

## Determination of neutralizing antibodies against RANKL inhibitors using *iLite*<sup>®</sup> 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*<sup>®</sup> platform offers a cell-based assay that enables immunogenicity studies of drug candidates targeting 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*<sup>®</sup> 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*<sup>®</sup> 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. In the absence of RANKL inhibitor activity and suspected NAb presence in test samples, a known amount of drug is added to quench the Firefly signal and the presence of NAb is measured as a restored signal. The *iLite*<sup>®</sup> RANKL Assay Ready Cells can therefore be utilized as an assay for determination of neutralizing antibodies against RANKL inhibitors in test samples, including human serum.

## Material and equipment needed

Material and equipment	Suggested supplier	Reference
<i>iLite</i> <sup>®</sup> 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)
Anti-denosumab antibody	Bio-rad	HCA282
Denosumab or analogues	NA	NA
RANKL or analogues	Immunotools	11343453
Firefly 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 <sub>2</sub>	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 neutralizing antibodies against RANKL inhibitor

An anti-denosumab antibody from Bio-rad has successfully been used to neutralize denosumab (RANKL inhibitor) and restore the RANKL regulated Firefly luciferase expression in *iLite*<sup>®</sup> RANKL Assay Ready Cells (refer to the table and graph below).

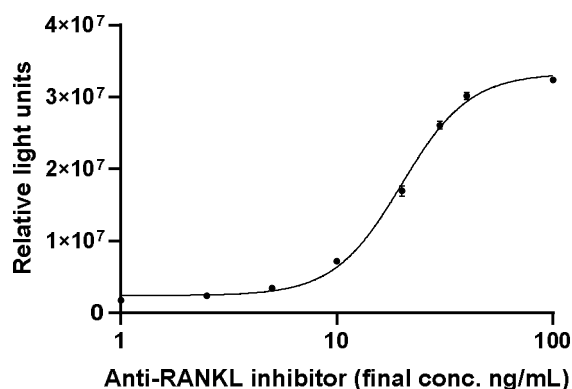


Figure 1. Example of anti-RANKL inhibitory curve

Final 50 ng/mL RANKL and 25 ng/mL Denosumab	Anti-denosumab Suggested solution concentrations, ng/mL
A	800
B	320
C	240
D	160
E	80
F	40
G	20
H	0

Table 1. Suggested calibrator solution concentrations for anti-denosumab

**Assay performance and incubation**

1. Design a plate layout. It is recommended to perform the test at least in duplicate.
2. Perform a serial dilution of the reference anti-denosumab antibody. Ensure matrix consistency between reference antibody solutions, control solutions, and sample solutions.
3. Add 20  $\mu\text{L}$  of the reference anti-denosumab antibody dilutions, controls, and samples to assigned wells (final concentration will be one-eighth of solution concentration).
4. Add 20  $\mu\text{L}$  of 200 ng/mL denosumab to all wells (final concentration will be 25 ng/mL denosumab).
5. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO<sub>2</sub>.
6. Add 40  $\mu\text{L}$  of 200 ng/mL RANKL to all wells (final concentration will be 50 ng/mL RANKL).
7. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO<sub>2</sub>.
8. Transfer references, controls, and samples to new wells, adding 40  $\mu\text{L}$  per well.
9. Thaw the vial of *iLite*<sup>®</sup> 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.
10. Dilute 250  $\mu\text{L}$  cells with 5.75 mL Diluent.
11. Add 40  $\mu\text{L}$  diluted cells to each well.
12. Place the lid on the plate, mix and incubate for 5 hours at 37 °C with 5% CO<sub>2</sub>.

**Adding substrate solutions**

13. Equilibrate the plate and the substrate solution to room temperature.
14. Prepare the **Firefly luciferase** substrate according to the manufacturer's instructions and add 80  $\mu\text{L}$  per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.
15. If appropriate, prepare the **Renilla luciferase** substrate according to the manufacturer's instructions and add 80  $\mu\text{L}$  per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.

