

***i*LITE® RANKL PERFORMANCE DATA**

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

INTRODUCTION TO *i*LITE REPORTER GENE TECHNOLOGY

Cell-based *i*Lite® 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 RANKL 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.

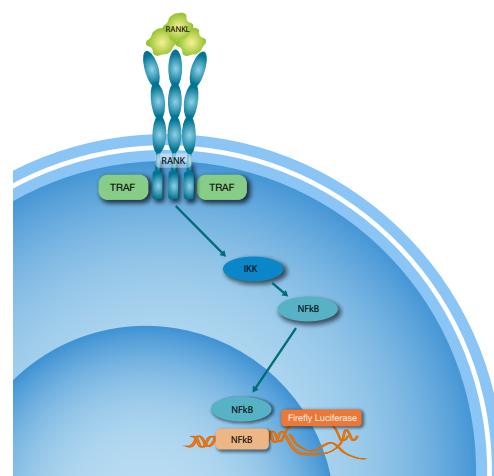
***i*LITE® CELL-BASED SOLUTIONS - RANKL ASSAY READY CELLS**

RANKL is a membrane protein belonging to the tumor necrosis factor (TNF) superfamily. It is expressed in a wide variety of tissues but predominately in bone tissue, lymphoid organs and the vascular system. RANKL was originally identified as a factor involved in the immune system, where it is expressed by T helper cells. In addition, it is believed to be involved in the maturation of dendritic cells.

The *i*Lite® RANKL assay is ideal for potency measurements of anti-RANKL candidate drugs and studies of RANKL or RANKL agonists. As an added benefit, the RANKL *i*Lite assay contains a luciferase normalization gene which can be used to compensate for differences in cell number and viability as well as serum matrix effects.

FIG. 1 - SCHEMATIC ILLUSTRATION OF *i*LITE RANKL

Schematic overview of the RANKL signal transduction pathway, engineered to give highly specific results.



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

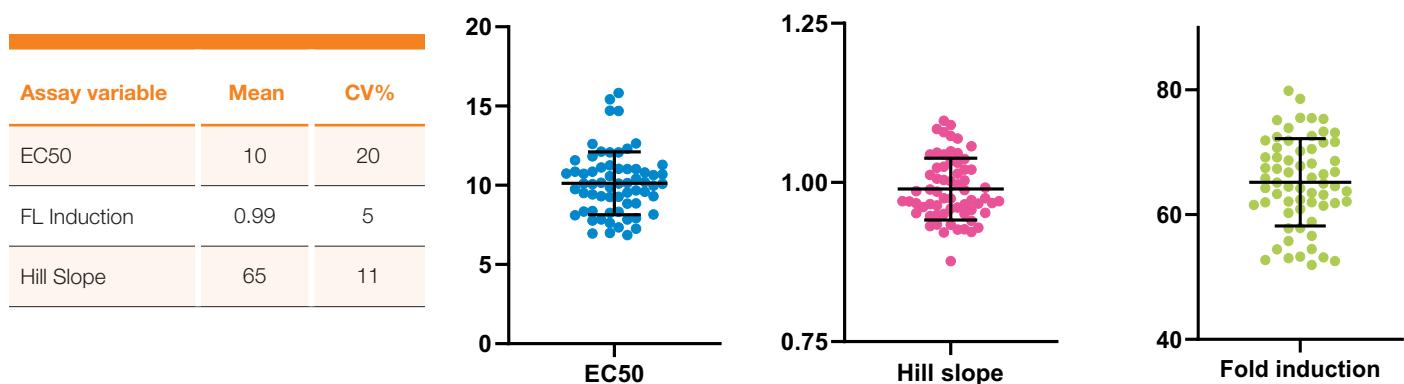


FIG. 2 - OVERALL CELL LINE VARIATION

Data were collected from tests with a RANKL functional bioassay analyzing a dose-response curve covering a concentration range from 0-500 ng/ml RANKL. The data represent results from 64 dose response curves using 3 different cell batches performed by 4 operators at 11 different days. Fold induction was calculated by: FL RLU 21 ng/ml/FL RLU 0.086 ng/ml.

