instaCELL TNF-α neutralization assay kit

Product Information

Cat N° SF310-01

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Content

1 Description

The instaCELL TNF-α neutralization assay kit is used to determine the potency of tumor necrosis factor-alpha (TNF-α) neutralizing/inhibiting therapeutics. These recombinant antibodies and soluble proteins are used to regulate excessive inflammatory responses in the body. A dysregulation of TNF-α is associated with diseases like Alzheimer's disease[1], cancer[2], major depression[3], psoriasis[4] and inflammatory bowel disease (IBD)[5].

Figure 1: instaCELL TNF-α neutralization assay kit

The assay utilizes the cytotoxic effect of TNF-α on the well-established mouse fibroblast cell line L-929 to determine the inhibitory efficacy[6]. Cells are incubated with a dilution series of a drug/TNF-α mixture, with a constant TNF-α concentration, and then analyzed via a resazurin cell viability assay. Based on a reference standard, which has a fixed, known effective unit size, the inhibitory antibody can be potentiated. The kit is in accordance with the verified method of the European Pharmacopoeia 10.3 for testing biosimilars.

The instaCELL TNF-α neutralization assay kit includes prequalified assay-ready L-929 cells as well as media, reagents, reference standard, and 96-well plate to perform the assay according to the European Pharmacopeia. Assay Ready Cells are frozen cell aliquots which can be used directly in the assay without prior cultivation, basically like a reagent.

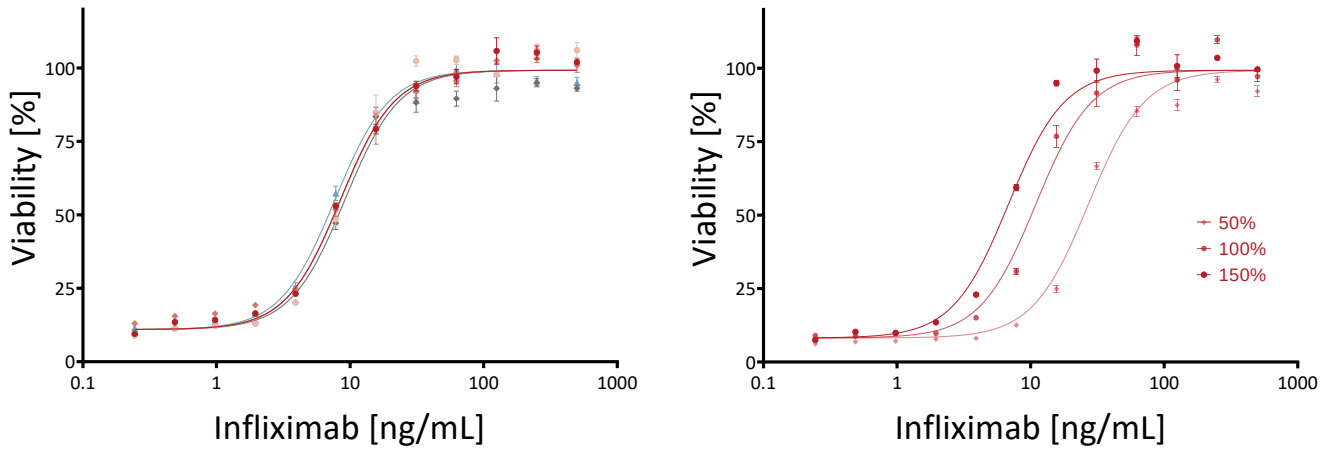

Figure 2: Precision & Accuracy of the instaCELL TNF-α neutralization assay kit

[A] Five independent experiments were performed, with individual aliquots of Assay Ready Cells, on different days, displaying precision for the IC_{50} value. The CV of the IC_{50} value is 9% for Infliximab confidence interval of 93% to 107%.

[B] The accuracy of the neuralization assay was tested with Infliximab. Nominal doses of 50%, 100% and 150% were applied and potencies of 44%, 100% and 145% were determined, which is an average accuracy of 92%.

