Determination of neutralizing antibodies against TNF-alpha inhibitors using *iLite™* TNF-alpha Assay Ready Cells

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

Background

TNF-alpha promotes inflammatory responses, which, in turn, contribute to the clinical problems associated with many inflammatory disorders, including rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis and refractory asthma. These diseases are sometimes treated with TNF-alpha inhibitors, including infliximab, adalimumab, etanercept, certolizumab pegol or golimumab. 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.

The *iLite™* TNF-alpha Assay Ready Cells can be used for measurements of TNF-alpha inhibitor activity and presence of neutralizing antibodies to TNF-alpha inhibitors.

Principle of the assay

The *iLite™* TNF-alpha Assay Ready Cells are engineered cells optimized to express Firefly luciferase under the control of a TNF-alpha responsive promoter and Renilla luciferase under the control of a constitutive promoter. TNF-alpha binds to the TNF receptor on the cell surface and activates the TNF-alpha regulated Firefly luciferase reporter gene construct. The Firefly luciferase signal can be measured in a luminometer following addition and incubation of the luciferase substrate. The Firefly luciferase signal is proportional to the functional activity of TNF-alpha in the sample.

In the presence of TNF-alpha inhibitor activity in test samples, the amount of free TNF-alpha is reduced, resulting in a decreased stimulation of Firefly luciferase production. The Firefly luciferase signal is inversely proportional to the amount of anti-TNF-alpha activity in the samples.

In the absence of TNF-alpha inhibitor activity and suspected NAb presence in test samples, a known amount of drug is added to quench the Firefly signal and the presence of NAbs is measured as a restored signal.

The constitutively expressed gene Renilla luciferase can be sequentially measured in the same wells as the Firefly luciferase, following addition and incubation of the Renilla substrate. This allows for normalization of results by compensating for cell number variation and eliminating potential matrix effects from a serum sample, for example. In samples with no suspected matrix effect, the Renilla reading may be excluded.

Specimen collection

iLite™ TNF-alpha Assay Ready Cells can be used for detecting presence of neutralizing antibodies to TNF-alpha inhibitors in test samples including human serum.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite™ TNF-alpha Assay Ready Cells	Euro Diagnostica	BM3044
TNF-alpha	Euro Diagnostica	BM3133, *iLite*™ TNF-alpha (16 ng/mL)
Diluent B, RPMI 1640	Euro Diagnostica	BM3134, *iLite™* Diluent B
Diluent C, RPMI 1640 with 40% heat inactivated NHS	Euro Diagnostica	BM3139, *iLite™* Diluent C
Reagent BLANK, RPMI 1640 with 30% heat inactivated FCS.	Euro Diagnostica	BM3135, *iLite*™ Reagent BLANK
Positive control infliximab neutralizing antibody	Euro Diagnostica	BM3136, *iLite*™ Infliximab NAb positive control
Positive control adalimumab neutralizing antibody	Euro Diagnostica	BM3159, *iLite*™ Adalimumab NAb positive control
Positive control etanercept neutralizing antibody	Euro Diagnostica	BM3177, *iLite*™ Etanercept NAb positive control
Positive control golimumab neutralizing antibody	Contact Euro Diagnostica for suggestions	NA
Positive control certolizumab pegol neutralizing antibody	Contact Euro Diagnostica for suggestions	NA
Firefly/Renilla luciferase substrate	Promega	E2920, Dual-Glo Luciferase Assay System
Firefly luciferase substrate only	Promega	E2610, Bright-Glo™ Luciferase Assay System
Infliximab/adalimumab/etanercept/golimumab/ certolizumab pegol	NA	NA
Plate 1; Polypropylene 96-well plate	Greiner	786201
Plate 2; White walled micro well plate suitable for luminescence	PerkinElmer	6005680
Microplate Luminometer with appropriate reading software – no filter on luminometer	Contact Euro Diagnostica 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 for dilution of cells	NA	NA
Single-use polypropylene reservoir	NA	NA
Plate shaker	NA	NA
Timer	NA	NA

Preparation of reference samples and controls

Prepare a reference sample by diluting the TNF-alpha inhibitors to relevant concentrations with Diluent C. Appropriate dilutions for 5 TNF-alpha inhibitors are given in Table 1 below, as well as the final assay concentration in the wells. The reference sample is used for all wells except the blank. For positive controls please see Materials table above. Diluent C is used as the negative control.