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**

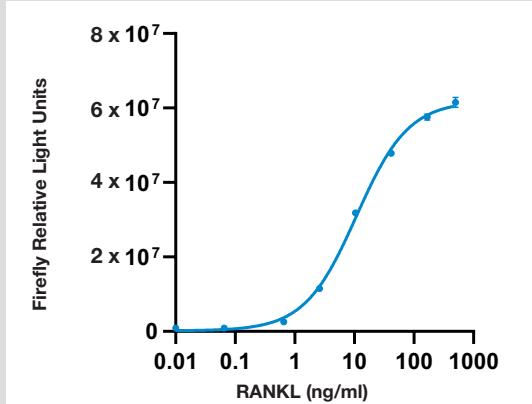
The dynamic range of the assay is determined by performing a dose-response curve from which we establish the range of the assay, i.e., where we see a signal from the inducible Firefly luciferase reporter gene, measured in this case in Relative Light Units (RLU).

- **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**

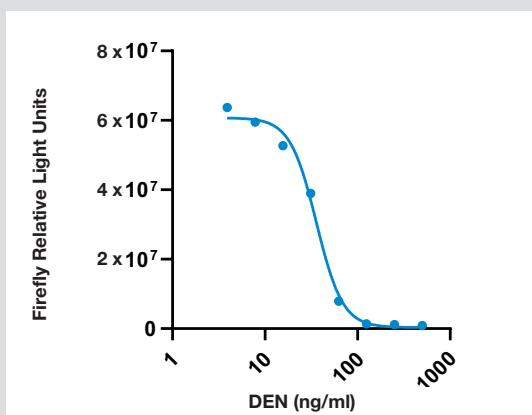
The Firefly luciferase signal is inversely proportional to the amount of inhibitory activity in a sample. In the absence of target inhibitor activity and suspected NAb presence in a test sample, a known amount of drug is added to quench the Firefly luciferase signal, resulting in a partially restored signal which can be used as a measure of the presence of NAb in the sample.



RANKL	In-assay (ng/ml)
A	500
B	167
C	42
D	10
E	2.6
F	0.65
G	0.065
H	0

FIG. 3 - DYNAMIC RANGE

Example of a functional RANKL bioassay application, were 0-500 ng/ml RANKL was used to achieve a dose-response curve.



DEN	In-assay (ng/ml)
A	500
B	250
C	125
D	62.5
E	31.3
F	15.6
G	7.8
H	0

FIG. 4 - DYNAMIC RANGE

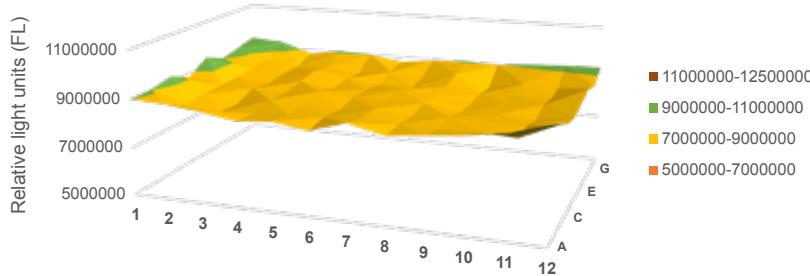
Example of an inhibitory RANKL bioassay application, were ng/ml anti-RANKL was used with 25 ng/ml RANKL to achieve a dose-response curve. DEN: Denosumab

PLATE HOMOGENEITY

For optimal results, it is important to use a plate design for the readout that provides the optimum conditions for consistency, minimizing edge effects and to use opaque plates when using substrates that produce fluorescent signals.

Readout of this type can be prone to common errors such as edge effects or inherent inconsistency of the actual cells.

