Instructions for use

Sanquin Reagents B.V.
Plesmanlaan 125
1066 CX Amsterdam
The Netherlands

Phone: +31 20 5123599
Fax: +31 20 5123570
info@essange-reagents.nl
www.sanquin.org/MAT

MAT Cell Set REF **M2016** Σ 288

302_v04 09/2023 (en) *For research use only*

1 General information

Pharmaceutical products intended for parenteral use must be free of pyrogens (fever–inducing substances), which can originate from bacteria, viruses and fungi. The monocyte activation test (MAT) is intended as an in vitro-based assay which determines pyrogenic activity in pharmaceutical products by measuring cytokine production of human monocytes. The MAT detects endotoxin and non-endotoxin pyrogens that are relevant to humans. The European Pharmacopoeia (Ph. Eur. chapter 2.6.30) considers the MAT to be suitable, after a product-specific validation, as a replacement for the rabbit pyrogen test[1].

2 Principle of the test

The MAT measures a response of the human innate immune system. Monocytes, the key cells of innate immunity, respond to the presence of pyrogens by producing a number of cytokines, which can be measured by Enzyme-Linked Immunosorbent Assays (ELISA). The pooled MAT (pMAT) Cells consist of a pool of cryo-preserved peripheral blood mononuclear cells (PBMC) isolated from 4 healthy human donors. The pMAT Cells are thawed and incubated with the test sample. Following incubation at 37°C, the cell culture supernatant is collected and analysed for the presence of the cytokine interleukin-6 (IL-6) by ELISA[2].

3 Package contents

pMAT Cells	3x1 mL	green caps	
MAT Culture Medium Supplement	3x2.1 mL	yellow caps	REF M201602

- The vial of pMAT Cells is labelled with two codes;
  - The first code (68-XXXX) refers to the lot org (original lot) number which can also be found on the label of the box and the Certificate of Analysis
  - The second code (P-XXX) refers to the individual vial code
- After opening, all reagents must be used immediately and cannot be re-frozen
- Consult the Certificate of Analysis for additional information

4 Additional materials and/or equipment

The following equipment and materials are required but not provided:

- Freezer (-80°C or lower)
- Laminar airflow cabinet
- CO_2-incubator at 37°C with 5% CO_2
- Water bath at 37°C
- Vortex mixer
- Microplate photometer for measuring absorbance at 450 nm, with a reference wavelength set between 540 and 590 nm
- Adjustable multichannel pipettor (30 - 300 µL)
- Adjustable pipettes (5 - 1000 µL)
- Appropriate sterile, non-pyrogenic pipette tips
- Sterile, non-pyrogenic polystyrene disposable pipettes
- Sterile, non-pyrogenic borosilicate glass tubes
- Sterile, non-pyrogenic 96-well flat bottom microplates suitable for cell culture
- 96-well round bottom, untreated microplate for harvesting supernatant from cell culture
- Sterile, non-pyrogenic 50 mL conical polypropylene centrifuge tubes
- Iscove's Modified Dulbecco's Media (IMDM; 40 mL from a new, unopened bottle) from Lonza (REF: BE12-722F)
- Endotoxin reference standard that has been calibrated against the International Standard, e.g. endotoxin standard Biological Reference Preparation (BRP) from the European Pharmacopoeia
- Sterile, non-pyrogenic distilled or deionised water to reconstitute endotoxin standard
- Human IL-6 ELISA, e.g.:
  - PeliKine compact™ human IL-6 kit (Sanquin REF M1916)
  - PeliKine-Tool™ set (additional reagents for application in PeliKine-compact™ ELISA kits) (Sanquin REF M1980)
- European Pharmacopoeia (Ph. Eur. chapter 2.6.30)

