instaCELL pyrogen detection kit

Product Information

Cat N° SF240-01

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Content

1 Description

The pyrogen detection assay is an *in vitro* test method to detect or quantify substances that activate human monocytes to release endogenous mediators such as pro-inflammatory cytokines, like tumor necrosis factor alpha (TNF-α), which play an important role in fever pathogenesis. The assay detects the presence of endotoxins as well as non-endotoxin pyrogens and is capable to replace the rabbit pyrogen test[1].

The pyrogenicity of a substance is determined by the instaCELL kit based on the release of TNF-α. For this purpose, Assay Ready THP-1 derived macrophages are incubated with the test substance and the supernatant is analyzed in a TNF-α specific ELISA. The Assay was established with heat killed staphylococcus aureus (HKSA), lipopolysaccharide (LPS), lipoteichoic acid (LTA) and Flagellin to ensure that all cell membrane toll-like receptors (TLR) are expressed. The amount of pyrogen can be determined using a standard curve of the European Pharmacopoeia Endotoxin Standard (BRP).

The instaCELL pyrogen detection kit includes prequalified assay-ready THP-1 derived macrophages as well as media, reagents, controls, and plates to perform the assay according to the European Pharmacopeia 2.6.30. Assay Ready Cells are frozen cell aliquots which can be used directly in the assay without prior cultivation, basically like a reagent.

Figure 1: instaCELL pyrogen detection kit (MAT)

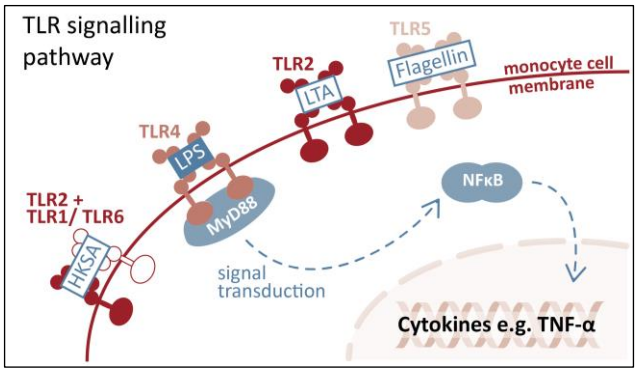

Figure 2: Toll-like receptor signaling pathway to release TNF-α.

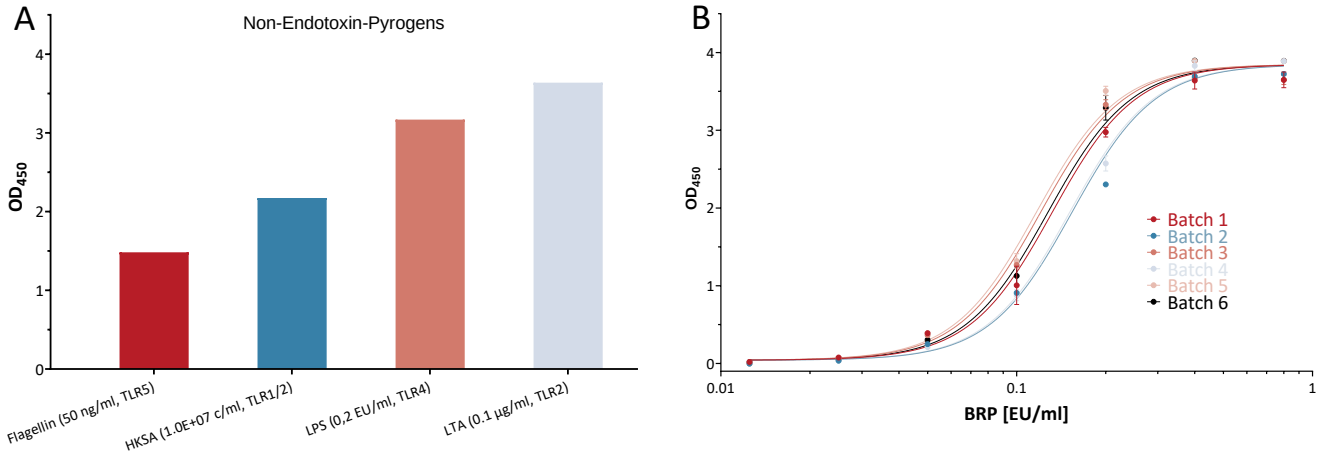

Figure 3: [A] instaCELL pyrogen detection kit, analyzing non-endotoxin pyrogens like Flagellin, Lipoteichoic acid (LTA), Lipopolysaccharide (LPS) and heat killed staphylococcus aureus (HKSA) using a TNF-α ELISA. Concluding, the macrophages express the toll like receptors TLR5 (Flagellin), TLR1/2 (HKSA), TLR4 (LPS) and TLR2 (LTA).

