Measuring neutralizing antibodies against TNF-alpha inhibitors in serum samples 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 can be treated with TNF-alpha inhibitors, such as **infliximab, adalimumab, etanercept,** analogs or biosimilars**.** TNF-alpha inhibitor therapy may lead to development of neutralizing antibodies (NAbs), which presents as a non-response to the therapeutic TNF-alpha antagonist activity of the inhibitors. (2)

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 NFκB responsive promoter. Binding of TNF-alpha to its receptor results in activation of the NFκB 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 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 functional assay for measurements of TNF-alpha inhibitor activity and determination of neutralizing antibodies against a TNF-alpha inhibitor in human serum. (3,4)

Specimen collection

iLite® TNF-alpha Assay Ready Cells can be used for detecting presence of neutralizing antibodies to TNF-alpha inhibitors in human serum samples. Collect serum according to standard protocol for antibody measurements. Samples with Infliximab ≥0.65 µg/mL should not be used, as the presence of drug will interfere with the measurement.

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Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® TNF-alpha Assay Ready Cells	Svar Life Science	BM3044
TNF-alpha (16 ng/ml)	Svar Life Science	BM3133
iLite® Diluent B (RPMI 1640)	Svar Life Science	BM3134
iLite® Diluent C (RPMI 1640 with 40% heat inactivated NHS)	Svar Life Science	BM3139
iLite® Reagent BLANK, (RPMI 1640 with 30% heat inactivated FCS)	Svar Life Science	BM3135
iLite® Positive control Infliximab NAb	Svar Life Science	BM3136
iLite® Positive control Adalimumab NAb	Svar Life Science	BM3159
iLite® Positive control Etanercept NAb	Svar Life Science	BM3177
Firefly/Renilla luciferase substrate	Promega	E2920, Dual-Glo Luciferase Assay System
TNF alpha inhibitor (Infliximab, Adalimumab, Etanercept or analogues)	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 Svar Life Science for a 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

Protocol

Preparation of reagents

Equilibrate reagents and samples to room temperature. **Do not thaw cells and substrate reagents at this stage!**

Dilute the TNF-alpha inhibitor to relevant concentrations with RPMI 1640 containing 30 % fetal calf serum. Suggested concentration for TNF-alpha inhibitors are given in Table 1 below, as well as the final assay concentration in the wells.

50 µl TNF-alpha inhibitor will be added to all wells except the blank. For positive control please see Materials table above. For negative control, Diluent C can be used.

Table 1. Preparation of TNF-alpha inhibitor

Infliximab or biosimilar		Adalimumab or biosimilar		Etanercept or biosimilar	
Solution conc (ng/mL)	Final assay conc (ng/mL)	Solution conc (ng/mL)	Final assay conc (ng/mL)	Solution conc (ng/mL)	Final assay conc (ng/mL)
320	40	240	30	80	10

Part 1 – Sample preparation and pre-incubation with plate 1

Neutralizing antibodies against TNF-alpha inhibitors can be measured with a **qualitative protocol** (one dilution per sample), or with a **semi-quantitative** protocol (8 dilutions per sample).

Qualitative protocol:

For qualitative protocol, dilute serum samples to 40% serum concentration, for example 20 µL serum sample and 30 µL Diluent B. This can be done directly in the polypropylene plate 1 used for the incubation steps performed before cells are added. An example of a plate layout is given in figure 1.

Design a plate layout for plate 1. One well per sample at this step, which will be split into duplicates when transferred to plate 2. Example of a plate layout (plate 1):

	1	2	3	4	5	6	7	8	9	10	11	12
A	REF	S1	S9	S17	S25	S33						
B	REF	S2	S10	S18	S26	S34						
C	REF	S3	S11	S19	S27	S35						
D	REF	S4	S12	S20	S28	S36						
E	BLANK	S5	S13	S21	S29	S37						
F	BLANK	S6	S14	S22	S30	S38						
G	PC	S7	S15	S23	S31	S39						
H	NC	S8	S16	S24	S32	S40						

Figure 1. Plate layout, qualitative protocol, plate 1

1. Add 50 µL Diluent C to the reference wells (REF) and Blank wells in plate 1. A minimum of two reference wells is recommended (equals to 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 average of the reference wells.
2. Add 50 µL Reagent BLANK to assigned Blank wells in plate 1 (BLANK).
3. Add 50 µL positive and negative control (DIL C) to the assigned control wells (PC/NC).
4. Add 20 µL serum sample and 30 µL Diluent B to the assigned sample wells.
5. Add 50 µL TNF-alpha inhibitor to all wells except the BLANK wells.
6. Cover the plate with a lid, mix by agitation approximately at 900 rpm for 10 seconds and then incubate 30 minutes at 37°C with 5% CO_2.

