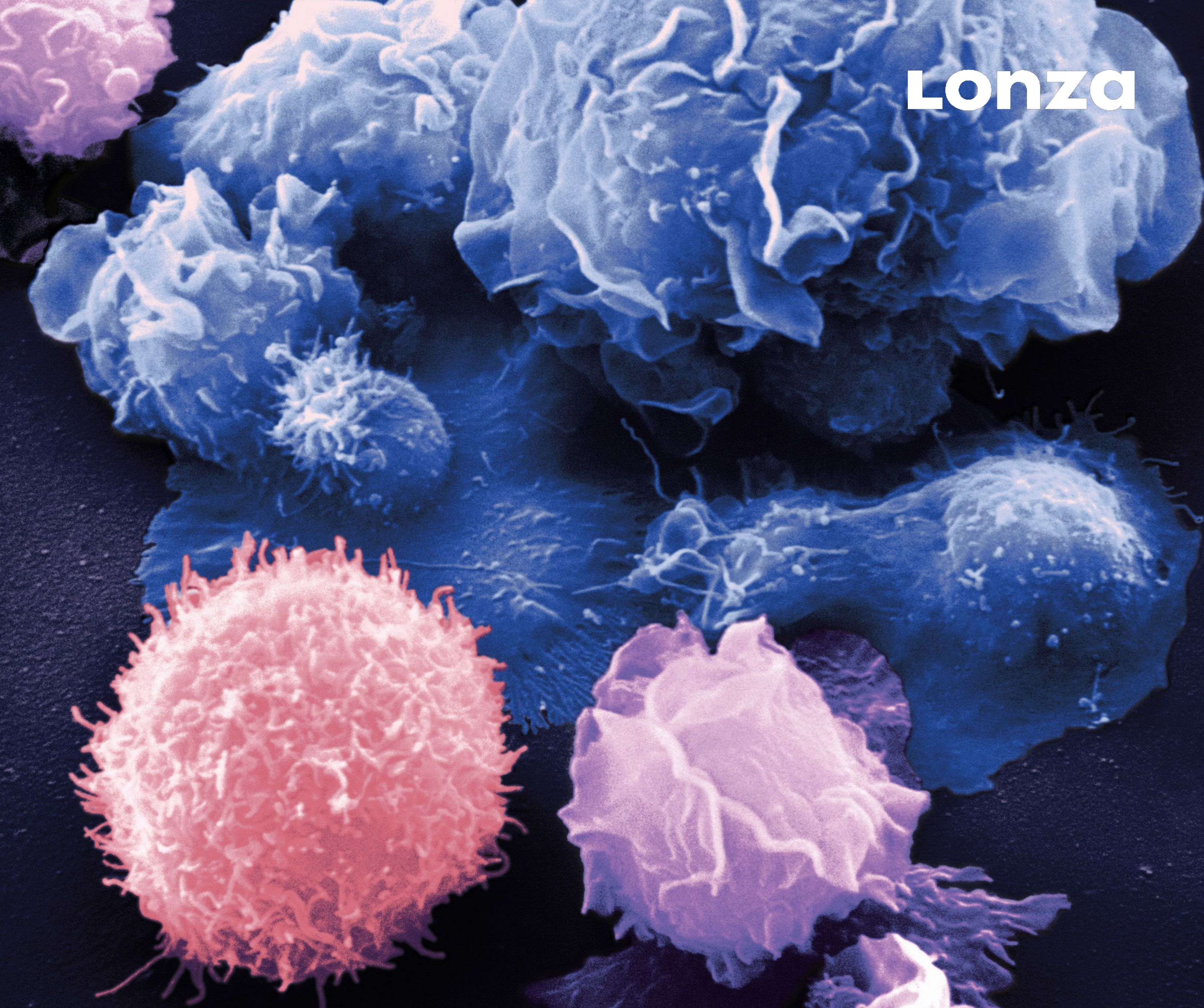


# PyroCell® MAT Rapid System

## Instructions for Routine Use

**Cat. # 296407** contains:
**Cat. # 249735** - PyroCell® MAT Kit
**Cat. # 296406** - PeliKine Human IL-6 ELISA Rapid Kit

RT-MN040 08/24

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.

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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 cytokine release as read-out for pyrogenic activity. Other cytokines may be suitable as read-out, but their use with the PyroCell® MAT 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.

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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 relevant compendia guidelines, e.g. the European Pharmacopoeia[1] 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.**

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## 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 by the European Pharmacopeia[1] as a compendial, *in vitro* pyrogenicity test for the detection of endotoxins and non-endotoxin pyrogens (NEP) in pharmaceutical preparations (e.g. general chapters 2.6.30 “Monocyte Activation Test”, 5.1.13 “Pyrogenicity”).

