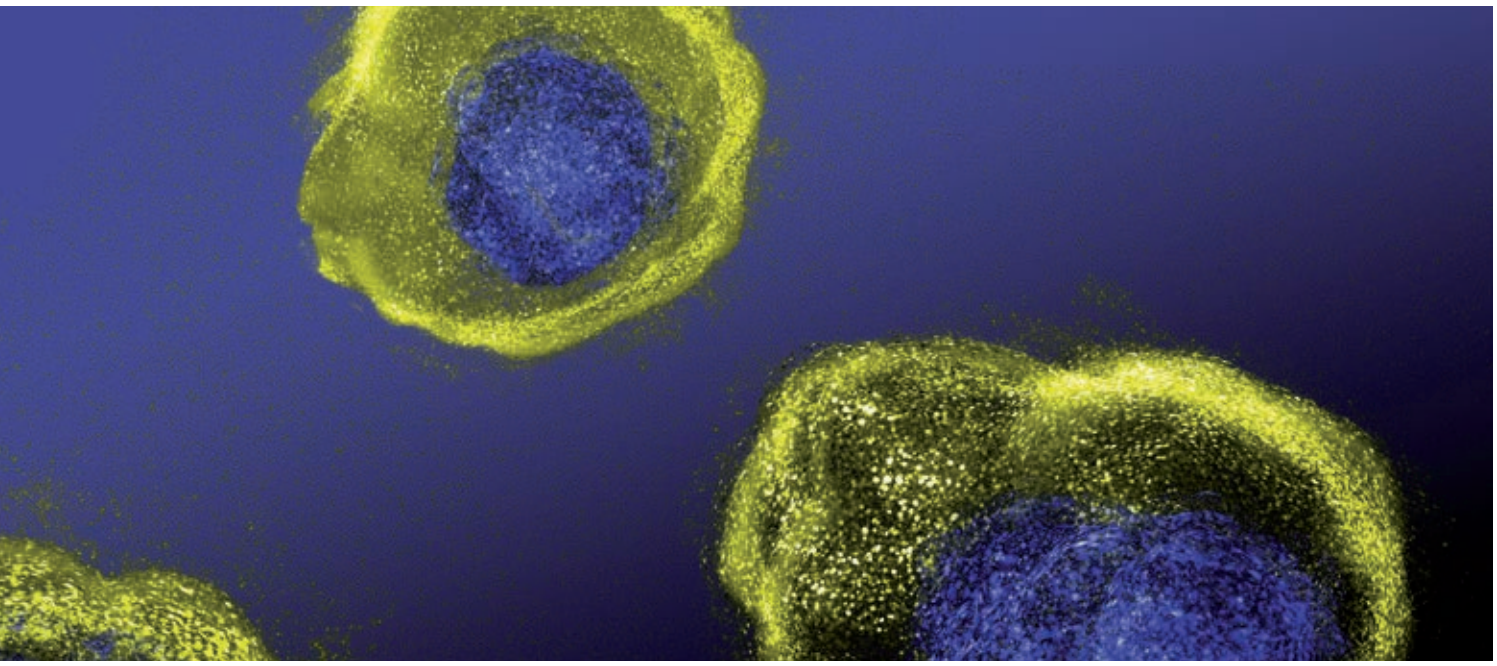


iLITE® TNF-α PERFORMANCE DATA

A QUALIFICATION OF THE MOST COMMON BIOANALYTICAL ASPECTS OF A CELL-BASED REPORTER GENE ASSAY

INTRODUCTION TO *iLITE* REPORTER GENE TECHNOLOGY

Cell-based *iLite*® assays are cleverly designed, sensitive, and specific reporter-gene assays with firefly luciferase readout. Most assays also contain a secondary reporter-gene that can be used for normalization purposes.

They can be developed for virtually any pharmaceutical target and allow an easy, rapid, and accurate test format for a wide range of applications – including measurement and quantification of drug activity, immunogenicity and ADCC activity.

Here, we present performance data for the TNF-α cell line. The generation of such data is an important step in the quality assessment to ensure that the assays are reliable, accurate, and reproducible and that errors resulting from biological variation and methodology are kept to a minimum.

iLITE® CELL-BASED SOLUTIONS - TNF-α ASSAY READY CELLS

Designed for use in anti-TNF-α drug activity and neutralizing antibody assays, the *iLite* TNF-α Assay Ready Cells are genetically engineered reporter gene cells that respond specifically to TNF-α through a luminescent readout in a highly sensitive and reproducible manner. A unique second reporter gene readout allows for normalization of results, effectively adjusting for serum matrix effects.

