User Guide

PyroDetect System Monocyte-Activation Test (MAT)

User Manual for routine tests (Method A, B and C)

The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the US and Canada.

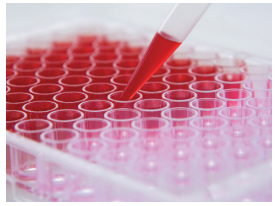
www.sigmaaldrich.com

Assay Overview

Cryoblood Incubation

Step 1

- Mix sample or endotoxin diluted with cryoblood in cell culture microplate
- Incubate for 8–24 hours for Interleukin-1β production at 37 °C



Interleukin-1β ELISA

Step 2

- Transfer cryoblood incubation mix into Interleukin-1β Detection Microplate
- Incubate for 2 hours at room temperature
- Remove the incubation liquid from the microtiter plate and wash it 3 times with Wash Buffer

Step 3

- Add enzyme-labeled antibody (Anti-human IL-1β Conjugate)
- Incubate for 1 hour at room temperature
- Remove the incubation liquid from the microtiter plate and wash it 3 times with Wash Buffer

Step 4

- Mix the enzyme substrate components (color reagent A and B) and add mixture to each well of the microtiter plate
- Incubate the micro titer plate for 20 minutes in the dark, at room temperature

Step 5

- Add Stop Solution

Read-out & Data Analysis

Step 6

- Read plate at 450 nm within 30 minutes

Step 7

- Analyze the results

Contents

This manual should be read in its entirety before using the product

1. Introduction

Pyrogenic substances in pharmaceutical products can induce life threatening fever reactions. The test methods used so far to control the absence of pyrogens are the rabbit pyrogen test (RPT) and the limulus amoebocyte lysate (LAL) test. Since 2010 the Monocyte Activation Test (MAT) has been an official test of the European Pharmacopoeia (EP 9.2 chapter 2.6.30). Unlike the other methods, the MAT does not require the use of animal testing.

This test allows the determination of the pyrogenic activity in a solution or suspension by inducing interleukin production in cell cultures with human monocytes.

1.1 Principle of the MAT

The MAT uses an innate immune defense reaction of the human monocytes. Monocytes present in preparations from human whole blood or a monocytic cell line respond to pyrogens by a quick and strong production of cytokines which are detected in an immunological assay (ELISA) involving specific anti-bodies and an enzymatic color reaction.

1.2 Principle of the MAT with the PyroDetect System

The PyroDetect System uses cryo-preserved human whole blood as a source of monocytes. The response to pyrogenic substances is determined by measurement of the Interleukin-1β (IL-1β) production. For this purpose, the ELISA-microplate (ELISA = enzyme-linked immunosorbent assay) in the PyroDetect System is precoated with an antibody specific for IL-1β. Interleukin molecules present in the culture supernatant transferred from the cryoblood cultures are bound by the immobilized antibody.

After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-1β is added. Excess antibody-enzyme complex is removed by an additional washing step. The IL-1β bound complex is then detected in a color reaction started by the addition of an appropriate substrate. The efficiency of IL-1β production in the pyrogen reaction is calculated from the data of a photometrical quantification of the colored product of the enzymatic reaction in an ELISA reader.

For the setup of the cryoblood incubation, the PyroDetect kit contains the appropriate culture medium and a cell culture plate. An endotoxin standard, the Reference Standard Endotoxin (RSE) (Cat. No. 1.44161.0001) or an equivalent reference standard can be used.

The kit is suitable for all MAT methods depicted in EP 9.2 chapter 2.6.30: the quantitative (Method A) and the semi-quantitative (Method B) tests and the reference lot comparison test (Method C). The ELISA is based on antibodies raised against recombinant human IL-1β through immunization. For control of detection reactions, a standard preparation of IL-1β is available: the PyroDetect Interleukin Standard (Cat. No. 1.44158.0001).

The PyroDetect Kit contains sufficient amounts of material to perform detection reactions in a 96 well micro titer plate:

- 3 samples with Method A
- 3 samples with Method B
- 3 samples with Method C

1.3 Kit Contents

To perform the MAT, we recommend using the PyroDetect System which consists of the PyroDetect Kit (Cat. No.1.44154.0001) and the PyroDetect Cryoblood (Cat. No. 1.44155.0001). Moreover, the use of an endotoxin standard is required to perform the MAT test: e.g.: Reference Standard Endotoxin (Cat. No. 1.44161.0001).