Optional

- Repeating pipette
- Automated microplate washer
- Microplate shaker

5 Precautions

- Not suitable for in vitro diagnostic use. The MAT Cell Set is not intended for the detection of pyrogens in clinical samples or as an aid in the diagnosis of human disease
- All MAT Cell Set reagents must be stored at -80°C or lower in the original packaging. Check your freezer for appropriate alarm settings
- Thawed reagents must be used immediately and cannot be refrozen
- Leaking or damaged vials cannot be used
- Reagents (unopened or opened) cannot be used beyond the expiration date, which is printed on the label of the box
- Do not use reagents with any evidence of turbidity or microbial contamination
- Components of MAT Cell Set contain foetal bovine serum
- The user must be trained and familiar with sterile and non-pyrogenic cell handling and with ELISA test procedures
- Although the components of MAT Cell Set are tested for infectious diseases and found negative, the reagents cannot be assumed to be free from infectious agents and must be handled with appropriate care
- Care must be taken in the use and disposal of each container and its contents. Waste disposal, after completion of the test, must be performed according to your laboratory regulations.

6 Abbreviations

BRP	Biological Reference Preparation
CLC	Contaminant Limit Concentration
ELISA	Enzyme-Linked Immunosorbent Assay
Ph. Eur.	European Pharmacopoeia
EU/mL	Endotoxin Units per millilitre
IL-6	Interleukin-6
IMDM	Iscove's Modified Dulbecco's Media
IPA	Isopropyl Alcohol
LOD	Limit Of Detection
MAT	Monocyte Activation Test
MVD	Maximum Valid Dilution
PBMC	Peripheral Blood Mononuclear Cell

7 Definitions

The MAT is used to demonstrate that the amount of pyrogens in the test product does not exceed the Contaminant Limit Concentration (CLC). Therefore it is necessary to calculate the Maximum Valid Dilution (MVD) based on the Limit Of Detection (LOD) of the test system.

7.1 Calculation of the Maximum Valid Dilution (MVD) and Contaminant Limit Concentration (CLC)

The Maximum Valid Dilution (MVD) is defined as the maximum allowable dilution of a sample at which the contaminant limit can be determined.[1] The MVD is calculated using the following expression:

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

CLC = Contaminant Limit Concentration
C = Concentration of test solution
LOD = Limit Of Detection

The CLC is calculated using the following expression:

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

K = threshold pyrogenic dose of endotoxin per kilogram of body mass
M = maximum recommended bolus dose of product per kilogram of body mass

7.2 Calculation of the Limit Of Detection (LOD)

The LOD of the MAT is determined by the values of the blank and the endotoxin standard curve. The LOD is the concentration in Endotoxin Units per millilitre (EU/mL), corresponding to the cut-off value. The cut-off value is calculated using the following expression[1]:

cut-off value $= \bar{x} + (3s)$

$\bar{x}$ = mean of the 4 replicates for the responses to the blank
s = standard deviation of the 4 replicates of the blank

The terms cut-off value and LOD depict the same information in different units. The cut-off value is expressed in optical density (OD) and the LOD is expressed in EU/mL. Each user has to establish the LOD in their laboratory based on the current experiment or historical data.[1] Using MAT Cell Set combined with the PeliKine compact™ human IL-6 kit from Sanquin, an LOD of 0.02 EU/mL may be expected. Consult the Certificate of Analysis for additional information.

NOTE: Endotoxin concentrations stated throughout this Instructions For Use are given as concentrations per sample (i.e. prior to addition of additional (spiked) media and pMAT Cells to the samples). The final concentration in the reaction well is half of the stated concentrations: The total reaction volume in a single well is 200 µL, which consists of 100 µL sample, 50 µL of media or spiked media and 50 µL of cells.

8 Test procedure

The MAT consists of two main steps:

1) the MAT cell incubation with the standards and test samples
2) the IL-6 ELISA

Refer to Ph. Eur. chapter 2.6.30 for a detailed description of the test methods.

During step 1, all equipment and materials coming into contact with the culture components need to be non-pyrogenic. Work in a laminar airflow cabinet, wear gloves, and clean the exterior of equipment and materials with 70% Isopropyl Alcohol (IPA) prior to the experiment. Step 2 (ELISA) can be performed on a regular lab bench (no requirement to work in a laminar airflow cabinet).

8.1 Preparation of complete medium

The complete medium used for the MAT cell incubation consists of IMDM supplemented with the provided MAT Culture Medium Supplement (M201602). Always use an unopened bottle of IMDM and prepare complete medium on the day of the experiment.