Semi-Quantitative protocol:

A serial dilution of each sample is performed in a polypropylene plate or tubes according to table 2 below. An example of a plate layout is given in figure 2.

Table 2. Suggested dilutions when semi-quantitative measurements are performed.

Dilution	Sample	Diluent	Final assay dilution
A	100 µL sample	150µL **Diluent B***	20x
B	100µL dilution A	100µL Diluent C	40x
C	100µL dilution B	100µL Diluent C	80x
D	100µL dilution C	100µL Diluent C	160x
E	100µL dilution D	100µL Diluent C	320x
F	100µL dilution E	100µL Diluent C	640x
G	100µL dilution F	100µL Diluent C	1280x
H	100µL dilution G	100µL Diluent C	2560x

*To ensure consistent sample matrix (serum concentration) diluents with different amounts serum are used in 1st dilution step and in the following dilution steps.

Design a plate layout for plate 1. One well per sample at this step, which will be split into duplicates when transferred to plate 2. Example of a plate layout (plate 1):

	1	2	3	4	5	6	7	8	9	10	11	12
A	REF	S1-A	S2-A	S3-A	S4-A	S5-A						
B	REF	S1-B	S2-B	S3-B	S4-B	S5-B						
C	REF	S1-C	S2-C	S3-C	S4-C	S5-C						
D	REF	S1-D	S2-D	S3-D	S4-D	S5-D						
E	BLANK	S1-E	S2-E	S3-E	S4-E	S5-E						
F	BLANK	S1-F	S2-F	S3-F	S4-F	S5-F						
G	PC	S1-G	S2-G	S3-G	S4-G	S5-G						
H	NC	S1-H	S2-H	S3-H	S4-H	S5-H						

Figure 2. Plate layout, semi-quantitative protocol, plate 1

1. Add 50 µL Diluent C to reference wells (REF) and Blank wells in plate 1. A minimum of two reference wells are recommended (equals to 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 average of the reference wells
2. Add 50 µL Reagent BLANK to the assigned Blank wells (BLANK)
3. Add 50 µL positive and negative control to the assigned control wells (PC/NC).
4. Add 50 µL patient samples from the dilutions to the assigned sample wells.
5. Add 50 µL TNF-alpha inhibitor to all wells except the BLANK wells.
6. Cover the plate with a lid, mix by agitation approximately at 900 rpm for 10 seconds and then incubate for 30 minutes at 37°C with 5% CO_2.

Incubation with TNF-alpha:
Add **100 µL TNF-alpha solution**, 16 ng/ml, to all wells (final assay concentration will be 4 ng/mL TNF-alpha). Cover the plate with a lid, mix by agitation approximately at 900 rpm for 10 seconds and then incubate 30 minutes at 37°C with 5% CO_2.

Part 2 – Cell preparation and incubation with plate 2

Cell thawing and dilution

1. Remove the *iLite*® TNF-alpha Assay Ready Cells and substrate reagents from the freezer.
2. Five minutes prior to completion of the incubation with TNF-alpha, thaw the cell vial in a 37°C water bath with gentle agitation and pre-warm the diluent B to 37°C.
3. When thawed, **immediately** transfer the entire content (1.5 mL) of the cell vial to 6 mL diluent B. Invert the vial with the diluted cell suspension approximately ten times to ensure homogeneous distribution of cells.

