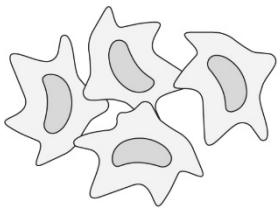
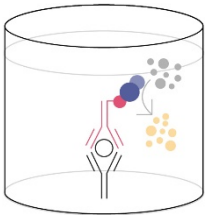
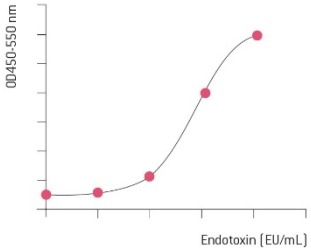
Lonza
Pharma & Biotech

PyroCell™ MAT System
Instructions for routine use

Monocyte Activation ▶ **IL-6 ELISA** ▶ **Analysis of results**







Lonza # : PyroCell™ MAT Kit: **00249735**
Lonza # : PeliKine compact human IL-6 ELISA kit: **00253325**

Revision A
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The PyroCell™ MAT Kit is a Lonza Bioscience product that includes pMAT Cells and MAT Culture Medium Supplement for the Monocyte Activation Test which are manufactured for Lonza Bioscience by Sanquin Reagents B.V.

The PeliKine compact human IL-6 kit, the PeliKine compact human IL-1 beta kit, and the PeliKine tool set 1 are manufactured by Sanquin Reagents B.V. and distributed by Lonza Bioscience for Monocyte Activation Test applications.

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The PyroCell™ MAT Kit has been designed and validated for reproducible detection of a variety of different pyrogens using IL-6 release as read-out for pyrogenic activity. Other cytokines may be suitable as read-out, but their use with the PyroCell™ MAT Kit would have to be validated by the user. A reference sample with a known concentration of a pyrogen (not included) may be used for quality control purposes.

pMAT Cells are found to be reactive towards the non-endotoxin pyrogens listed in the Certificate of Analysis (CoA). pMAT Cells are responsive to other non-endotoxin pyrogens, these have to be determined by the user.

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Lonza and Sanquin Reagents B.V. are not liable for any claims, including third party claims, due to or caused by pyrogenic activity in pharmaceutical products. Consult the relevant chapters in the European Pharmacopoeia (Ph. Eur. 2.6.30) as well as other documents relevant for your specific purpose for utilization of the test.

Do not use reagents beyond the expiry date. The expiry date is printed on the packaging or directly on the vial. **pMAT Cells can be used for up to 6 months from the date of shipment.**

Quality control

It is considered good laboratory practice to run laboratory samples according to instructions and specific recommendations included in the instructions for use of the test to be conducted. Failure to follow these instructions may result in erroneous test data.

Safety Data Sheets (SDS) are available from the Lonza product website.

Batch-specific Certificates of Analysis are available from the Lonza website, www.lonza.com/coa.

Regulatory information

The Monocyte Activation Test (MAT) is described in the European Pharmacopeia (Ph. Eur. chapter 2.6.30) as a compendial, *in vitro* test for the detection of pyrogens in pharmaceutical preparations. The MAT is therefore, suitable as a replacement test for the Rabbit Pyrogen Test (RPT) after a product-specific validation[1].

The MAT assay is recognized by other world pharmacopeias such as the United States Pharmacopeia[2], as an alternative method to the RPT. Alternative method validation may be required.[3] Please contact your local authorities for country-specific information.

Intended use statement

The PyroCell™ MAT Kit, consisting of cryopreserved human peripheral blood mononuclear cells (PBMC) pooled from four healthy donors (pMAT Cells), and MAT Culture Medium Supplement, is used as an *in vitro* assay for the detection of pyrogenic components in raw materials, intermediates, parenteral preparations or medical devices.

This PyroCell™ MAT Kit is for laboratory use only and not intended for diagnostic purpose, detection of pyrogens in man or animal, treatment of patients or patient management.

Product support & technical service

If your Lonza product fails to function properly, if you have questions about how to use or maintain our products, or if you need to send an instrument to Lonza for repair or other service, please contact our Scientific Support.

North America

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For technical support for countries outside of the USA or Europe, please contact your local Lonza sales office or your regional distributor. See contact information on back cover.

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Revision History

Rev	Date	Changes
A	2020	First issue

Document Conventions

	This icon identifies information that protects the **safety** of the operator and the integrity of data.
Warning!	A **Warning** indicates the potential for bodily harm and tells you how to avoid the problem.
Caution	A **Caution** indicates the potential for damage and tells you how to avoid the problem.

Note:	**Bold text** is primarily used for emphasis.
	This icon calls attention to **important** information.

Warnings and precautions

NOTE: All reagents described in this manual are intended for research use only.

Warning!	Not suitable for in *vitro* diagnostics or medical use.
Warning!	Not intended to detect pyrogens in man or animal or for diagnosis of human or animal diseases.
Warning!	Not intended for patient management.
Warning!	Components of the PyroCell™ MAT Kit contain fetal bovine serum (FBS) sourced in North America. The FBS component shall be handled in laboratory environments only.
Warning!	pMAT Cells are derived from human blood donations. Human blood used for preparation of pMAT Cells are tested for human infectious disease markers and are found to be negative. Please also refer to the CoA for the pMAT Cell lot. However, primary human cells cannot be assumed to be free from infectious agents and must be handled with appropriate care.
Caution	Handle all reagents according to Good Laboratory Practice (GLP) using appropriate precautions.
Caution	Apply aseptic and non-pyrogenic handling of the PyroCell™ MAT Kit. MAT assays shall be carried out by authorized and well-trained laboratory personnel only.
Caution	PyroCell™ MAT Kit components must be stored at ≤ -80°C under controlled conditions. Temperature excursion may impair the functionality of components. Thawed reagents must be used immediately and cannot be refrozen.
Caution	Do not use vials that show signs of temperature excursion such as ice crystals inside the vial or uneven surfaces. Please contact Lonza scientific support.
Caution	Do not use leaking or damaged vials.
Caution	Do not use vials of pMAT Cells where the medium color turned from pink to yellow.
Caution	Do not use reagents that show turbidity or other signs of microbial contamination.
Caution	**Expiry**. Do not use reagents beyond the expiry date. The expiry date is printed on the packaging or directly on the vial. **pMAT Cells can be used for up to 6 month from the date of shipment.**
Caution	**Disposal**. Dispose of reagent containers according to local regulations.
	Please refer to the Safety Data Sheet (SDS) for product safety information.

Abbreviations

Abbreviation	Description
BET	Bacterial Endotoxin Test
BRP	Biological Reference Preparation
CLC	Contaminant Limit Concentration
CO_2	Carbon dioxide
CoA	Certificate of Analysis
Complete IMDM	Iscove's Modified Dulbecco's Medium supplemented with MAT Culture Medium Supplement included in the PyroCell™ MAT Kit
ELISA	Enzyme-linked Immunosorbent Assay
Ph. Eur.	European Pharmacopeia
EU/mL	Endotoxin Units per Milliliter
EEU/mL	Endotoxin Equivalent Units per Milliliter
IL-6	Interleukin-6
IL-1beta	Interleukin-1 beta
HRP	Horseradish peroxidase
IMDM	Iscove's Modified Dulbecco's Medium
IPA	Isopropyl Alcohol
LAL	Limulus Amebocyte Lysate
LOD	Limit of Detection
MAT	Monocyte Activation Test
MVD	Maximum Valid Dilution
mL, µL	Milliliter, Microliter
N/A	Not applicable
NEP	Non-Endotoxin Pyrogens
NM	Nanometer
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cells
Pcs	Pieces
Ph. Eur.	European Pharmacopeia
pMAT Cells	Pooled, cryopreserved PBMC qualified for the MAT assay
RPT	Rabbit Pyrogen Test
RSE	Reference Standard Endotoxin
SDS	Safety Data Sheet

Table of Contents

1. Background – Monocyte Activation Test (MAT)

Pyrogenic substances in parenterally administered pharmaceutical products can lead to fever, and life-threatening reactions in patients. In order to ensure patient safety, it is required by regulatory agencies to demonstrate that parenteral preparations do not contain harmful levels of pyrogens. Pyrogenic substances can originate from microorganisms such as bacteria or fungi, from viruses, or from non-organic sources such as chemicals and primary packaging materials. The most potent pyrogens are endotoxins derived from gram negative bacteria. All pyrogens that are not endotoxins are summarized under the name non-endotoxin pyrogens (NEP). The purpose of a pyrogen test is to demonstrate that the amount of pyrogenic contaminants contained in a preparation is below the contaminant limit concentration (CLC) in order to release the product as being safe. Different tests have been acknowledged by regulatory agencies and are available to determine whether a product or test preparation is free from pyrogenic contaminants (Fig. 1).