The MAT is recognized by other world pharmacopeias such as the United States Pharmacopeia, either as a suitable replacement test for the Rabbit Pyrogen Test (RPT) or as an alternative method to the RPT. Alternative method validation may be required[6]. 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 FBS-based 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 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.

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Customer Service: 800 638 8174 (toll free)

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Online ordering: bioscience.lonza.com

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 |
|---|---|
| 2023 | First issue |
| 2024 | Adoption to revised general chapter 2.6.30 (Ph. Eur. edition 11.5) |

## 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. |

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## 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 Kits contain fetal bovine serum (FBS) sourced in North America. FBS components shall be handled in laboratory environments only. |
| **Warning!** | pMAT Cells are derived from human blood donations.<br>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 Kit. The MAT 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 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.<br>**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. |

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## 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 Culture Medium Supplement (included in the PyroCell® MAT Kit) |
| Cut-off | Limit of detection, absorbance (OD) |
| ELISA | Enzyme-linked Immunosorbent Assay |
| EE, EE/mL | Endotoxin Equivalents, Endotoxin Equivalents per Milliliter |
| EEU/mL | Endotoxin Equivalent Units per Milliliter |
| EU, EU/mL | Endotoxin Units, Endotoxin 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, unit of reference standard (e.g. EU/mL) (calculated by conversion of the cut-off value into EU or EE) |
| 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 |
| Test Sensitivity | Lowest endotoxin reference concentration on the standard curve whose response exceeds the cut-off value. |
| USP | United States Pharmacopeia |
| 4-PLM | Four parameter logistic model |
| 5-PLM | Five parameter logistic model |

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## 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)**<br>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)**<br>USP <151> | *In vivo*: measures raise in body temperature after injection of a drug | |
| Endotoxin | **Recombinant Factor C Assay (rFC)**<br>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)**<br>USP <85>, Ph. Eur. 2.6.14 | *In vitro*: measures the initiation of the clotting cascade in horseshoe crab blood amebocytes by endotoxin | |

**Fig. 1**: Compendial analytical methods for endotoxin and pyrogen detection.

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 first qualified and validated by the European Center for the Validation of Alternative Methods (ECVAM) in 2005[3] and by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)[4] in 2008. It was adopted by some main pharmacopeias, for example, the European Pharmacopeia (Ph. Eur. Chapter 2.6.30)[2] as a compendial method. Other pharmacopeias describe the MAT as a suitable, alternative method to the RPT. For example, the United States Pharmacopeia (USP) requests the MAT to be validated according to USP <1225>[5,6]. Please contact your local authorities for more information.

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## 2. PyroCell® MAT Rapid System – Assay Principle

The PyroCell® MAT 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 are 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. Interleukin-6 (IL-6) cytokines released by the monocytes into the supernatants is then detected with the PeliKine Human IL-6 ELISA Rapid Kit and measured in a microplate absorbance reader. Using a dose-response curve generated with reference standard endotoxin (RSE) dilutions (endotoxin standard curve), the measured OD values are finally converted into Endotoxin Equivalents (EE/mL).