PyroDetect System	Cont.
1. PyroDetect Kit, Cat. No. 1.44154.0001, store at 2–8 °C	
Cell Culture Microplate for 96 microcultures, with lid	1 plate
Cell Culture Medium—RPMI with L-Glutamine and HEPES	50 mL
IL-1β Microplate—12 strips of 8 wells each, coated with a monoclonal antibody specific for IL-1β	1 plate
IL-1β Conjugate—IL-1β specific, polyclonal antibody, conjugated to horseradish peroxidase	21 mL
Wash Buffer Concentrate—25-fold concentrated solution of buffered surfactant	21 mL
Color Reagent A—stabilized hydrogen peroxide	12.5 mL
Color Reagent B—stabilized chromogen tetramethylbenzidine	12.5 mL
Stop Solution—sulfuric acid	11 mL
Plate—adhesive clear sealing tapes	4 tapes
2. PyroDetect Cryoblood, Cat. No. 1.44155.0001, store at –80 °C or lower	
Cryoblood—deep frozen human blood preparation	2 x 2 mL

PyroDetect Standards	Cont.
1. Reference Standard Endotoxin, Cat. No. 1.44161.0001, store at –20 °C	
Endotoxin Standard, lyophilized	1 µg
2. PyroDetect Interleukin-1β Standard*, Cat. No. 1.44158.0001, store at 2–8 °C	
Human IL-1β Standard—recombinant human IL-1β in a buffered protein base, lyophilized	1.25 ng
Calibrator Diluent—dilution reagent for the standard	21 mL

*Optional (to perform control reactions in the IL-1β ELISA)

PyroDetect Data Analysis

A data analysis tool has been developed with Gen5 Software. You can download the files from our website for free.

Please note that you will need to be equipped with Gen5 Software from BioTek company to be able to use our data analysis tool.

1.4 Additional Equipment Required

- Incubator, 37 °C
- Microplate photometer for measuring absorbance at 450 nm, with the reference wavelength set between 600 and 690 nm. We recommend the ELx808™ reader from BioTek®
- Multichannel pipettes with suitable pyrogen free containers
- Adjustable pipettes: (10–100 µL; 100–1,000 µL)
- Suitable sterile, pyrogen-free pipette tips
- Pyrogen-free glass tubes
- Deionized or distilled water
- Cryo-freezer (≤ 80 °C)
- Vortex
- Automated microplate washer (optional)

2. Warnings and Precautions

Not suitable for *in vitro* diagnostic use.

Caution: The test must be performed by well-trained and authorized laboratory personnel.

Caution: All reagents should be handled in accordance with good laboratory practice using appropriate precautions.

Caution: Do not use reagents past the expiration date printed on the label.

Caution: Do not use reagents with any evidence of turbidity or microbial contamination.

Caution: IL-1β is detectable in saliva and sweat. Take precautionary measures to prevent contamination of the kit reagents while running the assay.

Caution: The Stop Solution provided with this kit contains sulfuric acid. Wear eye, hand, face, and clothing protection when using this material.

Limitation of Liability

Notwithstanding our attempts to observe the rules specified in national and international guidelines, we cannot guarantee the proper calculations and subsequent interpretations. Please refer to the relevant chapters in the European Pharmacopoeia (EP 9.2 chapter 2.6.30) as well as other documents relevant for your specific purpose for utilization of the test.

3. Storage

3.1 PyroDetect Cryoblood

Important!

PyroDetect Cryoblood should be stored immediately at –80 °C or lower! Higher storage temperatures than –80 °C can lead to a loss of functionality!

Higher storage temperatures (e.g. –60 °C, –70 °C, –75 °C, –78 °C) are not sufficient to fully maintain the activity of PyroDetect Cryoblood. Please check your freezer for appropriate alarm settings.

The following example shows Endotoxin Standard curves with improperly stored PyroDetect Cryoblood.

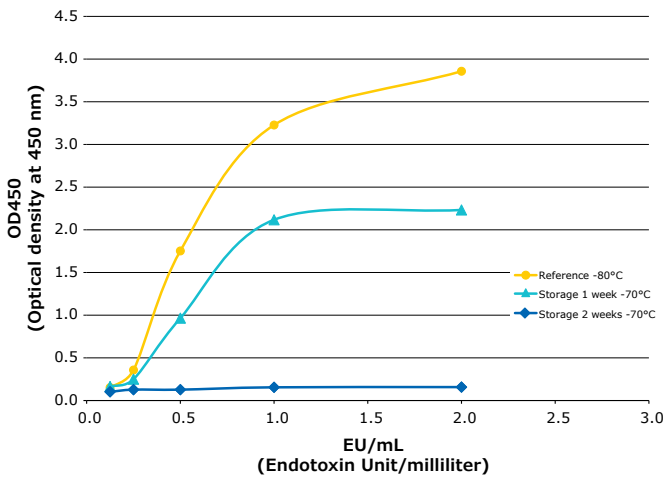


Fig. 1: Examples of improper storage at –70 °C after one and two weeks of storage.