		Test type	Mechanism	Identification
Pyrogen		**Monocyte Activation Test (MAT)** Ph. Eur. 2.6.30, USP <1085>	*In vitro*: measures inflammatory cytokines released by human blood monocytes	Bacterial endotoxins and non-endotoxin pyrogens (e.g. bacteria, viruses, yeasts and molds)
		**Rabbit Pyrogen Test (RPT)** Ph. Eur. 2.6.8, USP <151>	*In vivo*: measures raise in body temperature after injection of a drug	
Endotoxin		**Recombinant Factor C Assay (rFC)** Ph. Eur. 2.6.32, USP <1085>	*In vitro*: recombinant alternative to LAL. Based on factor C, the first component of the LAL clotting cascade	Specific for bacterial endotoxin (gram-negative bacteria)
		**Limulus Amebocyte Lysate (LAL) Test (= BET Assay)** Ph. Eur. 2.6.14, USP <85>	*In vitro*: measures the initiation of a clotting cascade by horseshoe crab blood amebocytes	

Fig. 1: Compendial endotoxin and pyrogen detection methods

The **Monocyte Activation Test (MAT**) is an *in vitro* assay based on the detection of pro-inflammatory cytokines released by human blood monocytes upon stimulation with pyrogenic substances. The MAT assay is capable of detecting endotoxins as well as non-endotoxin pyrogens in raw materials, intermediates, process samples and manufactured biomedical products. The MAT assay has been qualified and validated by the European Center for the Validation of Alternative Methods (ECVAM) in 2005[2] and by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)[3] in 2008. It has been adopted by some main pharmacopeias, for example, the European Pharmacopeia (Ph. Eur. Chapter 2.6.30)[1] as a compendial method and is considered to be suitable, after a product-specific validation, to replace the rabbit pyrogen test (RPT)[1]. Other pharmacopeias are describing the MAT as an alternative method to the RPT. For example, the United States Pharmacopeia (USP) requests the MAT assay to be validated according to USP <1225>[4,5]. Please contact your local authorities for more information.

2. PyroCell™ MAT system – Assay principle

The PyroCell™ MAT system measures a response of the human innate immune system. Monocytes, the key cells of innate immunity, respond to the presence of pyrogens (endotoxin and non-endotoxin pyrogens) by secreting pro-inflammatory cytokines, which can be measured by Enzyme-Linked Immunosorbent Assays (ELISA). The pooled, cryopreserved MAT cells (pMAT Cells) consist of equal amounts of peripheral blood mononuclear cells (PBMC) prepared from blood donations of four healthy human individuals. At the day of the experiment, the pMAT Cells are thawed and incubated with the product or test samples in an overnight cell culture at 37°C in a humidified cell incubator. On the next day, the cell culture supernatants are harvested and the cytokine interleukin-6 (IL-6) released by the monocytes is detected with the PeliKine compact human IL-6 ELISA kit. Presence of IL-6 is then measured in an absorbance reader as optical density (OD). Using a standard curve generated with reference standard endotoxin (RSE), the OD values are finally converted into Endotoxin Equivalent Units (EEU/mL).

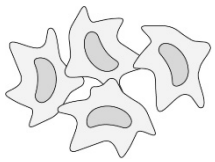
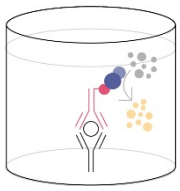
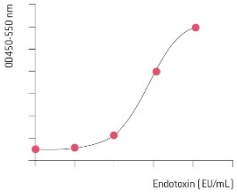
	Process	
Monocyte Activation	• Prepare endotoxin dilutions • Prepare test samples on the cell culture plate • Thaw pMAT Cells, transfer to plate. • Incubate for 18-24h	• See section 5.3 
IL-6 ELISA	• Overnight coating of the ELISA plate • Harvest the cell culture supernatant • Detect IL-6 cytokines with an IL-6 ELISA • Read results in a microplate absorbance reader	• See section 5.4 
Analysis	• Convert the measured OD values into concentrations (EEU/mL) • Determine the pyrogen content in the test sample	• See section 6 

Fig. 2: MAT assay overview

3. Material information and storage conditions

The PyroCell™ MAT system, IL-6 combines all reagents required to conduct the MAT assay in a ready-to-use kit (# 00254714). The following reagents are included: the PyroCell™ MAT Kit (# 00249735) and the PeliKine compact human IL-6 ELISA kit (# 00253325, comprising the PeliKine compact human IL-6 kit and the PeliKine tool set).

3.1 PyroCell™ MAT Kit

pMAT Cells are manufactured and qualified according to the compendial requirements of Ph. Eur. chapter 2.6.30. Each preparation of pMAT Cells is generated by pooling equal amounts of peripheral blood mononuclear cells (PBMC) isolated from four qualified blood donors, and subsequent cryopreservation of the pooled PBMC under controlled conditions (pMAT Cells). Please refer to the Certificate of Analysis (CoA) for more information on quality assurance.

To ensure traceability, each vial of pMAT Cells is labelled with two codes; the first code (68-XXXX) refers to the original production lot number (lot org) which can also be found on the CoA. The second code (P-XXX) refers to the individual vial. The lot org number of the MAT Culture Medium Supplement is printed on the vial and on the CoA.

3.1.1 Storage and stability

- **Important! Components of the PyroCell™ MAT Kit need to be stored at ≤ -80°C.** Temperature excursion will impair the functionality of the vial content.
- **Important!** pMAT Cells expiry is 6 months from the shipment date.
- **Important!** Components cannot be re-frozen after thawing and must be used immediately.
- Consult the CoA for additional information.

The MAT Culture Medium Supplement contains an antibiotics mix. Consult SDS for Product Safety Information.

3.1.2 Package content

Amount	Component	Closure	Volume	Lot-org #
3 vials	pMAT Cells	Green	3 x 1 mL	68-xxxx
3 vials	MAT Culture Medium Supplement	Yellow	3 x 2.1 mL	8000-xxxxxx

3.2 PeliKine compact human IL-6 ELISA kit

The PeliKine compact human IL-6 ELISA kit comprises two reagent kits, the PeliKine compact human IL-6 kit and the Pelikine tool set 1.

- Only use reagents and microtiter plates supplied with the PeliKine compact human IL-6 kit. Do not mix reagents from different lots.
- Sodium azide inactivates the horseradish peroxidase (HRP). Do not use solutions containing sodium azide, nor add sodium azide to the supplied materials.
- All reagents contains merthiolate (0.001% w/v).
- Mix all reagents thoroughly before use (avoid foaming).
- Briefly spin all vials prior to use to prevent reagent loss (1 min at 3000 x g).
- Do not allow wells to stand uncovered or dry for extended periods of time between incubation steps. Cover plate with adhesive seal.

3.2.1 PeliKine compact human IL-6 kit

The PeliKine compact human IL-6 kit is a "sandwich-type" enzyme immunoassay. The target cytokine IL-6 is bound to a pre-coated microtiter plate and is detected by an enzymatic reaction forming a colored product in proportion to the amount of cytokine present in the sample.

3.2.1.1 Storage and stability

Store the PeliKine compact human IL-6 kit at -18°C to -32°C. The expiration date is shown on the box label. Do not use reagents beyond the expiration date.

3.2.1.2 Package content

The PeliKine compact human IL-6 kit contains all material sufficient for 3 plates (288 tests). The following reagents are contained:

Amount	Component	SDS	Closure	Volume	Concentrate
1 vial	Coating antibody		Red	375 µL	100-fold
1 vial	Blocking reagent		Transparent	2 mL	50-fold
1 vial	Biotinylated antibody		Yellow	375 µL	100-fold
1 vial	Streptavidin-poly-HRP conjugate		Brown	20 µL	10,000-fold
1 vial	IL-6 standard		Black	750 µL	See label
1 bottle	HPE buffer	⚠	-	55 mL	55 mL
3 pcs	96-well microtiter plate with lid		-	-	-
10 pcs	Plate seals		-	-	-

3.2.1.3 Sensitivity

The assay sensitivity can be increased by using a horizontal plate shaker at 700 ± 100 rpm for all incubations including the enzymatic color development.