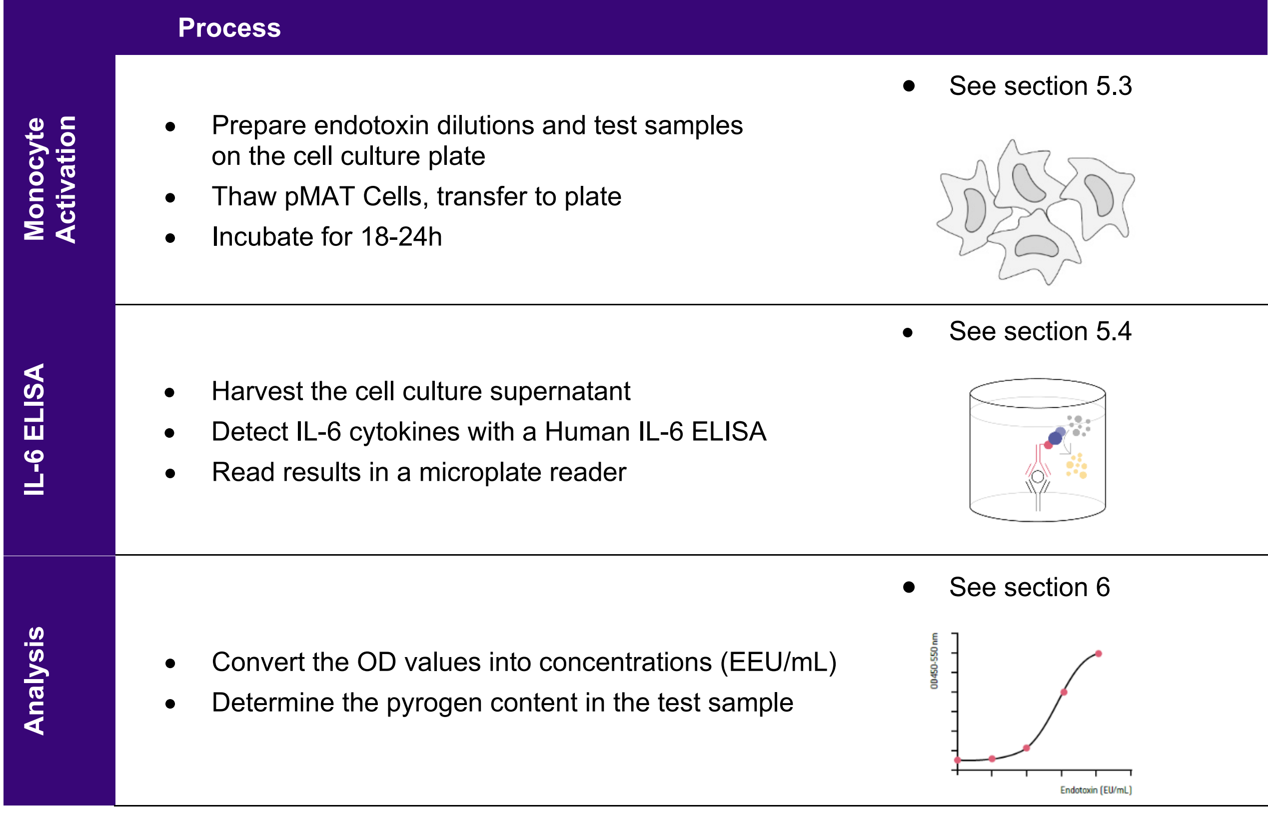


**Fig. 2**: PyroCell® Monocyte Activation Test - Assay overview

# 3. Material Information and Storage Conditions

The PyroCell® MAT Rapid System (# 296407) combines reagents to conduct the MAT in a ready-to-use kit. The following materials are included: the PyroCell® MAT Kit (# 249735), comprising 3 vials pMAT cells (M2016LC), and 3 vials MAT Culture Medium Supplement, (M2016LS) and the PeliKine Human IL-6 ELISA Rapid Kit (# 296406, comprising the PeliKine Human IL-6 ELISA Rapid Set A (M2018) and the PeliKine Human IL-6 ELISA Rapid Set B (M2019).

## 3.1 PyroCell® MAT 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 Culture Medium Supplement (M2016LS) | Yellow | 3 x 2.1 mL | 8000-xxxxxx |

### 3.1.2 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!** 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.

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The MAT Culture Medium Supplement contains an antibiotics mix. Consult SDS for Product Safety 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 test wells). 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 ELISA 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)[7]; 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.

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### 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 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 | 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:

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| 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 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<br>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 (700±100 rpm) | For incubation of ELISA plates |
| Microplate reader (read out: 450nm; reference wavelength: 540-590nm), e.g.<br>Nebula® Multimode Reader, Cat.# 25-375S<br>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*
2. European Directorate for the Quality of Medicines (EDQM, edition 11.5 or later). *European Pharmacopeia chapter* 2.6.30: Monocyte Activation Test
3. 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)
4. 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)
5. United States Food and Drug Administration (FDA), 2012: Guidance for Industry Pyrogen and Endotoxins Testing: Questions and Answers
6. United States Pharmacopeia (USP), 2007: Validation of Compendial Procedures <1225>. Rockville, MD. USP 41–NF:1-5.
7. 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.
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 term test sensitivity has been introduced as of Ph. Eur. 11.5/ 2.6.30. It represents the lowest endotoxin reference standard concentration on the standard curve whose response exceeds the cut-off value. The test sensitivity is used for calculation of the maximum valid dilution (MVD).

The cut-off value is expressed in the unit of response, i.e. optical density (OD). The cut-off value and the "limit of detection" (LOD) represent the same information but use different units. LOD is typically expressed as a concentration of the unit of reference, e.g. EU/mL. For the purpose of the MAT test the unit EE (endotoxin equivalent) is also used.