Measuring the functional activity of TNF-α, the assay can be used for both potency and immunogenicity assessments of TNF-α inhibitors in the same assay. As such, the *iLite* TNF-α Assay Ready Cells also provide a means of directly comparing a biosimilar and innovator product in the same assay.

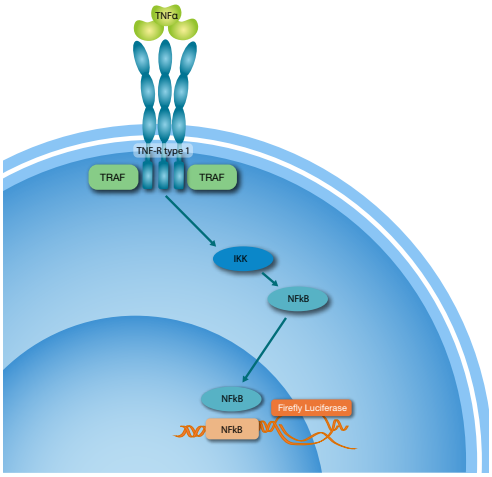


FIG. 1 - SCHEMATIC ILLUSTRATION OF TNF-α

Schematic overview of the TNF-α signal transduction pathway, engineered to give a highly specific readout.

VALIDATION OF MANUFACTURING PROCESS & DETERMINATION OF THE OVERALL CELL LINE VARIATION

Our procedures for establishing a new cell line include several pre-determined steps:

- Risk assessment to identify any potential hazards to the operating personnel, the environment or regarding the product as a whole
- Development phase with process optimization
- Manufacturing of at least 3 validation batches
- Assessment of performance criteria such as EC50, Hill slope, and fold induction to determine the overall cell line variation

Assay variable	Mean	CV%
EC50	0.9	11
IFX FL Reduction	26	8.2
IFX Pos. Control	5	21

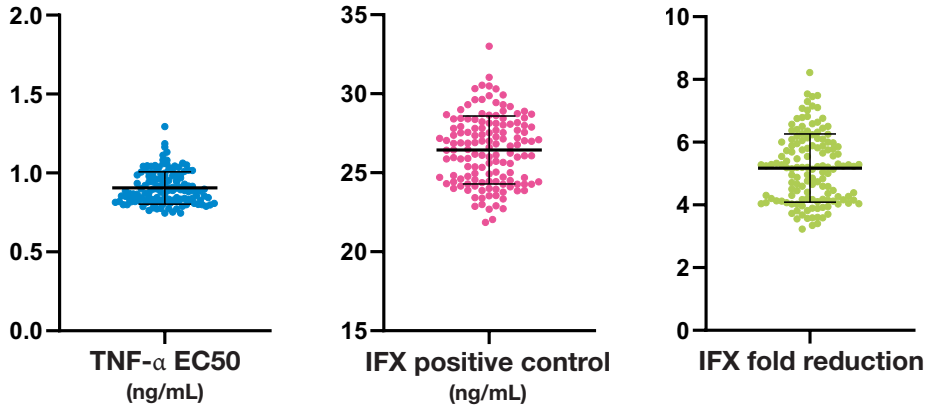


FIG. 2 - OVERALL CELL LINE VARIATION

Data were collected from tests of a TNF-α functional bioassay with a dose-response curve of 0-100 ng/ml TNF-α and tests with a TNF-α inhibition bioassay with Infliximab (IFX), using 4 ng/ml TNF-α and 0-50 ng/ml Infliximab. The data represent results from 69 different cell batches performed by 4 operators at 43 different days. Infliximab fold reduction was calculated as FL 5ng/ml / FL 50 ng/ml.

REAL TIME STABILITY STUDY

Another important parameter is the stability of the cell line over time. We perform real time stability studies where the **defined QC parameters are monitored over time**. Our goal is to exceed a shelf life of 36 months on all our Assay Ready Cell lines, when stored at -80°C.