2 Cell Information

Cell Type:	Fibroblast
Tissue:	Connective Tissue
Species:	Mouse
Growth:	Adherent
Gender:	Male
Biosafety Level:	1

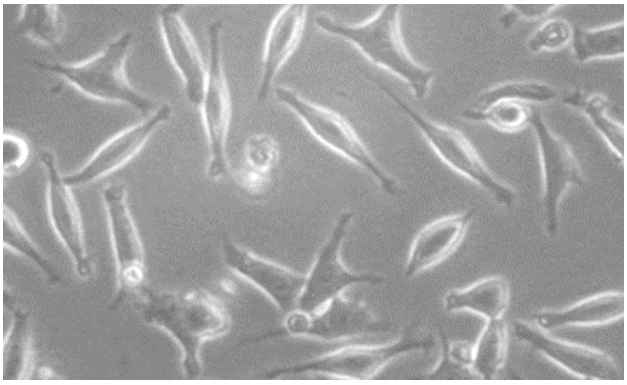

Figure 3: Morphology of Assay Ready L-929 Cells

3 Kit Content

• L-929 cells	1 vial (3E+06 cells)	RE772K
• Recovery Buffer A	1 bottle (10ml)	MD163-01
• Assay Buffer A	1 bottle (60ml)	MD363-06
• TNF-α – Infliximab Stock Solution	1 vial (25µl)	RX516-01
• TNF-α – TNF-α Stock Solution	1 vial (25µl)	RX517-01
• Actinomycin D	1 vial (1mg)	10710.01 (Serva)
• Resazurin Solution	1 bottle (5ml)	RX718-01
• 96-well plate	1 plate	83.3924.300 (Sarstedt)

Additionally required but not provided with the kit:

DMSO

4 Protocol of Use

4.1 Day I: Seeding of L-929 Cells

- Keep the cells on dry ice before thawing and process quickly.
- 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 50ml 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 30ml of assay buffer. Mix cell suspension before each pipetting step. Dispense 150µl of the cell suspension into each well of the provided assay plate, except the wells in row A.
- Incubate for 24h in a humidified incubator at 37°C and 5% CO_2.

4.2 Day II: Preparation and addition of test sample and controls to the cells

Caution: Actinomycin is sensitive to light, switch off the light of the sterile hood!

4.2.1 Preparation of the working medium:

- Dissolve Actinomycin D in 1ml DMSO, mix well. Dilute the solution 10-fold in assay buffer (50µl + 450µl).
- Dilute the solution again 100-fold (270µl+26.73ml) in assay buffer to obtain the working medium (1µg/ml).

4.2.2 Preparation of the TNF-α working solution:

- Dilute the TNF-α stock solution **two times** 10-fold in working medium. First 10µl stock solution in 90µl working medium (dilution 1) and then 20µl of dilution 1 in 180µl working medium (dilution 2).
- Further dilute 100µl of dilution 2 in 12.56ml working medium to obtain the TNF-α working solution. This solution is 2x concentrated and contains 7 IU/ml (0.158 ng/ml).

4.2.3 Preparation of the reference standard:

Use an inhouse reference standard according to your protocol or the instaCELL reference standard (36.5mg/ml Infliximab) and procced with the following:

- Dilute the reference standard solution **three times** 10-fold in working medium (10µl + 90µl).
- Further dilute 22.5µl of the third dilution in 779µl working medium to obtain the top concentration (C1) of the infliximab standard curve.
- Perform a 2-fold serial dilution series of 12 concentrations for the infliximab standard curve. Add 400µl of C1 to 400µl of working medium, mix well and continue this process up to C12. Discard 400µl from C12 to have 400µl of Infliximab solution in each micro tube.

4.2.4 Preparation of the test sample and transfer to the assay plate:

- Prepare a 2x concentrated dilution series of your test sample in working medium, analogue to the infliximab standard dilution series.
- Add 400µl of TNF-α working solution to each micro tube of the infliximab and test sample dilution series, mix well and incubate for 1h at room temperature. Make sure to protect the dilutions from light.
- Check the cell viability and confluence under a microscope.
- Aspirate the medium from the assay plate and add 150µl of reference standard (rows A-D) and test sample (rows F-H) to the corresponding well. To avoid any edge effect, use the inverse concentration order from the plate layout below (Fig. 4).
- Add 150µl of TNF-α working solution to wells E1-E4 for a TNF-α control; 150µl of working medium to the wells E5-E8, for Actinomycin D control; 150µl of assay buffer to the wells E9-E12, for medium control. Shake the plate for 15s on an orbital shaker
- Incubate for 24h in a humidified incubator at 37°C and 5% CO_2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	~~no cells C12~~	~~no cells C10~~	~~no cells C8~~	~~no cells C6~~	~~no cells C4~~	~~no cells C2~~	~~no cells C1~~	~~no cells C3~~	~~no cells C5~~	~~no cells C7~~	~~no cells C9~~	~~no cells C11~~
B	STD C12	STD C10	STD C8	STD C6	STD C4	STD C2	STD C1	STD C3	STD C5	STD C7	STD C9	STD C11
C	STD C12	STD C10	STD C8	STD C6	STD C4	STD C2	STD C1	STD C3	STD C5	STD C7	STD C9	STD C11
D	STD C12	STD C10	STD C8	STD C6	STD C4	STD C2	STD C1	STD C3	STD C5	STD C7	STD C9	STD C11
E	TNF-α control				Actinomycin D control				medium control			
F	sample C12	sample C10	sample C8	sample C6	sample C4	sample C2	sample C1	sample C3	sample C5	sample C7	sample C9	sample C11
G	sample C12	sample C10	sample C8	sample C6	sample C4	sample C2	sample C1	sample C3	sample C5	sample C7	sample C9	sample C11
H	sample C12	sample C10	sample C8	sample C6	sample C4	sample C2	sample C1	sample C3	sample C5	sample C7	sample C9	sample C11