[B] Six different macrophage batches have been compared and the results show an inter-assay CV of 16.2% at the LOD level (0,05 EU/ml) and 2.8% at the upper level (0.8EU/ml) of the BRP (European Pharmacopoeia Standard) standard curve. The spike recovery tests displayed an average recovery rate between 71% and 107%. These results prove that the batches are precise and accurate on a reproducible level.

2 Cell Information

Cell Type:	Acute monocytic leukemia
Tissue:	Peripheral blood
Species:	Human
Morphology:	Macrophage
Growth:	Adherent
Host Cell Line:	THP-1
Biosafety Level:	1

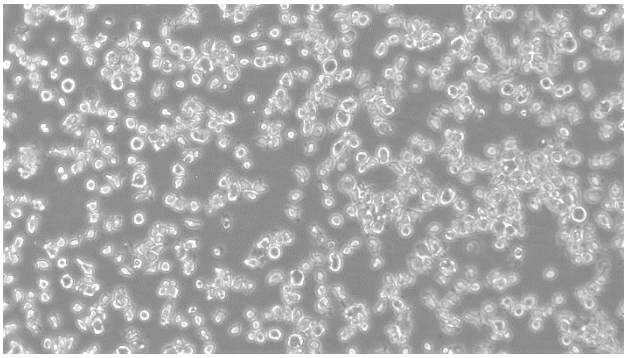

Figure 2: Morphology of assay-ready THP-1 macrophages.

3 Kit Content

• THP-1 macrophages	1 vial (1E7 cells)	RE514
• Recovery Buffer A	1 bottle (10ml)	MD163-01
• Assay Buffer A	1 bottle (60ml)	MD363-06
• Capture Antibody	1 vial (50µl)	RX509-01
• Detection Antibody	1 vial (50µl)	RX510-01
• Coating Buffer	1 vial (1.3ml)	RX511-01
• Streptavidin-HRP	1 vial (125µl)	RX512-01
• ELISA buffer	1 bottle (14ml)	RX513-01
• TMB Solution	1 bottle (11ml)	RX514-01
• BRP-Standard	1 vial (45µl)	RX515-01
• 96-well ELISA plate	1 plate	LM071-00
• 96-well Assay plate	1 plate	ZG14-08

Additionally required but not provided with the kit:

beta-mercaptoethanol, Tween 20, DBPS, conc. H_2SO_4, 15ml centrifugation tube

4 Protocol of Use

The instaCELL pyrogen detection kit can be conducted in three different versions:

1. Quantitative Test
2. Semi-Quantitative Test
3. Reference Lot Comparison Test

There is a specific protocol for each method in the following sections. For more details, please refer to the **European Pharmacopeia:** 2.6.30. Monocyte Activation test

Prior routine testing of a sample, a product specific validation must be performed to ensure that the sample does not interfere with the assay system. Possible interferences can be removed by diluting the sample up to the maximum valid dilution (MVD). The MVD is the maximum dilution factor which can be calculated using equation 1. The product specific validation must be performed according to the desired method for routine testing.

$$MVD = \frac{CLC \bullet C}{LOD} \quad \textbf{(Eq. 1)}$$

CLC = Contaminant Limit Concentration

C = Concentration of test solution

LOD = Limit of Detection

The LOD of the instaCELL pyrogen detection kit is **0.05 EU/ml** based on historical data.

4.1 Day I: Preparation of cells

- Keep the cells on dry ice before thawing and process quickly.
- Add 60µl of 50mM beta-mercaptoethanol to the assay buffer.
- Equilibrate all media and buffer to 37°C.
- Thaw one vial of Assay Ready Cells in a water bath at 37°C for 3min. Prepare 8ml of recovery buffer in a 15ml centrifugation tube. Dispense the cells completely into the prepared tube. Rinse the cryovial once with 1ml recovery buffer.
- Centrifuge for 3min at 200xg and carefully aspirate the supernatant. Resuspend the cell pellet in 10ml of assay buffer. Dispense 100µl of the cell suspension into each well of the provided assay plate.
- Incubate for 24h in a humidified incubator at 37°C and 5% CO_2.