Table 1. Preparation of reference samples

Reference sample	Infliximab		Adalimumab		Etanercept		Golimumab		Certolizumab pegol	
	Reference conc (ng/mL)	Final conc (ng/mL)	Reference conc (ng/mL)	Final conc (ng/mL)	Reference conc (ng/mL)	Final conc (ng/mL)	Reference conc (ng/mL)	Final conc (ng/mL)	Reference conc (ng/mL)	Final conc (ng/mL)
Ref	320	40	240	30	80	10	160	20	320	40

Protocol

Sample dilution and pre-incubation

Neutralizing antibodies against TNF-alpha inhibitors can be measured with a screening protocol (one dilution per sample and 40 samples per plate), or with a quantitation protocol (8 dilutions per sample and 5 samples per plate).

Screening protocol:

1. Design a plate layout for plate 1. One well per sample at this step, to be split into duplicates when transferred to plate 2.
2. Add 50 µL Diluent C to the reference wells in plate 1. Two reference wells are recommended (equals quadruple wells when transferred to plate 2), as this gives a more reliable mean result. The presence of NAbs in every sample is calculated in relation to the reference well mean.
3. Add 50 µL Diluent C and 50 µL Reagent BLANK to Blank wells in plate 1 (not used for calculations, can be excluded).
4. Add 50 µL positive control and negative control to the assigned control wells.
5. Add 20 µL patient sample and 30 µL Diluent B to the assigned sample wells.
6. Add 50 µL of the reference sample (containing TNF-alpha inhibitor) to all wells except the Blank wells.
7. Cover the plate with a lid, mix by gently swirling and incubate for 30 minutes at 37°C with 5% CO_2.

Quantitation protocol:

For quantitative measurement of neutralizing antibodies to TNF-alpha inhibitors, a serial dilution of each sample is performed in a polypropylene plate or tubes according to the table below.

Table 2. Suggested dilutions when quantitative measurements are performed.

Dilution	Infliximab /Adalimumab/ Certolizumab pegol /Etanercept /Golimumab		
	Sample	Diluent	Final assay dilution
1	100 µL sample	150µL Diluent B	20x
2	100µL dilution 1	100µL Diluent C	40x
3	100µL dilution 2	100µL Diluent C	80x
4	100µL dilution 3	100µL Diluent C	160x
5	100µL dilution 4	100µL Diluent C	320x
6	100µL dilution 5	100µL Diluent C	640x
7	100µL dilution 6	100µL Diluent C	1280x
8	100µL dilution 7	100µL Diluent C	2560x

1. Design a plate layout for plate 1. One well per sample at this step, to be split into duplicates when transferred to plate 2.
2. Add 50 µL Diluent C to Reference wells and Blank wells in plate 1.
3. Add 50 µL positive control and negative control to the assigned control wells.

4. Add 50 µL patient samples from the dilutions to the assigned sample wells.
5. Add 50 µL of the reference sample (containing TNF-alpha inhibitor) to all wells except the Blank wells.
6. Add 50 µL Reagent BLANK to the Blank wells
7. Cover the plate with a lid, mix by gently swirling and incubate for 30 minutes at 37°C with 5% CO_2.

Incubation with TNF-alpha:

8. Add 100 µL TNF-alpha to all wells.
9. Cover the plate with a lid, mix by gently swirling and incubate for 30 minutes at 37°C with 5% CO_2

Cell dilution

10. Cells and substrate reagents are removed from the freezer. Thaw the vial of cells in a 37°C water bath 5 minutes prior to completion of the incubation. Invert the vial a minimum of 10 times to ensure a uniform cell suspension. Add the entire contents (1.5 mL) of the *iLite*™ TNF-alpha Assay Ready Cell vial to 6 mL Diluent B. Invert the tube gently a few times.

Cell incubation

11. Transfer calibrators, controls and samples in duplicate from plate 1 to plate 2 (white plate), 50 µL per well.
12. Add 50 µL diluted cells to each well.
13. Place the lid on the plate, mix and incubate for 3 hours at 37 °C with 5% CO_2.

Adding substrate solutions

14. Equilibrate plate 2 and the substrate solutions to room temperature.
15. Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. Read in a luminometer after 10 minutes incubation at room temperature.
16. 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. Read in a luminometer after 10 minutes incubation at room temperature.

Calculations

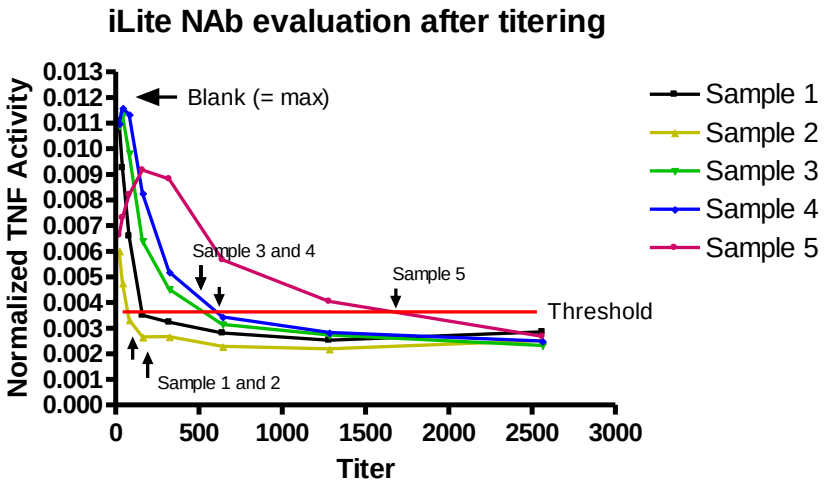
Calculate the normalized TNF-alpha activity for each well by dividing the values of the Firefly Luciferase reading with the values of the Renilla Luciferase reading.