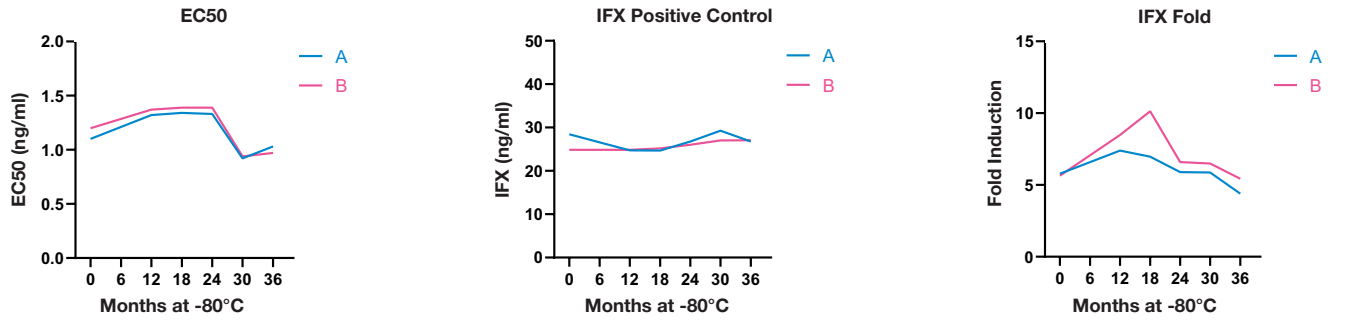


FIG. 3 - REAL TIME STABILITY AT -80°C.

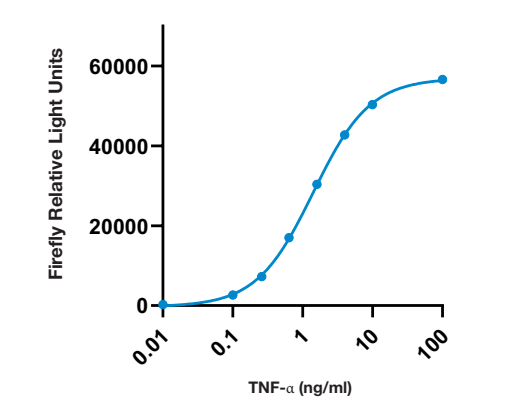
The stability of the TNF-α assay ready cell line over 36 months was tested by performing the functional TNF-α bioassay and the inhibitory TNF-α bioassay regulary.

DYNAMIC RANGE

ASSAY APPLICATION EXAMPLE

When validating the cell lines, different potential applications are assessed using a standardized stimuli or commercial drug compound. The cell lines are evaluated based on:

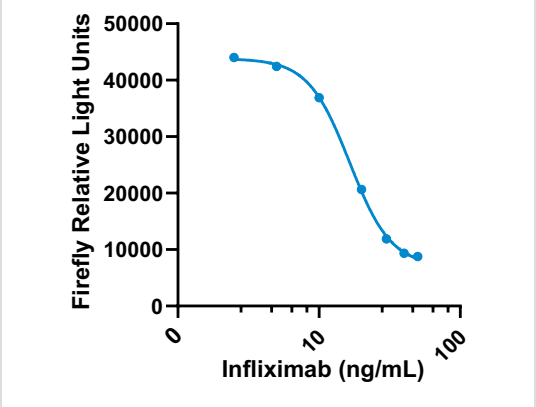
- **Functional Dose-Response Range**
  The dynamic range of the assay is determined by performing a dose-response curve from which we establish the range of the assay, i.e., where we see a signal from the inducible Firefly luciferase reporter gene, measured in this case in Relative Light Units (RLU).
- **Inhibition Dose-Response Range**
  In the presence of inhibitory activity against the target, the amount of free target is reduced, resulting in a decreased stimulation of Firefly luciferase expression. Accordingly, the Firefly luciferase signal is inversely proportional to the amount of inhibitory activity in a sample.
- **Anti-Inhibitor (Neutralizing Antibodies, NAb) Dose-response Range**
  The Firefly luciferase signal is inversely proportional to the amount of inhibitory activity in a sample. In the absence of target inhibitor activity and suspected NAb presence in a test sample, a known amount of drug is added to quench the Firefly luciferase signal, resulting in a partially restored signal which can be used as a measure of the presence of NAbs in the sample.



TNF-α	In-assay (ng/ml)
A	100
B	10
C	4.0
D	1.6
E	0.64
F	0.26
G	0.10

FIG. 4a - DYNAMIC RANGE

Example of a functional TNF-α bioassay application, where 0-100 ng/ml TNF-α was used to achieve a dose-response curve.



