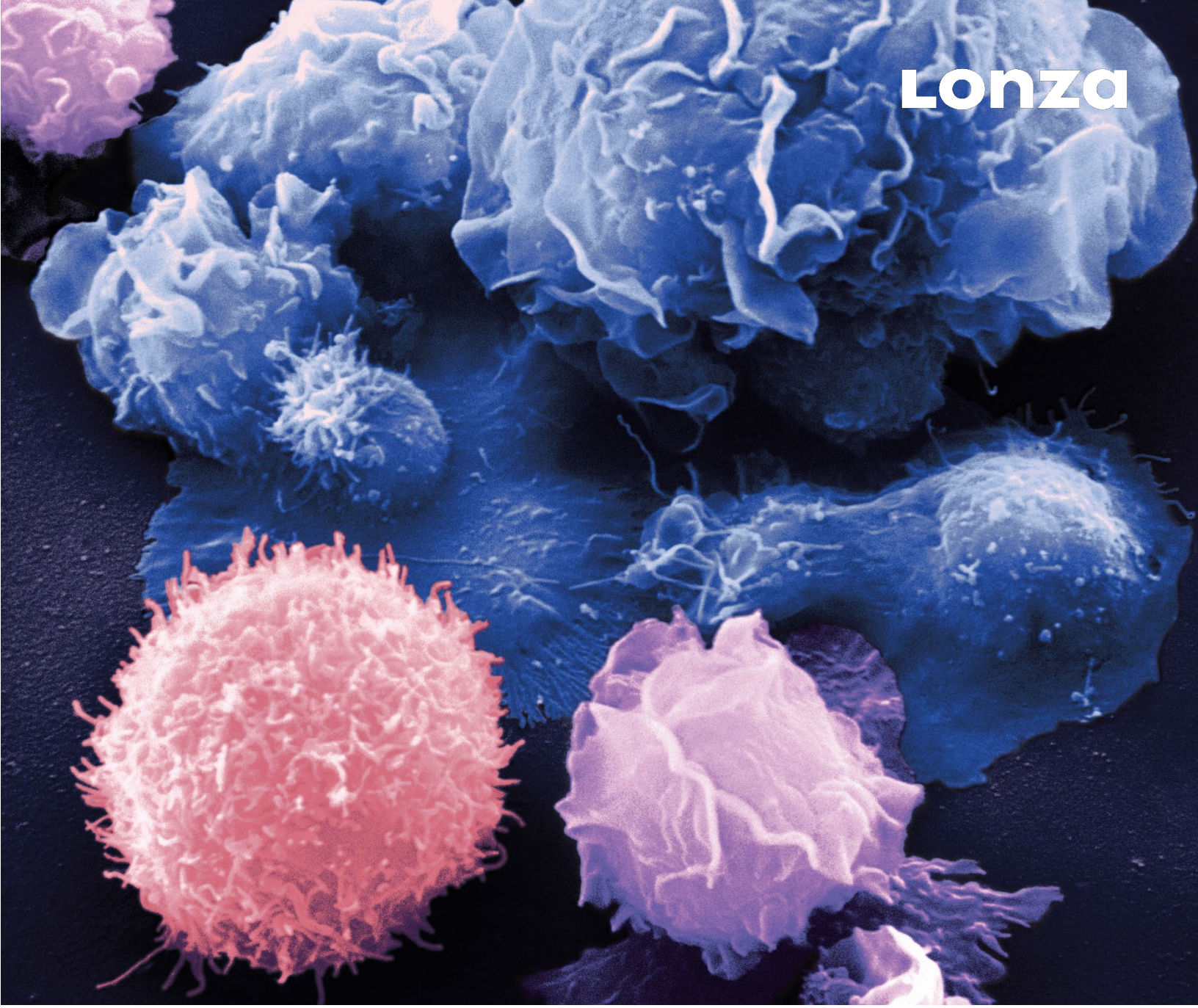

PyroCell® MAT HS Rapid System

Instructions for Routine Use

Cat. # 296408 contains:
Cat. # 279770 - PyroCell® MAT HS Kit
Cat. # 296406 - PeliKine Human IL-6 ELISA Rapid Kit

RT-MN038 09/23

The PyroCell® MAT Kit and the PyroCell® MAT HS Kit are Lonza Bioscience products for the Monocyte Activation Test. Components are manufactured by Sanquin Reagents B.V. for Lonza Bioscience.

The PeliKine Human IL-6 ELISA Rapid Set A and PeliKine Human IL-6 ELISA Rapid Set B are manufactured by Sanquin Reagents B.V. and distributed by Lonza Bioscience for Monocyte Activation Test applications.

Notices

Trademarks

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Restrictions and liabilities

Information in this document is subject to change and does not represent a commitment by Lonza. Changes made to the information in this document will be incorporated in new editions of the publication. No responsibility is assumed by Lonza for the use or the reliability of reagents, software or equipment that are not supplied by Lonza or its affiliated dealers.

The PyroCell® MAT HS Kit has been designed and validated for reproducible detection of a variety of different pyrogens using IL-6 cytokine release as read-out for pyrogenic activity. Other cytokines may be suitable as read-out, but their use with the PyroCell® MAT HS Kit needs 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 multiple non-endotoxin pyrogens, the specific reactivity has to be determined by the user.

The applicable warranty for Lonza reagents and products stipulated in this user guide is found at www.bioscience.lonza.com. This warranty contains the obligations of Lonza, and no other warranties, expressed, implied, or statutory are given. Other than the express warranty contained herein, Lonza shall not be responsible for any direct or consequential damages of any kind. This warranty shall not be changed or modified in any way without the express written permission of Lonza.

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 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 HS Kit, consisting of cryopreserved human peripheral blood mononuclear cells (PBMC) pooled from four healthy donors (pMAT Cells), and MAT Human Serum (HS) 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 HS 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 and 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

Customer Service: 800 638 8174 (toll free)

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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.

