Quantification of RANKL inhibitor 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

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)

Primary tumors, in breast and prostate cancers for example, commonly metastasize into the bone. Many cancers utilize the RANKL/RANK/OPG system to promote migration and implantation of cancer cells in the bone and support downregulation of the body's tumor immune surveillance mechanism. (2,3)
Denosumab (trade names Prolia and Xgeva), approved by the FDA in 2010, is a fully human monoclonal antibody to RANKL. It decreases bone turnover markers by blocking the RANKL/RANK pathway. The first approved indication for denosumab was osteoporosis and treatment-induced bone loss. Further clinical studies have led to the approval of denosumab for the prevention of skeletal-related events from bone metastases in cancer. RANKL inhibition is also being investigated for use in combination with other cancer immunotherapies to improve the effect of immune-checkpoint inhibitors targeting CTLA-4, PD-1, or PD-L1. (4,5)

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

RANK - receptor activator of nuclear factor kappa B. RANKL – RANK ligand. OPG - Osteoprotegerin

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. The luciferase signal can be measured in a luminometer following addition and incubation of luciferase substrate.

The Firefly luciferase signal is proportional to the functional activity of RANKL in the sample. In the presence of inhibitory activity against RANKL, the amount of free RANKL is reduced, resulting in a decreased stimulation of Firefly luciferase production. Thus, the Firefly luciferase signal is inversely proportional to the amount of inhibitory activity against RANKL in a sample. The *iLite*® RANKL Assay Ready Cells can therefore be utilized as an assay for quantification of RANKL inhibitor activity 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)
Denosumab	NA	NA
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	Revvity	6055680
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 RANKL inhibitor

Denosumab from Amgen has successfully been used to neutralize soluble RANK Ligand and inhibit the RANKL regulated Firefly luciferase expression in *iLite*® RANKL Assay Ready Cells (refer to the table and graph below)

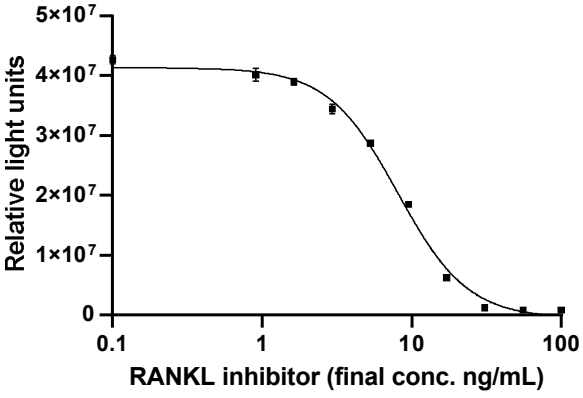


Figure 1. Example of RANKL inhibitory curve

Final 50 ng/mL RANKL	Denosumab
	Suggested calibrator solution concentrations, ng/mL
1	100
2	56
3	31
4	17
5	10
6	5.3
7	2.9
8	1.6
9	0.91
10	0

Table 1. Suggested calibrator solution concentrations for RANKL inhibitor

Assay preparation and incubation

1. Design a plate layout. It is recommended to perform the test at least in duplicates.
2. Perform a serial dilution of the reference RANKL inhibitor. Ensure matrix consistency between reference antibody solutions, control solutions, and sample solutions.
3. Add 20 µL of the reference RANKL inhibitor dilutions, controls and samples to assigned wells (final concentration will be a quarter of solution concentration).
4. Add 20 µL of 200 ng/ml RANKL to all wells (final concentration will be 50 ng/mL RANKL).
5. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2
6. 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.
7. Dilute 250 µL cell suspension with 5.75 mL Diluent.
8. Add 40 µL diluted cells to each well.
9. Place the lid on the plate, mix and incubate for 5 hours at 37 °C with 5% CO_2.

Adding substrate solutions

10. Equilibrate the plate and the substrate solution to room temperature.
11. 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.
12. 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.