MEAN calculated zero signal + 3 SD: 0.2 – 0.4 pg/mL (shake – static incubation)
2x (MEAN calculated zero signal): 0.5 – 1.0 pg/mL (shake – static incubation)

3.2.1.4 Reference standard

The recombinant human IL-6 standard has been calibrated against the WHO International Standard (IL-6 89/548)[6]; National Institute for Biological Standards and Control, Potter Bar, Hertfordshire, U.K. 1 WHO Unit = 10 pg IL-6.

3.2.2 PeliKine tool set 1

The PeliKine tool set 1 is complementing the PeliKine compact human IL-6 kit by providing all chemicals and solutions used for the PeliKine compact human IL-6 ELISA: coating and washing buffers, as well as substrate and stop solutions.

- Only use reagents from one set. Do not mix reagents from different lots.
- Do not add any preservative to the reagents. Preservatives may impair the color development of the HRP substrate system.
- The washing buffer contains merthiolate (0.001% w/v). Handle with appropriate care.
- Avoid exposure of TMB substrate solution and stop solution to metals or metal ions to prevent unintended color formation.

3.2.2.1 Storage and stability

- Store the PeliKine tool set 1 at 2-8°C. The expiration date is shown on the box label. Do not use reagents beyond the expiration date.
- Store prepared buffers (working strength buffer) at 2-8°C in closed containers and use within time frame indicated in the table below. Bring the buffer to room temperature (18-25°C) prior to use.

Reagent (ready-to-use)	Storage	Application
Coating buffer	2-8°C, up to 1 week	18-25 °C
PBS solution	2-8°C, up to 1 week	18-25 °C
Washing buffer	2-8°C, up to 2 months	18-25 C

3.2.2.2 Package content

The PeliKine tool set 1 contains all materials sufficient for one PeliKine compact human IL-6 kit (288 tests, 3 plates). The following reagents are contained:

Amount	Component	SDS	Volume	Preparation
1 bottle	Coating buffer capsules		3 pieces	3 x 100 mL buffer
1 bottle	PBS tablets		3 pieces	3 x 200 mL buffer
2 bottles	Washing Buffer		2 x 50 mL	2 x 1 L buffer
1 bottle	TMB substrate solution		40 mL	Ready-to-use
1 bottle	Stop solution	⚠	40 mL	Ready-to-use

3.3 Further reagents and consumables

The following reagents have been optimized for use with the PyroCell™ MAT System kits. Alternative reagents may be used however, their qualification for the intended purpose is recommended.

Reagent	Volume	Cat. #
Iscove's Modified Dulbecco's Medium (IMDM)	500 mL	12-722F
Reference standard endotoxin (RSE), e.g. preparations from USP or EP (BRP)	10,000 U	E700 (USP)
Sterile, non-pyrogenic water (LAL water) for reconstitution of RSE	100 mL	W50-100
Distilled or deionized water for buffer preparation	1250 mL	N/A
(optional) Two relevant non-endotoxin pyrogens	N/A	N/A

The following standard laboratory consumables shall be appropriate for cell culture and endotoxin testing, i.e. they need to be sterile and non-pyrogenic:

Consumable	Volume	Cat. #
Borosilicate glass tubes	30 pcs/pack	N207
(optional) De-pyrogenated sample container	25 pcs/box	80-507U
Reservoirs	10 pcs/pack	00190035
96-well flat bottom microplates (cell culture)	50 pcs/pack	25-340
Pipette tips	as appropriate	See Catalog
Polystyrene disposable pipettes	as appropriate	See Catalog
96-well round bottom untreated microplates for harvesting the cell culture supernatant	as appropriate	See Catalog
Conical polypropylene centrifuge tubes, 50 mL	as appropriate	See Catalog

3.4 Laboratory equipment needed

The following equipment is required to conduct the assay.

Equipment	Step
Freezer (-80°C or lower, alarm setting)	Storage of PyroCell™ MAT Kit
Freezer (-20°C or lower)	Storage of PeliKine compact human IL-6 kit
Refrigerator (2-8°C)	Storage of PeliKine Tool Set 1 Storage of working strength buffer
Laminar airflow cabinet with HEPA filter	Aseptic preparation of cell culture plate
CO_2-incubator (humidified, 37°C, 5% CO_2)	Overnight incubation of cell culture plate
Water bath, 37°C	Controlled thawing of pMAT Cells
Vortex mixer	Preparation of Reference Standard Endotoxin (RSE) **Do not vortex dilutions containing complete medium and/ or pMAT Cells**
Adjustable multichannel pipettor (30-300 µL)	Preparation of cell culture and ELISA plate
Adjustable pipettors for accurate delivery of liquid volumes (diverse, 1-1000 µL)	Reagent preparation, preparation of the cell culture plate and ELISA plate
Beakers, flasks, cylinders	Preparation and storage of working strength buffer for ELISA
(optional) Device for delivery of washing buffer	Supports washing steps for the ELISA
(optional) Automated microplate washer	Supports washing steps for the ELISA
(optional) Microplate shaker	Increases sensitivity of the ELISA

Microplate reader, e.g. ELx808™ reader containing a 450nm filter and a 550nm (540-590 nm) reference filter	Cat. # 25-315S

3.6 Recommended literature

1. European Directorate for the Quality of Medicines (EDQM). *European Pharmacopeia chapter* 2.6.30: Monocyte Activation Test, Edition 9
2. European Centre for Validation of Alternative Methods (ECVAM), 2006: *In Vitro* Pyrogen Test using Human Whole Blood/IL-6. Test method validation report (TM 2002-05)
3. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), 2008: Validation Status of Five In Vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products. Test method evaluation report (NIH No. 08-6392)
4. United States Food and Drug Administration (FDA), 2012: Guidance for Industry Pyrogen and Endotoxins Testing: Questions and Answers
5. United States Pharmacopeia (USP), 2007: Validation of Compendial Procedures <1225>. Rockville, MD. USP 41–NF:1-5.
6. RE Gaines Das, S Poole: The International Standard for interleukin-6. Evaluation in an International Collaborative Study J Immunol Methods (1993) 160(2):147-53.
7. Helle M, Boeije L, de Groot E, de Vos A, Aarden L: Sensitive ELISA for interleukin-6. Detection of IL-6 in biological fluids: synovial fluids and sera. *J.Immunol.Methods.* 1991 Apr 8;138(1):47-56.
8. Solati S, Aarden L, Zeerleder S, Wouters D: An improved monocyte activation test using cryopreserved pooled human mononuclear cells. *Innate Immunity*. 2015 Oct;21(7):677-84.

4. Definitions and calculations

	• The terms cut-off value and LOD represent the same limit information but use different units. The cut-off value is expressed in the unit of response, i.e. optical density (OD) while the LOD is expressed as a concentration, e.g. EU/mL. • **Important!** Historical data of the PyroCell™ MAT Kit suggest an LOD ≤ 0.02 EU/mL if used with the PeliKine compact human IL-6 ELISA**.** • **Important!** Endotoxin concentrations stated throughout this user guide are given as concentrations per sample, i.e. prior to addition of additional (spiked) media and suspended pMAT Cells. The final concentration in the reaction well of the cell culture plate is half of the stated concentrations: The total reaction volume in a single well is 200 µL which consists of 100 µL sample, 50 µL of media or spiked media and 50 µL of pMAT Cells. • **Important!** Consult the Certificate of Analysis for additional information.

The MAT assay is used to demonstrate that the amount of pyrogenic contaminants in the product tested does not exceed the contaminant limit concentration (CLC). To assure that the CLC can be detected in the assay, it is necessary not to exceed the Maximum Valid Dilution (MVD) based on the limit of detection (LOD) of the test system.