Revision History

Rev	Date	Changes
	2023	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!	pMAT Cells contain traces of fetal bovine serum (FBS) sourced in North America, as a stabilizer. FBS components shall be handled in laboratory environments only.
Warning!	pMAT Cells and MAT Human Serum Supplement are derived from human blood donations. Human blood used for preparations was tested negative for human infectious disease markers. Please 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 HS Kit. The MAT shall be carried out by authorized and well-trained laboratory personnel only.
Caution	PyroCell® MAT HS Kit components must be stored at ≤ -80°C under controlled conditions. Temperature excursions 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 changed 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 with added MAT Human Serum Supplement (included in the PyroCell® MAT HS Kit)
ELISA	Enzyme-linked Immunosorbent Assay
EU/mL	Endotoxin Units per Milliliter
EEU/mL	Endotoxin Equivalent Units per Milliliter
HPE	High performance buffer
HRP	Horseradish peroxidase
HS	Human serum (if used in combination with PyroCell® Kits)
IMDM	Iscove's Modified Dulbecco's Medium
IL-6	Interleukin-6
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 PBMC qualified for the MAT
RPT	Rabbit Pyrogen Test
RSE	Reference Standard Endotoxin
SDS	Safety Data Sheet
4-PLM	Four parameter logistic model
5-PLM	Five parameter logistic model

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 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 pyrogen is bacterial endotoxin derived from gram negative bacteria. All other pyrogens are summarized under the term 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 acknowledged by regulatory agencies 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	*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 <86>	*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 the clotting cascade in horseshoe crab blood amebocytes by endotoxin	

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 is capable of detecting endotoxins as well as non-endotoxin pyrogens in raw materials, intermediates, process samples and manufactured products. The MAT 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 describe the MAT as an alternative method to the RPT. For example, the United States Pharmacopeia (USP) requests the MAT to be validated according to USP <1225>[4,5]. Please contact your local authorities for more information.

2. PyroCell® MAT HS Rapid System – Assay Principle

The PyroCell® MAT HS Rapid 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 an Enzyme-Linked Immunosorbent Assay (ELISA). On 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 Human IL-6 ELISA Rapid Kit. Presence of IL-6 is then measured in a microplate 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).

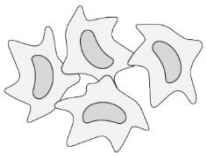
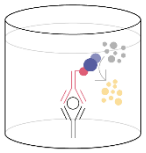
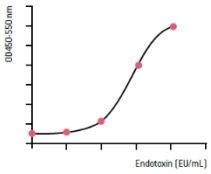
	Process	
Monocyte Activation	• Prepare endotoxin dilutions • Prepare test samples on the cell culture plate • Thaw pMAT Cells, transfer to plate • Incubate for 18-24h	• See section 5.3 
IL-6 ELISA	• Harvest the cell culture supernatant • Detect IL-6 cytokines with a Human IL-6 ELISA • Read results in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

Fig. 2: Monocyte Activation Test - Assay overview

3. Material Information and Storage Conditions

The PyroCell® MAT HS Rapid System (# 296408) combines reagents to conduct the MAT in a ready-to-use kit. The following materials are included: the PyroCell® MAT HS Kit (# 279770), comprising 3 vials pMAT cells (M2016LC), and 3 vials Human Serum Supplement, (M2017LS) and the PeliKine Human IL-6 ELISA Rapid Kit (# 296406, comprising the PeliKine Human IL-6 ELISA Rapid Set A (Sanquin, M2018) and the PeliKine Human IL-6 ELISA Rapid Set B (Sanquin, M2019).

3.1 PyroCell® MAT HS Kit

pMAT Cells (M2016LC) 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 human blood donations, and subsequent cryopreservation of the pooled PBMC under controlled conditions. 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 number of the MAT Human Serum Supplement is printed on the vial label, and on the CoA.

3.1.1 Package content

Amount	Component	Closure	Volume	Lot #
3 vials	pMAT Cells (M2016LC)	Green	3 x 1 mL	68-xxxx (Lot org.)
3 vials	MAT Human Serum Supplement (M2017LS)	White	3 x 2 mL	8000-xxxxxx

3.1.2 Storage and stability

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

3.2 PeliKine Human IL-6 ELISA Rapid Kit

The PeliKine Human IL-6 ELISA Rapid Kit (# 296406) is comprised of two reagent kits, the PeliKine Human IL-6 ELISA Rapid Set A (M2018) and the PeliKine Human IL-6 ELISA Rapid Set B (M2019).

- Only use reagents and microtiter plates supplied with the PeliKine Human IL-6 ELISA Rapid 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 contain 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).

3.2.1 PeliKine Human IL-6 ELISA Rapid Set A (M2018)

The PeliKine Human IL-6 ELISA Rapid Set A is a "sandwich-type" enzyme immunoassay. The target cytokine human 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 Package content

The PeliKine Human IL-6 ELISA Rapid Set A contains material sufficient for 3 plates (288 tests). The following reagents are included:

Amount	Component	SDS	Closure	Volume	Concentrate
1 vial	Biotinylated antibody		Yellow	375 µL	100x
1 vial	Streptavidin-poly-HRP conjugate		Brown	20 µL	10,000x
1 vial	IL-6 standard		Black	750 µL	4000 pg/mL
1 bottle	HPE buffer	⚠	Transparent	55 mL	5x

3.2.1.2 Storage and stability

Store the components of the PeliKine Human IL-6 ELISA Rapid Set A at -18°C to -32°C. The expiration date is shown on the box label. Do not use reagents beyond the expiration date.

Store the prepared HPE buffer (working strength buffer) at 2 to 8°C in a closed container and use within 1 week. Bring the buffer to room temperature (18 to 25°C) prior to use.

3.2.1.3 Sensitivity

The limit of detection (LOD) can be calculated as follows:

Mean calculated zero signal + 3 SD: ≤ 1 pg/mL (shake or static incubation).

The assay sensitivity may be increased by longer incubation times (up to 2 hours).