4.2 Day II: Preparation of test sample and ELISA plate

4.2.1 Quantitative Test

The quantitative method involves a comparison of the preparation being examined with a standard endotoxin dose-response curve. The contaminant concentration of the preparation being examined is to be less than the CLC to pass the test[1].

Plate Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BRP, 0.8 EU/ml				Sample 1, C1				Sample 2, C1			
B	BRP, 0.4 EU/ml				Sample 1, C1 (spiked)				Sample 2, C1 (spiked)			
C	BRP, 0.2 EU/ml				Sample 1, C2				Sample 2, C2			
D	BRP, 0.1 EU/ml				Sample 1, C2 (spiked)				Sample 2, C2 (spiked)			
E	BRP, 0.05 EU/ml				Sample 1, C3				Sample 2, C3			
F	BRP, 0.025 EU/ml				Sample 1, C3 (spiked)				Sample 2, C3 (spiked)			
G	BRP, 0.0125 EU/ml				Blank				Blank			
H	Blank				Blank				Blank			

Figure 3: Plate layout of the quantitative test. BRP is given in final concentrations per well.

- Dilute the BRP stock solution 1:25 in assay buffer to obtain the BRP working solution. Dilute the working solution 1:50 to obtain the highest concentration of the standard curve and perform 2-fold serial dilutions for a total of 7 dilutions to obtain the BRP standard concentrations (see plate layout).
- Each sample is tested in three concentrations. Use the concentration previously determined in the test for interfering factors as the highest sample concentration (C1) and prepare two-fold serial dilutions for concentrations 2 and 3 (C2 and C3), not exceeding the MVD. Prepare your sample in assay buffer at 2x of the final concentration.
- Dilute the BRP working solution 1:4 to obtain the spiking solution. Add 10µl of spiking solution to 990µl of the test sample dilutions to spike your samples.
- Add 100µl of BRP standard series, samples, spiked samples and blank in quadruplicates to the respective wells of the assay plate. Use Assay Buffer for the blank.
- Incubate for 18h in a humidified incubator at 37°C and 5% CO_2.

- Dilute coating buffer 1:10 in ddH_2O (1.1ml + 9.9ml).
- Dilute capture antibody 1:250 in coating buffer (42µl + 10458µl).
- Coat the provided 96-well ELISA plate by adding 100µl of coating solution to each well. Incubate overnight at 4°C.

4.2.2 Semi-Quantitative Test

The semi-quantitative method involves a comparison of the preparation being examined with a standard endotoxin dose-response curve. The contaminant concentration of the preparation being examined is to be less than the CLC to pass the test[1].

	1	2	3	4	5	6	7	8	9	10	11	12
A	BRP, 0.2 EU/ml				Sample 1, C2				Sample 2, C3			
B	BRP, 0.1 EU/ml				Sample 1, C2 (spiked)				Sample 2, C3 (spiked)			
C	BRP, 0.05 EU/ml				Sample 1, C3				Sample 3, C1			
D	BRP, 0.025 EU/ml				Sample 1, C3 (spiked)				Sample 3, C1 (spiked)			
E	BRP, 0.0125 EU/ml				Sample 2, C1				Sample 3, C2			
F	Blank				Sample 2, C1 (spiked)				Sample 3, C2 (spiked)			
G	Sample 1, C1				Sample 2, C2				Sample 3, C3			
H	Sample 1, C1 (spiked)				Sample 2, C2 (spiked)				Sample 3, C3 (spiked)			

Figure 4: Plate layout of the semi-quantitative test. BRP is given in final concentrations per well.