3.2 Unopened PyroDetect System

PyroDetect Kit (Cat. No. 1.44154.0001)	Storage at 2–8 °C. Do not use kits past the expiration date!
PyroDetect Cryoblood (Cat. No. 1.44155.0001)	Store at –80 °C or lower!
PyroDetect Interleukin Standard (Cat. No. 1.44158.0001)	Store at 2–8 °C
PyroDetect Endotoxin Standard (Cat. No. 1.44161.0001)	Store at –20 °C

3.3 Opened/Reconstituted Reagents

Microplate	Return unused strips to the foil pouch and reseal along entire edge of zip-seal. May be stored for up to 1 month at 2–8 °C.*
Cryoblood	Thawed cryoblood should not be refrozen for security reasons!
Endotoxin Standard	Reference Standard Endotoxin reconstituted at –40 °C or below. For other Endotoxin standard, please refer to the included manufacturer instructions.
Stop Solution	May be stored for up to 1 month at 2–8 °C*
up to 1 month at 2–8 °C*	
Anti Human IL-1β Conjugate	
Unmixed Color Reagent A (not mixed with B)	
Unmixed Color Reagent B (not mixed with A)	
Diluted Wash Buffer	
Interleukin-1β Standard and its Calibrator Diluent	

*within the expiration date of the kit.

4. Planning Test Execution

4.1 Term Definition

The intention of the MAT is to prove that the amount of pyrogens in the test product does not exceed the limit of contamination (Contaminant Limit Concentration, CLC).

Prior to the test, it is necessary to calculate the Maximum Valid Dilution (MVD) based on the Limit of Detection (LOD) of the test system.

4.1.1 Calculation of the Limit of Detection (LOD)

When performing a test according to Method A (quantitative method), the limit of detection of the PyroDetect system is determined by the values of the negative control of the endotoxin standard curve for each assay. This is why it cannot be exactly determined before the test. The LOD is the concentration determined by the endotoxin standard curve in Endotoxin Units per milliliter (EU/mL), corresponding to the cut-off value. The cut-off value according to the European Pharmacopoeia is defined as the mean of 4 independent negative control samples (blanks) increased by the 3-fold standard deviation of these values. Therefore, the terms cut-off value and LOD depict the same information in different units. The cut-off value is expressed in OD (Optical Density) and the LOD is expressed in EU/mL.

In addition to the LOD defined in the European Pharmacopoeia, we guarrantee by the release tests performed within the quality control, a minimum sensitivity of 0.25 EU/mL (based on the LOD-calculation of the European Pharmacopoeia). This LOD is used when performing the test according to Method B. As there is no endotoxin standard curve performed in Method B (semi-quantitative method), we check that the OD value of the standard at 0.25 EU/mL is above the cut-off value. This LOD criterion must be valid to allow further analysis of the assay.

4.1.2 Calculation of the Maximum Valid Dilution (MVD)

Before testing a pharmaceutical product for interfering factors, the appropriate dilution, i.e. The maximum valid dilution of the test sample in which you can still detect the specified pyrogen limit must be calculated.

$$\mathbf{MVD} = \frac{\mathbf{CLC \times C}}{\mathbf{LOD}}$$

CLC = Endotoxin Limit (Contaminant Limit Concentration, EU/mL)
C = Concentration of the test solution (product weight/mL)
LOD = Limit of detection of the test system (Limit of Detection, EU/mL)

For the Method A, the MVD is calculated using the LOD of the assay, determined using the endotoxin standard curve.
For Method B, the MVD is calculated using the theoretical LOD of 0.25 EU/mL.

4.2 Product Specific Validation

Before routine testing of a pharmaceutical product with MAT, a product specific validation (PSV) must be performed to ensure that the tested sample will not interfere with the assay, namely that the endotoxins and non-endotoxin pyrogens in the product will be detected using the chosen method. The PSV is intended to determine the valid product dilutions, not exceeding the MVD, that will be tested in routine.