**Important!** Historical data suggests a test sensitivity ≤ 0.02 EU/mL for the PyroCell® MAT Kit if used with the PeliKine Human IL-6 ELISA Rapid Kit**.**

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

**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 test sensitivity of the 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.[2]

The MVD is calculated using the following expression:

$$MVD = \frac{\text{CLC x C}}{\text{Test Sensitivity}}$$

CLC = Contaminant Limit Concentration (e.g. EU/mg).
C = Concentration of test solution (e.g. mg/mL).
Test sensitivity = the lowest endotoxin reference standard concentration on the standard curve whose response exceeds the cut-off value.

Since test sensitivity is an actual point on the standard curve, it must be greater than the cut-off value. Confirm the test sensitivity for each test to assure correctness of the MVD calculation.

The CLC is calculated using the following expression:

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

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 cut-off value

The cut-off value is expressed as optical density (OD) and is calculated using the following expression[2]:

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

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

The LOD of the measured response corresponds to the conversion of the cut-off value into a concentration in Endotoxin Units per milliliter (EU/mL, EE/mL).

## 5. Test Procedure

### 5.1 Use of qualified cells

pMAT Cells contained in the PyroCell® MAT Kit consist of a cryopreserved pool of human peripheral blood mononuclear cells (PBMC) serving as the monocytic cell source[2] 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 6-5 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).[1,2] Preparatory testing is required to establish the preparation of the endotoxin standard curve in the laboratory, to confirm that the test solution 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 1 – Semi-quantitative test, or method 2 – Reference lot comparison test). Preparatory testing is repeated whenever there is a significant change in the experimental conditions that likely influence the results of the test.

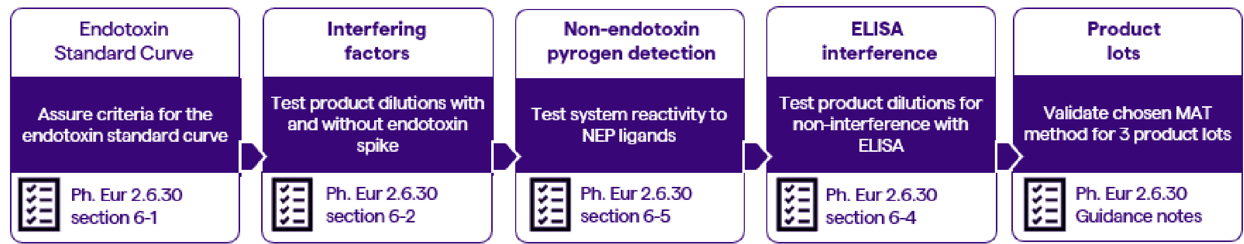


**Fig. 3**: Preparatory testing requirements.

An endotoxin standard curve prepared from reference standard endotoxin (e.g. RSE, # E700) is required on each 96-well test plate during preparatory testing and for method 1[2]. It is used to convert the measured OD signals into endotoxin equivalents (EE). Establish the endotoxin standard curve during preparatory testing by performing the MAT on 7 ascending RSE dilutions, and a blank (negative control).

For data analysis, chose an appropriate regression model for curve fitting. For the PyroCell® MAT Kit a 4-parameter logistic model (4-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, optionally reduce the number of endotoxin reference dilutions to 5 in order to fit more test samples on the same microtiter plate.

The test for interfering factors requires a positive product control (PPC) at a concentration at or near the middle of the standard curve to be recovered from product dilutions within the range of 50 – 200% of the spike.[2] All product dilutions shall not exceed the product MVD in order to ensure that the contaminant can still be detected. If product interferences cannot be overcome by dilution, method 2 is recommended over method 1.

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 test solution with all dilutions not exceeding the MVD.
- For all test dilutions, generate a PPC by spiking with an endotoxin concentration near the estimated middle of the standard curve, i.e. 0.08 EU/mL for the PyroCell® MAT Kit.
- 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 sample concentration with a valid spike recovery.