Cell incubation

4. Transfer content of plate 1 (REF, BLANK, control or sample, TNF-alpha inhibitor and TNF-alpha) to plate 2 (white plate), in duplicates, 50 µL per well (see figure 3 and 4 below).
5. Add 50 µL diluted cell suspension to each well.
6. Cover the plate with a lid, mix by agitation approximately at 900 rpm for 10 seconds and then incubate for 3 hours at 37 °C with 5% CO_2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	REF	REF	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	REF	REF	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	REF	REF	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	REF	REF	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	BLANK	BLANK	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	BLANK	BLANK	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	PC	PC	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	NC	NC	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

Figure 3. Plate layout, qualitative protocol, plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	REF	REF	S1-A	S1-A	S2-A	S2-A	S3-A	S3-A	S4-A	S4-A	S5-A	S5-A
B	REF	REF	S1-B	S1-B	S2-B	S2-B	S3-B	S3-B	S4-B	S4-B	S5-B	S5-B
C	REF	REF	S1-C	S1-C	S2-C	S2-C	S3-C	S3-C	S4-C	S4-C	S5-C	S5-C
D	REF	REF	S1-D	S1-D	S2-D	S2-D	S3-D	S3-D	S4-D	S4-D	S5-D	S5-D
E	BLANK	BLANK	S1-E	S1-E	S2-E	S2-E	S3-E	S3-E	S4-E	S4-E	S5-E	S5-E
F	BLANK	BLANK	S1-F	S1-F	S2-F	S2-F	S3-F	S3-F	S4-F	S4-F	S5-F	S5-F
G	PC	PC	S1-G	S1-G	S2-G	S2-G	S3-G	S3-G	S4-G	S4-G	S5-G	S5-G
H	NC	NC	S1-H	S1-H	S2-H	S2-H	S3-H	S3-H	S4-H	S4-H	S5-H	S5-H

Figure 4. Plate layout, semi-quantitative protocol, plate 2

Adding substrate solutions

7. Prepare the Firefly luciferase substrate according to the supplier's instructions.
8. Equilibrate the plate and the substrate solutions to room temperature.
9. Add 80 µL Firefly luciferase substrate per well. Mix by agitation at approximately 900 rpm for 10 seconds and then incubate 10 minutes at room temperature. Protect the plate from light during incubation. Read the plate in the luminometer.
10. If appropriate, prepare the Renilla luciferase substrate according to the supplier's instructions and add 80 µL per well. Mix by agitation at approximately 900 rpm for 10 seconds and then incubate 10 minutes at room temperature. Protect the plate from light during incubation. Read the plate in the luminometer.

Calculations

Calculate the mean Firefly Luciferase luminescence and Renilla Luciferase luminescence values for all references, controls and samples. Then calculate the normalized TNF-alpha activity for each well by dividing the results of the Firefly Luciferase luminescence with the results of the Renilla Luciferase luminescence.

$$\textbf{Normalized TNF alpha activity in well} = \frac{\text{Firefly Luciferase luminescence}}{\text{Renilla Luciferase luminescence}}$$

Qualitative protocol

When the **qualitative protocol** is followed, calculate a ratio of the normalized TNF-alpha result between samples and the reference (REF).

$$\textbf{Result Ratio} = \frac{\text{Normalized TNF alpha activity of sample}}{\text{Normalized TNF alpha activity of REF}}$$

Semi-quantitative protocol

When the **semi-quantitative protocol** is followed, a graph should be constructed by plotting normalized sample values (Y-axis) against dilution factors (X-axis), Figure 1.

The threshold factor for calculation of presence or absence of NAbs to TNF-alpha inhibitors should be established by each laboratory. The threshold is then calculated at each assay run. (see section "Threshold and threshold factor")

The titer of the sample 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 considered negative for NAbs to the TNF-alpha inhibitor. If the entire dilution curve for a sample is above the threshold line, the sample NAb titer is above the highest sample dilution and the sample should be diluted further.

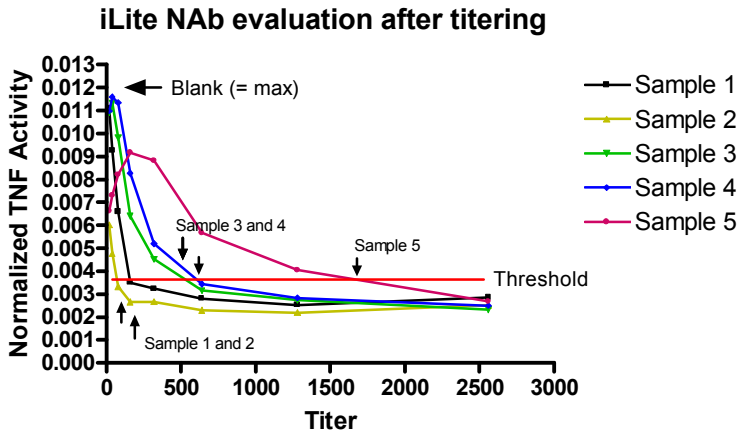

Figure 1. Semi-quantitative measurement of NAbs against TNF-alpha inhibitors. Sample values plotted against sample dilution. Both axis linear. The figure is an example and should not be used for actual sample interpretation.