With the *i*Lite Assay Ready Cells, we see very low variance across the plate, allowing the entire plate layout to be used in the plate design.

**FIG. 5 - HOMOGENEITY**

One single sample, 2 ng/ml RANKL, representing the lower part of the linear range of the functional bioassay, was tested in all 96 wells of the plate.

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.

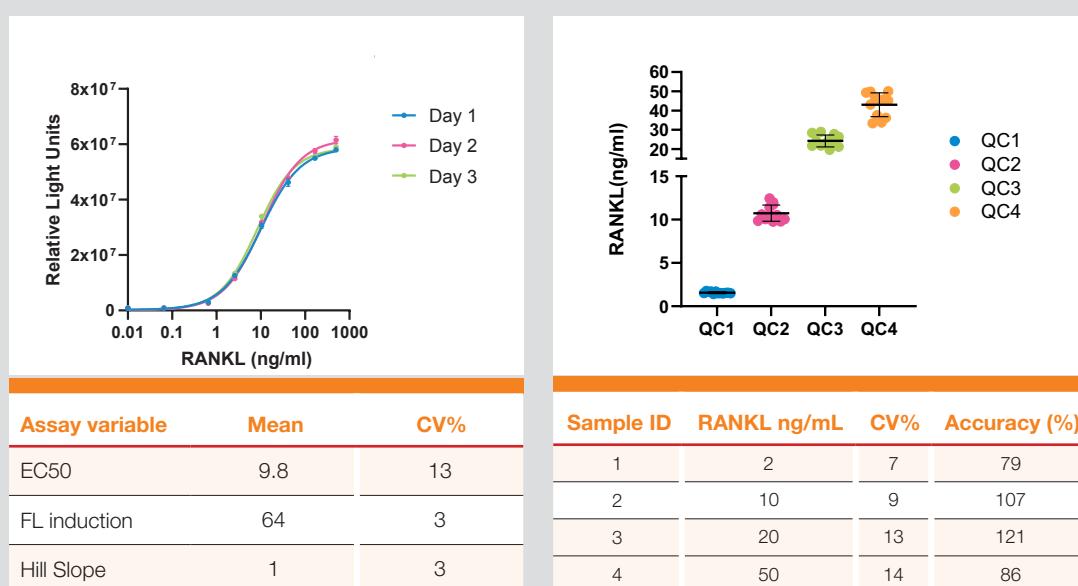


FIG. 6 - DAY-TO-DAY

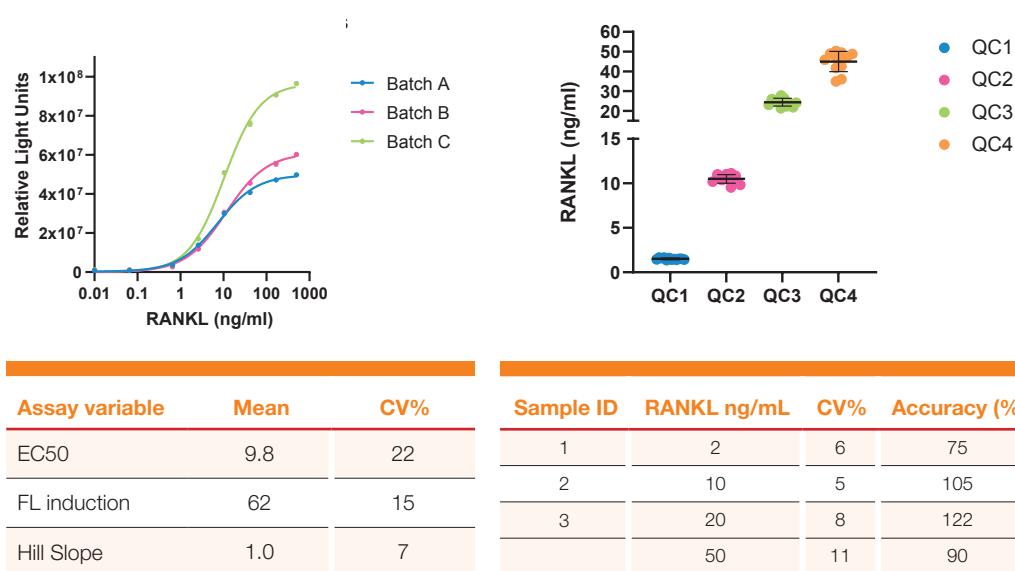
The *i*Lite RANKL functional bioassay was performed with a dose-response curve and four technical samples with known amount of RANKL. Data shown is generated from assays performed at three separate days with the same batch of RANKL cells.