1. Thaw MAT Culture Medium Supplement in a water bath at 37ºC and add the entire contents (2.1 mL) to a sterile, non-pyrogenic 50 mL tube containing 33 mL IMDM and mix gently by inversion of the closed tube

IMPORTANT: a minimum of 1 mL IMDM **without** addition of the culture medium supplement mix is needed to make the first dilution of the endotoxin standard. Preparing the first dilution with complete medium will result in a non-accurate endotoxin standard curve.

2. Allow the complete medium to reach room temperature (18 - 25°C) and mix gently before use. Use complete medium within eight hours.

8.2 Preparation of the endotoxin standard curve

The endotoxin (not included) standard curve is used to determine the LOD and to estimate the amount of pyrogen in the test sample. Include an endotoxin standard in each microplate as the OD values of the endotoxin standard dilutions and samples may vary from plate to plate. Chapter 2.6.30 of the European Pharmacopoeia requires a standard curve that consists of at least 4 replicates of 4 different endotoxin concentrations and a blank. We recommend to use 5 different endotoxin concentrations starting from 0.16 EU/mL with twofold dilutions down to 0.01 EU/mL and a blank. These concentrations are based on a historical LOD of ≤ 0.02 EU/mL. A suggested plate layout is given below:

row ID	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												

Figure 1: Suggested plate layout for the endotoxin standard curve.
Standard (R0 – R5) with the final endotoxin concentrations in EU/mL.

Reconstitute the endotoxin standard to 2000 Endotoxin Unit (EU)/mL following the manufacturer's instructions. **Prepare the endotoxin standard curve on the day of the experiment.** Example for preparation of the endotoxin standard curve with range of 0.01-0.16 EU/mL:

1. Add 950 µL **IMDM** (WITHOUT MAT CULTURE MEDIUM SUPPLEMENT) to a sterile, non-pyrogenic borosilicate glass tube and to this add 50 µL of 2000 EU/mL endotoxin to reach a concentration of 100 EU/mL. Vortex thoroughly for 3 minutes.
2. Add 980 µL **complete medium** to a sterile, non-pyrogenic borosilicate glass tube and to this add 20 µL of 100 EU/mL endotoxin to reach a final concentration of 2 EU/mL. Mix thoroughly by pipetting up and down for 10 times, with a volume of at least 500 µL.
   NOTE: Do not vortex and prevent the formation of air bubbles as this will result in decreased cell reactivity towards endotoxin.
3. Add 920 µL **complete medium** to a sterile, non-pyrogenic borosilicate glass tube and to this add 80 µL of 2 EU/mL endotoxin to reach a final concentration of 0.16 EU/mL. Mix thoroughly by pipetting up and down for 10 times, with a volume of at least 500 µL.
   NOTE: Do not vortex and prevent the formation of air bubbles as this will result in decreased cell reactivity towards endotoxin.
4. Add 200 µL of 0.16 EU/mL endotoxin to well A1 to D1 (see Figure 1).
5. Add 100 µL of **complete medium** to the other wells used for the standard curve.
6. Make a twofold dilution by pipetting 100 µL from R5 (0.16 EU/mL) to R4 (0.08 EU/mL) and mix thoroughly by pipetting up and down for 10 times. Prevent the formation of air bubbles.
7. Repeat this until R1 (0.01 EU/mL) and discard the final 100 µL
8. Save the remainder of the 2 EU/mL endotoxin solution prepared at step 2 for preparation of endotoxin spiked samples (see Chapter 8.3).

