Determination of neutralizing antibodies against TNF-alpha inhibitor using *iLite*® TNF-alpha Assay Ready Cells

For research and professional use only. Not for use in diagnostic procedures.

This application note contains a suggested protocol and performance data.
Each individual laboratory must set up their own method and perform relevant validations.

Background

TNF-alpha promotes inflammatory responses, which in turn contribute to the clinical symptoms associated with many inflammatory disorders, including rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis and refractory asthma. (1) These diseases are in many cases treated with TNF-alpha inhibitors, such as **infliximab, adalimumab, or etanercept** to mention a few. Prolonged therapies with these TNF-alpha inhibitors may lead to development of neutralizing antibodies (NAbs), which may counteract the TNF-alpha antagonist activity of the inhibitors. (2)

The *iLite*® TNF-alpha Assay Ready Cells can be used for measurements of functional TNF-alpha, TNF-alpha inhibitor activity and presence of neutralizing antibodies to TNF-alpha inhibitors. (3,4)

Principle of the assay

The *iLite*® TNF-alpha Assay Ready Cells are engineered cells, optimized to express Firefly luciferase (FL) under the control of a NFkB responsive promoter. Binding of TNF-alpha to its receptor results in activation of the NFkB regulated Firefly luciferase reporter gene construct. *iLite*® TNF-alpha Assay Ready Cells also contain the Renilla Luciferase (RL) reporter gene, under the control of a constitutive promoter. The constitutive expression of RL allows normalization of TNF-alpha induced FL activity, and renders assay results independent of variations in cell number or serum matrix effects. The Firefly luciferase signal is inversely proportional to the amount of inhibitory activity in a sample. In the absence of TNF-alpha inhibitor activity and suspected NAb presence in test samples, a known amount of TNF-alpha inhibitor is added to quench the Firefly signal and the presence of NAbs is measured as a restored signal.

The *iLite*® TNF-alpha Assay Ready Cells can therefore be utilized as a highly sensitive assay for determination of neutralizing antibodies against TNF-alpha inhibitor in human serum. (3,4) In the following outline, Infliximab is used as example – other TNF inhibitors can be used as well, but with changed dose scheme.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® TNF-alpha Assay Ready Cells	Svar Life Science	BM3044
Diluent (RPMI containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin).	Gibco	61870-044 (RPMI 1640) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Anti-infliximab antibody	Bio-Rad	HCA213
Infliximab	NA	NA
TNF-alpha or analogues	R&D Systems	210-TA/CF
Firefly/Renilla luciferase substrate	Promega	E2920, Dual-Glo Luciferase Assay System

Plate; White walled micro well plate suitable for luminescence	PerkinElmer	6005680
Microplate Luminometer with appropriate reading software – no filter on luminometer	Contact Svar Life Science for list of recommended suppliers	NA
Incubator, 37 °C with 5% CO_2	NA	NA
Water bath, 37 °C	NA	NA
Single-channel and multi-channel pipettes with polypropylene disposable tips	NA	NA
Polypropylene tubes or plate for dilution	NA	NA
Single-use polypropylene reservoir	NA	NA
Plate shaker	NA	NA
Timer	NA	NA

Protocol

Preparation of neutralizing antibodies against TNF-alpha inhibitor

An anti-infliximab antibody from Bio-Rad has successfully been used to neutralize infliximab (TNF-alpha inhibitor) and restore the TNF-alpha regulated Firefly luciferase expression in *iLite*® TNF-alpha Assay Ready Cells (refer to the table and graph below).

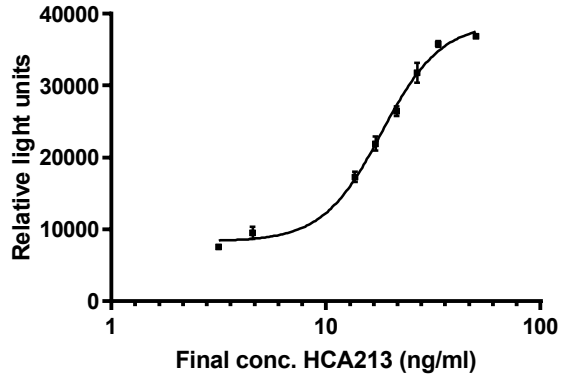

Figure 1. Example of Infliximab inhibitory curve

Final 4 ng/mL TNFa and 40 ng/mL Infliximab	Anti- infliximab Suggested solution concentrations, ng/mL
A	400
B	267
C	213
D	171
E	137
F	109
G	36
H	0

Table 1. Suggested calibrator **solution concentrations** for anti-infliximab.