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 Human IL-6 ELISA Rapid Set B (M2019)

The PeliKine Human IL-6 ELISA Rapid Set B completes the PeliKine Human IL-6 ELISA Rapid Set A by providing the pre-coated plates, the plate seals, the washing buffer concentrate, and the ready-to-use 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 Package content

The PeliKine Human IL-6 ELISA Rapid Set B contains all materials sufficient for one PeliKine compact human IL-6 kit (288 tests, 3 plates). The following reagents are included:

Amount	Component	SDS	Appearance	Volume	Preparation
2 bottles	Washing Buffer, 20-fold concentrate		White bottle	2 x 50 mL	2 x 1 L buffer
1 bottle	TMB substrate solution		Brown bottle	40 mL	Ready-to-use
1 bottle	Stop solution	⚠	White bottle	40 mL	Ready-to-use
3 pcs	Human IL-6 ELISA pre-coated plates		Vacuum sealed, desiccant	3 x 96 wells	Ready-to use
12 pcs	Plate seals		Transparent foil	-	Ready-to use

3.2.2.2 Storage and stability

- Store the PeliKine Human IL-6 ELISA Rapid Set B at 2 to 8°C. The expiration date is shown on the box label. Do not use reagents beyond the expiration date.
- Store the prepared washing buffer (working strength buffer) at 2 to 8°C in closed containers and use within 2 month.

3.3 Further reagents and consumables

Reagents in the table below are recommended for use with the PyroCell® MAT HS Rapid System. Alternative reagents may be used however, their suitability for the intended purpose needs to be verified.

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	refer to CoA

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
Reagent Reservoirs, non-pyrogenic	10 pcs/pack	00190035
96-well flat bottom microplates (cell culture)	50 pcs/pack	25-340
Pipette tips, non-pyrogenic	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 HS Kit
Freezer (-20°C or lower)	Storage of PeliKine Human IL-6 ELISA Rapid Set A
Refrigerator (2 to 8°C)	Storage of PeliKine Human IL-6 ELISA Rapid Set B 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)
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, or cylinders	Preparation and storage of working strength buffer for the ELISA
(optional) Device for delivery of washing buffer or automated plate washer	Supports washing steps for the ELISA
(optional) Microplate shaker	Increases sensitivity of the ELISA
Microplate reader (read out: 450nm; reference wavelength: 540-590nm), e.g. Nebula® Multimode Reader, Cat.# 25-375S Nebula® Absorbance Reader, Cat. # 25-365S	ELISA assay read-out

3.6 Recommended literature

1. European Directorate for the Quality of Medicines (EDQM). *European Pharmacopeia chapter* 2.6.30: Monocyte Activation Test, Edition 10
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. Molenaar-de Backer, M. W. A., Gitz, E., Dieker, M., Doodeman, P. and ten Brinke, A. (2021) “Performance of monocyte activation test supplemented with human serum compared to fetal bovine serum”, ALTEX - Alternatives to animal experimentation, 38(2), pp. 307–315. doi: 10.14573/altex.2008261.
9. 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 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 HS Kit suggest an LOD ≤ 0.08 EU/mL if used with the PeliKine Human IL-6 ELISA Rapid Kit**.**

Important! Endotoxin concentrations stated throughout this user guide, such as LOD, are calculated as concentration per sample. The concentration in the reaction well of the cell culture plate is half of the stated concentration, e.g. half LOD. Use the LOD per sample to calculate the MVD of the product to be tested.

Important! Consult the Certificate of Analysis for additional information.

The MAT 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) for the sample.

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 each user has to establish the LOD in their laboratory based on the current experiment or historical data[1]. Using the PyroCell® MAT HS Kit combined with the PeliKine Human IL-6 ELISA Rapid Kit an LOD of ≤ 0.08 EU/mL per sample can be expected. Consult the Certificate of Analysis (CoA) for additional information.

5. Test Procedure

5.1 Use of qualified cells

pMAT Cells contained in the PyroCell® MAT HS Kit consist of a cryopreserved pool of human peripheral blood mononuclear cells (PBMC) serving as the monocytic cell source[1] for the MAT. PBMCs are isolated from blood donations of healthy volunteers. 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 negative for common infectious disease marker. 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 as of the blood donation. After thawing, the pMAT Cells are qualified with the PeliKine Human IL-6 ELISA Rapid 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).

On the day of the experiment the pMAT Cells are thawed and immediately diluted in complete medium 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 (= product-specific validation) should be performed (Fig. 3).[1] Preparatory testing is required to establish the preparation of the endotoxin standard curve in the laboratory, to confirm that the specific product to be examined does not interfere with the assay, or that interference can be overcome by product dilutions not exceeding the MVD of the product. It further assures that both bacterial endotoxin and non-endotoxin pyrogens (NEP) are detected. Finally, testing of three manufactured product lots using the optimal product dilutions determined during product validation confirms the choice of the appropriate MAT method (method A – Quantitative test, method B – Semi-quantitative test, or method C – Reference lot comparison test).

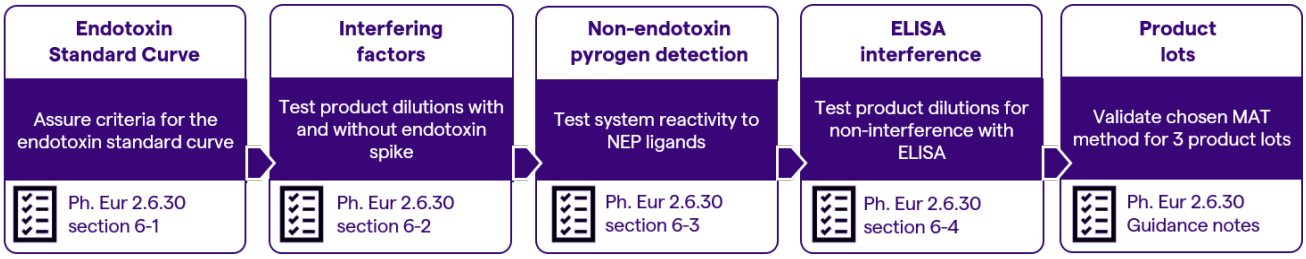


Fig. 3: Preparatory testing requirements.

An endotoxin standard curve prepared from reference standard endotoxin (RSE, # E700) is required for preparatory testing and for method A[1]. It is used to convert the OD signal measured for product samples into endotoxin equivalent units (EEU). Establish the endotoxin standard curve by performing the MAT on 7 ascending RSE dilutions, and a blank (negative control). Prepare each dilution in 4 replicates on a 96-well microtiter plate.

For data analysis, chose an appropriate regression model for curve fitting. For the PyroCell® MAT HS Kit a 5-parameter logistic model (5-PLM) is recommended. Lonza is offering an excel based analysis template as an easy-to-use tool that automatically performs all necessary calculations. For more information on data analysis please also refer to section 6.