IFX	In-assay (ng/ml)
A	50
B	40
C	30
D	20
E	10
F	5
G	2.5

FIG. 4b - DYNAMIC RANGE

Example of a TNF-α inhibition bioassay application using Infliximab. 4 ng/ml of TNF-α was used with 0-50 ng/ml of anti-TNF-α.

2. BIOASSAY VARIATION AND PERFORMANCE

In addition to characterizing the cell line, validating the manufacturing process and creating Application Notes, we also perform an overall Bioassay Characterization – where accuracy and variance, between days and lots are determined.

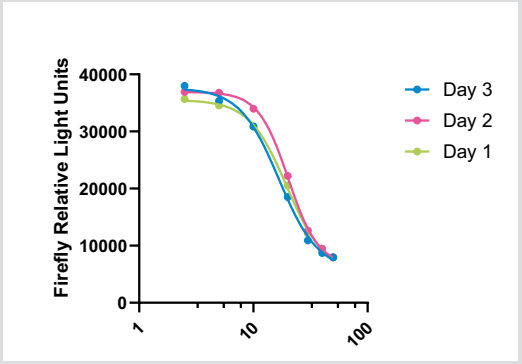
To assess the impact of any variance on the actual bioassays, key performance parameters such as:

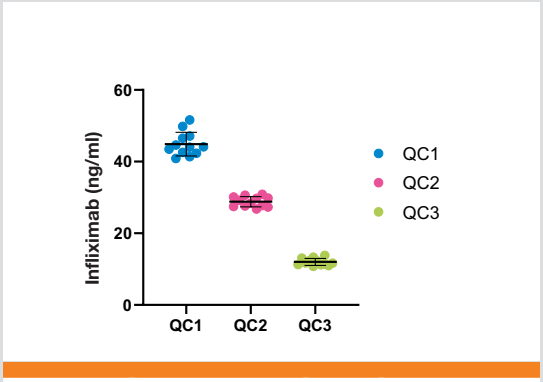
- EC50
- Fold induction
- Hill slope

are determined using technical samples. All this is performed to establish the necessary information regarding how they perform in their designated assays/applications.

ACCURACY & VARIATIONS – DAY-TO-DAY

The following assays are performed to determine how the results of the assay varies over time. This is important to ensure that the results are reproducible and that there is no drift in the results over time.





Assay variable	Mean	CV%
IC50	19	9.7
FL reduction	4.5	2.9
Hill Slope	-2.8	15

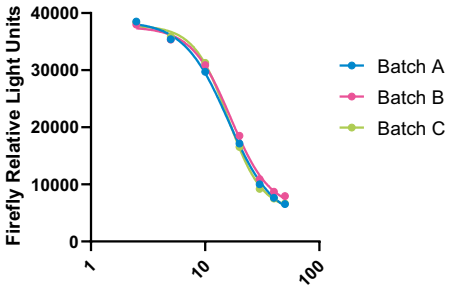
Sample ID	IFX (ng/mL)	CV%	Accuracy (%)
1	40	6.7	112
2	25	5.0	115
3	10	8.8	120

FIG. 5 - DAY-TO-DAY

The iLite Anti-TNF-α bioassay was performed with a dose-response curve and three technical samples with known amount of infliximab. Data shown is generated from assay performed at three separate days with the same batch of TNF-α cells.

ACCURACY & VARIATIONS – LOT-TO-LOT

Another important accuracy measurement is the variance between lots, to ensure a consistent readout of the assays over a longer period of time and for multiple lots.



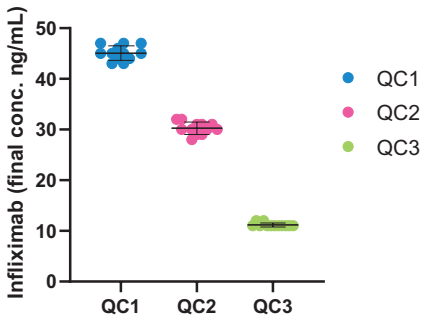


FIG. 6 - LOT-TO-LOT

The iLite anti-TNF-α bioassay was performed with a dose-response curve and three technical samples with known amount of infliximab. Data shown is generated from using three different batches of TNF-α cells at the same assay occasion.