4.1 Calculating the Maximum Valid Dilution (MVD) and the Contaminant Limit Concentration (CLC)

The Maximum Valid Dilution (MVD) is defined as the maximum allowable dilution of a product or test sample at which the contaminant limit can be determined.[1]

The MVD is calculated using the following expression:

$$MVD = \frac{\text{CLC x C}}{\text{LOD}}$$

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

The CLC is calculated using the following expression:

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

K = Threshold pyrogenic dose per kilogram of body mass (e.g. EU/kg)
M = Maximum recommended bolus dose of product per kilogram of body mass (e.g. mg/kg)

4.2 Calculating the Limit of Detection (LOD)

The LOD is determined by the mean value of the replicates of the responses to the blank and the endotoxin standard curve. The LOD is the concentration in Endotoxin Units per milliliter (EU/mL), corresponding to the cut-off value of the measured response (optical density, OD). The cut-off value is calculated using the following expression[1]:

$$cutoff\ value = x + (3s)$$

x = mean of the four replicates of the response of the blank
s = standard deviation of the four replicates of the responses to the blank

According to the European Pharmacopeia[1] each user has to establish the LOD in their laboratory based on the current experiment or historical data. Using the PyroCell™ MAT Kit combined with the PeliKine compact human IL-6 ELISA kit an LOD of < 0.02 EU/mL may be expected.

5. Test procedure

5.1 Use of qualified cells

pMAT Cells contained in the PyroCell™ MAT Kit consist of a pool of cryopreserved human peripheral blood mononuclear cells (PBMC) and serve as the monocytic cell source[1]. PBMCs are isolated from blood donations of healthy volunteers. The blood donors are qualified to satisfy the criteria described in Ph. Eur. chapter 2.6.30, section 5.3 prior to blood donation. All donations are tested and found negative for common infectious disease marker (see CoA). On the day of donation equal amounts of PBMC from four donors are pooled and cryopreserved in a controlled-rate freezer within a validated time frame. After thawing pMAT Cells are qualified with the PeliKine compact human IL-6 ELISA kit for conformance to section 5-5 of Ph. Eur. chapter 2.6.30 and to meet the criteria outlined in section 6-1 and 6-3 regarding the reactivity towards reference standard endotoxin (RSE) and non-endotoxin pyrogens (NEP). In addition, the averaging effect of pooling is considered by comparing the reactivity of the pool and each of the four individual donors towards endotoxin. For details on the individual cell lot please consult the respective certificate of analysis (CoA).

For monocyte activation the pMAT Cells are thawed on the day of the experiment and diluted in complete medium as stipulated below in order to achieve the optimal cell concentration per reaction well.

5.2 Preparatory testing - Choosing the right method

Before proceeding to routine testing of a product with MAT, preparatory testing as well as a product-specific validation should be performed.[1] Preparatory testing is required to confirm that the product to be examined does not interfere with the MAT system or that interference can be overcome by product dilutions not exceeding the MVD of the product. Further, it needs to be verified that both, endotoxin and non-endotoxin pyrogens (NEP) are detected. Finally, the testing of three manufactured product lots using the optimal product dilutions determined during preparatory testing confirms the choice of the appropriate MAT method (method A, B or C). For further information on data analysis please also refer to section 6.

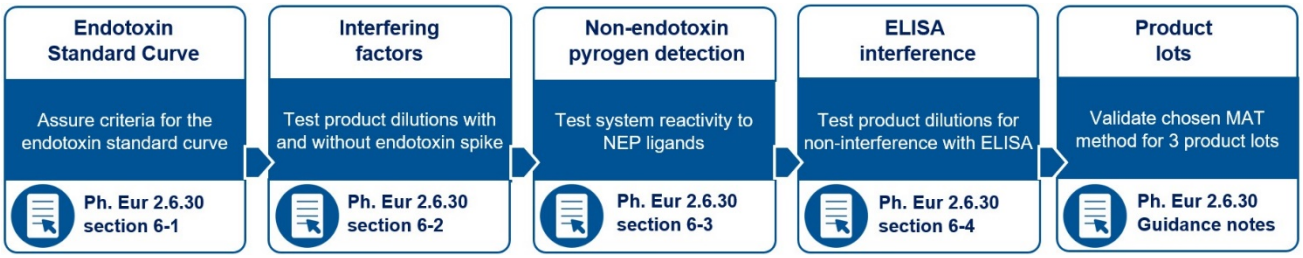


Fig. 3: Preparatory testing requirements

Test samples are compared to an endotoxin standard curve (≥ 4 concentrations) prepared from reference standard endotoxin (RSE, # E700), and a blank. Each endotoxin dilution, the blank, and product sample dilution is measured in 4 replicates.

Product dilution is recommended if a positive product control (PPC) cannot be recovered within the specified range of 50-200% of the spike.[1] However, all product dilutions shall not exceed the maximum valid dilution (MVD) for the product in order to ensure that the contaminant can still be detected at the CLC (contaminant limit concentration). The MVD is linked to the limit of detection (LOD) of the system. A more sensitive system allows for higher product dilutions. If product interferences cannot be overcome by dilution, method C (batch comparison) is recommended over methods A and B.

Determining the appropriate product dilution can be approached as follows:

- Prepare endotoxin dilutions for the endotoxin standard curve.
- Prepare geometric dilutions of the product with all dilutions not exceeding the MVD.
- Using the same product dilutions, generate a positive product control (PPC) by spiking each dilution with endotoxin at a concentration near the estimated middle of the standard curve, i.e. 0.04 EU/mL (method A) or twice the LOD (method B).
- Test both dilution series in the same experiment on the same microtiter plate.
- Calculate the mean recovery of the spiked endotoxin by subtracting the mean concentration of unspiked dilutions from spiked dilutions. Compare to the expected values by using the endotoxin standard curve to calculate the concentration.
- Determine the lowest dilution that shows a valid spike recovery.

Within the same experiment, evaluate the dilution series of the product to be parallel to the endotoxin standard curve. If this is not the case, use of method B is recommended.

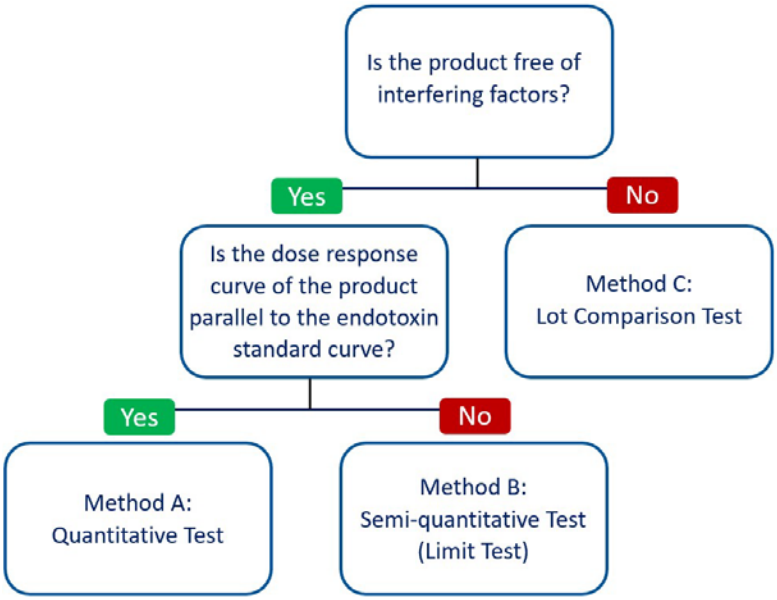


Fig 4: Simplified decision tree to support the choice of the MAT method.

Test the system reactivity towards NEP by using either a historic product batch that contains NEP contaminants (or elicited a positive response in the rabbit pyrogen test) or use at least two different NEP ligands for toll-like receptors that may reflect the most likely contaminants of the product. It is recommended to test commercial NEPs for absence of endotoxin prior to use. At least one relevant NEP ligand should be spiked into the lowest product dilution and recovered within a range of 50-200%

Once the lowest product dilution that is showing a valid spike recovery has been determined, interference of the product dilution with the chosen read-out system must be excluded.[1] OD values obtained in the IL-6 ELISA for an IL-6 standard dilution series in the presence and absence of the product preparation should not differ by more than 20%.