Once the endotoxin standard curve is established or if method B is used during routine testing, we recommend to use 5 endotoxin reference dilutions in order to fit more test samples on the same microtiter plate.

The test for interfering factors requires a positive product control (PPC, endotoxin spike at a concentration at the middle of the standard curve) to be recovered from product dilutions within the range of 50-200% of the spike.[1] All product dilutions shall not exceed the MVD for the product in order to ensure that the contaminant can still be detected at the CLC. If product interferences cannot be overcome by dilution, method C is recommended over methods A and B.

Each product dilution, the endotoxin dilutions, and the blank are prepared in 4 replicates on a 96-well microtiter plate. Determine the appropriate product dilution as follows:

- Prepare dilutions for the endotoxin standard curve.
- Prepare geometric dilutions of the product with all dilutions not exceeding the MVD.
- For all product dilutions, generate a PPC by spiking with an endotoxin concentration near the estimated middle of the standard curve, i.e. 0.32 EU/mL (method A) or twice the LOD, i.e. 0.16 EU/mL (method B).
- Test the dilution series with and without PPC in the same experiment on the same microtiter plate.
- Calculate the mean recovery of the spiked endotoxin by subtracting the mean concentration of dilutions without endotoxin from PPC dilutions. Compare to the expected values by using the endotoxin standard curve.
- Determine the highest product sample concentration with 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.

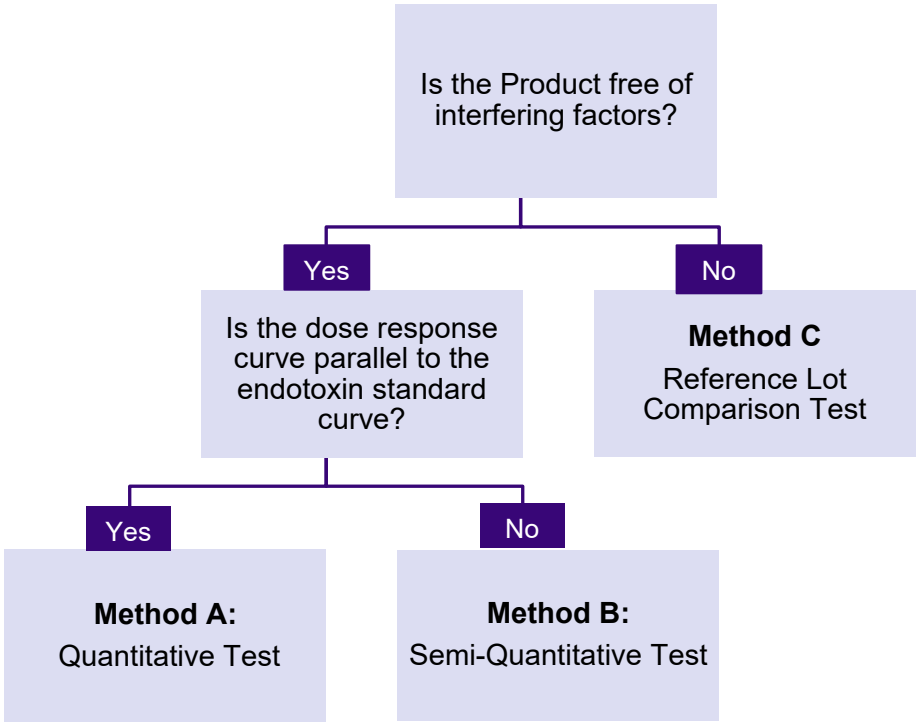


Fig 4: Simplified decision tree to support the choice of the MAT method.

Test the system reactivity towards NEP by using either a historic product batch with 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 highest risk for the product. At least one relevant NEP ligand should be spiked into the highest valid concentration of the test product that was determined during interference testing, and must be recovered within the range of 50-200% of the spike. It is recommended to test commercial NEPs for absence of bacterial endotoxin, e.g. with a BET prior to use.

Finally, use the highest valid test product concentration to exclude interference of the product with the chosen read-out system.[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, select the suitable method 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!** 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**.**
- 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.

5.3.1 Preparation of complete medium

- Ensure sterility of the IMDM, e.g. by using an unopened bottle.
- **Important!** A minimum of 1 mL IMDM without MAT Human Serum Supplement is needed for the first RSE dilution. Preparing the first RSE dilutions with complete medium may result in an inaccurate endotoxin standard curve.
- Equilibrate the complete medium to room temperature (18 to 25 °C).

The complete medium used for the pMAT cell incubation consists of IMDM with added MAT Human Serum Supplement (M2017LS) that is included in the PyroCell® MAT HS Kit.

1. Thaw the **MAT Human Serum Supplement** vial in a water bath at 37°C.
2. Add the entire vial content (2 mL) to a sterile, non-pyrogenic 50 mL tube containing 38 mL IMDM.
3. Mix gently by inverting the closed tube. **Use the complete medium within 8 hours**.

5.3.2 Preparation of reference endotoxin dilutions

- **Important!** Prepare all endotoxin dilutions shortly before the experiment.
- **Important!** Do not vortex samples containing complete medium to prevent the formation of air bubbles.
- Vortex the RSE stock solution (2,000 EU/mL) for 30 min before use.

Reference endotoxin dilutions are used to determine the LOD of the test system and to estimate the amount of pyrogen in test sample. Include reference endotoxin dilutions, and a blank on each microtiter plate. Each dilution is prepared in 4 replicates.

When working with the PyroCell® MAT HS kit we recommend to prepare a standard curve with 7 endotoxin dilutions, and a blank for the quantitative test method A, and 5 endotoxin dilutions, and a blank for the semi-quantitative test method B. Based on the label sensitivity of ≤ 0.08 EU/mL for the PyroCell® MAT HS system prepare geometric dilutions from 0.04 (1/2 LOD) to 0.64 EU/mL (5-point standard curve) or 0.02 to 1.28 EU/ml (7-point standard curve) respectively. Example plate layout are illustrated in **Fig 5.**

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.28 EU/mL (R7)	0.64 EU/mL (R6)	0.32 EU/mL (R5)	0.16 EU/mL (R4)								
B												
C												
D												
E	0.08 EU/mL (R3)	0.04 EU/mL (R2)	0.02 EU/mL (R1)	Blank (R0)								
F												
G												
H												

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.64 8xLOD (R5)	0.32 4xLOD (R4)	0.16 2xLOD (R3)									
B												
C												
D												
E	0.08 LOD (R2)	0.04 1/2 LOD (R1)	Blank (R0)									
F												
G												
H												

Fig. 5: (A) Suggested plate layout for method A (R0-R7). (B) Suggested plate layout for method B (R0-R5). Concentrations are based on a historical LOD of ≤ 0.08 EU/mL.