8.3 Preparation of the test samples

Ph. Eur. chapter 2.6.30 describes a number of test methods: the test for interfering factors, the quantitative (Method A), the semi-quantitative (Method B) tests or the reference lot comparison test (Method C). All tests are run in 4 replicates. An example of a plate layout suitable for either the quantitative (A) or semi-quantitative method (B) is given below:

A)

row ID	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)	S1-1	S1-1/2	S1-1/4						
C												
D												
E												
F	0.02 EU/mL (R2)	0.01 EU/mL (R1)	0 EU/mL (R0)	S1-1 + spike	S1-1/2 + spike	S1-1/4 + spike						
G												
H												

B)

row ID	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	R5 8x LOD	R4 4x LOD	R3 2x LOD	S1-1	S1-1/2	S1-1/4						
C												
D												
E												
F	R2 1x LOD	R1 0.5x LOD	R0 0	S1-1 + spike	S1-1/2 + spike	S1-1/4 + spike						
G												
H												

Figure 2: Suggested plate layout for the quantitative (A) or semi-quantitative (B) method. Endotoxin reference standards (R0 – R5) with endotoxin concentration in EU/mL (A) or LOD (B). Sample (S1) in 3 twofold dilutions without and with spiked endotoxin (0.04 EU/mL for method A, 2xLOD for method B).

Using this layout, a maximum of 3 products can be tested on a single plate. **Prepare the test samples on the day of the experiment.** Preparation of the sample dilutions (NOTE: standard curve and spike concentrations are based on a historical LOD of 0.02 EU/mL):

1. Add 200 µL of sample (S1-1) in A4 to H4 (see Figure 2).
2. Add 100 µL of complete medium to the other wells used for the sample dilutions
3. Make a twofold dilution by pipetting 100 µL from S1-1 to S1-1/2 and mix thoroughly by pipetting up and down for 10 times. Prevent the formation of air bubbles.
4. Repeat this until S1-1/4 and discard the final 100 µL
5. Add 50 µL of complete medium to the standards and samples without endotoxin spike.
6. Add 1920 µL complete medium to a sterile, non-pyrogenic borosilicate glass tube and to this add 80 µL of 2 EU/mL endotoxin to reach a final concentration of 0.08 EU/mL. This is sufficient to spike 3 products with endotoxin. Mix thoroughly by pipetting up and down for 10 times, with a volume of at least 1 mL. Do not vortex and prevent the formation of air bubbles.
7. Add 50 µL of 0.08 EU/mL endotoxin to the spiked samples (+ spike). The endotoxin spike concentration in the sample corresponds to the 0.04 EU/mL value from the endotoxin standard curve (see also the note in chapter 7.1 for more information)

8.4 MAT Cell incubation

1. Take the vial of pMAT Cells from the -80°C freezer and **immediately thaw it in a water bath at 37°C until a small clump of ice remains (<5 minutes)**
2. Transfer the entire contents of the vial (1 mL) into a 50 mL tube and immediately but slowly add 5 mL complete medium (at room temperature) while gently swirling the tube (<5 minutes). Do not vortex or vigorously pipet, take care not to form air bubbles.
3. Immediately add 50 µL of the pMAT Cells to the appropriate wells
4. Incubate the microplate with lid in a CO_2-incubator at 37°C with 5% CO_2 for 18-24 hours
    - (Coat ELISA plate with coating antibody if necessary, for more information see Chapter 8.5)
5. Harvest 100-150 µL of supernatant by carefully placing the pipette tip at the left or right bottom side of the well and transfer this to a new 96-well round bottom, untreated microplate
6. Measure IL-6 production by ELISA. Alternatively, the supernatant may be frozen at -20°C or -80°C. Storage time of the frozen supernatant may be product-dependent and needs to be determined by the user.

8.5 IL-6 ELISA procedure

The pMAT Cells have been validated using the PeliKine compact™ human IL-6 kit[3] (Sanquin REF M1916) and PeliKine Toolset (Sanquin REF M1980) (not included). Using this kit, an LOD of 0.02 EU/mL may be expected. Consult the Certificate of Analysis for additional information on LOD. Follow the manufacturer's instructions for use of the ELISA kit. In our experience using the PeliKine compact™ human IL-6 kit from Sanquin, a sample dilution of 3-5 fold gives the optimal result when using the recommended endotoxin standard curve. Please establish your own optimal dilution. An IL-6 standard curve may be included in the ELISA as a quality control, but is not necessary to determine the pyrogenic activity of the sample. As an option, the test procedure for the IL-6 ELISA using the PeliKine compact™ human IL-6 kit and Toolset from Sanquin is given in Chapter 8.6.