Based on the results from preparatory testing and the product-specific validation, the suitable method is selected for routine testing:

- Method A: Quantitative test
- Method B: Semi-quantitative test
- Method C: Reference lot comparison test

For Method C, the dilution of the product and the reference lot depends on the type of analysis used to make the comparison between the two and is determined by the user.

5.3 Routine testing - Monocyte activation

	• **Important!** Ensure aseptic handling of cells. Use sterile, endotoxin-free materials only. • **Important!** Monocyte Activation: Adhere to GCCP (good cell culture practices). Work in a laminar airflow cabinet, wear gloves, and clean the exterior of equipment and materials with disinfectant, e.g. 70% isopropyl alcohol (IPA) prior to use**.** • Adhere to GLP (good laboratory practices) for ELISA. An ELISA can be performed on a regular laboratory bench. • Refer to Ph. Eur., chapter 2.6.30 for description of MAT test methods.

Stimulation of pMAT cells with product is carried out by an overnight cell culture step at 37 ± 1°C in an appropriate atmosphere (5% CO_2, humidified air). The stimulation period shall allow for sufficient accumulation of the IL-6 cytokine and is typically achieved within 18-24h. The response to the product sample is then compared in an ELISA to the response towards RSE (method A or B), or to a reference lot of the same product (method C).

5.3.1 Preparation of complete medium

	• **Important!** Ensure aseptic handling of cells. Use sterile, endotoxin-free materials only. • Always use an unopened bottle of IMDM and prepare the complete medium shortly before the experiment. • **Important!** A minimum of 1 mL IMDM without MAT Culture Medium Supplement is needed for the first dilution of the reference standard endotoxin (RSE). • **Important!** Do not prepare the first RSE dilutions with complete medium as it may result in an inaccurate endotoxin standard curve.

The complete medium used for the pMAT cell incubation consists of IMDM supplemented with the MAT Culture Medium Supplement contained in the PyrCell™ MAT Kit.

1. Thaw the **MAT Culture Medium Supplement** vial in a water bath at 37 °C.
2. Add the entire vial content (2.1 mL) to a sterile, non-pyrogenic 50 mL tube containing 33 mL IMDM.

3. Mix gently by inverting the closed tube.
4. Equilibrate the complete medium to room temperature (18-25 °C).
5. Mix gently before use. **Use the complete medium within 8 hours**.

5.3.2 Preparation of endotoxin dilutions

- **Important!** Prepare all endotoxin dilutions shortly before the experiment.
- **Important!** Do not vortex samples containing complete medium. Prevent the formation of air bubbles.
- It is recommended to vortex the stock solution of the reference standard for 30 min before use.

Reference standard endotoxin dilutions (RSE, Lonza # E700) are used to determine the LOD of the test system and the amount of pyrogen in the test samples. Include reference endotoxin dilutions for each microplate.

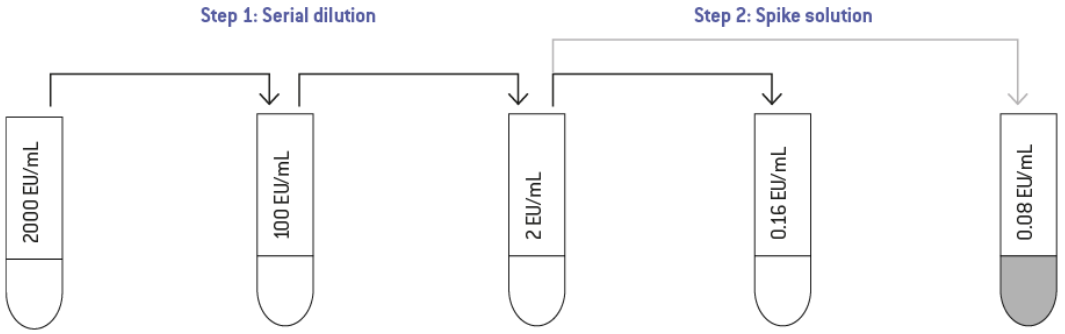
According to Ph. Eur. chapter 2.6.30[1] an appropriate endotoxin standard curve consists of 4 replicates of at least four geometrically diluted RSE concentrations and additionally, a blank (negative control). Based on the historical LOD of 0.02 EU/mL for the PyroCell™ MAT System we recommend a dilution series starting at 0.16 EU/mL with two-fold dilutions down to 0.01 EU/mL (0.16 – 0.08 – 0.04 – 0.02 – 0.01 EU/mL), and a blank. A suggested plate layout is illustrated in **fig. 5** below:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	0.16 EU/ml (R5)	0.08 EU/ml (R4)	0.04 EU/ml (R3)									
C												
D												
E												
F	0.02 EU/ml (R2)	0.01 EU/ml (R1)	0 EU/ml (R0)									
G												
H												

Fig. 5: Suggested plate layout (R0-R5, also see Ph. Eur. 2.6.30).

Prepare an endotoxin stock solution of 2,000 Endotoxin Unit (EU)/mL following the user instructions of the reference standard endotoxin. Aliquots of the stock solution can be stored frozen at -80°C for future use. An example for endotoxin dilutions to prepare an endotoxin standard curve with range of 0.01-0.16 EU/mL is given in **fig 6** below:

1. Add 5 mL of endotoxin-free LAL water to a vial of RSE.
2. Reconstitute RSE by vortexing for 30 min at maximum speed.
3. The resulting solution (2,000 EU/ mL) is used as a stock solution.
4. Endotoxin dilutions for one plate are prepared with 50 µL stock solution. Upon thawing vortex an aliquot for 3 min before use.



Aliquot RSE, 2,000 EU/ml in LAL water	50 µL RSE, (2,000 EU/mL) in 950 µL IMDM	20 µL RSE, (100 EU/mL) in 980 µL complete medium	80 µL RSE, (2 EU/mL) in 920 µL complete medium	80 µL RSE, (100 EU/mL) in 1920 µL complete medium
Upon thawing, vortex for 3 min	Vortex 1 min	Mix by pipetting up and down 10x	Mix by pipetting up and down 10x	Mix by pipetting up and down 10x
	100 EU/mL	**2 EU/mL**	**0.16 EU/mL**	**0.08 EU/mL**

Fig. 6: Serial dilution, prepare 0.16 EU/mL endotoxin dilution and 0.08 EU/mL spike solution.

Proceed with the preparation of endotoxin dilutions:

5. Add 950 µL of IMDM (WITHOUT MAT CULTURE MEDIUM SUPPLEMENT) to a sterile, non-pyrogenic borosilicate glass tube; add 50 µL of RSE stock solution (2000 EU/ mL) to reach a concentration of 100 EU/mL. Vortex thoroughly for 1 min.
6. Add 980 µL of **complete medium** to a sterile, non-pyrogenic borosilicate glass tube, add 20 µL of 100 EU/mL RSE dilution to reach a final concentration of 2 EU/mL. Mix thoroughly by pipetting up and down 10x, with a volume of at least 500 µL.

   Do not vortex samples containing complete medium and prevent the formation of air bubbles as this may decrease the cells' reactivity towards endotoxin.
7. Add 920 µL of **complete medium** to a sterile, non-pyrogenic borosilicate glass tube, add 80 µL of 2 EU/mL RSE dilution to reach a final concentration of 0.16 EU/mL. Mix thoroughly by pipetting up and down 10x, with a volume of at least 500 µL.
8. Add 200 µL of 0.16 EU/mL endotoxin to wells A1 to D1 (see fig 5).
9. Add 100 µL of **complete medium** to the other wells used for the standard curve (E1-H1, A2-H2 and A3-H3).

10. Make a two-fold dilution by pipetting 100 µL from R5 (0.16 EU/mL, A1-D1) to R4 (0.08 EU/mL, A2-D2) using a multi-channel pipettor (4 lines). Mix thoroughly by pipetting up and down 10x.

    Prevent the formation of air bubbles.

11. Repeat dilution as described in point 10 for transferring 100 µL from R4 to R3 (A3-D3), from R3 to R2 (E1-H1) and R2 to R1 (E2-H2). Discard the final 100 µL from R1.
12. Save the remaining 2 EU/mL RSE solution prepared at step 2 for preparation of endotoxin spiked samples (see section 5.3.3).