Prepare an endotoxin stock solution of 2,000 Endotoxin Unit (EU)/mL by following the user instructions:

1. Add 5 mL of endotoxin-free LAL water to a vial of RSE (Lonza # E700).
2. Reconstitute RSE by vortexing for 30 min at maximum speed. The resulting solution (2,000 EU/mL) is the stock solution.

Use 50 µL stock solution to prepare endotoxin dilutions for one 96-well microtiter plate. Aliquots of the stock solution can be stored frozen at -80°C for future use. Upon thawing vortex the aliquot for 3 min before use.

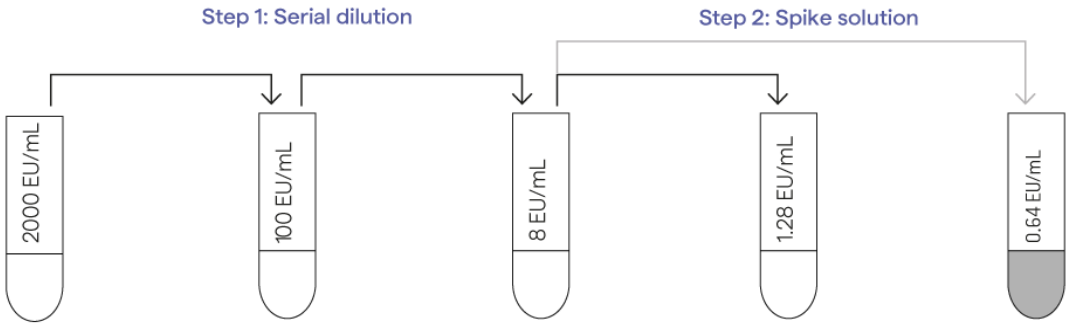
5.3.2.1 Method A

Prepare a serial dilution starting at 1.28 EU/mL with two-fold dilutions down to 0.02 EU/mL (1.28 – 0.64 – 0.32 – 0.16 – 0.08 – 0.04 – 0.02 EU/mL), and a blank in sterile, non-pyrogenic borosilicated glass tubes (# N207) as described below (also refer to **Fig 6A**):

3. Add 50 µL of RSE stock solution (2000 EU/ mL) to 950 µL **IMDM** (WITHOUT MAT CULTURE MEDIUM SUPPLEMENT). The final concentration is 100 EU/mL. Vortex thoroughly for 3 min.
4. Add 80 µL of 100 EU/mL RSE dilution to 920 µL **complete medium.** The final concentration is 8 EU/mL. Mix 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.
5. Add 160 µL of 8 EU/mL RSE dilution to 840 µL **complete medium.** The final concentration is 1.28 EU/mL. Mix by pipetting up and down 10x, with a volume of at least 500 µL.
6. Add 200 µL of 1.28 EU/mL endotoxin to wells A1 to D1 (see **Fig 5**).

7. Add 100 µL of **complete medium** to the other wells used for the standard curve (E1-H1, A2-H2, A3-H3, and A4-H4).
8. Make a two-fold dilution by pipetting 100 µL from R7 (1.28 EU/mL, A1-D1) to R6 (0.64 EU/mL, A2-D2) using a multi-channel pipettor with 4 lines. Mix thoroughly by pipetting up and down 10x. **Prevent the formation of air bubbles.**
9. Repeat dilution as described in point 6 for transferring 100 µL from R6 to R5 (A3-D3), from R5 to R4 (A4-D4), from R4 to R3 (E1-H1), from R3 to R2 (E2-H2), and from R2 to R1 (E3-H3). Discard the final 100 µL from R1.
10. Save the remaining 8 EU/mL RSE solution prepared at step 4 for preparation of endotoxin spiked samples (see section 5.3.3 and **Fig. 6A**).



Aliquot RSE, 2,000 EU/ml in LAL water	50 µL RSE, (2,000 EU/mL) in 950µL IMDM	80 µL RSE, (100 EU/mL) in 920µL complete medium	160 µL RSE, (8 EU/mL) in 840µL complete medium	160 µL RSE, (8 EU/mL) in 1840 µL complete medium
Upon thawing, vortex for 3 min	Vortex 3 min	Mix by pipetting up and down 10x	Mix by pipetting up and down 10x	Mix by pipetting up and down 10x
2,000 EU/mL	**100 EU/mL**	**8 EU/mL**	**1.28 EU/mL**	**0.64 EU/mL**

Fig. 6A: Serial dilution for method A. Prepare 1.28 EU/mL endotoxin dilution and 0.64 EU/mL spike solution.

5.3.2.2 Method B

Prepare a dilution series starting at 0.64 EU/mL with two-fold dilutions down to 0.04 EU/mL (0.64 – 0.32 – 0.16 – 0.08 – 0.04 EU/mL), and a blank in sterile, non-pyrogenic borosilicated glass tubes (# N207). An example for a dilution series is shown in **Fig 6B** below:

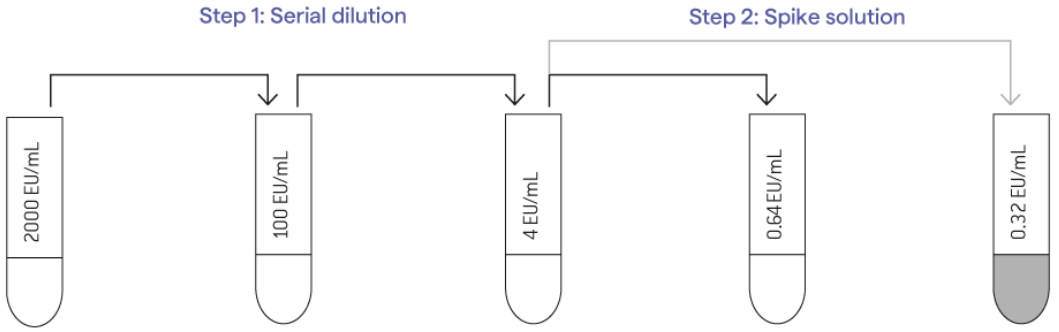
Proceed with the preparation of endotoxin dilutions:

1. Add 50 µL of RSE stock solution (2000 EU/ mL) to 950 µL of **IMDM** (WITHOUT MAT CULTURE MEDIUM SUPPLEMENT). The final concentration is 100 EU/mL. Vortex thoroughly for 3 min.
2.