If the interference cannot be removed by dilution of the product within the MVD range or a specific sample preparation, Method C (reference lot comparison) is preferred over Method A and B.

The following plan is one possible way to run the MAT. The calculations of the following steps reflect the specifications as described in the European Phamacopoeia (EP 9.2 chapter 2.6.30 “Monocyte-Activation Test”).

1. Calculation of the MVD (Maximum Valid Dilution) using therotical LOD

2. Product Specific Validation to determine validity of the standard curve and the LOD, to test the interference of the product with the method and to assess the detection of non-endotoxins contaminants

3. Method selection (quantitative/semi-quantitative/Reference lot comparison)

4. Test according to quantitative/semi-quantitative method or reference lot comparison test

We can provide support for the Product Specific Validation. Please refer to our website Service page or contact your local customer service or Sales Representative.

The following chapters contain information about how to perform the different test methods and analyze the results. The shape of the pyrogen response curve (slope and maximum OD values) depends on the time of cryoblood incubation. The EP allows incubation times of 8–24 hours. For reasons of standardization we recommend incubating all the tests under the same conditions as the respective standard curve.

5. Test Execution

5.1 Endotoxin Standard solutions

The endotoxin standard solutions are needed to quantify or estimate the amount of pyrogens in the samples and to assess the validity of the LOD.

As the OD values of the endotoxin standard dilutions and samples may vary from test to test, it is recommended to perform an endotoxin standard curve for each test plate.

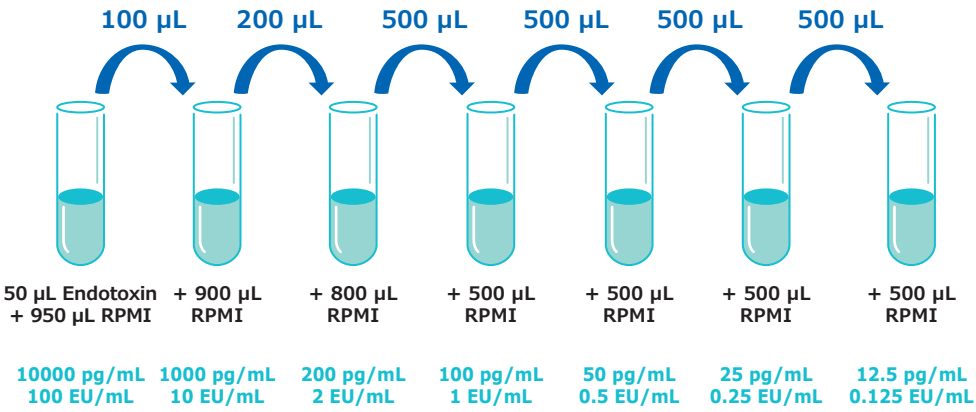
For the quantitative test (Method A), a regression model, based on a 4-parameters logistic model is used to generate an endotoxin standard curve to convert the OD signal into concentrations (EU/mL).

For the semi-quantitative test (Method B), only the OD signal of the endotoxin standard dilutions are considered for the analysis (no back calculation of concentration).

5.1.1 Preparation of endotoxin dilutions

The preparation of different endotoxin dilutions should be done in pyrogen-free and non-endotoxin absorbing glass tubes. The endotoxin standard is diluted in the RPMI culture medium provided in the kit. The following dilution series is an example comprising the concentrations used in the pipetting scheme.

The first dilution should be mixed with a vortex for 3 min. For the following steps, each dilution should be mixed with a vortex for 30 seconds.



EU = Endotoxin Units

Fig. 2: Endotoxin dilution series.

Note: The first two dilution levels are pre-dilutions that are not part of the standard curve.

5.1.2 Generation of the Endotoxin Standard Curve

The endotoxin standard curve results from cultures containing different endotoxin concentrations. According to EP requirements, standard curves are calculated from the analysis of at least 4 replicates of 4 different endotoxin concentrations and a negative control. Therefore, a total amount of 5 x 4 = 20 microcultures are required.

For optimal utilization of the microplate, we recommend doing 5 different endotoxin concentrations and a negative control. In that case, the total amount will be 6 x 4 = 24 microcultures.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 EU/mL											
B	0.125 EU/mL											
C	0.25 EU/mL											
D	0.5 EU/mL											
E	1 EU/mL											
F	2 EU/mL											
G												
H												

Fig. 3: Plate layout for 5 endotoxin concentrations and a negative control.

For the generation of the endotoxin standard curve, the mean of the 4 replicates is used.

