Quantification of 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.

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 quantification of TNF-alpha inhibitor activity in different 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 A, RPMI 1640 with 8% heat inactivated NHS	Euro Diagnostica	BM3132, *iLite*™ Diluent A
Diluent B, RPMI 1640	Euro Diagnostica	BM3134, *iLite*™ Diluent B
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 and 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 calibrators and controls

Dilute the TNF-alpha inhibitors to relevant calibrator dilutions with Diluent A. Appropriate calibrator dilutions for 5 TNF-alpha inhibitors are given in Table 1 below, as well as the final assay concentration in the wells. Prepare a positive control with Diluent A according to the table below. Use Diluent A as the negative control.

Table 1. Preparation of calibrators and controls

Calibrator/ control	Infliximab		Adalimumab		Etanercept		Golimumab		Certolizumab pegol	
	Calibrator conc (ng/mL)	Final conc (ng/mL)	Calibrator conc (ng/mL)	Final conc (ng/mL)	Calibrator conc (ng/mL)	Final conc (ng/mL)	Calibrator conc (ng/mL)	Final conc (ng/mL)	Calibrator conc (ng/mL)	Final conc (ng/mL)
A	200	50	200	50	48	12	120	30	160	40
B	160	40	160	40	40	10	80	20	120	30
C	120	30	120	30	32	8	60	15	80	20
D	80	20	80	20	24	6	40	10	40	10
E	40	10	40	10	16	4	20	5	12	3
F	20	5	20	5	8	2	10	2.5	6	1.5
Pos	100	25	100	25	20	5	48	12	68	17

Protocol

Sample dilution and pre-incubation

Add calibrators and controls to plate 1 (polypropylene 96-well plate) according to a pre-decided layout, 100 µL per well. Prepare one well per calibrator/control/sample at this step and split to duplicates when transferring to plate 2.

Screening of samples: If analysing Infliximab, Adalimumab or Certolizumab pegol add 8 µL of sample to plate 1. Add 92 µL of Dilution B to each well containing a sample. Etanercept should be pre-diluted with 20 µL sample and 230 µL of Dilution B before transferring 25 µL to plate 1 and adding 75 µL of Dilution A. Golimumimab is prediluted with 20 µL sample and 230 µL of Dilution B before transferring 40 µL to plate 1 and adding 60 µL of Dilution A. The total number of samples that can be analysed in a screening test is 40 per plate. Final sample dilution is 50x/200x/125x respectively. If a quantitative titration is preferred, proceed to the next section. Dilution B is used in the first dilution step and Dilution A in the consecutive steps to ensure matrix conformity.

Quantitation of samples:
For quantitative measurement of TNF-alpha inhibitor activity, a serial dilution of each sample is performed according to the table below. The total number of samples that can be assayed in a quantitative test is 10 per plate. Final sample dilutions are in Table 2 below.

Table 2. Suggested dilutions when quantitative measurements are performed.

Dilution	Infliximab /Adalimumab/ Certolizumab pegol			Etanercept			Golimumab		
	Sample	Diluent	Final dilution	Sample	Diluent	Final dilution	Sample	Diluent	Final dilution
0	NA	NA	NA	20µL sample	230µL Dil B	Not analysed	20µL sample	230µL Dil B	Not analysed
I	20µL sample	230µL Dil B	50x	75µL Dilution 0	225µL Dil A	200x	100µL Dilution 0	150µL Dil A	125x
II	100 µL Dilution I	150 µL Dil A	125x	100 µL Dilution I	150 µL Dil A	500x	100 µL Dilution I	150 µL Dil A	313x
III	100 µL Dilution II	150 µL Dil A	313x	100 µL Dilution II	150 µL Dil A	1250x	100 µL Dilution II	150 µL Dil A	781x
IV	100 µL Dilution III	150 µL Dil A	781x	100 µL Dilution III	150 µL Dil A	3125x	100 µL Dilution III	150 µL Dil A	1953 x

- Add 100 µL of the sample dilutions to plate 1. Prepare one well per sample at this step and split to duplicates when transferring to plate 2.
- Add 100 µL TNF-alpha to all wells containing sample dilutions, calibrators and controls.
- Place the lid on plate 1 and mix.
- Incubate for 30 minutes at 37 °C with 5% CO_2.

Cell dilution

- 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 content (1.5 mL) of the *iLite*™ TNF-alpha Assay Ready Cell vial to 6 mL Dilution B.
- Invert the tube gently a few times.

Cell incubation

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

Adding substrate solutions

- Equilibrate plate 2 and the substrate solutions to room temperature.

- Prepare the **Firefly luciferase** substrate according to the suppliers 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.