5.3.3 Preparation of product sample dilutions

	• **Important!** Prepare the product sample at the day of the experiment • Using the example layout below, 3 product preparations can be tested on a single plate
	• Standard curve and spike concentrations are based on a historical LOD of 0.02 EU/mL

For routine application of Method A (quantitative) and Method B (semi-quantitative) it is requested to fit the following preparations on the same 96-well plate: A fresh preparation of an endotoxin standard curve (method A) or endotoxin dilutions (method B) and up to three dilutions of a product preparation, each measured with and without an endotoxin spike. Each dilution is tested in 4 replicates suggesting 3 samples to fit on one plate. Plate layout examples for method A and B with one product sample is given below (fig. 7):

(A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.16 EU/ml (R5)	0.08 EU/ml (R4)	0.04 EU/ml (R3)	S1	S1-1/2	S1-1/4						
B												
C												
D												
E	0.02 EU/ml (R2)	0.01 EU/ml (R1)	blank (R0)	S1 + spike	S1-1/2 + spike	S1-1/4 + spike						
F												
G												
H												

(B)

	1	2	3	4	5	6	7	8	9	10	11	12
A	8x LOD (R5)	4x LOD (R4)	2x LOD (R3)	S1	S1-1/2	S1-1/4						
B												
C												
D												
E	LOD (R2)	0.5x LOD (R1)	blank (R0)	S1 + spike	S1-1/2 + spike	S1-1/4 + spike						
F												
G												
H												

Fig. 7: Suggested plate layout for the quantitative (A) or semi-quantitative (B) MAT method. Endotoxin reference standards (R0 – R5) with endotoxin concentrations in EU/mL (A) or LOD (B). Sample (S1) in 2 successive two-fold dilutions (S1-1/2 and S1-1/4) without and with spiked endotoxin. A spike concentration at the middle or the standard curve (0.04 EU/mL, method A) corresponds to 2x LOD (method B).

1. Add 200 µL of sample (S1) in wells A4 to H4 (see Figure 7).
2. Add 100 µL of complete medium to the other wells used for the sample dilutions.
3. Prepare a two-fold dilution by pipetting 100 µL from S1 (A4-H4) to S1-1/2 (A5-H5) and mix by pipetting up and down 10x. Prevent formation of air bubbles.
4. Repeat for transfer from S1-1/2 to S1-1/4 and discard the final 100 µL.
5. Add 50 µL of complete medium to endotoxin dilutions and samples without an endotoxin spike.
6. Transfer 1920 µL complete medium (see 5.3.2) in a sterile, non-pyrogenic borosilicate glass tube. Add 80 µL of 2 EU/mL endotoxin (see 5.1.4) to reach a final concentration of 0.08 EU/mL. Mix by pipetting up and down 10x, with a volume of at least 1 mL.

   Do not vortex! Prevent formation of air bubbles.
7. Add 50 µL of 0.08 EU/mL endotoxin to wells for spiked samples (marked with + spike). The endotoxin spike concentration in the sample corresponds to the 0.04 EU/mL value from the endotoxin standard curve.

5.3.4 Incubation of test samples with pMAT Cells

	• **Important!** Appropriate handling of cryopreserved cells is critical to maintain full functionality of the cells. • **Important!** Ensure aseptic handling of cells. Adhere to GCCP (good cell culture practices). Work in a laminar airflow cabinet, wear gloves, and clean the exterior of equipment and materials with disinfectant, e.g. 70% isopropyl alcohol (IPA) prior to use**.**

1. Take one vial of pMAT Cells from the -80°C freezer.

Immediately thaw the vial in a water bath at 37ºC until a small clump of ice remains visible (<5 min).

2. Transfer the entire contents of the vial (1 mL) into a 50 mL conical tube and immediately but slowly add 5 mL complete medium (equilibrated to room temperature) while gently swirling the tube (<5 min).

Do not vortex or vigorously pipet, take care not to form air bubbles.

3. Transfer mixture to a sterile reservoir. Immediately transfer 50 µL of the suspended pMAT Cells to each well containing prepared test dilutions.

4. Incubate the microplate with lid in a CO_2-incubator at 37°C (humidified air, 5% CO_2) for 18-24 hours.

5. On the next day, harvest 100-150 µL of the supernatant by carefully placing the pipette tip at the side of the well. Avoid turbulence to leave the pMAT Cells at the bottom of the plate. Transfer the supernatant to a fresh 96-well round bottom, untreated microplate

Only part of the supernatant is used for a routine ELISA (see section 5.2), the remaining supernatant can be used for further experiments or stored frozen at -20°C or -80°C for future experiments. The storage time of the frozen supernatant may be product-dependent and needs to be determined by the user.

5.4 Routine testing - IL-6 ELISA

- **Important!** The pMAT Cells have been validated using the PeliKine compact human IL-6 ELISA kit.
- **Important!** The optimal dilution of the cell culture supernatant may be specific for the test preparation. We recommend to start with a 5-fold dilution if using the recommended endotoxin standard curve.
- Generation of an IL-6 standard curve may be included as a quality control for the IL-6 ELISA but is not required to determine the pyrogenic activity of the sample in routine use.

The PeliKine compact human IL-6 ELISA kit is a "sandwich-type" enzyme immunoassay in which a monoclonal anti-IL-6 antibody is pre-coated to polystyrene microtiter wells. The IL-6 cytokines contained in the cell culture supernatant are bound by the IL-6 antibody on the microtiter plate. Unbound material is removed by washing. Subsequently, a biotinylated antibody is added. This antibody binds to the cytokine-antibody complex present in the microtiter well. Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the cytokine sandwich. After removal of unbound streptavidin-HRP conjugate by washing, a substrate solution is added to the wells. A colored product is formed in proportion to the amount of cytokine present in the sample or standard. After the reaction is terminated by the addition of a stop solution, the absorbance is measured in a microtiter plate reader. The absorbance (optical density, OD) of the reference endotoxin dilutions is then plotted against the concentration (EU/ mL) and resulting standard curve is used to calculate the concentration of the contaminant in the product samples (see section 6). The contaminant concentration is expressed in endotoxin equivalent units (EEU).

For more information PeliKine compact human IL-6 kit you may also refer to the package insert of the kit as well as to the original instructions for use for the PeliKine compact human IL-6 kit (Sanquin, # M1916) and PeliKine tool set 1 (Sanquin, # M1980).

5.4.1 Preparation of working strength buffers

- All buffers provided with the PeliKine tool set 1 are concentrates. Working strength buffers may be prepared prior to the day of experiment. For storage information of working strength buffers refer to section 3.2.2.1 “storage information”.
- All volumes stated refer to one ELISA plate. Coating of the supplied ELISA plate with coating antibody is ideally performed 1 day prior to conducting the ELISA**.**
- Substrate solution contains a mixture of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB). Protect the substrate solution from prolonged exposure to light.
- Protect TMB substrate solution and stop solution from contact with metal or metal ions. This may lead to unspecific color formation.
- The TMB substrate solution should be color-less. Do not use TMB substrate solution that appears blue. Replace solution.

Stop solution contains sulfuric acid solution (0.18 M) in water

Consult the SDS for Product Safety Information

Reagent	Buffer Preparation
Coating buffer	Dissolve **contents of one capsule** in 100 mL of distilled water. Incubate 5 min, mix contents **Do not dissolve or rinse the outer capsule as this will interfere with the functionality of the coating buffer**
PBS solution	Dissolve **one PBS tablet** in 200 mL of distilled water Incubate 5 min, mix contents
Washing buffer	Dissolve **50 mL of the concentrate** in 950 mL of distilled water
HPE buffer	Dissolve **15 mL of the concentrate** in 60 mL of distilled water, mix contents
TMB Substrate Solution	Ready-to-use
Stop Solution	Ready-to-use

5.4.2 Overnight coating of the microtiter plate (day 1)

	• **Important!** Equilibrate coating antibody and working strength coating buffer to 18-25°C prior to use

Preparation	Add to each wells	Incubation
• Prepare working strength coating buffer (see section 5.2.1) • Dilute 120 µL coating antibody (red cap) in 12 mL coating buffer. • Mix by inverting the tube 10 times.	• 100 µL of coating antibody solution • Cover plate with a lid	• Overnight at 18-25°C.