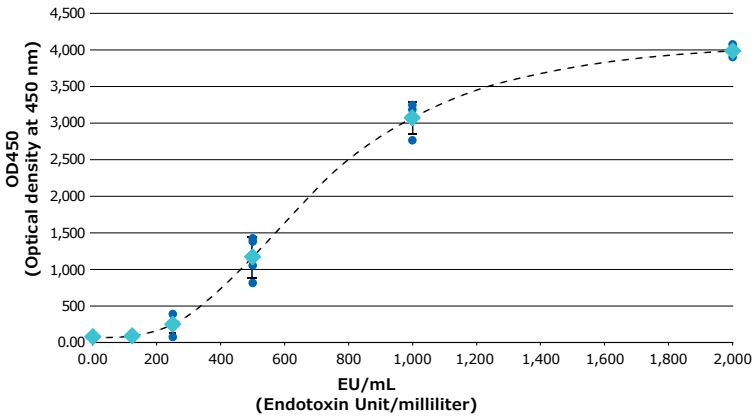


Fig. 4: Example of an endotoxin standard curve.

5.1.3 Validity criteria of the Endotoxin Standard Curve

The endotoxin standard curve is a tool to quantify the amount of pyrogen in the test solution. In order to assure the validity of the standard curve for this function, there are two acceptance criteria (p-values) specified in the Pharmacopoeia. The criteria are met when:

1. **Effect of dose: $p < 0.01$**
   The regression of responses is statistically significant on a logarithmic scale.
2. **Goodness of fit: $p > 0.05$**
   The regression of responses on a logarithmic scale shall not deviate significantly from linearity (refer to EP 6.7 Chapter 5.3).

5.2 Quantitative Test (Method A)

A quantitative test comprises a standard curve, including a negative control, and samples at different dilutions. The appropriate sample dilutions (see plate layout) have to be done using the RPMI culture medium. In addition, samples at each dilution inoculated with an endotoxin spike corresponding to the middle dose of the standard curve concentration (i.e. 0.5 EU/mL) are included. Through these endotoxin spiked samples, a test for interfering factors is performed in each test run.

Using the quantitative method, up to 3 samples can be tested on one plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 EU/mL				Sample 1, dilution 2				Sample 2, dilution 3			
B	0.125 EU/mL				Sample 1, dilution 2 + spike RSE 0.5				Sample 2, dilution 3 + spike RSE 0.5			
C	0.25 EU/mL				Sample 1, dilution 3				Sample 3, dilution 1			
D	0.5 EU/mL				Sample 1, dilution 3 + spike RSE 0.5				Sample 3, dilution 1 + spike RSE 0.5			
E	1 EU/mL				Sample 2, dilution 1				Sample 3, dilution 2			
F	2 EU/mL				Sample 2, dilution 1 + spike RSE 0.5				Sample 3, dilution 2 + spike RSE 0.5			
G	Sample 1, dilution 1				Sample 2, dilution 2				Sample 3, dilution 3			
H	Sample 1, dilution 1 + spike RSE 0.5				Sample 2, dilution 2 + spike RSE 0.5				Sample 3, dilution 3 + spike RSE 0.5			

Fig. 6: Plate layout of a test according to the quantitative method.

5.2.1 Analysis of results for the quantitative method

The analysis of the results of method A can be performed using the data analysis tool (.prt file) available on our website, to be used with Gen5 Software.

1. **Validity of the endotoxin standard curve**

   The standard curve is used to determine the endotoxin concentration (EU/mL) from the measured OD values.

   For each test, the assurance criteria for the standard curve are checked:

   a. Effect of dose criteria: a statistical test that confirms a positive dose/effect response.

   b. Goodness of fit criteria: a statistical test that confirms the suitability of the regression model to describe the raw data. The data are modeled with a 4-parameter logistics regression model.

   c. LOD criteria: the test is valid if a LOD ≤ 0.25 EU/mL is reached

2. **Validity of the sample dilutions**

   For each sample dilution that does not exceed the MVD, the endotoxin spike recovery is calculated based on the EEU/mL for the spiked and non-spiked samples.

   The spike recovery is valid if it is within the range 50–200%. Dilutions that do not fulfill this criterion are not valid and are excluded from further analysis.

3. **Pyrogenicity of the sample**

   The test preparation fulfills the test requirements if the mean of the endotoxin concentration from the replicates of the 3 different sample dilutions after the correction of dilution and concentration factors is below the CLC. The limit of contamination depends on the applied amount of the product or is given by the product monograph when available (see chapter 4.1.1). One valid dilution is the minimum required for a valid test.