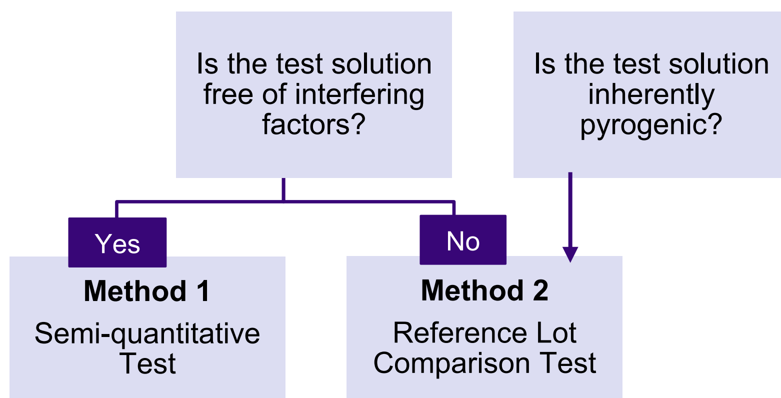


**Fig 4**: Simplified decision tree to support the choice of the MAT method.

Test the system reactivity towards NEP by using either a historic product lot with NEP contaminant (or use a batch that elicited a positive response in the rabbit pyrogen test), or use at least two different NEP ligands for toll-like receptors reflecting a risk for the product. Spike NEPs into the most concentrated test sample that passed interference testing. NEPs must be recovered in 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 same optimal dilution to exclude interference of the test sample with the read-out system.[2] OD values obtained in the IL-6 ELISA for a dilution series with an IL-6 reference standard in the presence and absence of the test sample should not differ by more than 20%.

Based on the results from preparatory testing, select the suitable method for routine testing:

- Method 1: Semi-quantitative test
- Method 2: Reference lot comparison test

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Method 2 involves the comparison of a test sample with a validated reference lot of the same substance or a reference substance.[2] The type of analysis selected to compare the two is to be justified and validated by the user for each product and has to include test validity criteria. Method 2 is to be also performed in cases where a preparation to be examined shows marked interference that cannot be diluted out within the MVD limits. If an IL-6 ELISA is used for the analysis, we recommend using an IL-6 reference standard. Preparation of reference standard endotoxin is not required.

## 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 test substances 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 – 24 hours.

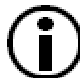
### 5.3.1 Preparation of complete medium

- Ensure sterility of the IMDM, e.g. by using an unopened bottle.
- **Important!** A minimum of 1 mL IMDM without MAT Culture Medium 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 Culture Medium Supplement (M2016LS) that is included in the PyroCell® 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. **Use the complete medium within 8 hours**.

### 5.3.2 Preparation of reference endotoxin dilutions



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

A dose-response curve prepared from reference endotoxin dilutions (endotoxin standard curve), and a blank (negative control) should be included on every microtiter plate for method 1. The endotoxin standard curve is used to determine that the pyrogen contents in the test sample are below the contaminant limit concentration (CLC). The calculated test sensitivity further confirms the MVD calculation. The basal content of the blank may indicate unspecific cell stimulation and should be optimized to be as low as possible.

During initial qualification of the PyroCell® MAT Kit, we recommend to prepare a standard curve with 7 endotoxin dilutions (7-point standard curve), and a blank (negative control). For routine testing 5-point standard curve may be applied in order to fit more test dilutions to the microtiter plate. Based on the test sensitivity of ≤ 0.02 EU/mL for the PyroCell® MAT system prepare geometric dilutions from 0.01 to 0.16 EU/mL (5-point standard curve) or 0.01 to 0.64 EU/ml (7-point standard curve) respectively. Example plate layouts are illustrated in **Fig 5.**

A

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | 0.64 EU/ml (R7) | 0.32 EU/ml (R6) | 0.16 EU/ml (R5) | 0.08 EU/ml (R4) | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | 0.04 EU/ml (R3) | 0.02 EU/ml (R2) | 0.01 EU/ml (R1) | Blank (R0) | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

B

| | 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 | | | | | | | | | | | | |