Assay variable	Mean	CV%
IC50	16	2.9
FL reduction	5.1	11
Hill Slope	-2.6	13

Sample ID	IFX (ng/mL)	CV%	Accuracy (%)
1	40	1.1	113
2	25	4.0	121
3	10	2.3	111

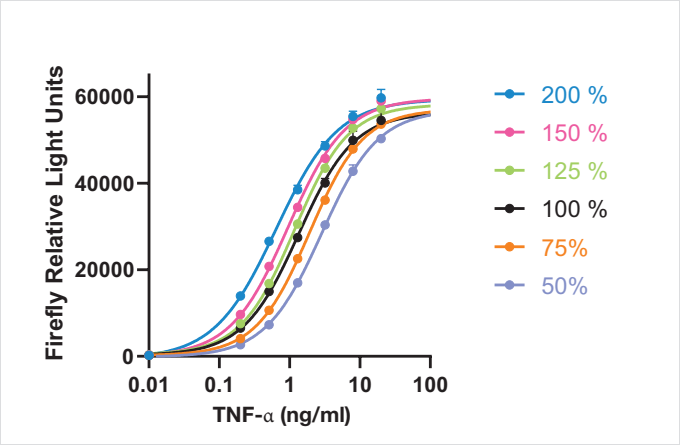
As shown in figure 6, the precision and accuracy of the technical samples are well within acceptable limits, with CVs generally below 10%, even though the absolute Firefly (FL) signal may vary. These results show that there are only small differences between assays performed on different days or using different batches, which is most probably due to the closely controlled production process and the assay ready format of the cells.

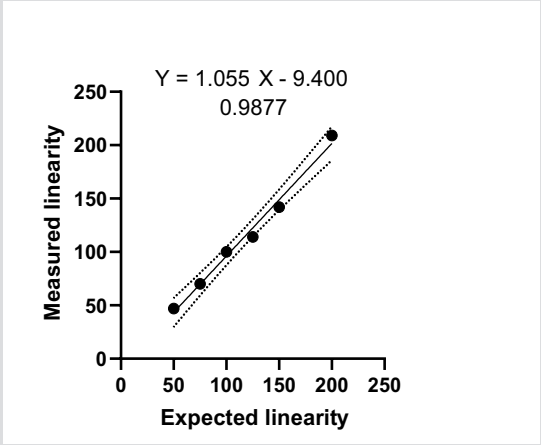
LINEARITY (PARALLELISM) FUNCTIONAL / LINEARITY (PARALLELISM) INHIBITORY

To show linearity between ligand/inhibitor concentrations and induced firefly expression, we perform a linearity assay using different starting concentrations of the ligand/inhibitor.

The results are plotted against a 100% reference curve, which can be used to calculate the linearity between the expected EC50/IC50 and the measured EC50/IC50.

An important aspect of any Linearity/Potency assay is to establish the assay's ability to provide measured values that are proportional to the concentration of the sample.

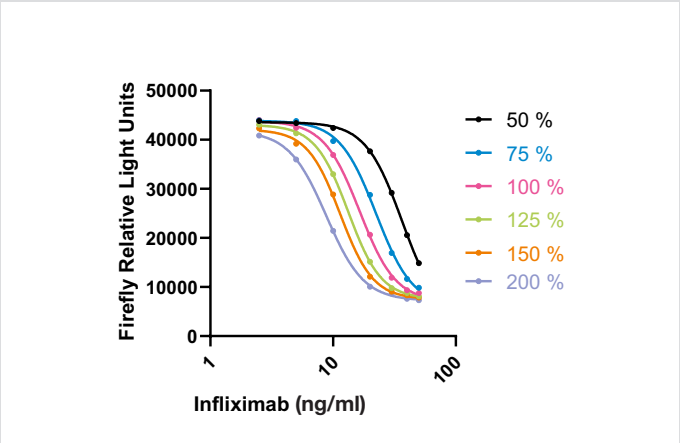


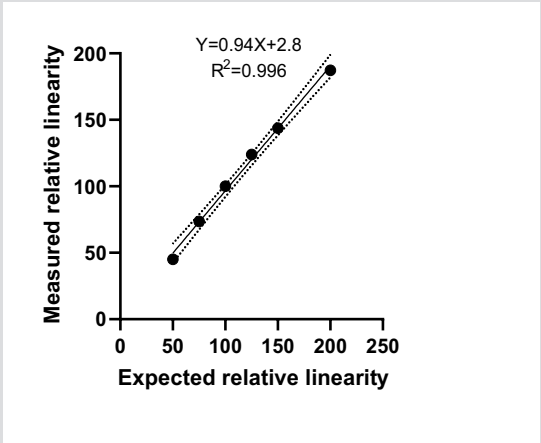


Expected linearity	50%	75%	100%	125%	150%	200%
EC50	2.86	1.93	1.35	1.18	0.951	0.645
Accuracy	94%	93%		91%	95%	105%
Hill Slope	1.08	1.13	1.10	1.13	1.06	0.987
FL induction	19	13	8	8	6	4