- If appropriate, prepare the **Renilla luciferase** substrate according to the suppliers 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 activity for each well by dividing the values of the Firefly Luciferase reading with the values of the Renilla Luciferase reading. Construct a calibrator curve by plotting the normalized TNF activities (Y-axis) against the log concentrations of the six calibrators (X-axis). A 4 parameter logistic curve fit is recommended (Fig. 1).

Read the values of controls and samples from the calibrator curve as ng/mL. The concentration in undiluted samples is calculated by multiplying the read concentration (ng/mL) with the sample dilution. In the quantitative assay, a mean concentration is calculated from the different dilutions of each sample (from results in the linear range between Cal B and Cal E). Transfer the concentration from ng/mL to µg/mL by dividing by 1000.

Example:
Read value 13ng/mL
Sample dilution 50x
TNF-alpha inhibitor activity in undiluted sample 13 x 50 = 650ng/mL or 0.65 µg/mL

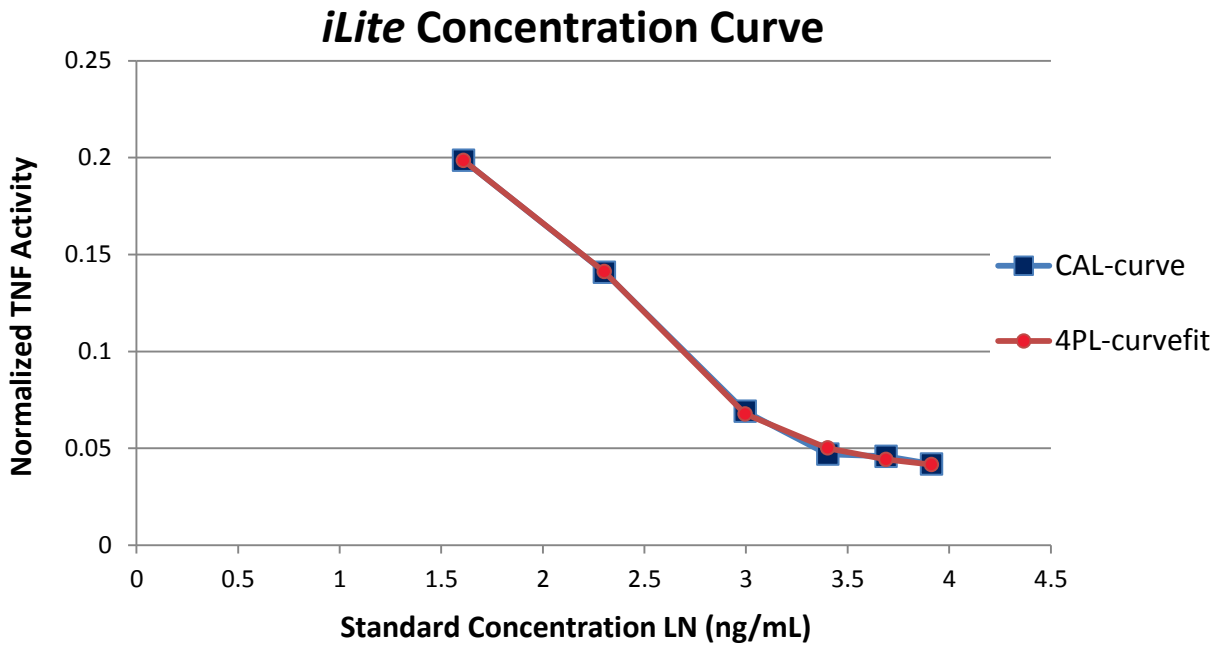

Figure 1. TNF-alpha inhibitor calibrator with 4 parameter logistic curve fit. The curve is an example and should not be used for actual sample interpretation.

Reference range

If the value of an undiluted sample is < 0.65 µg /mL => The sample is negative for TNF-alpha inhibitor activity.
If the value of an undiluted sample is ≥ 0.65 µg /mL => The sample is positive for TNF-alpha inhibitor activity.

Quality Control

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

Positive control: nominal value ± 30%

Negative control: < 0.65 µg/mL

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 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 materials and instruments referenced according to the supplier's/manufacturer's instructions or product specifications accompanying the individual materials 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 TNF-alpha inhibitor biological activity

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.
- 100 µL calibrators, controls and samples are added to plate 1 (polypropylene). One well per sample at this step, to be split to duplicates in step 3 (plate 2)
- 100 µL of TNF-alpha is added to all wells in use (calibrators, controls and samples)
- Cover the wells and mix the contents

2 Incubation 1 30 min

- Incubate at 37 °C with 5% CO_2 for 30 min.
- 5 minutes before the end of the incubation, thaw and dilute the cells; add entire cell contents to 6 mL Diluent B
- Commence thawing of substrate reagents

3 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

4 Incubation 2 3 h

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

5 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, FAQ and Contacts

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