Quantification of functional RANKL using *iLite*® RANKL 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

Receptor activator of nuclear factor kappa-B ligand (RANKL) is a cytokine of the tumor necrosis factor (TNF) superfamily and is expressed in many kinds of tissue, including bone, lung, lymph nodes and mammary glands. It exists in two forms, as a transmembrane protein or in a soluble form, and binds to the receptor activator of nuclear factor kappa B (RANK) which is mainly found at osteoclasts and immune cells. Osteoprotegerin (OPG) is a soluble decoy factor regulating the RANKL-RANK interaction by binding RANKL and thereby decreasing the binding to RANK. (1) The RANK/RANKL/OPG cytokine system was discovered in the 1990s and identified for its key role in the bone metabolism through regulation of osteoclastogenesis. Besides bone remodeling, this cytokine system has been shown to play important roles in adaptive immunity, mammary gland development, thermoregulation of the central nervous system as well as tumor cell development and migration. (2)

The *iLite*® platform offers a cell-based assay that enables studies of RANKL, its receptor and their interaction.

Principle of the assay

The *iLite*® RANKL Assay Ready Cells are engineered cells, optimized to express Firefly luciferase (FL) under the control of a RANKL responsive promoter. Binding of soluble RANK Ligand to the human RANK receptor (human TNFRSF11A isoform 1) results in activation of the RANKL regulated Firefly luciferase reporter gene construct. *iLite*® RANKL 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 RANKL induced FL activity, and renders assay results independent of variations in cell number or serum matrix effects (there is a slight dose dependency trend for the normalization signals and a shift downwards can be detected in higher RANKL concentrations – see section “Normalization”). The luciferase signal can be measured in a luminometer following addition and incubation of luciferase substrate. The Firefly luciferase signal is proportional to the concentration of functional RANKL in a sample (Fig.1).

Specimen collection

The *iLite*® RANKL Assay Ready Cells can be used for measuring concentration of RANKL in test samples including human serum.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® RANKL Assay Ready Cells	Svar Life Science	BM4052
Diluent (DMEM containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin)	Gibco	31966-021 (DMEM) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
RANKL or analogues	Immunotools	11343453
Firefly/Renilla luciferase substrate	Promega	E2920, Dual-Glo Luciferase Assay System
Plate; 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 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 or plate for dilution	NA	NA
Single-use polypropylene reservoir	NA	NA
Plate shaker	NA	NA
Timer	NA	NA

Protocol

Preparation of calibrators (RANKL)

Recombinant human soluble RANKL from Immumotools has successfully been used to stimulate the *iLite*® RANKL Assay Ready Cells. The below table shows the dilutions of RANKL, used for QC release of the *iLite*® RANKL Assay Ready Cells.

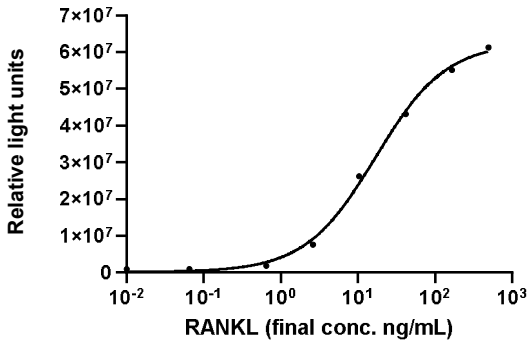


Figure 1. Example of RANKL calibration curve.

Calibrator	RANKL
	Suggested calibrator solution conc. (ng/ml)
A	1000
B	333
C	83
D	21
E	5.2
F	1.3
G	0.13
H	0

Table 1. Suggested calibrator solution concentrations for RANKL.

Assay preparation and incubation

1. Design a plate layout. It is recommended to perform the test at least in duplicates.
2. Dilute calibrators, controls, and samples to fall within the expected **in assay values** of 0 - 500 ng/mL.
3. Add 40 µL calibrators, controls, and samples in duplicate to assigned wells (final concentration will be half of solution concentration).
4. Thaw the vial of *iLite*® RANKL Assay Ready Cells in a 37°C water bath with gentle agitation. The cell suspension is mixed very carefully ten times with pipette to ensure a homogeneous distribution of cells.
5. Dilute 250 µL cell suspension with 5.75 mL Diluent.
6. Add 40 µL diluted cells to each well.
7. Place the lid on the plate, mix and incubate for 5 hours at 37 °C with 5% CO_2.