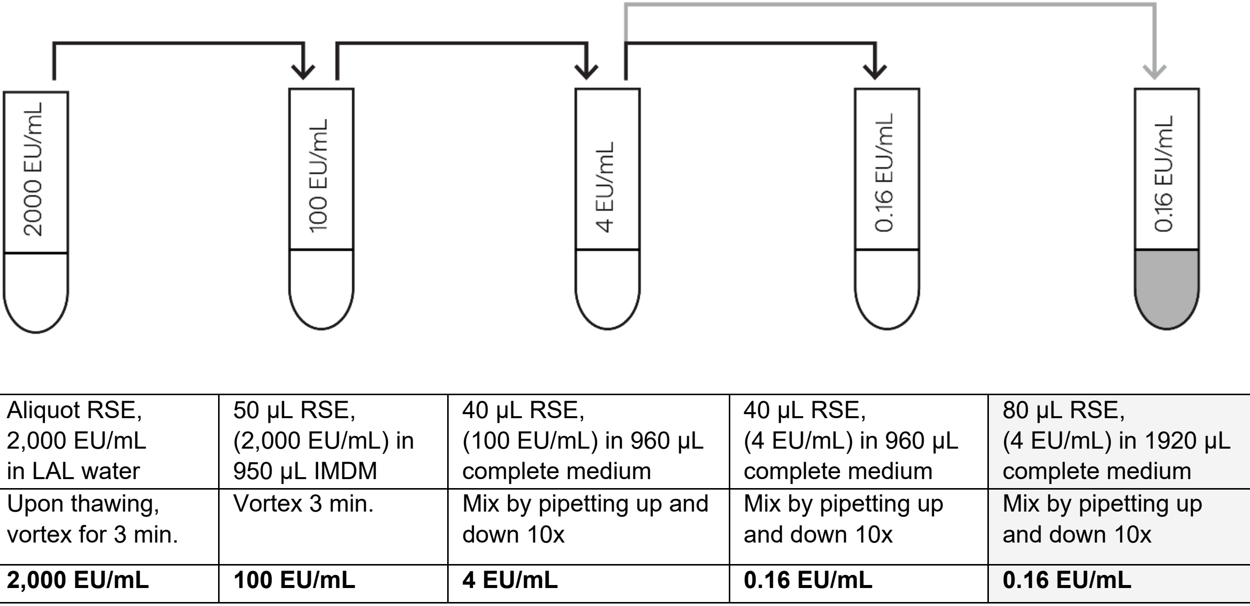
**Fig. 5**: (A) Suggested plate layout for 7-point standard curve (R0-R7). (B) Suggested plate layout for a 5-point standard curve (R0-R5). Concentrations are based on a test sensitivity of ≤ 0.02 EU/mL.

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.

An example for endotoxin dilutions for the preparation of a 5-point endotoxin standard curve, and a spike solution is given in **Fig 5**. Use sterile, non-pyrogenic borosilicated glass tubes (# N207) to dilute the endotoxin stock solution into the highest concentration of the endotoxin standard curve in complete medium as shown in **Fig 6** (step 1).




| Aliquot RSE,<br>2,000 EU/mL<br>in LAL water | 50 µL RSE,<br>(2,000 EU/mL) in<br>950 µL IMDM | 40 µL RSE,<br>(100 EU/mL) in 960 µL<br>complete medium | 40 µL RSE,<br>(4 EU/mL) in 960 µL<br>complete medium | 80 µL RSE,<br>(4 EU/mL) in 1920 µL<br>complete medium |
|---|---|---|---|---|
| Upon thawing,<br>vortex for 3 min. | Vortex 3 min. | Mix by pipetting up and<br>down 10x | Mix by pipetting up<br>and down 10x | Mix by pipetting up<br>and down 10x |
| **2,000 EU/mL** | **100 EU/mL** | **4 EU/mL** | **0.16 EU/mL** | **0.16 EU/mL** |

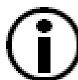
**Fig. 6**: Serial dilution to generate a 5-point standard curve. Prepare 0.16 EU/mL endotoxin dilution and 0.16 EU/mL spike solution. The final PPC is 0.08 EU/mL

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 40 µL of 100 EU/mL RSE dilution to 960 µL complete medium. The final concentration is 4 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 40 µL of 4 EU/mL RSE dilution to 960 µL **complete medium.** The final concentration is 0.16 EU/mL. Mix by pipetting up and down 10x, with a volume of at least 500 µL.
6. Add 200 µL of 0.16 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, and A3-H3).

8. On the plate, prepare a serial dilution from R5 to R0 (**Fig. 5**) 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). Transfer 100 µL from R5 (10.16 EU/mL, A1-D1) to R4 (0.08 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 R4 to R3 (A3-D3), from R3 to R2 (E1-H1), and from R2 to R1 (E2-H2). Discard the final 100 µL from R1.
10. Save the remaining 4 EU/mL RSE solution prepared at step 4 for preparation of endotoxin spiked samples (see **Fig. 6**).

### 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
- A sample dilution series contains the optimum dilution determined during preparatory testing (f) and two geometric dilutions ($f_1$ and $f_2$)[2]
- Using the example layout below, 2 – 3 product preparations can be tested on a single plate

For routine application of Method 1 it is required to fit the following preparations on a 96-well plate: A fresh preparation of an endotoxin standard curve and three dilutions of a product preparation, each with and without an endotoxin spike. Each dilution is tested in 4 replicates suggesting 2 – 3 test samples fitting on a plate. Optional, an NEP control can be used instead of a third sample. Examples for a plate layout is given below (**Fig. 7**):