5.4.3 Prepare dilutions of the cell culture supernatants

	• Harvest the cell culture supernatant as described in section 5.3.4 • When using the PeliKine compact human IL-6 ELISA kit, a 5 fold supernatant dilution is recommended. The optimal dilution can vary between different test products and may be determined by the user • The protocol below refers to a 1:5 dilution of the cell culture supernatant

- Pipette 100 µL of HPE buffer into each well of a fresh, round bottom 96 well microtiter plate (= HPE plate)
- Homogenize the harvested supernatants by pipetting up and down 5 times. Add 25 µL of cell culture supernatant to the corresponding well on the HPE plate and mix by pipetting.

5.4.4 (Optional) Preparation of an IL-6 standard curve

	• Adding an IL-6 standard curve is requiring 1-2 “empty” columns on the microtiter plate

Preparation of an IL-6 standard curve is optional for routine MAT assays. The IL-6 standard curve will contain 450, 150, 50, 16.7, 5.6, 1.9, 0.6 and 0 pg/mL IL-6 in HPE buffer.

- Label 7 tubes, one tube for each dilution: 450, 150, 50, 16.7, 5.6, 1.9 and 0.6 pg/mL.
- Pipette 497 µL of working-strength HPE buffer into the tube for 450 pg/mL.
- Pipette 400 µL of working strength dilution buffer into all other tubes.
- Transfer 63 µL of the IL-6 standard (4000 pg/mL, black cap) into the first tube for 450 pg/mL. Mix well.
- Transfer 200 µL of this dilution into the second tube labelled 150 pg/mL.
- Repeat the serial dilution steps by adding 200 µL of the previous tube of diluted standard to the 400 µL of dilution buffer. Mix well.

5.4.5 ELISA incubation and wash steps

- Equilibrate all buffers and antibody dilutions to 18-25°C before use.
- Keep the streptavidin-HRP conjugate at -18 to -32°C to ensure stability. Thaw shortly before use
- Always empty wells completely before adding new solutions. A plate washer is recommended for all wash steps.
- Always mix contents in wells by tapping the edge of the microtiter plate for a few seconds. Alternatively, use a plate shaker at 700 ± 100 rpm. Use of a plate shaker may increase the sensitivity of the assay.
- In the buffer concentrate salt crystals may appear. Before preparing the working-strength buffer, briefly warm the buffer concentrate to 37°C in a water bath to dissolve any precipitates.
- Avoid repeated freeze-thawing of the IL-6 standard. Experimental data have shown that up to 3 freeze-thaw cycles have no effect on the IL-6 levels of the IL-6 standard.

Preparation (1 plate)	Add to each well	Incubation/ Wash
Continue from step 5.1.2 “coating of microtiter plate”		
Wash with PBS	≥ **300 µL** PBS (working strength)	4 times
Blocking • Dilute 500 µL blocking reagent (transparent cap) in 25 mL PBS • Mix by inverting the tube 10 times.	• Add 200 µL blocking buffer dilution • Cover plate with adhesive seal	1 hour at 18-25 °C **Prepare supernatant dilutions**

Preparation (1 plate)	Add to each well	Incubation/ Wash
Wash with washing buffer	≥ **300 µL** washing buffer (working strength)	5 times
Supernatant dilutions (1:5) • Pipette 100 µL of HPE buffer into each well of a fresh, round bottom 96 well microtiter plate (= HPE plate) • Homogenize the harvested supernatant by pipetting up and down 5 times. Add 25 µL to the HPE plate and mix by pipetting up and down.	• Add **100 µL** of supernatant dilutions • (Optional), add IL-6 standard dilutions (see section 5.2.4) • Cover plate with adhesive seal	1 hour at 18-25 °C **Prepare biotinylated antibody dilution immediately before wash step**
Wash with washing buffer	≥ **300 µL** washing buffer (working strength)	5 times
Biotinylated antibody • Dilute 120 µL biotinylated antibody (yellow cap) in 12 mL working-strength HPE dilution buffer. Mix by inverting tube 10 times	• Add 100 µL of biotinylated antibody dilution • Cover plate with adhesive seal	1 hour at 18-25 °C **Prepare streptavidin-HRP conjugate dilution immediately before wash step**
Wash with washing buffer	≥ 300 µL washing buffer (working strength)	5 times
Streptavidin-HRP conjugate • Dilute 1.2 µL streptavidin-HRP (brown cap) conjugate in 12 mL of working-strength HPE dilution buffer. Mix by inverting tube 10 times	• Add 100 µL of Streptavidin-HRP conjugate dilution • Cover plate with adhesive seal	30 min at 18-25 °C **Equilibrate TMB substrate solution and stop solution to 18-25°C before wash step**
Wash with washing buffer	≥ 300 µL washing buffer (working strength)	5 times
TMB substrate solution • Ready to use	• 100 µL TMB substrate solution (ready to use) • Cover plate with adhesive seal **Protect TMB substrate from exposure to light!**	10 min at 18-25 °C in the dark, e.g. use a drawer. **Do not cover plate with aluminum foil**!
Stop solution • Ready to use	Add 100 µL of stop solution	**The color is stable for max. 30 min**

5.5 Microtiter plate absorbance reading

	• The absorbance reader must be equipped with a 450 nm filter and a reference wavelength filter between 540 and 590 nm. We recommend the ELx808™ Absorbance Reader with a 450 nm filter and a 550 nm reference filter (Lonza # 25-315S) • If the substrate reaction appears too intense, reduce the incubation time. Do not dilute the TMB substrate.

- Place the microtiter plate in an ELISA reader and record absorbance (OD) at 450 nm, and at a reference wavelength (e.g. 550 nm)
- Subtract the reference wavelength measurement at 550 nm from the measurement at 450 nm before proceeding with further analysis of the ELISA data. For some software packages this may be performed automatically.

6 Analysis of results

	• The analysis and interpretation of MAT data depends on the chosen MAT method. Refer to Ph. Eur. chapter 2.6.30 for a detailed description of the three compendial MAT methods. • Any appropriate software available in-house or on-line may be used for the analysis of MAT data. • The software CombiStats, developed by the European Directorate for the Quality of Medicines and Healthcare (EDQM, www.edqm.eu) may assist you in statistical analysis according to the Ph. Eur chapter 5.3. • Lonza is offering an Excel-based MAT Analysis Tool to assist in the analysis of MAT data. Please contact Lonza scientific support for more information.

Preparatory testing of the product to be examined with the PyroCell™ MAT system is providing the basis for choosing the appropriate MAT method for testing and data analysis. Preparatory testing needs to be conducted to ensure that criteria for precision and validity of the test for the respective test method are met (see section 5.2).

An overview on how to assess the pyrogen content in a product sample is given below:

1. For each sample dilution, calculate the net absorbance values by subtracting the reference OD550 nm reading from the OD450 nm reading.
2. (optional) Remove outliers caused by documented deviations to work instructions, e.g. pipetting errors.
3. (optional) Apply a sound statistical method to identify outliers within each group of replicates (e.g. Dixon method with 95% Q-critical value).
4. Calculate the average OD value for each test replicate.
5. Perform a log transformation of the reference endotoxin (RSE) concentrations and the OD values.
6. Plot the OD values on the y-axis and the RSE values on the x-axis and perform a regression.
7. With the regression equation calculate the concentration of each product dilution. Alternatively, locate the average net value for each product dilution on the y-axis and follow a horizontal line intersecting the regression line. At the intersection read the endotoxin concentration from the x-axis on a vertical line.
8. Multiply the obtained endotoxin concentration with the dilution factor of the product sample to obtain the contaminant concentration in the sample.
9. Determine the LOD (see section 4).

It is recommended to set a limit for the maximally acceptable relative standard deviation (max. %CV) of the replicates of each dilution in order to identify potential issues during data acquisition that lead to variability in results, e.g. CV ≤ 25%.

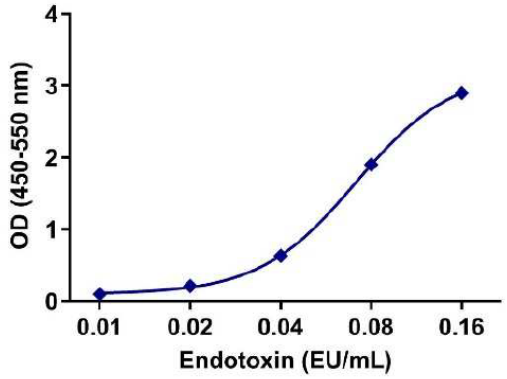


Fig. 8: Typical sigmoidal endotoxin standard curve obtained using the PyroCell™ MAT Kit with the PeliKine compact human IL-6 ELISA as a detection system.