FIG. 7 - PARALLELISM / LINEARITY

The iLite TNF-α functional bioassay was performed with the one reference dose-response curve, called 100% and with concentrations at 50, 75, 125, 150 and 200% of the reference.





Expected linearity	50%	75%	100%	125%	150%	200%
IC50	37	22	16	13	11	9
Accuracy	90%	98%		99%	96%	94%
Hill Slope	-2.909	-2.908	-2.955	-3.217	-3.234	-2.837
FL reduction	2.9	4.4	4.8	5.1	5.0	2.9

FIG. 8 - PARALLELISM / LINEARITY

The iLite anti-TNF-α bioassay was performed with one reference dose-response curve, called 100% and with concentrations at 50, 75, 125, 150 and 200% of the reference.

IMMUNOGENICITY CHARACTERIZATION

Assessment of undesirable immunogenicity of therapeutic proteins and peptides is a key element in biological drug development. These assays are more complex since they are performed using clinical samples with complicated matrixes and therefore need to reflect the mechanism of actions of the drug.

With our cell-based functional bioassays, screening for NAbs is a key application area. Here, it is important to determine how the composition of the samples, i.e., the serum tolerance, affects the assay outcome.

Two major parameters influence the outcome and robustness of an assay:

NAb assay sensitivity

In order to determine the levels of neutralizing antibodies, a fixed concentration of the target is mixed with a clinical sample containing antibodies against the target. Upon the addition of increasing amounts of anti-target antibodies, the signal is gradually quenched and an inverted dose-response curve can be plotted.

Serum tolerance

To establish if the serum matrix with the clinical samples has any effect on the outcome of the assay, experiments were performed using a range of serum concentrations. This allows us to determine the effect of serum on the assays and establish the maximum serum concentration (tolerance) for the assay.

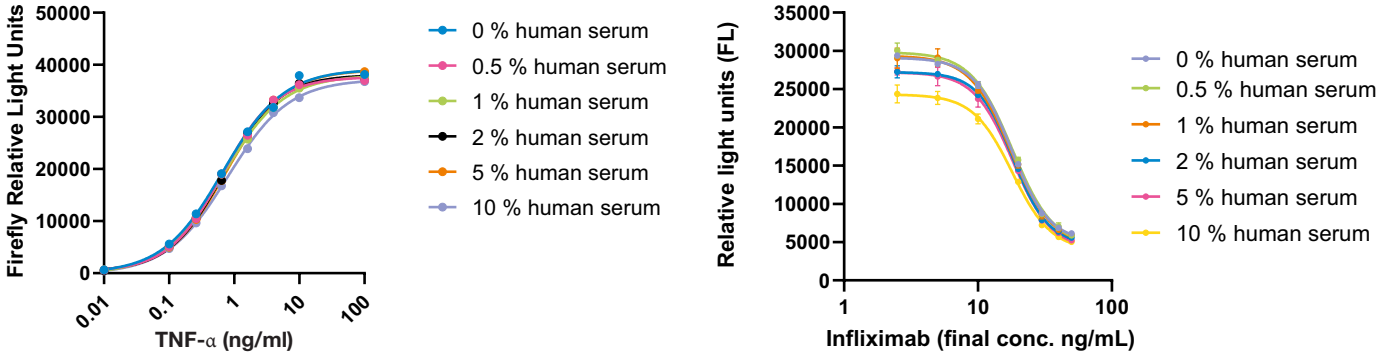


FIG. 9 - SERUM EFFECT / TOLERANCE

The iLite TNF-α functional bioassay was performed with a dose-response curve including human serum, up to 10%.