- Dilute the BRP stock solution 1:25 in assay buffer to obtain the BRP working solution. Dilute the working solution 1:10 and again 1:20 to obtain the highest concentration of the standard curve and perform 2-fold serial dilutions for a total of 5 dilutions to obtain the BRP standard concentrations (see plate layout).
- Each sample is tested in three concentrations. Use the concentration of the interfering test as highest concentration(C1) and prepare two-fold serial dilutions for concentrations 2 and 3 (C2 and C3), not exceeding the MVD. Prepare your sample in assay buffer at 2x of the final concentration.
- Dilute the BRP working solution 1:4 to obtain the spiking solution. Spike your sample by a 1:100 dilution of the spiking solution in each of the three sample dilutions.
- Add 100µl of BRP standard series, samples, spiked samples and blank in quadruplicates to the respective wells of the assay plate. Use Assay Buffer for the blank.
- Incubate for 18h in a humidified incubator at 37°C and 5% CO_2.

- Dilute coating buffer 1:10 in ddH_2O (1.1ml + 9.9ml).
- Dilute capture antibody 1:250 in coating buffer (42µl + 10458µl).
- Coat the provided 96-well ELISA plate by adding 100µl of coating solution to each well. Incubate overnight at 4°C.

4.2.3 Reference Lot Comparison Test

The reference lot comparison test involves a comparison of the preparation being examined with a validated reference lot of that preparation. The type of analysis selected to compare the two is to be justified and validated for each product and is to include assay validity criteria. The reference lot is also selected according to criteria that have been justified and authorized. The test is intended to be performed in cases where a preparation being examined shows marked interference but cannot be diluted within the MVD to overcome the interference or because it contains or is believed to contain non-endotoxin contaminants. Response 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. The test procedure is described below and includes an example of a type of analysis used for the comparison of a test lot and reference lot [1].

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ref 1, C1				Ref 2, C1				Ref 3, C1			
B	Ref 1, C2				Ref 2, C2				Ref 3, C2			
C	Ref 1, C3				Ref 2, C3				Ref 3, C3			
D	Sample 1, C1				Sample 2, C1				Sample 3, C1			
E	Sample 1, C2				Sample 2, C2				Sample 3, C2			
F	Sample 1, C3				Sample 2, C3				Sample 3, C3			
G	BRP, 0.2 EU/ml				Blank				Blank			
H	Blank				Blank				Blank			

Figure 5: Plate layout for the lot comparison test. BRP is given in the final concentration per well.

For the Lot comparison test, the sample to be analyzed is compared with a known similar reference substance. Both the reference and the test sample are tested in three dilutions. The dilutions to be tested can be derived from the preliminary test to check for interfering factors. It is advisable to select concentrations that are around the lowest still clearly positive concentration and do not exceed the MVD.

- Dilute the BRP stock solution 1:25 in assay buffer to obtain the BRP working solution. Dilute the working solution 1:10 and again 1:20 to obtain the BRP concentration used as positive control (see plate layout).
- Add 100µl of BRP standard, samples, and blank in quadruplicates to the respective wells of the assay plate. Use Assay Buffer for the blank.
- Incubate for 18h in a humidified incubator at 37°C and 5% CO_2.

- Dilute coating buffer 1:10 in ddH_2O (1.1ml + 9.9ml).
- Dilute capture antibody 1:250 in coating buffer (42µl + 10458µl).
- Coat the provided 96-well ELISA plate by adding 100µl of coating solution to each well. Incubate overnight at 4°C.

4.3 Day III: ELISA Assay

- Prepare 150ml of wash buffer (0.05% Tween20 in PBS).
- Dilute ELISA Buffer 1:5 to 50ml in ddH_2O.
- Aspirate wells of the ELISA plate and wash with 200µl/well of wash buffer.
- Add 200µl ELISA buffer to each well of the ELISA plate. Incubate for 1h at room temperature.
- Aspirate wells and wash with 200µl/well of wash buffer.
- Transfer 100µl from the assay plate to appropriate wells of the ELISA plate. Incubate for 2h at room temperature.
- Dilute the detection antibody 1:250 in ELISA buffer (42µl+ 10.458ml).
- Aspirate and wash once with 200µl washing buffer. Let the buffer soak in for 1min.
- Add 100µl detection antibody to all wells and incubate for 1h at room temperature.
- Dilute Streptavidin-HRP 1:100 in ELISA buffer (110µl + 10.890ml).
- Aspirate and wash once with 200µl washing buffer. Let the buffer soak in for 1min.
- Add 100µl Streptavidin-HRP to each well and incubate for 30min at room temperature.
- Aspirate and wash thrice with 200µl washing buffer. Let the buffer soak in for 1min during every step.
- Add 100µl TMB solution to each well and incubate for 15min at room temperature while shaking in the dark.
- Add 100µl of stop solution (1M H_2SO_4) to each well.
- Measure OD at 450nm and 570nm for reference.