3. Add 40 µL of 100 EU/mL RSE dilution to 960 µL of **complete medium.** The final concentration of 4 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.

4. Add 160 µL of 4 EU/mL RSE dilution to 840 µL of **complete medium.** The final concentration of 0.64 EU/mL. Mix thoroughly by pipetting up and down 10x, with a volume of at least 500 µL.
5. Add 200 µL of 0.64 EU/mL endotoxin to wells A1 to D1 (see Fig 5).
6. Add 100 µL of **complete medium** to the other wells used for the standard curve (E1-H1, A2-H2 and A3-H3).
7. Make a two-fold dilution by pipetting 100 µL from R5 (0.64 EU/mL, A1-D1) to R4 (0.32 EU/mL, A2-D2) using a multi-channel pipettor with 4 lines. Mix thoroughly by pipetting up and down 10x. **Prevent the formation of air bubbles.**
8. Repeat dilution as described in point 6 for transferring 100 µL from R4 to R3 (A2-D2), from R3 to R2 (E1-H1) and R2 to R1 (E2-H2). Discard the final 100 µL from R1.
9. Save the remaining 4 EU/mL RSE solution prepared at step 2 for preparation of endotoxin spiked samples (see section 5.3.3 and **Fig. 6B**).



Aliquot RSE, 2,000 EU/mL in LAL water	50 µL RSE, (2,000 EU/mL) in 950µL IMDM	40 µL RSE, (100 EU/mL) in 960µL complete medium	160 µL RSE, (4 EU/mL) in 840µL complete medium	160 µL RSE, (4 EU/mL) in 1840 µL complete medium
Upon thawing, vortex for 3 min	Vortex 3 min	Mix by pipetting up and down 10x	Mix by pipetting up and down 10x	Mix by pipetting up and down 10x
2,000 EU/mL	**100 EU/mL**	**4 EU/mL**	**0.64 EU/mL**	**0.32 EU/mL**

Fig. 6B: Serial dilution, prepare 0.64 EU/mL endotoxin dilution and 0.32 EU/mL spike solution.

5.3.3 Preparation of product sample dilutions

- **Important!** Prepare the product sample on the day of the experiment.
- **Important!** Prepare RSE dilutions in borosilicated glass tubes
- Using the example layout below, 2-3 product preparations can be tested on a single plate

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 three dilutions of a product preparation, each measured with and without an endotoxin spike. Each dilution is tested in 4 replicates suggesting 2 different samples fitting on a plate if method A is used and 3 different samples for method B. Optional, an NEP control can be prepared. Examples for a plate layout 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	1.28 EU/mL (R7)	0.64 EU/mL (R6)	0.32 EU/mL (R5)	0.16 EU/mL (R4)	S1	S1-1/2	S1-1/4					
B												
C												
D												
E	0.08 EU/mL (R3)	0.04 EU/mL (R2)	0.02 EU/mL (R1)	Blank (R0)	S1 + RSE spike	S1-1/2 + RSE spike	S1-1/4 + RSE spike					
F												
G												
H												

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.64 8xLOD (R5)	0.32 4xLOD (R4)	0.16 2xLOD (R3)	S1	S1-1/2	S1-1/4						
B												
C												
D												
E	0.08 LOD (R2)	0.04 1/2 LOD (R1)	Blank (R0)	S1 + RSE spike	S1-1/2 + RSE spike	S1-1/4 + RSE spike						
F												
G												
H												

Fig. 7: Suggested plate layout for the quantitative (A) or semi-quantitative (B) MAT method. Endotoxin reference standards with concentrations in EU/mL (A, R0 – R7) or x LOD (B, R0 – R5). Sample (S1) in 2 successive two-fold dilutions (S1-1/2 and S1-1/4) without and with spiked endotoxin (PPC, row E-H, indicated by darker color). The spike concentration should be near the middle or the standard curve for method A (0.32 EU/mL) or at 2x LOD for method B (0.16 EU/mL).

1. Add 200 µL of the product test sample S1-1 (highest concentration with valid PPC recovery, determined during preparatory testing) in wells A5 to H5 (Method A), or A4 to H4 (Method B).
2. Add 100 µL of complete medium into the other wells used for the sample dilutions.
3. Prepare a two-fold dilution by pipetting 100 µL from S1 to S1-1/2 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. If a further samples are to be tested: repeat step 1-4 for each additional sample.

6. Add 50 µL of complete medium to endotoxin dilutions and product test samples without an endotoxin spike.
7. Prepare the endotoxin spike solution (PPC) from the saved dilutions of step 5.3.2.1 or 5.3.2.2, respectively.

   Method A: Add 160 µL of 8 EU/mL endotoxin to 1840 µL complete medium. The final concentration is 0.64 EU/mL.

   Method B: Add 160 µL of 4 EU/mL endotoxin to 1840 µL complete medium. The final concentration is 0.32 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.
8. Add 50 µL of the PPC preparation to wells for spiked samples (Ex to Hx, marked with + RSE spike).

   Method A: The endotoxin spike concentration in the sample corresponds to 0.32 EU/mL value from the standard curve.

   Method B: The endotoxin spike concentration in the sample corresponds to the endotoxin dilution with 0.16 EU/mL.

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 and transfer 50 µL of the suspended pMAT Cells to each well containing prepared test dilutions using a multi-channel pipette with 8 lines.
4. Incubate the microplate with lid in a CO_2-incubator at 37°C (humidified air, 5% CO_2) for 18-24 hours.

5.4 Routine testing – Human IL-6 ELISA

- Adhere to GLP (good laboratory practices) for ELISA. An ELISA can be performed on a regular laboratory bench.
- **Important!** The Pelikine Human IL-6 ELISA Rapid Kit has been validated with the PyroCell® MAT Kit and the PyroCell® MAT HS Kit.