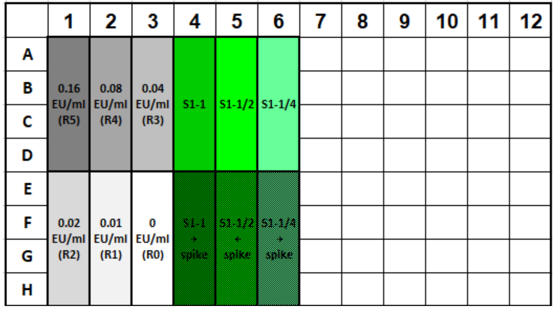



**Fig. 7**: Example plate layout for MAT method 1. Endotoxin reference standards with concentrations in EU/mL (R0 – R5). Sample (f = S1) in 2 successive two-fold dilutions ($f_1$ = S1-1/2 and $f_2$ = S1-1/4) with and without spiked endotoxin (PPC, row E-H, indicated by darker color). The spike concentration should be at or near the middle or the standard curve.

1. Add 200 µL of the product test sample S1-1 (highest concentration with valid PPC recovery, determined during preparatory testing) in wells A4 to H4.

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 S1-1 to S1-1/2 (A5 to 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 (A6 to H6) 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 4 EU/mL dilution of step 5.3.2. Add 80 µL of 4 EU/mL endotoxin to 1920 µL complete medium. The final concentration is 0.16 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). The endotoxin spike concentration in the sample corresponds to the 0.08 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 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 Immuno-sorbent 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 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 equivalents (EE).

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

| 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. |

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

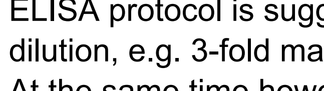
**Consult the SDS for Product Safety Information.**

- Working strength dilutions of the HPE buffer and the washing buffer may 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.

### 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 approximately 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. At the same time however, more concentrated endotoxin reference dilutions may fall 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 2. It is optional for routine MAT methods 1.
- 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).

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| | 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.

| Preparation (1 plate) | Add to each well | Incubation/ Wash |
|---|---|---|
| • Homogenize the harvested supernatant by pipetting up and down 5 times.<br><br>**Biotinylated IL-6 antibody**<br>• Dilute 120 µL biotinylated antibody (yellow cap) in 9.6 mL HPE buffer (working strength).<br>• Mix by inverting tube 10 times. | **(Supernatant dilutions: 1:5)**<br>• Add **20 µL** supernatant or IL-6 standard to the pre-coated plate. Keep the plate layout.<br>• (Optional) add 20 µL of IL-6 standard dilutions to “empty” wells.<br>• Add **80 µL** of biotinylated IL-6 antibody dilution. Mix 3 times by gently pipetting up and down.<br>• Cover plate with adhesive seal. | 1 hour at 18 to 25°C<br><br>**Prepare streptavidin-HRP conjugate dilution immediately before wash step.** |
| **Wash with washing buffer** | ≥ **300 µL** washing buffer (working strength). | 5 times. |
| **Streptavidin-HRP conjugate**<br>• Dilute 2 µL streptavidin-HRP (brown cap) in 20 mL of HPE buffer (working-strength).<br>• Mix by inverting tube 10 times. | • Add **100 µL** of Streptavidin-HRP conjugate dilution.<br>• Cover plate with adhesive seal.<br>• Gently agitate the plate by tapping the edge for a few seconds | 30 min at 18 to 25°C.<br><br>**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**<br>• Ready to use. | • Add **100 µL** TMB substrate solution<br>• Gently agitate the plate by tapping the edge for a few seconds<br><br>**Protect TMB substrate from exposure to light!** | Approximately 10 min at 18 to 25°C in the dark, e.g. use a drawer.<br><br>**Do not cover plate with aluminum foil!** |
| **Stop solution**<br>• 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 ensure a read-out at 450 nm and at a reference wavelength between 540 and 590 nm (e.g. Nebula® multimode reader, # 25-375S or standard absorbance reader).
- 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[2].
- 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.

A product-specific validation provides 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:

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1. For each test 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. Plot the OD values on the y-axis and the log of the endotoxin concentrations used for the standard curve on the x-axis and perform a regression.
5. Calculate the average OD value for each test replicate. With the regression equation calculate the concentration (EE/ mL) of each product dilution.
6. Multiply the obtained endotoxin concentration with the dilution factor of the product sample to obtain the contaminant concentration in the sample.
7. Determine the LOD and thereof, confirm the test sensitivity (next, actual point on the standard curve, 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 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-parameter logistic model (4-PLM) is typically used for a symmetrical curve, the 5-parameter logistic model (5-PLM) is applied if the curve from one asymptote to the other is not symmetrical. For statistical data analysis with the PyroCell® MAT system, a 4-PLM model is recommended.