Precautions

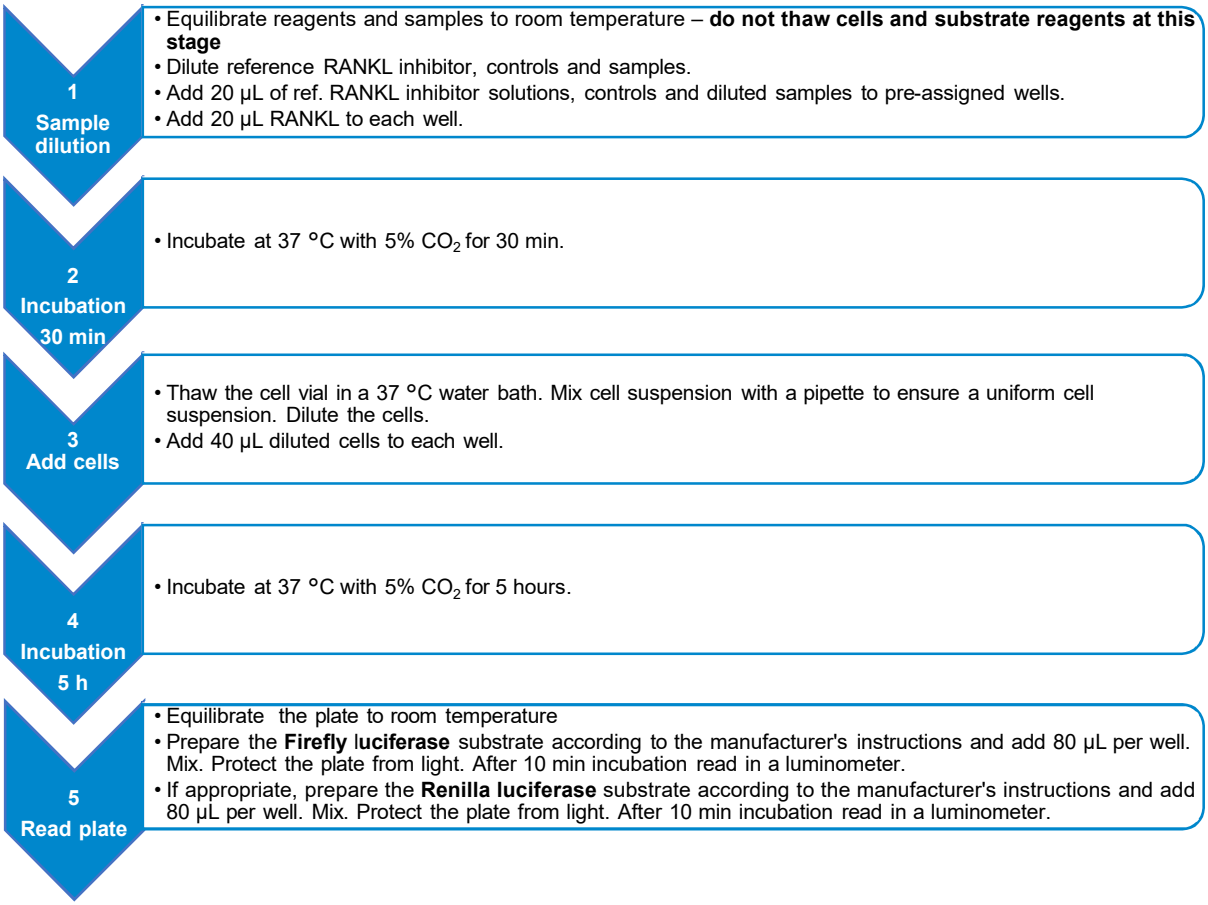
- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according 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.

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. *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

Quantification of RANKL inhibitor activity using *iLite*® RANKL Assay Ready Cells



Troubleshooting and FAQ

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

References

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