The Pelikine Human IL-6 ELISA Rapid Kit is a "sandwich-type" Enzyme-linked Immunosorbent Assay (ELISA) for fast and specific quantification of the human IL-6 cytokines (IL-6) in MAT assays. Secreted IL-6 contained in the cell culture supernatants are simultaneously bound to anti-IL-6 capture antibodies coated to the polystyrene microtiter wells of a 96-well plate, and biotinylated anti-IL-6 antibodies added in the same incubation step. Unbound material is removed by washing. Subsequently, a horseradish peroxidase (HRP) conjugated streptavidin is added that binds onto the biotinylated side of the cytokine sandwich. After removal of unbound material by washing, a substrate solution is added.

A colored product is formed in proportion to the amount of IL-6 from supernatants, or IL-6 reference dilutions. After the reaction is terminated by adding a stop solution, the absorbance (optical density, OD) is measured in a microtiter plate reader at 450nm and a reference wavelength (540 to 590nm). The OD of the endotoxin standard dilutions is then plotted against their concentration (EU/ mL) and the resulting standard curve is used to calculate the concentration of a contaminant in the product samples (see section 6). The contaminant concentration is expressed in endotoxin equivalent units (EEU).

For more information about the PeliKine human IL-6 ELISA Rapid Kit you may also refer to the additional package inserts of the kit.

5.4.1 Preparation of working strength buffer

- Working strength dilutions of the HPE buffer and the washing buffer may be be prepared prior to the day of experiment. For storage information refer to section 3, “storage information”.
- Before preparing the working-strength buffer, briefly warm the buffer concentrates to 37°C in a water bath to dissolve any precipitates.
- All stated volumes refer to one ELISA plate.
- The 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
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 Harvest the cell culture supernatant and prepare dilutions

- Only part of cell culture supernatants is used for one routine ELISA. Remaining supernatant may be stored frozen at ≤ 20°C for future experiments.
- The storage time of the frozen supernatant may vary between products and needs to be determined by the user.
- Frozen supernatants/ samples should be thawed as quickly as possible in a water bath at 18-25°C. Do not use temperatures above 25°C.
- **Important!** The protocol refers to a 1:5 dilution of cell culture supernatants.

Following a 18-24 hour stimulation of pMAT Cells, harvest 100-150 µL of the supernatant by carefully placing the pipette tip at the side of the well. Avoid turbulence in order to leave the pMAT Cells at the bottom of the plate. Transfer the supernatant to a fresh 96-well round bottom, untreated microplate. Keep the original plate layout.

The optimal dilution of cell culture supernatants may vary between products. The below ELISA protocol is suggesting a 1:5 dilution that is providing the largest dynamic range. Less dilution, e.g. 3-fold may increase the accuracy for the lowest reference endotoxin dilutions and may eventually lead to a lower experimental LOD. At the same time however, the more concentrated endotoxin reference dilutions may reside outside the reader's detection range. When stimulating monocytes with inherently pyrogenic test samples, higher dilution of the supernatants is often required. Here, we recommend pre-dilution in a fresh, untreated microtiter plate.

Examples:

Supernatant Dilution	Dilution factor	Volume supernatant	Volume HPE buffer (1x)	Volume applied to ELISA
Pre-coated plate	3x	-	-	33,3 µL
	5x	-	-	20 µL
Pre-dilution, untreated plate	10x	30 µL	30 µL	20 µL
	20x		90 µL	20 µL
	50x		270 µL	20 µL

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

- An IL-6 standard curve may be included to control the IL-6 ELISA, and as reference for method C. It is optional for routine MAT methods A or B.
- Adding an IL-6 standard curve requires 8-16 “empty” wells (fig. 8) on the microtiter plate layout.
- It is recommended to prepare two separate dilution series (duplicate) per assay.
- Mix each dilution well before proceeding to the next step, e.g. by pipetting up and down 10x with at least 75% of volume.

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 representing the final IL-6 standard concentration in the ELISA plate.
- Pipette 49 µL of working-strength HPE buffer into the tube labelled 450 pg/mL and 80 µL into all other tubes.
- Transfer 63 µL of the IL-6 standard (4000 pg/mL, black cap) into the tube labeled 450 pg/mL. Mix well.
- Serial dilution: Transfer 40 µL of the 450 pg/mL dilution into the second tube labelled 150 pg/mL. Mix well. Repeat the dilution step 5 more times by adding 40 µL of the previous diluted standard to the next tube. Always mix well before proceeding to the next serial dilution step.
- Use the HPE buffer as a blank (negative control).

	1	2	3	4	5	6	7	8	9	10	11	12
A											450 pg/mL	
B											150 pg/mL	
C											50 pg/mL	
D											16.7 pg/mL	
E											5.6 pg/mL	
F											1.9 pg/mL	
G											9.6 pg/mL	
H											Blank	

Fig. 8: Suggested plate layout for the IL-6 standard curve. Preparation of a duplicate series is recommended. Ensure availability of 8-16 “empty wells” (1-2 columns) on the plate layout.

5.4.4 ELISA incubation and wash steps

- All 96 wells of each plate are pre-coated with anti-human IL-6 capture antibodies. Use pre-coated plates immediately after opening the vacuum sealed pouch.
- Always empty wells completely before adding a new solution. Do not allow wells to stand uncovered or dry for extended periods of time between incubation steps. Cover plate with an adhesive seal.
- Equilibrate all buffers and antibody dilutions to 18 to 25°C before use.
- Avoid repeated freeze-thawing of the IL-6 standard. Up to 3 freeze-thaw cycles have no effect on the IL-6 levels of the IL-6 standard.
- Keep the streptavidin-HRP conjugate at -18 to -32°C to ensure stability. Thaw shortly before use.
- Mix contents in wells by tapping the edge of the microtiter plate for a few seconds.
- The protocol below is for static incubation.