8.6 OPTIONAL: IL-6 ELISA procedure using the PeliKine compact™ human IL-6 kit and Toolset (not included) from Sanquin

Refer to the original IFU of the PeliKine compact™ human IL-6 kit[3] (Sanquin REF M1916) and PeliKine Toolset (Sanquin REF M1980) for information on principle of the test, storage and stability, contents of the kit, precautions for use, and additional information.

Components marked with "[(a)]" are part of the PeliKine compact™ human IL-6 ELISA kit, components marked with "[(b)]" are part of the PeliKine Toolset. The stated volumes are for *one* ELISA plate.

Day 0: Coating plate with coating antibody

1. Bring coating antibody[(a)] to 18-25°C
2. Prepare coating buffer.
    - Dissolve **only the contents** of one coating buffer capsule[(b)] in 100 mL distilled water (NOTE: the outer coating of the capsule (gelatine) will stop the coat buffer working properly, so do not dissolve or rinse the outer capsule). Wait for 5 minutes, mix contents and the working strength buffer is ready for use. The prepared buffer can be stored in a closed container for up to 1 week at 2-8°C. Bring buffer to 18-25°C before use.
3. Add 120 μL coating antibody[(a)] to 12 mL coating buffer and mix by inverting the tube 10 times.
4. Add 100 μL of this solution to all wells of the supplied 96-well plate. Cover the plate with lid and incubate overnight at 18-25°C.

Day 1

5. Bring all reagents to 18-25°C, with the exception of the streptavidin-HRP conjugate which has to be kept at -18°C to -32°C to ensure stability.
6. Prepare working-strength PBS
    - Dissolve 1 PBS tablet[(b)] in 200 mL distilled water. Wait for 5 minutes, mix contents and the working strength PBS is ready for use. The prepared buffer can be stored in a closed container for up to 1 week at 2-8°C. Bring buffer to 18-25°C before use.
7. Prepare blocking buffer
    - Add 500 μL blocking reagent[(a)] to 25 mL PBS and mix by inverting the tube 10 times.

Wash step with PBS

8. Discard the supernatants from the wells and completely fill the wells (volume > 300 μL) with working-strength PBS and empty. Repeat this four times. After the final emptying the plate should be dry.

Blocking step

9. Add 200 μL blocking buffer to all wells and cover the microplate with adhesive seal. Gently agitate the microplate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 1 hour at 18-25°C.** For increased sensitivity incubate on a plate shaker (700 ± 100 rpm).

Preparation of IL-6 standard and sample dilutions

10. Prepare working-strength HPE-dilution buffer
    - Add 15 mL 5-fold concentrated HPE-dilution buffer[(a)] to 60 mL distilled water. The opalescent working-strength HPE-dilution buffer can be stored for up to one week at 2-8°C. Bring buffer to 18-25°C before use.

    NOTE: In the concentrated buffer salt crystals may appear. Before preparing the working-strength buffer, first warm the concentrated buffer BRIEFLY to 37°C in a water bath to dissolve the precipitate.
11. Prepare IL-6 standard in working-strength HPE-dilution buffer (Optional: An IL-6 standard curve may be included in the ELISA as a quality control, but is not necessary to determine the pyrogenic activity of the sample)

    NOTE: Avoid repeated freeze-thawing of the standard, although experimental data have shown that up to 3 freeze-thaw cycles have no effect on the IL-6 levels of the standard.
    - Label 7 tubes, one tube for each dilution: 450, 150, 50, 16.7, 5.6, 1.9 and 0.6 pg/mL
    - Pipette 497 μL of working-strength dilution buffer into the tube labelled 450 pg/mL and 400 μL of working-strength dilution buffer into the other tubes
    - Transfer 63 μL of the IL-6 standard[(a)] (4000 pg/mL) into the first tube labelled 450 pg/mL, mix well and transfer 200 μL of this dilution into the second tube labelled 150 pg/mL
    - Repeat the serial dilutions five more times by adding 200 μL of the previous tube of diluted standard to the 400 μL of dilution buffer
    - The standard curve will contain 450, 150, 50, 16.7, 5.6, 1.9, 0.6 and 0 pg/mL IL-6
    - It is recommended to prepare two separate series (duplicate) for each assay
12. Prepare the dilution of the endotoxin standard and sample supernatants in working-strength HPE-dilution buffer. We recommend a dilution of 1:5:
    - In a 96-well round bottom, untreated microplate pipet 100 μL working-strength HPE-dilution buffer in the wells used for the samples
    - Homogenise the supernatant by pipetting up and down for 5 times and add 25 μL to the corresponding well containing 100 μL of working-strength HPE-dilution buffer. Mix by pipetting up and down for 5 times.

