyroMAT® System to detect pyrogens in hormone drug substances and drug products was shown and is comparable to the results from the MAT system evaluation on drugs from other laboratories.[25,26,27]

The data shows that the PyroMAT® System is suitable for detecting both endotoxin and non-endotoxin pyrogenic contaminations in drug products. Moreover, plate repetitions carried out using two different PyroMAT® cell lots, increasing experiment variability, demonstrate that the test results are cell batch independent.

The examined recombinant hormones (drug products) occasionally led to inhibition of the monocyte reaction in the undiluted sample (see hGH drug product experiments), but this could be overcome by dilution within the authorized dilution range (not exceeding the MVD).

In conclusion, the PyroMAT® System is a valid system for pyrogens detection in hormonal pharmaceutical products.

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