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. 7 - LOT-TO-LOT

The *i*Lite RANKL functional bioassay was performed with a dose-response curve and four technical samples with known amount of RANKL. Data shown is generated from using three different batches of RANKL cells at the same assay occasion.



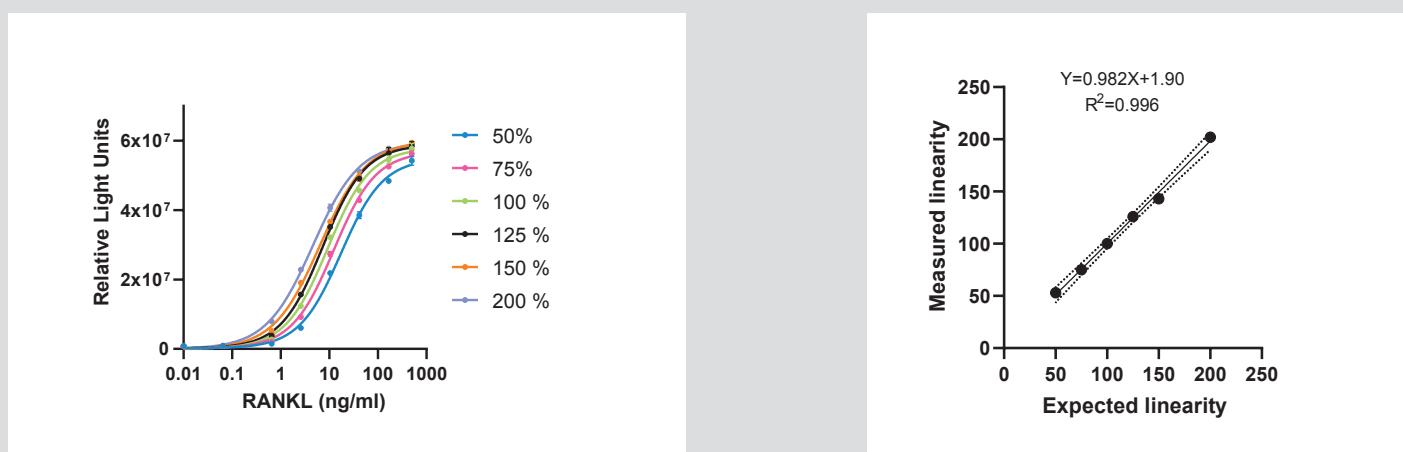
As shown in the figure, the precision and accuracy of the technical samples are well within acceptable limits, with CVs generally below 10%, even though the absolute Firefly (FL) signal may vary. These results show that there are only small differences between assays performed on different days or using 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.