Preparation (1 plate)	Add to each well	Incubation/ Wash
• Homogenize the harvested supernatant by pipetting up and down 5 times. **Biotinylated IL-6 antibody** • Dilute 120 µL biotinylated antibody (yellow cap) in 9.6 mL HPE buffer (working strength). • Mix by inverting tube 10 times.	**(Supernatant dilutions: 1:5)** • Add **20 µL** supernatant to the pre-coated plate. Keep the plate layout. • (Optional) add 20 µL of IL-6 standard dilutions to "empty" wells. • Add **80 µL** of biotinylated IL-6 antibody dilution. Mix 3 times by gently pipetting up and down. • Cover plate with adhesive seal.	1 hour at 18 to 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 2 µL streptavidin-HRP (brown cap) in 20 mL of HPE buffer (working-strength). • Mix by inverting tube 10 times.	• Add **100 µL** of Streptavidin-HRP conjugate dilution. • Cover plate with adhesive seal. • Gently agitate the plate by tapping the edge for a few seconds	30 min at 18 to 25°C. **Equilibrate TMB substrate solution and stop solution to 18 to 25°C before wash step.**
Wash with washing buffer	≥ **300 µL** washing buffer (working strength).	5 times.
TMB substrate solution • Ready to use.	• Add **100 µL** TMB substrate solution • Gently agitate the plate by tapping the edge for a few seconds **Protect TMB substrate from exposure to light!**	Approximately 10 min at 18 to 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 reading

- The microtiter plate reader must be equipped with filters at wavelength 450 nm and a reference wavelength filter between 540 and 590 nm (e.g. ELx808™ Reader, Lonza # 25-315S). Alternatively, a multimode reader may be used (e.g. Nebula® Multimode Reader, Lonza # 25-375S)
- If the substrate reaction appears too intense, reduce the incubation time. Do not dilute the TMB substrate.
- If WinKQCL® Software is used, perform a “quick-read” at each recommended wavelength. Export the data into the MAT Analysis Tool.

- Place the microtiter plate into 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 depend on the chosen MAT method. Refer to Ph. Eur. chapter 2.6.30 for a detailed description[1].
- 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 offers an Excel-based MAT Analysis Tool to assist in the analysis of MAT data. Please contact Lonza scientific support for more information.

Product-specific validation is providing the basis for choosing the appropriate MAT method for testing and data analysis (see section 5.2). It ensures that the validity criteria for the respective test method are met. 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 90 to 99% Q-critical value).
4. Calculate the average OD value for each test replicate.
5. Perform a log transformation of 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.
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 acceptable relative standard deviation (max. %CV, e.g. CV ≤ 25%) of the replicates of each dilution in order to identify potential issues during data acquisition that lead to variability in results.

For statistical evaluation of the MAT, logistic regression models like the 4-parameter logistic model (4-PML) or 5-parameter logistic model (5-PML) are most frequently used. Bioassays, e.g. ELISA, usually start as an asymptote at low doses, increase linear into an “S”-shaped curve, and end up at another asymptote at high doses meaning that they are only linear across a specific range of concentration magnitudes. While the 4-PLM is used for a symmetrical curve, the 5PLM is used if the curve from one asymptote to the other is not symmetrical. For statistical data analysis with the PyroCell® MAT HS system, a 5-PML model is recommended.

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 free of detectable endotoxin (i.e. endotoxin content lower than the assay sensitivity). Critically assess procedures and the environment when preparing the assay. Repeat the experiment with fresh, endotoxin-free materials.
- The “historical LOD” is defined as the endotoxin concentration that reproducibly shows an OD reading above the cut-off value with different batches of pMAT Cells. Initially, it is recommended to use the historical LOD for calculating, e.g. the MVD of the product. The LOD may be adjusted based on laboratory data or the current experiment[1].
- 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 ($p < 0.01$), and (2) the regression of response on log10 dose does not deviate significantly from linearity ($p > 0.05$ (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 highest product concentration that was 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 microtiter 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 ascending OD values with increasing endotoxin concentrations. All OD values of endotoxin 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 their concentrations.
- Using an appropriate analysis tool, e.g. the Lonza MAT Analysis template, a dose-response curve is fitted through the data points.
- Determine the LOD (see section 4).

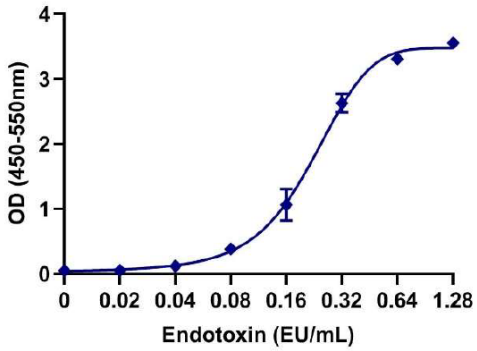


Fig. 9A: Typical sigmoidal 7-point endotoxin standard curve obtained with the PyroCell® MAT HS Kit.

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 HS Kit (0.08 EU/mL) is used to calculate the MVD of the product, the test is valid if LOD ≤ 0.08 EU/mL.
- **Dose response**: the dose-response of the product dilutions must be parallel to the Endotoxin Standard Curve.
- **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 preparation procedures and repeat the experiment with endotoxin-free materials.
- The term “historical LOD” is defined as the endotoxin concentration that reproducibly demonstrates an OD reading above the cut-off value with different 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 highest concentration 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 to or higher than the historical LOD must be above the blank. The 0.5x LOD concentration can be higher than 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).

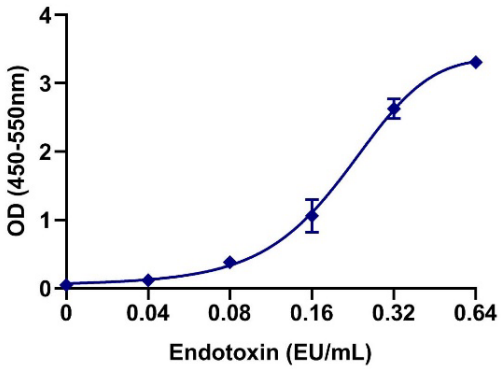


Fig. 9B: Typical sigmoidal 5-point endotoxin standard curve obtained with the PyroCell® MAT HS Kit and the PeliKine compact human IL-6 ELISA as a read-out system.

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: the historical LOD of the PyroCell® MAT HS Kit is used to calculate the MVD of the product. The test is valid if LOD ⩽ 0.08 EU/mL. The mean OD signal for the endotoxin standard solutions at 1x LOD 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 → not 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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