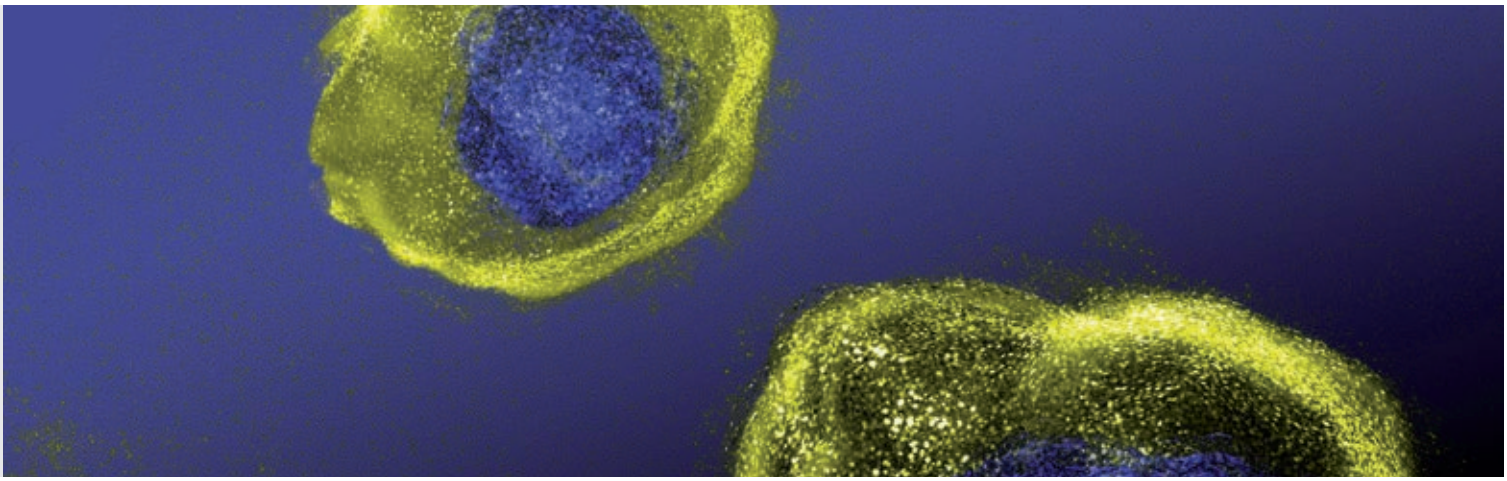


PLATE HOMOGENEITY

For optimal results, it is important to use a plate design for the readout that provides the optimum conditions for consistency and minimizes edge effects. Readout of this type can be prone to common errors such as edge effects or inherent inconsistency of the actual cells. With the *iLite* Assay Ready Cells, we see very low variance across the plate, allowing the entire plate layout to be used in the plate design.

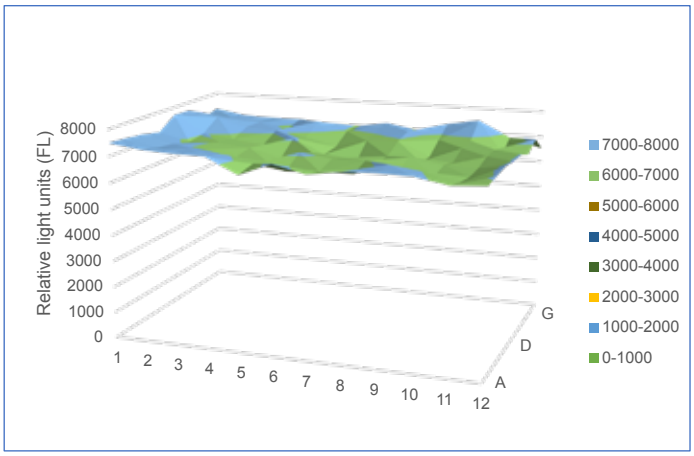


FIG. 10 - HOMOGENEITY

One single sample, 50 ng/ml infliximab + 4 ng/ml of TNF-α, representing the lower part of the linear range of the anti-TNF-α functional bioassay, was tested in all 96 wells of the plate

Svar Life Science

Svar is a Swedish life science company that invents, develops and applies the best assay technology for drug development and clinical diagnostics with the goal of delivering new solutions tailored to customer requirements.

We establish practical platforms for clinical routine testing, work to secure relevant competences by sharing best practices and knowledge and our partnerships enable us to deliver flexible solutions depending on specific needs.

Svar Life Science AB

Mail address:
P.O. Box 50117
SE - 202 11 Malmö
Sweden

T +46 40 53 76 00
F +46 40 43 22 88
E info@svarlifescience.com
W www.svarlifescience.com

Doc No: S-051-GB03, Sep 2022