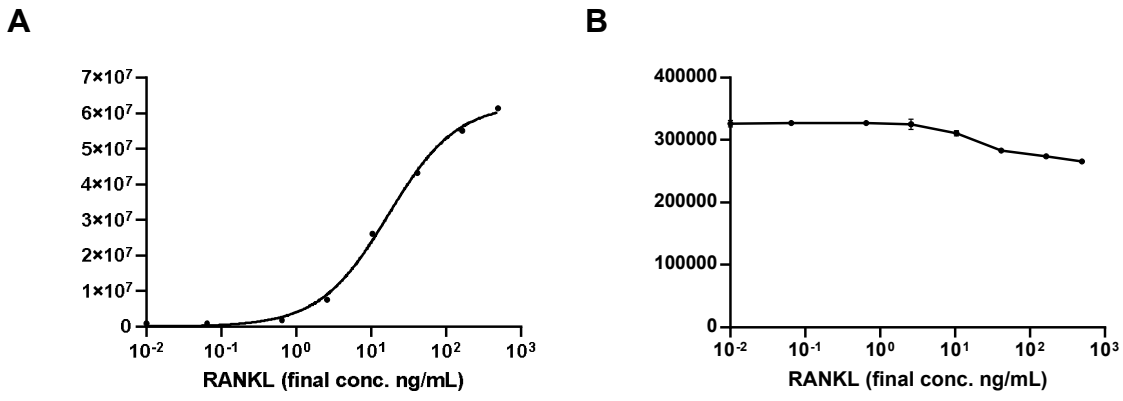
Adding substrate solutions

8. Equilibrate the plate and the substrate solution to room temperature.
9. Prepare the **Firefly luciferase** substrate according to the manufacturer's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.
10. If appropriate, prepare the **Renilla luciferase** substrate according to the manufacturer's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.

Normalization

The reporter gene used for normalization, Renilla luciferase, is under the control of a tyrosine kinase promoter, and is thus constitutively expressed. Unspecific effects such as serum matrix effects or differences in cell number can be obviated by relating the specific Firefly signal with the Renilla normalization signal through simple division.

In the case of metabolic factors such as RANKL, high concentrations can result in a quantifiable effect on the general machinery of the cell, such as the transcription rate of polymerases or the activity of certain elongation factors. This highly reproducible effect is seen as a decrease in the normalization gene readout, inversely proportional to the increase of RANKL concentration (see Figure 2 below). Normalization against the Renilla signal will compensate the effects of RANKL on the cellular machinery as well as non-specific effects such as serum matrix effects or differences in cell number, the result can be seen below.



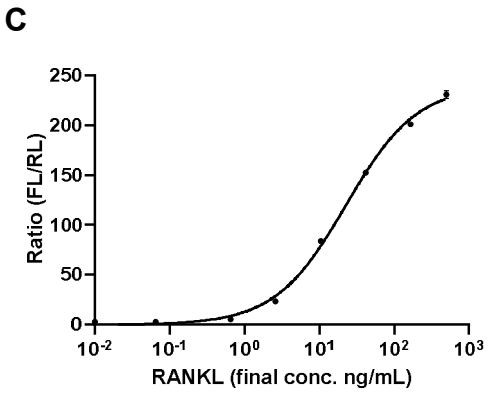


Figure 2.
A: Measurement of the specific Firefly (FL) signal. Cells were stimulated with increasing concentrations of RANKL.
B: Measurement of the Renilla (RL) signal for normalization, from the identical assay as in A.
C: Dose response curve after normalization of the specific Firefly signal with the according Renilla signal.

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.

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.