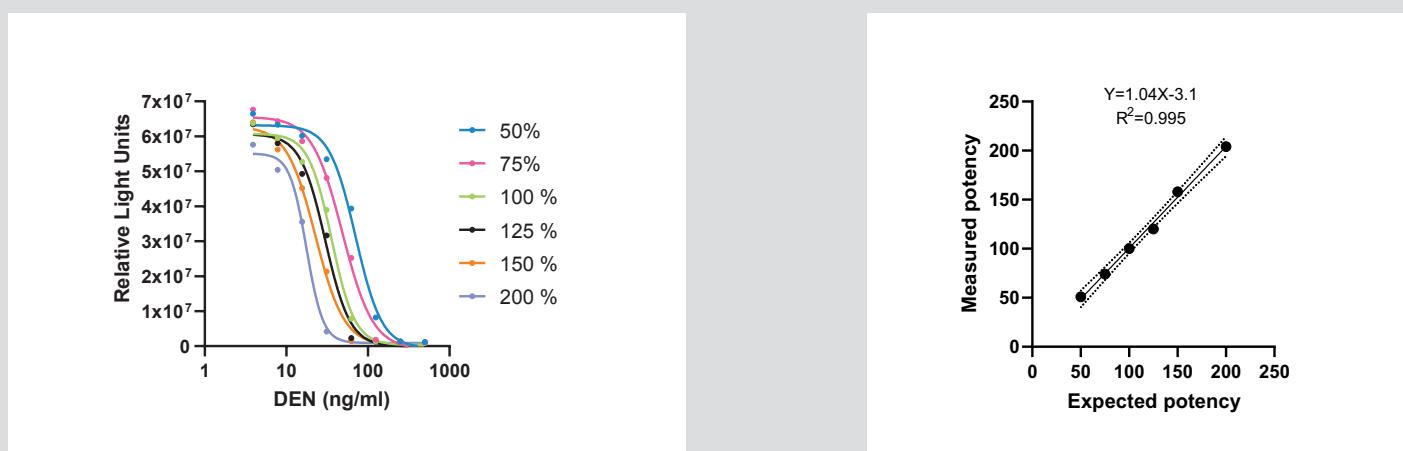
An important aspect of any Linearity/Potency assay is to establish the assay's ability to provide measured values that are proportional to the concentration of the sample.



Expected linearity	50%	75%	100%	125%	150%	200%
EC50	17.4	12.4	9.28	7.37	6.51	4.59
Accuracy	107%	100%		101%	95%	101%
Hill Slope	1.0	1.0	0.99	1.0	0.90	0.90
FL induction	59	64	67	67	65	60

FIG. 8 - PARALLELISM / LINEARITY

The iLite RANKL 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	71	49	36	30	23	18
Accuracy	102%	98%		96%	105%	102%
Hill Slope	-2.7	-2.3	-3.1	-2.9	-2.5	-4.3
FL reduction	46	49	52	50	51	50

FIG. 9 - PARALLELISM / LINEARITY

The iLite RANKL inhibitory bioassay was performed with one reference dose-response curve, called 100% and with concentrations at 50, 75, 125, 150 and 200% of the reference. DEN; Denosumab

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 *in vivo* 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.

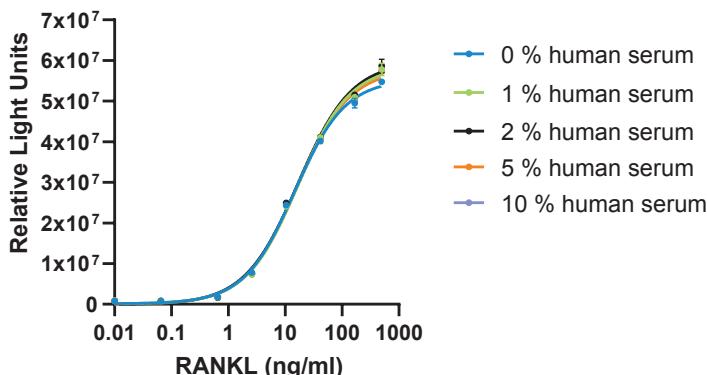
Two major parameters influence the outcome and robustness of an assay:

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.



Serum concentration	0%	1%	2%	5%	10%
Hill Slope	0.98	0.95	0.93	0.95	0.93
EC50	15	17	17	16	16
FL induction	61	63	64	60	62

FIG. 10 - SERUM EFFECT / TOLERANCE

The *i*Lite RANKL functional bioassay was performed with a dose-response curve including human serum, up to 10%.

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.