5 analysis and assay acceptance criteria

5.1 Quantitative Test

The quantitative test uses the measured standard curve to convert the OD signal to a concentration in EEU/ml. The obtained values are compared to the CLC for the tested matrix.

- The OD signal should increase progressively with the used standard concentrations.
- The mean signal of the LOD standard solution (0.05 EU/ml) should be above the cut-off value (Eq. 3).
- Calculate the average OD for each concentration of the standard curve and plot the values on the y-axis against the endotoxin concentrations on a $\log_{10}$ x-axis.
- Perform a regression using a 4-paramter fit and use the resulting regression equation to calculate the concentration of each sample dilution and correct the value by the dilution factor.
- The Dose-Response of the product must be parallel to the Endotoxin Standard Curve.
- The basal rate should be as low as possible → OD of blank < 0.1
- The regression of response shall be statistically significant on a 5% level of significance
  Use a statistical program like GraphPad Prism and perform a 4-paramter fit and use a statistical test (Runs-Test) that confirms the suitability of the regression model.
- To test for statistical relevance, use the equation below (Eq.2). For the instaCELL kit the ratio should be > 3,747 using a 4-parameter fit.

$$S_{Ratio} = \frac{Top-0}{SD_{Top}} \quad \textbf{(Eq. 2)}$$

- The spike recovery must be in a range of 50% – 200%. Dilutions not fulfilling that argument must be excluded from further evaluation. One valid dilution is the minimum required for a valid test.
- For each valid dilution, convert the OD value in EEU/ml, corrected with the dilution factor and compare it to the CLC.

5.2 Semi-quantitative test

The semi-quantitative test compares the measured sample dilution OD value with the cut-off value. For all OD-values below the cut-off value the sample dilution is to be considered negative. This test does not require a full endotoxin standard curve.

- The OD signal should increase progressively with the used standard concentrations.
- The mean signal of the LOD standard solution (0.05 EU/ml) should be above the cut-off value.
- The spike recovery must be in a range of 50% – 200%. Dilutions not fulfilling that argument must be excluded from further evaluation. One valid dilution is the minimum required for a valid test.
- If the OD-value of the sample dilution is below the cut-off value, the pyrogen concentration is below the LOD.
- If the OD-value is above the cut-off value, the result is non-conclusive since a pyrogen contamination is present but cannot be evaluated with respect to the CLC.
- If the OD-value at the MVD is above the cut-off value, the pyrogen level of the sample is above the CLC.

$$cut\text{-}off = mean_{OD_{blank}} + 3 \times SD_{OD_{blank}} \quad \textbf{(Eq. 3)}$$

5.3 Lot comparison test

The Lot comparison test is based on a comparison of the test sample with a reference lot of the same preparation. The reference lot must be carefully justified and validated.

- For assay acceptance at least one dilution of the test sample and the positive control must have a mean OD-value above the blank.
- For evaluation an OD ratio is calculated, corresponding to the sum of the mean response of the 3 dilutions of the test sample divided by the sum of mean response of the dilutions of the reference lot. The Pharmacopoeia gives an acceptance criterion of 2.5 for the OD ratio.
- If the OD ratio is below the acceptance criterion, the sample is to be considered not pyrogenic compared to the reference.

6 Stability & Storage

Performance of the kit is guaranteed within the specifications as defined in the certificate of analysis only before the expiration date as indicated on the packaging and only if stored and handled according to the instruction of this datasheet.

- Store Assay Ready Cells below -140°C (e.g., in vapor phase of liquid nitrogen).
- Store all other reagents and media as indicated on the label.

7 Literature & Related Documents

1. **European Pharmacopeia:** 2.6.30. Monocyte Activation test

8 Support

https://www.accellerate.me/support/contact.html

Phone: +49 (40) 33 464 73 20

9 Disclaimer

The product is sold under the terms of a Limited Use Label License attached to the product. By breaking the seal of the product package, the user explicitly agrees to the license terms. Assay Ready Cells are for immediate assay use only. The user shall not propagate, passage, or refreeze the cells.

This product is intended for research use only. Do not use for diagnostic or therapeutic purposes.