5.3 Semi-Quantitative Test Method (Method B)

In contrast to the quantitative method referring to fixed endotoxin concentration for generation of a standard curve, the semi-quantitative method uses the OD values to conclude on the pyrogenicity of the sample. In comparison to the quantitative method, the semi-quantitative method does not allow a precise quantification. The result provides the information whether the pyrogen content of the product exceeds or remains below the limit (CLC).

A semi-quantitative test comprises a series of endotoxin dilutions, including a negative control, and samples at different concentrations with and without spikes of endotoxin. The appropriate sample dilutions (see plate layout) have to be done using the RPMI culture medium. As in the quantitative method, the endotoxin-spiked samples serve as a limited test for interfering factors.

Using the semi-quantitative method, up to 3 samples can be tested on one plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 EU/mL				Sample 1, dilution 2				Sample 2, dilution 3			
B	0.125 EU/mL				Sample 1, dilution 2 + spike RSE 0.5				Sample 2, dilution 3 + spike RSE 0.5			
C	0.25 EU/mL				Sample 1, dilution 3				Sample 3, dilution 1			
D	0.5 EU/mL				Sample 1, dilution 3 + spike RSE 0.5				Sample 3, dilution 1 + spike RSE 0.5			
E	1 EU/mL				Sample 2, dilution 1				Sample 3, dilution 2			
F	2 EU/mL				Sample 2, dilution 1 + spike RSE 0.5				Sample 3, dilution 2 + spike RSE 0.5			
G	Sample 1, dilution 1				Sample 2, dilution 2				Sample 3, dilution 3			
H	Sample 1, dilution 1 + spike RSE 0.5				Sample 2, dilution 2 + spike RSE 0.5				Sample 3, dilution 3 + spike RSE 0.5			

Fig. 8: Plate layout of a test according to the semi-quantitative method.

5.3.1 Analysis of results for the semi-quantitative method

The analysis of the results of Method B can be performed using the protocol available on our website, to be used with Gen5 Software. For the semi-quantitative method, 2 validity criteria have to be fulfilled for the standard solutions.

1. **Validity of the endotoxin standard solutions**

   a. Effect of dose criteria: the mean response of the various endotoxin standard solutions should increase progressively with concentration

   b. LOD criteria: the average response of the used test system (cryoblood) to a standard endotoxin at 1 x LOD is greater than the cut-off (OD of the 0.25 EU/mL solution > cut-off).

2. **Validity of the sample dilutions**

   For each sample dilution that does not exceed the MVD, the endotoxin spike recovery is calculated based on the OD value for the spiked and non-spiked samples.

   The spike recovery is valid if it is within the range 50–200%. Dilutions that do not fulfill this criterion are not valid and are excluded from further analysis.

3. **Pyrogenicity of the sample**
   For each valid dilution, not exceeding the MVD, the sample OD value is compared to the cut-off value. If it is below the cut-off, the pyrogen concentration is considered to be below the LOD.

- If OD value of the diluted sample is < Cut–off → PASS, meaning that the pyrogen level in the pure sample is below the CLC.
- If OD value of the diluted sample is > Cut-off → NOT CONCLUSIVE, meaning that a contamination is present in the pure sample but the pyrogen level cannot be evaluated with regards to the CLC.
- Note: at the MVD, if the sample signal is above the cut-off and above the endotoxin standard solution signal at the LOD (0.25 EU/mL), it is a FAIL, meaning that pyrogen level is above the CLC.

5.4 Reference Lot Comparison Test (Method C)

Method C is preferred if in the test for interfering factors, the endotoxin concentration determined in the spiked test solution is not in the range of 50–200% of the added endotoxin concentration. In this test the (semi) quantification of endotoxin is replaced by a reference lot comparison test. For this test, different dilutions of the test product are compared to the equivalent dilutions of a reference lot. The appropriate dilutions of the test sample and of the reference lot (see plate layout) have to be done using the RPMI culture medium. The reference lot comparison test comprises dilutions of the reference lot, sample dilutions, as well as a positive and a negative control. Using the Reference Lot Comparison test, up to 3 samples can be tested per plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank (RPMI)											
B	positive control (endotoxin standard)											
C	Sample 1 - Ref : dilution 1				Sample 1 - Ref : dilution 1				Sample 1 - Ref : dilution 1			
D	Sample 1 - Ref : dilution 1				Sample 1 - Ref : dilution 1				Sample 1 - Ref : dilution 1			
E	Sample 1 - Ref : dilution 1				Sample 1 - Ref : dilution 1				Sample 1 - Ref : dilution 1			
F	Sample 1 - Lot 1 : dilution 1				Sample 1 - Lot 1 : dilution 1				Sample 1 - Lot 1 : dilution 1			
G	Sample 1 - Lot 1 : dilution 1				Sample 1 - Lot 1 : dilution 1				Sample 1 - Lot 1 : dilution 1			
H	Sample 1 - Lot 1 : dilution 1				Sample 1 - Lot 1 : dilution 1				Sample 1 - Lot 1 : dilution 1			