    NOTE: Prior to the assay, frozen samples should be thawed as quickly as possible in tap water (18-25°C), do not use 37°C or 56°C water baths for this purpose.
13. Prepare wash buffer
    - Prepare working-strength wash buffer by adding 50 mL of the wash buffer concentrate[(b)] (total content of the bottle) to 950 mL distilled water. The working-strength wash buffer can be stored up to 2 months at 2-8°C. Bring buffer to 18-25°C before use.

Wash step 1 with wash buffer

14. After 1 hour of blocking wash the plate 5 times with wash buffer (volume > 300 μL) manually or on a plate washer. After the final emptying the plate should be dry.

Incubation step with standard and samples

15. Transfer 100 µL of the prepared standards and samples into the appropriate wells and cover the microplate with adhesive seal. Gently agitate the microplate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 1 hour at 18-25°C.** For increased sensitivity incubate on a plate shaker (700 ± 100 rpm).
16. Just before the next washing step, prepare the working-strength biotinylated antibody
    - Add 120 µL 100-fold concentrated biotinylated antibody[a] to 12 mL working-strength HPE-dilution buffer. Mix by inverting the tube 10 times

Wash step 2 with wash buffer

17. After 1 hour of incubation wash the plate 5 times with wash buffer (volume > 300 µL) manually or on a plate washer. After the final emptying the plate should be dry.

Incubation with biotinylated antibody

18. Add 100 µL of working-strength biotinylated antibody to the appropriate wells and cover the microplate with adhesive seal. Gently agitate the microplate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 1 hour at 18-25°C.** For increased sensitivity incubate on a plate shaker (700 ± 100 rpm).
19. Just before the next washing step, prepare the working-strength streptavidin-HRP conjugate
    - Add 1.2 µL 10,000-fold concentrated streptavidin-poly-HRP conjugate[a] to 12 mL working-strength HPE-dilution buffer. Mix by inverting the tube 10 times.

Wash step 3 with wash buffer

20. After 1 hour of incubation wash the plate 5 times with wash buffer (volume > 300 µL) manually or on a plate washer. After the final emptying the plate should be dry.

Incubation with streptavidin-HRP conjugate

21. Add 100 µL of working-strength streptavidin-HRP conjugate to the appropriate wells and cover the microplate with adhesive seal. Gently agitate the microplate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 30 minutes at 18-25°C**. For increased sensitivity incubate on a plate shaker (700 ± 100 rpm).
22. Just before the next washing step, make sure the TMB substrate solution[b] and stop solution[b] is at 18-25°C. Protect TMB substrate solution from prolonged exposure to light.

Wash step 4 with wash buffer

23. After 30 minutes of incubation wash the plate 5 times with wash buffer (volume > 300 µL) manually or on a plate washer. After the final emptying the plate should be dry.

Incubation with TMB substrate solution

24. The TMB substrate solution[b] is ready for use. Add 100 µL to all wells and cover the microplate with adhesive seal. Gently agitate the microplate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 10 minutes (recommended) at 18-25°C in the dark** (do not use aluminium foil, but put the microplate e.g. in a cupboard) under static conditions.

Stop enzymatic reaction with stop solution

25. After 10 minutes of incubation add 100 µL of ready for use stop solution[b] to all wells. **After stopping the substrate reaction the colour is stable for maximally 30 minutes.**
26. Put the microplate in an ELISA reader and record absorbance at 450 nm, with a reference wavelength set between 540 and 590 nm (subtract the reference wavelength measurement from the measurement at 450 nm if the software does not subtract this automatically).