Assay preparation and incubation

1. Design a plate layout. It is recommended to perform the test at minimum in duplicates.
2. Perform a serial dilution of the reference anti-infliximab antibody. Ensure matrix consistency between reference antibody solutions, control solutions, and sample solutions.
3. Add 20 µL of the reference anti-infliximab antibody dilutions, controls and samples to assigned wells (final concentration will be one-eighth of solution concentration).
4. Add 20 µL of 320 ng/mL infliximab to all wells (final concentration will be 40 ng/mL infliximab).
5. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
6. Add 40 µL of 16 ng/mL TNF-alpha to all wells (final concentration will be 4 ng/mL TNF-alpha).
7. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
8. Transfer references, controls and samples to new wells, adding 40 µL per well.
9. Thaw a vial of *iLite*® TNF-alpha Assay Ready Cells in a 37 °C water bath with gentle agitation.
10. Add the entire content of the cell vial to 6 mL Diluent. Invert the vial containing diluted cell suspension approximately ten times in order to ensure a homogeneous distribution of cells.
11. Add 40 µL diluted cells to each well.

12. Place the lid on the plate, mix and incubate for 3 hours at 37 °C with 5% CO_2.

Adding substrate solutions

13. Equilibrate the plate and the substrate solutions to room temperature.
14. Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.
15. If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.

Precautions

- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according to the suppliers'/manufacturers' instructions or product specifications accompanying the individual material and instruments.
- Dispose of all sample specimens, infected or potentially infected material in accordance with good microbiological practice. All such materials should be handled and disposed as though potentially infectious.
- Residues of chemicals and preparations are generally considered as biohazardous waste and should be inactivated prior to disposal by autoclaving or using bleach. All such materials should be disposed of in accordance with established safety procedures.

Proprietary Information

In accepting delivery of *iLite*® Assay Ready Cells the recipient agrees not to sub-culture these cells, attempt to sub-culture them or to give them to a third-party recipient, and only to use them directly in assays. *iLite*® cell-based products are covered by patents which are the property of Svar Life Science AB and any attempt to reproduce the delivered *iLite*® Assay Ready Cells is an infringement of these Patents.

QUICK GUIDE

Determination of neutralizing antibodies against TNF-alpha inhibitor using *iLite*® TNF-alpha Assay Ready Cells

1 Plate #1

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage**
- Serial dilute reference anti-infliximab antibody
- Add 20 µL of ref. anti-infliximab antibody solutions, controls and samples to pre-assigned wells
- Add 20 µL of infliximab to each well

2 Incubation #1 30 min

- Incubate at 37 °C with 5% CO_2 for 30 minutes

3 Incubation #2 30 min

- Add 40 µl of TNF-alpha to all wells
- Incubate at 37 °C with 5% CO_2 for 30 minutes

4 Plate #2 Add cells

- Transfer references, controls and samples to new wells
- Thaw the cell vial in a 37 °C water bath. Dilute the cells. Invert the cell suspension in order to ensure a homogeneous cell solution
- Add 40 µL diluted cells to each well

5 Incubation #3 3 hours

- Incubate at 37 °C with 5% CO2 for 3 hours

6 Read plate

- Equilibrate the plate to room temperature
- Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer
- If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer

Svar Life Science AB
Lundavägen 151
SE-212 24 Malmö
Sweden
P.O. Box 50117
SE-202 11 Malmö
Sweden
info@svarlifescience.com
+46 40 53 76 00

Troubleshooting and FAQ

Please consult the Svar Life Science website www.svarlifescience.com

References

1. Kalliolias GD, Ivashkiv LB. *TNF biology, pathogenic mechanisms and emerging therapeutic strategies.* Nat Rev Rheumatol. 2016 Jan;12(1):49-62.
2. Kalden JR, Schulze-Koops H. *Immunogenicity and loss of response to TNF inhibitors: implications for rheumatoid arthritis treatment.* Nat Rev Rheumatol. 2017 Nov 21;13(12):707-718.
3. Lallemand C, Tovey MG. et al. *Reporter gene assay for the quantification of the activity and neutralizing antibody response to TNF-alpha antagonists.* J Immunol Meth. 2011, 373: 229-239.
4. Pavlov I, Delgado JC et al. *Clinical laboratory application of a reporter-gene assay for measurement of functional activity and neutralizing antibody response to infliximab.* Clinica Chimica Acta. 2016, 453:147-153.