Fig. 10: Plate layout of a test according to the reference lot comparison test (Method C).

5.4.1 Analysis of results of the Reference Lot Comparison Test method

1. **Validity of the test**
   The positive control and at least one dilution of the reference lot should be above the mean OD value of the negative control (blank).

2. **Pyrogenicity of the sample**
   The reference lot comparison test complies with the requirements if the sum of the endotoxin averages of all concentrations of the test lot divided by the sum of the endotoxin averages of all concentrations of the reference lot results in a value which does not exceed a defined OD ratio (eg 2.5).

The sample is non-pyrogenic if:

$$\frac{\text{Sum mean ODs Sample Lot}}{\text{Sum mean ODs Reference}} \leq 2.5$$

The sample is pyrogenic if:

$$\frac{\text{Sum mean ODs Sample Lot}}{\text{Sum mean ODs Reference}} > 2.5$$

6. Test Protocol

The MAT consists of 3 main steps: the cryoblood incubation (or fresh blood incubation), the IL-1β ELISA, and the read-out & data analysis. According to EP, all tests should be run with 4 replicates. Some substances may have a negative impact on the ELISA test results. Therefore, it might be necessary to test the sample material in different dilutions.

6.1 Cryoblood Incubation/Stimulation of IL-1β Production

Perform incubation of blood samples in 240 µL microcultures in sterile, pyrogen-free microculture plates. It is recommended to prepare microcultures in a sterile environment. All solutions, consumables, and instrument parts coming into contact with the culture components have to be sterile and pyrogen-free. The basal release of the chosen read-out (blank) in the absence of added standard endotoxin is to be optimized to be as low as possible.

The preparation of the endotoxin dilutions is described in chapter 5.5.1.

The preparation of the sample (dilution using RPMI from the kit) is described in the Section 5 "Test Execution" (please refer to the method that you have chosen).

The PyroDetect Cryoblood is delivered in 2 x 2 mL vials, sufficient for the reactions of one entire microculture plate. For the preparation of half of the plate (max. 6 strips) one vial is sufficient. In this case please thaw only one vial of the cryoblood.

1. Transfer 20 µL of the culture medium (RPMI) into each well (the wells of the negative control contain 40 µL).
2. Transfer 20 µL of the different endotoxin concentrations into the respective wells of the microculture plate according to the plate layout of the chosen method.
3. Transfer 20 µL of the different sample dilutions into the respective wells according to the plate layout of the chosen method.
4. Thaw required number of cryoblood vials in your hand. Immediately after thawing (small frozen particles can remain), transfer the contents (2 mL) into 8.5 mL culture medium and mix the cell suspension gently by inversion of the closed tube.
5. Transfer 200 µL of the cell suspension into each well of the microculture plate.
6. Incubate the microculture plate with lid for 8 to 24 hours at 37 °C ± 1 °C in a humidified atmosphere.
7. After incubation, the contents of the microcultures may be immediately analyzed in the IL-1β ELISA. Alternatively the culture samples may be frozen in the microculture plate at –20 °C or –80°C for testing at a later time, but this procedure is not validated.

6.2 Fresh blood incubation

It is possible to use fresh blood instead of cryoblood for the blood incubation step. Fresh blood for this use is stable for 4 hours and should therefore be used as quickly as possible after donation.

1. Transfer 20 µL of endotoxin controls and/or of culture medium and/or of sample into the respective wells of the microculture plate according to the plate layout of the chosen method.
2. Transfer 200 µL of the cell suspension (180 µL RPMI and 20 µL fresh blood) into each well of the microculture plate.
3. Incubate the microculture plate for 8 to 24 hours at 37 °C ± 1 °C in a humidified atmosphere.
4. After incubation, the contents of the microcultures may be immediately analyzed in the IL-1β ELISA or be frozen at –20 °C or –80 °C (procedure not validated) for later tests.