6.1 Method A – Quantitative test

	• **Important!** If the basal release of the IL-6 cytokine measured in the blank is OD ≥ 0.1, inappropriate handling of the pMAT cells or an assay contamination with a pyrogen is indicated. Make sure to use materials tested for low endotoxin content, critically assess procedures and the environment when preparing the assay, and repeat the experiment with fresh, endotoxin-free materials. • The “historical LOD” is defined as the lowest tested endotoxin concentration that reproducibly shows an OD reading above the cut-off value with different manufactured batches of pMAT Cells. It is recommended to use the historical LOD for calculations, e.g. the MVD of the product. • When applying method A, the analysis software used should be capable to demonstrate that (1) the regression of response on log10 dose is statistically significant if $p < 0.01$ and (2) the regression of response on log10 dose does not deviate significantly from linearity if $p > 0.05$ (see Ph. Eur. chapter 2.6.30 and 5.3 “Statistical analysis” for more information).

Method A is based on the conversion of the OD signals for the product dilutions tested into a contaminant concentration (EEU/ mL) using the endotoxin standard curve. After applying the dilution factor correction, the contaminant concentration of the undiluted product (EEU/mL) is compared to the CLC for that product. Routine testing of products with method A is performed with the lowest dilution that was been demonstrated for a valid spike recovery in preparatory testing (see section 5) and two additional 2-fold dilutions of this dilution not exceeding the MVD. Each dilution is tested on the same plate with and without an endotoxin spike at or near the middle of the standard curve.

First data inspection of the average OD of the endotoxin standard curve dilutions should confirm increasing OD values with increasing endotoxin concentrations. All OD values of endotoxin standard dilutions must be greater than the OD value of the blank.

- Perform a log transformation and plot the average net absorbance (mean OD450nm – OD550nm) of the endotoxin dilutions against concentrations.
- Using an appropriate analysis software or the Lonza MAT Analysis Tool, a dose-response curve is fitted through the data points. The choice of the appropriate statistical model may be dependent on the product to be tested (see Ph. Eur. chapter 5.3). We recommend a 4-parameter logistic model for symmetric data, or a 5-parameter logistic model for asymmetric data. Alternatively, a linear regression model may be appropriate.
- Determine the LOD (see section 4).

6.1.1 Acceptance criteria

Applying method A requires that the endotoxin standard curve meets certain statistical fit criteria that are used as curve acceptance specifications (system suitability). Furthermore, method A should be applied only upon demonstration that the dose-response curve of the product is parallel to the dose-response curve of the endotoxin reference standard (sample suitability).

- **Blank:** the average of blank should be ≤ 0.1 OD.
- **LOD:** the experimental LOD ≤ historical LOD.
  Example: If the historical LOD of the PyroCell™ MAT Kit (0.02 EU/mL) is used to calculate the MVD of the product, the test is valid if LOD ≤ 0.2 EU/mL.
- **Dose response**: the dose-response of the product dilutions must be parallel to the Endotoxin Standard Curve.
- **Linear regression/ Goodness of fit** (statistical evaluation of the fitted curve): confirm the quality of the standard curve by statistical analysis:
  - Regression of responses (appropriately transformed if necessary) on log10 dose shall be statistically significant ($p < 0.01$).
  - Regression of responses on log10 dose must not deviate significantly from linearity ($p > 0.05$).

6.1.2. Calculation of the pyrogen content

- For each sample dilution not exceeding the MVD, calculate the endotoxin spike recovery. Dilutions with an invalid spike recovery (outside the range of 50-200%) are excluded from further analysis.
- For each valid sample dilution, the OD value is converted in EEU/mL, corrected with the dilution factor and compared to the CLC.
  - If value in EEU/ml in the pure sample is < CLC → PASS
  - If value in EEU/mL in the pure sample is > CLC → FAIL
  - If an NEP PPC is used it has to be detected above the cut off value

6.2 Method B - Semi-quantitative test

	• **Important!** If the basal release of the IL-6 cytokine measured in the blank is OD ≥ 0.1, inappropriate handling of the pMAT cells or an assay contamination with a pyrogen is indicated. Make sure to use materials tested for low endotoxin content, critically assess procedures and the environment when preparing the assay, and repeat the experiment with fresh, endotoxin-free materials. • The term “historical LOD” is defined as the lowest tested endotoxin concentration that reproducibly demonstrates an OD reading above the cut-off value with different manufactured batches of pMAT Cells. It is recommended to use the historical LOD for necessary calculations, e.g. the MVD of the product.

Method B compares the OD signal of the product sample with the CLC of the product. Sample data below the cut-off value is considered negative (= pass, meaning a pyrogen level below CLC). Routine testing of products with method B is performed with the lowest dilution that was been demonstrated for a valid spike recovery in preparatory testing (see section 5) and two additional 2-fold dilution of this dilution not exceeding the MVD. Each dilution is tested on the same plate with and without an endotoxin spike at 2x LOD.

First data inspection of the averages of the reference endotoxin dilutions (0.5x, 1x, 2x, and 4x historical LOD) should confirm increasing OD values with increasing endotoxin concentrations. OD values found for endotoxin dilutions with concentrations equal or higher than the historical LOD must be above the blank. The 0.5x LOD concentration can be higher or equal to the blank.

- Plot the average net absorbance (mean OD450nm – OD550nm) of the endotoxin dilutions against endotoxin concentrations.
- Read the endotoxin content of product samples and confirm a valid spike recovery.
- Determine the LOD (see section 4).

6.2.1 Acceptance criteria

The following criteria must be valid:

- **Blank:** the average of blank should be ≤ 0.1 OD.
- **LOD:** The experimental LOD ≤ historical LOD. Example: If the historical LOD of the PyroCell™ MAT Kit (0.02 EU/mL) is used to calculate the MVD of the product, the test is valid if LOD ≤ 0.2 EU/mL.
  The mean OD signal for the endotoxin standard solutions at 1xLOD should be above the cut-off value

- **Dose response**: the mean response of 4 endotoxin standard dilutions should increase progressively with increasing concentrations.

6.2.2 Calculation of pyrogen content

- For each product dilution not exceeding the MVD, the endotoxin spike recovery (PPC) is calculated. Dilutions with an invalid spike recovery (outside the range of 50-200%) are excluded from further analysis.
- For each valid product dilution the OD value is compared to the cut-off value. If the OD is below the cut-off value the pyrogen concentration is considered to be below the LOD of the detection system.
    - OD value (product dilution) < Cut-off value → Pass (pyrogen level of the undiluted sample is below CLC).
    - OD value (product dilution) > Cut-off value → non conclusive (a contamination is present in the undiluted sample but the pyrogen level cannot be evaluated with respect to the CLC).
    - OD value (product dilution at MVD) > Cut-off value → Fail (Pyrogen level is above CLC).

6.3 Method C - Lot comparison test

Method C is comparing the OD signals or IL-6 contents from 3 dilutions of the product to be examined to a reference lot of the same formulation or the same class of formulation. A reference lot must be carefully justified. Ideally, clinical data is available. The OD ratio of the product should not exceed a justified acceptance criterion which is defined by the user.
For a lot comparison samples of the reference lot and the lot to be evaluated must be tested on the same plate.

6.3.1 Acceptance criteria

The positive control and at least one dilution of the reference lot should be above the mean OD value of the blank. Ph. Eur. 2.6.30 recommends that a lot difference should not be higher or lower than 2.5-fold to pass the test.

6.3.2 Calculation of pyrogenicity

For each sample an OD ratio is calculated, corresponding to the sum of the mean response of the 3 dilutions of the lot being examined divided by the sum of the mean response of the 3 dilutions of the reference lot.

- OD ratio < acceptance criterion → pass (lot examined not pyrogenic as compared to the reference lot)
- OD ratio > acceptance criterion → fail (lot examined pyrogenic as compared to the reference lot)

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