When the **screening protocol** is followed, a ratio between the normalized sample value and the normalized value of the reference sample wells is calculated. It is important that every lab establish its own threshold for presence of NAbs. At Biomonitor the thresholds have been calculated according to the values in Table 3. Ratios below the threshold should be reported as negative for NAbs and ratios above the threshold should be reported as positive for the presence of NAbs.

Table 3. Biomonitor thresholds for the determination of neutralizing antibodies against TNF-alpha inhibitors

Anti-TNF-alpha inhibitor	Threshold at 99.5%CI
Infliximab	1.4
Adalimumab	1.3
Etanercept	1.3
Golimumab	1.3
Certolizumab pegol	1.4

When a **quantitation protocol** is followed, a graph should be constructed by plotting the normalised sample values (Y-axis) against the dilution factors (X-axis), Figure 1. The threshold for presence or absence of NAbs to the TNF-alpha inhibitors should be established by every lab. At Biomonitor the thresholds have been calculated according to the values in Table 3. The titre of the samples is defined as the dilution where the sample dilution curve intersects with the threshold line. If the entire curve for a sample is below the threshold line, the sample is negative for NAbs to the TNF-alpha inhibitor. If the entire dilution curve for a sample is above the threshold line, the sample titre is above the highest sample dilution and the sample can be diluted further.



Sample	Normalized TNF-alpha activity
Ref. sample	0.00268
Threshold	0.00375
Blank	0.0121 (max)
	Titre
Sample 1	150
Sample 2	60
Sample 3	480
Sample 4	580
Sample 5	1500

Figure 1. Quantitation of neutralizing antibodies against TNF-alpha inhibitors. Sample values plotted against sample dilution. Both axis linear. The figure is an example and should not be used for actual sample interpretation.

Quality Control

If the following criteria are met, the assay is considered valid:

Positive control: Positive for NAb

Negative control: Negative for NAb

If the following criteria are met, a sample result may be considered valid:

% CV of duplicates ≤ 20% (normalized values)

% CV of interference ratio ≤ 30%

Interference ratio: The Renilla activity in every sample is expected to be the same as the Renilla activity in the assay calibrators, if the sample does not contain interfering factors and the number of cells is constant throughout the plate. If the mean Renilla activity of a sample is > 30% different from the mean Renilla activity of the assay calibrators, the sample is considered to contain interfering factors (or the cell number is too variable) and the determination is considered invalid.

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 supplier's/manufacturer's 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.

Propriety 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. Biomonitor *iLite™* cell-based products are covered by patents which are the property of Euro Diagnostica AB and any attempt to reproduce the delivered *iLite™* Assay Ready Cells is an infringement of these patents.

Quick Guide – quantification of neutralizing antibodies against TNF-alpha inhibitors

1 Plate 1

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage**
- Dilute samples according to Table 2.
- Add 50 µL Diluent C to Reference wells and Blank wells in plate 1
- Add 50 µL positive control and negative control to the assigned control wells.
- Add 50 µL patient samples from the dilutions to the assigned sample wells.
- Add 50 µL of the reference sample (containing TNF-alpha inhibitor) to all wells except the Blank wells.
- Add 50 µL Reagent BLANK to the Blank wells

2 Incubation 1 30 min

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

3 Incubation 2 30 min

- Add 100 µL TNF-alpha to all wells.
- Incubate for 30 minutes at 37°C with 5% CO_2
- 5 minutes before the end of the incubation, thaw and dilute the cells
- Commence thawing of substrate reagents

4 Plate 2

- 50 µL sample/TNF alpha solutions are transferred to plate 2 (white plate) in duplicate
- 50 µL diluted cells are added to all wells in plate 2
- Cover the wells and mix the contents

5 Incubation 3 3 h

- Incubate at 37 °C with 5% CO_2 for 3 hours.

6 Read plate 2

- Equilibrate plate 2 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. Read in a luminometer after 10 min incubation.
- 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. Read in a luminometer after 10 min incubation.

Troubleshooting and FAQ

Please consult Euro Diagnostica's website www.eurodiagnostica.com

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