6.3 IL-1β ELISA Procedure

All components must be at room temperature (15–28 °C) before use. The IL-1β ELISA is carried out at room temperature. Dilute the required volume of 25-fold concentrated Wash Buffer with the appropriate volume of deionized or distilled water. Washing a complete microplate requires about 30 mL of wash solution for each washing step. For all washing steps of one plate dilute 10 mL Wash Buffer with 240 mL water.

1. After incubation mix the microcultures by repeated up and down pipetting and transfer 200 µL from each microculture into the wells of the IL-1β ELISA plate. Take care to transfer every sample from the culture plate to the identical position in the ELISA plate and change tips for each column.
2. Incubate 2 hours at room temperature.
3. After incubation, aspirate or decant the plate content from each well. Then wash the plate of each well 3 times with 300 µL diluted Wash Buffer. The washing steps can be done manually or by a plate washer.

   If these steps are performed manually, remove as much moisture as possible by inverting the washed microplate and carefully tapping out the residual Wash Buffer on blotting paper towel after aspiring or decanting. Please make sure that the single strips are not dislodged from the plate.
4. Add 200 µL of enzyme-labeled antibody Anti-human IL1-β (conjugate) to each well of the plate.
5. Incubate the plate for 1 hour at room temperature.
6. After incubation, aspirate or decant the plate contents from each well. Then wash the plate of each well 3 times with 300 µL of diluted Wash Buffer as in step 3.
7. Pull reagent A into the reagent B bottle or B into A bottle, invert and pull it in a container. Add 200 µL of the mixture to each well.
8. Incubate the plate for 20 minutes (protected from light) at room temperature.
9. Add 100 µL of Stop Solution to each well. Agitate slightly for even distribution of the Stop Solution in the wells. This results in an even yellow color.
10. Measure the results within 30 minutes in a microplate photometer at 450 nm with reference wavelength set between 600 and 690 nm.

Assay Procedure Summary

Cryoblood Incubation

1. Distribute 20 µL of the endotoxin controls (performed in RPMI) and 20 µL of dilutions of the test sample (performed in RPMI).

2. Thaw the cryoblood and dilute it in RPMI.

3. Transfer 200 µL of the diluted cryoblood into microculture plate.

4. Incubate microculture plate in a humidified atmosphere at 37 °C for 8–24 h.

5. Run the IL-1β ELISA.

IL-1β ELISA

1. Mix the content of each microculture by pipetting up and down, and transfer 200 µL of the mix into an IL-1β ELISA plate.

2. Incubate the plate for 2 hours at room temperature.

3. Remove the liquid from the wells and wash the plate 3 times with 300 µL of Wash Buffer.

4. Add 200 µL of Anti-human IL1-β conjugate to each well.

5. Incubate the plate for 1 hour at room temperature.

6. Remove the liquid from the wells and wash the plate 3 times with 300 µL of Wash Buffer as in step 3.

7. Mix equal volumes of Color Reagents A and B and add 200 µL of the mixture to each well.

8. Incubate the plate for 20 minutes protected from light and at room temperature.

9. Add 100 µL of Stop Solution to each well.

Read-out and Data Analysis

10. Measure the results within 30 minutes in a microplate photometer at 450 nm with a reference wavelength set between 600 and 690 nm.

11. Analyze the data with the Gen5 protocol.

7. Supplementary Information

7.1 License

The *in vitro* pyrogen test method with cryo-preserved blood is protected by US patent 5,891,728, EP 0 741 294 and EP 0 851 231.

The ELISA part of this product is covered by one or more of the following US patents held by R&D Systems, Inc. 614 McKinley Place NE, Minneapolis, MN 55413 USA: US 5,510,462; 5,681,933; 5,474,899; 5,789,185; 5,484,887.

7.2 Glossary

Abbreviation	Definition
CLC	Contaminant Limit Concentration
EC	Endotoxin concentration in EU/mL
ELISA	Enzyme-Linked Immunosorbent Assay
EP	European Pharmacopoeia
EU/mL	Endotoxin Units per milliliter
IL-1β	Interleukin-1β
LAL	Limulus Amoebocyte Lysate
LOD	Limit of Detection
MAT	Monocyte-Activation Test
MVD	Maximum Valid Dilution
OD	Optical Density
RPT	Rabbit Pyrogen Test

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MilliporeSigma
400 summit Drive
Burlington, MA 01803

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