Threshold and threshold factor

It is recommended that each laboratory establishes a threshold factor for presence of NAbs with samples commonly used since it may vary between different sample panels.
Serum samples from 50 TNF-alpha inhibitor treatment-naïve samples (Samples from 25 individuals with rheumatoid arthritis and 25 individuals with Crohn's disease) were tested for NAbs with the TNF-alpha inhibitor infliximab and the threshold factor at 99.5%CI was calculated (Table 3).

Anti-TNF-alpha inhibitor	Threshold factor at 99.5%CI
Infliximab	1.4

Table 3. Svar Life Science threshold factor for the determination of neutralizing antibodies against TNF-alpha inhibitor Infliximab

The threshold is then calculated at each assay run according to:

Threshold = Threshold Factor X Normalized TNF alpha activity of REF

Sample result ratios below the threshold are considered negative for NAbs and ratios above the threshold are considered positive for presence of NAbs.

Quality Control

The assay run is considered valid when following criteria are met:

- Positive control: Positive for NAb
- Negative control: Negative for NAb

The individual sample result is considered valid when following criteria are met:

- % CV of duplicates ≤ 20% (normalized values)
- % CV of interference ratio ≤ 30%

Interference ratio:
The Renilla activity in every sample is expected to be at the same level as the Renilla Luciferase activity in the assay reference and blank (REF and BLANK). If a sample contains interfering factors or deviating number of cells per well throughout the plate, this will be reflected by varying Renilla Luciferase activity.

Therefore, calculate the interference ratio of each sample by a ratio of the Renilla Luciferase activity result between samples and the reference (REF) and BLANK.

$$\textbf{Interference Ratio} = \frac{\text{Renilla Luciferase activity of sample}}{\text{Renilla Luciferase activity of REF and BLANK}}$$

If the Renilla activity of a sample deviates ≥ 30% from the mean Renilla activity of the assay reference the sample may contain interfering factors or the cell number is inconsistent, and the measurement is considered invalid. Presence of endogenous TNF-alpha inhibitor drug in collected serum samples may interfere with the measurement of NAbs.

LIMITATIONS

This Application Note and suggested protocol is for research and professional use only and not intended for use in diagnostic procedures or in human therapeutic applications. The protocol has been tested with serum samples only. Other matrices have not been tested.
The effect of interfering substances as hemoglobin, bilirubin, lipids and rheuma factor (RF) has not been tested. Endogenous serum concentration of Infliximab ≥0.65 µg/mL interfere with the application.

QUICK GUIDE -

Measurement of neutralizing antibodies in serum samples against TNF-alpha inhibitors 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.**
- Dilute samples according to qualitative or semi-quantitative protocol.
- Add 50 µL Diluent C to Reference wells and Blank wells in plate 1.
- Add 50 µL Reagent BLANK to the assigned Blank wells.
- Add 50 µL positive control and negative control to the assigned control wells.
- Add 50 µL diluted serum samples to the assigned sample wells.
- Add 50 µL TNF-alpha inhibitor to all wells except the Blank wells.
- Cover the wells and mix.

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 prior the end of the incubation, thaw and dilute the cells in Diluent B.
- Begin thawing substrate reagents.

4 Plate 2

- Transfer 50 µL sample/TNF-alpha inhibitor/TNF alpha solutions from plate 1 to plate 2 (white plate) in duplicates.
- Add 50 µL diluted cells are added to each well, plate 2.
- Cover the wells and mix.

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. 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. Add 80 µL per well. Mix. Protect the plate from light. Read in a luminometer after 10 min incubation.

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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

Troubleshooting and FAQ

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

References

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