Figure 4: Recommended plate layout

test sample (triplicates of 12 dilutions), reference standard (triplicates of 12 dilutions), ☒ reference standard no cells
TNF-α control (quadruplicate), Actinomycin D control (quadruplicate), medium control (quadruplicate)

4.3 Day III: Readout by Resazurin Viability Assay

- Equilibrate Resazurin Solution to room temperature.
- Add 20µl of Resazurin Solution to each well of the assay plate.
- Incubate for 4h in a humidified incubator at 37°C and 5% CO_2.
- Shake the plate for 15s on an orbital shaker. Afterwards measure the fluorescence at $540_{EX}/590_{EM}$.

5 Analysis and Assay Acceptance Criteria

5.1 Calculation of Viability and EC_{50}

- Calculate the mean of the wells of row A and subtract it from every other well for baseline correction.
- Calculate the mean of all three control quadruplicates.
- Calculate the viability of every concentration for the test sample and the reference standard with the following equation:

$$\frac{\text{Mean of triplicate}}{\text{Mean of Actinomycin D control}} \text{ x } 100 = \text{Viability [\%]}$$

Equation 1: Calculation of the cell viability

- Calculate the EC_{50} with a four-parameter fit model, the X axis contains the concentration of the Infliximab and the Y axis the calculated viability.

5.2 Acceptance Criteria

- Observe the "cell + TNF-α control", the value must be between the lowest concentration of Infliximab and close to the basis signal.
- The mean of Actinomycin D cell control is divided by the mean of the medium control, then multiply by 100, to obtain a value between 50% - 80% activity of the Actinomycin D treated cells.
- The coefficient of determination calculated for the standard curve (r^2) is not less than 0.97.
- Maximum Value (Actinomycin D control) to minimum value (cell + TNF-α control) ratio must be >3.
- The potency achieved by the TNF-α test must not be less than 80% and no more than 120% of the reference standard.
- The confidence limits (P = 0.95) must not be below 80% and not above 125%.
- The range of the EC_{50} of the instaCELL reference standard curve should be between 2 ng/ml and 15 ng/ml.

If the value is outside of these limits, the assay must be repeated.

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] Swardfager W, Lanctôt K, Rothenburg L, Wong A, Cappell J, Herrmann N (2010). "A meta-analysis of cytokines in Alzheimer's disease". Biol Psychiatry. 68 (10): 930–941. PMID 20692646

[2] Locksley RM, Killeen N, Lenardo MJ (2001). "The TNF and TNF receptor superfamilies: integrating mammalian biology". Cell. 104 (4): 487–501. PMID 11239407.

[3] Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lanctôt KL (2010). "A meta-analysis of cytokines in major depression". Biol Psychiatry. 67 (5): 446–457. PMID 20015486.

[4] Victor FC, Gottlieb AB (2002). "TNF-alpha and apoptosis: implications for the pathogenesis and treatment of psoriasis". J Drugs Dermatol. 1 (3): 264–75. PMID 12851985

[5] Brynskov J, Foegh P, Pedersen G, Ellervik C, Kirkegaard T, Bingham A, Saermark T (2002). "Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease". Gut. 51 (1): 37–43. PMID 12077089

[6] Humphreys DT, Wilson MR. Modes of L929 cell death induced by TNF-alpha and other cytotoxic agents. Cytokine. 1999 Oct;11(10):773-82. PMID: 10525316.

European Pharmacopoeia 10.3 (01/2021)

8 Support

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

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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.