The endotoxin standard curve is valid if there is a good fit between the data points and the chosen regression model. This can be evaluated visually (e.g. by assessment of regression and/or residual plots) or by means of a statistical test (lack-of-fit test, $p > 0.05$).[2] Furthermore, the coefficient of determination should not be less than 0.975.

### 6.1 Method 1 – Semi-quantitative test

- **Important!** The basal release of the IL-6 cytokine measured in the blank in the absence of added standard endotoxin should be optimized to be as low as possible.[2] If 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 term "test sensitivity" defines an actual point on the endotoxin standard curve.[2] The OD reading should be reproducibly above the cut-off value. The test sensitivity is used to calculate the MVD of the product and its validity has to be checked for each experiment.[1] The test sensitivity may need to be adjusted if a different standard curve is used.

Method 1 involves a comparison of the test sample with a standard endotoxin dose-response curve. To pass the test, the contaminant concentration of the preparation to be examined has to be lower than the CLC for the product The OD signals for the test dilutions are converted into contaminant concentration (EE/ mL) and corrected for the dilution factor. The final value is compared to the product CLC.

Routine testing of products with method 1 is performed with the highest concentration that was demonstrated for a valid spike recovery in preparatory testing (see section 5) and two additional geometric dilution of this test dilution. No dilution is allowed to exceed 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.

#### 6.1.1 Acceptance criteria for the endotoxin standard curve

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

- Plot the net absorbance (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 test sensitivity to confirm the MVD calculation

Confirm that the endotoxin standard curve meets certain statistical fit criteria that are used as curve acceptance specifications (system suitability).

- **Blank:** average of blank ≤ 0.1 OD.
- **Confirm MVD calculation:** Test sensitivity > cut-off value
  Example: the test sensitivity of the PyroCell® MAT Kit, 0.02 EU/mL, is used to calculate the MVD of the product. The test is valid if the converted cut-off value (= LOD) < test sensitivity (actual point on the standard curve closest to the LOD). For the purpose of the test, test sensitivity and LOD can be expressed as EE (endotoxin equivalent)/mL.
- **Goodness of fit** (evaluation of the fitted curve):
  - Confirm good fit between data points and the chosen regression model AND/ OR perform the Lack-of-fit test, $p > 0.05$
- **Coefficient of determination ($R^2$)**: $R^2 > 0.975$.

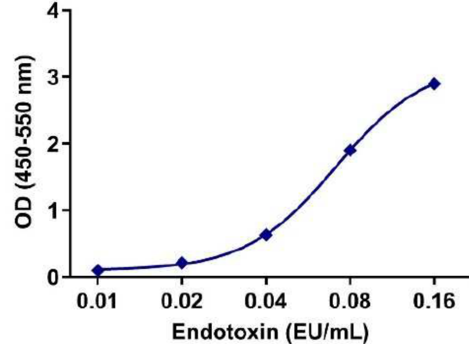


**Fig. 9B**: Typical sigmoidal 5-point endotoxin standard curve.

### 6.1.2. Calculation of the pyrogen content

- For each test sample dilution not exceeding the MVD, calculate the endotoxin spike recovery. The test is not valid unless at least one of the dilutions displays a spike recovery within 50 – 200%

- For each test sample dilution, the mean OD value is converted in EE/mL, corrected with the dilution factor and compared to the CLC. The test does not conform if the mean concentration of any dilution exceeds the CLC regardless of the spike recovery.

  - If all three sample dilutions display a value in EE/ml < CLC → PASS.
  - If at least one sample dilution display a value in EE/ml > CLC → FAIL.
  - If an NEP PPC is used it has to be detected above the cut off value.

### 6.2 Method 2 - Lot comparison test

Method 2 is recommended in cases where a preparation to be examined shows marked interference and cannot be diluted within the MVD to overcome such interferences. Responses to non-endotoxin contaminants may dilute out more rapidly than responses to endotoxin, which makes it necessary to perform the test at a range of dilutions that include minimum dilution. Method 2 is comparing the OD signals or IL-6 contents from 3 dilutions of the test sample to a reference lot of the same formulation or the same class of formulation. For the read-out an IL-6 standard curve is prepared with at least 4 geometrically diluted concentrations, and a blank in duplicate.

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 the test 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 exceeding a user-defined, justified value, for example 2.5-fold to pass the test.

#### 6.3.2 Calculation of pyrogenicity

For each sample an OD ratio (or IL-6 concentration equivalent 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 < user defined acceptance criterion → pass (lot examined not pyrogenic as compared to the reference lot).
- OD ratio > user defined acceptance criterion → fail (lot examined pyrogenic as compared to the reference lot).

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