9 Results

Every in-house or on-line available software method for calculation of endotoxin concentrations may be used. A general method for calculation is given below.

1. Plot the absorbance on the Y-axis and the endotoxin concentration of the standards on the X-axis on a log scale and draw the best fitting curve.
2. Locate the net average absorbance value found for each sample on the vertical axis and follow a horizontal line intersecting the standard curve.
3. Draw a vertical line from the intersection of the standard curve towards the X-axis.
4. At the intersection with the X-axis, read the endotoxin concentration from the horizontal axis.
5. Multiply the obtained endotoxin concentration with the dilution factor of the sample, this is the actual concentration in the sample.
6. Calculate the average of the quadruplicate values.
7. Determine the LOD by calculating the average of 4 independent blanks multiplied by the 3-fold standard deviation of these values.

A typical endotoxin standard curve using the PeliKine compact™ human IL-6 kit from Sanquin is shown in Figure 3. This standard curve was generated for demonstration purposes only. A pyrogen standard curve must be run with each plate.

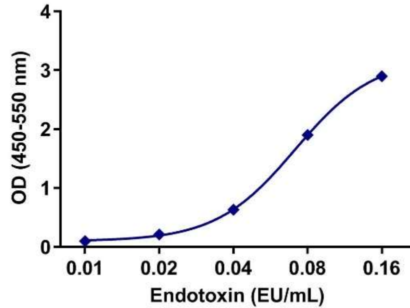

Figure 3: Example of an endotoxin standard curve using the PeliKine compact™ human IL-6 kit from Sanquin.

According to Ph. Eur. 2.6.30, the standard curve is valid when the following two acceptance criteria are met[1]:

1. The regression of responses (appropriately transformed if necessary) on log dose shall be statistically significant ($p < 0.01$)
2. The regression of responses on log dose must not deviate significantly from linearity ($p > 0.05$)

The required p-values can be calculated using appropriate statistics software, e.g. Combistats™ from the European Directorate for the Quality of Medicines (EDQM).

10 Interpretation

The interpretation of the results depends on the chosen test method. Refer to Ph. Eur. 2.6.30 for a detailed description.

11 Specifications

The values cited for specific performance characteristics of the test represent typical results obtained using the PeliKine compact™ human IL-6 kit (Sanquin REF M1916) and PeliKine Toolset (Sanquin REF M1980). The values are not to be viewed as specifications for this specific MAT Cell Set, consult the Certificate of Analysis for additional information.

11.1 Performance characteristics endotoxin

Limit of detection	:	≤0.02 EU/mL	
Precision		Total precision*	Between-run precision*
0.016 EU/mL	:	9.2%	7.9%
0.033 EU/mL	:	5.9%	4.7%
0.042 EU/mL	:	11.5%	11.4%
Tested working range	:	0.01 – 0.16 EU/mL	

*Precision was evaluated in three independent experiments performed by two technicians. Each assay was carried out with 12 replicates of 3 samples.

11.2 Performance characteristics of tested non-endotoxin pyrogens:

Consult the Certificate of Analysis on performance characteristics of non-endotoxin pyrogens.

12 Limitations

- MAT Cell Set has been designed and validated using IL-6 as read-out for pyrogenic activity. Other cytokines may be suitable as read-out, but have to be validated by the user.
- A reference sample with a known concentration of pyrogen (not included) may be used for quality control purposes.
- pMAT Cells are reactive towards the non-endotoxin pyrogens listed in the Certificate of Analysis. pMAT Cells may be responsive to other non-endotoxin pyrogens, but these have to be tested by the user.

13 References

1. European Directorate for the Quality of Medicines. *Ph. Eur.* chapter 2.6.30: Monocyte Activation Test.
2. 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.
3. 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.

Sanquin Reagents products are guaranteed to perform as described in the original manufacturer's instructions for use. Sanquin Reagents declines all responsibility arising from any deviation thereof. The user is responsible for proper calculations and subsequent interpretations of the results. Sanquin Reagents is 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.