**Precautions**

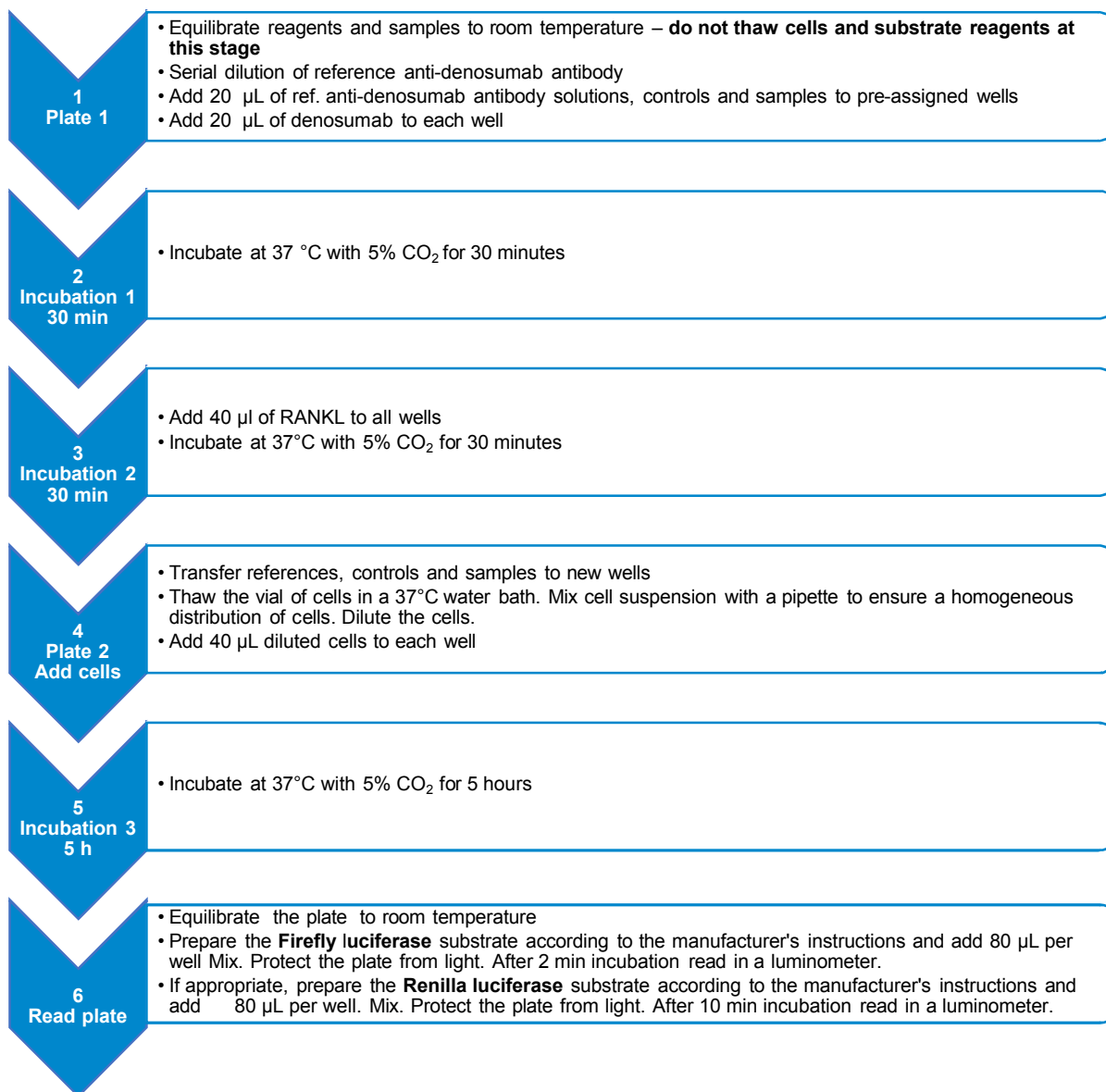
- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according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

**Proprietary Information**

In accepting delivery of *iLite*<sup>®</sup> 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*<sup>®</sup> cell-based products are covered by patents which are the property of Svar Life Science AB and any attempt to reproduce the delivered *iLite*<sup>®</sup> 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](http://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.
3. Sisay M et al. *The RANK/RANKL/OPG system in tumorigenesis and metastasis of cancer stem cell: potential targets for anticancer therapy*. Onco Targets Ther. 2017 Jul 27;10:3801-3810.
4. Dempster DW et al. *Role of RANK ligand and denosumab, a targeted RANK ligand inhibitor, in bone health and osteoporosis: a review of preclinical and clinical data*. Clin Ther. 2012 Mar;34(3):521-36.
5. Ahern E et al. *Roles of the RANKL-RANK axis in anti-tumour immunity - implications for therapy*. Nat Rev Clin Oncol. 2018 Nov;15(11):676-693.