QUICK GUIDE

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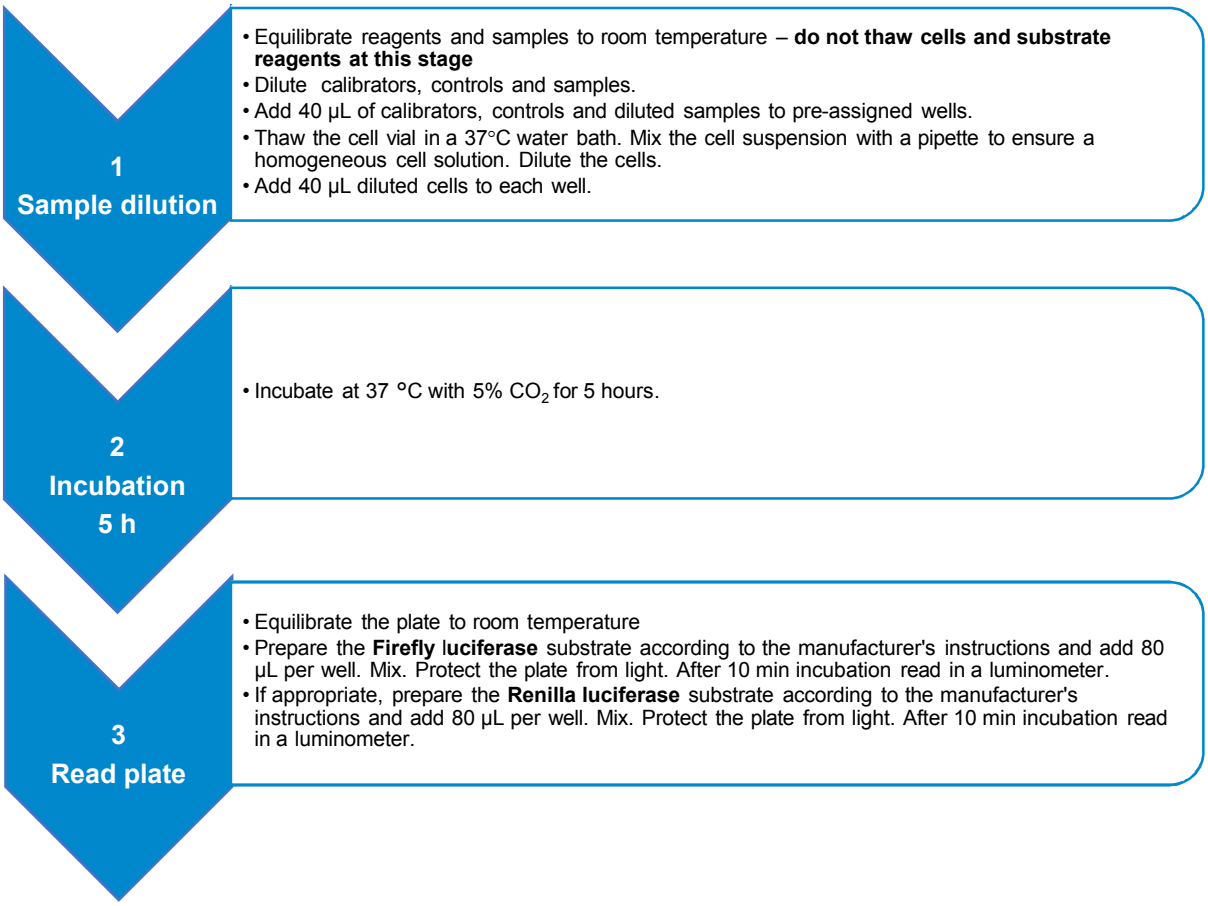

Troubleshooting and FAQ

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

References

1. Wu X et al. *RANKL/RANK System-Based Mechanism for Breast Cancer Bone Metastasis and Related Therapeutic Strategies.* Front Cell Dev Biol. 2020 Feb 11;8:76.
2. Antonio G et al. *Immune system and bone microenvironment: rationale for targeted cancer therapies.* Oncotarget. 2020 Jan 28;11(4):480-487.