Development and Evaluation of a Novel Bioassay for Denosumab Activity

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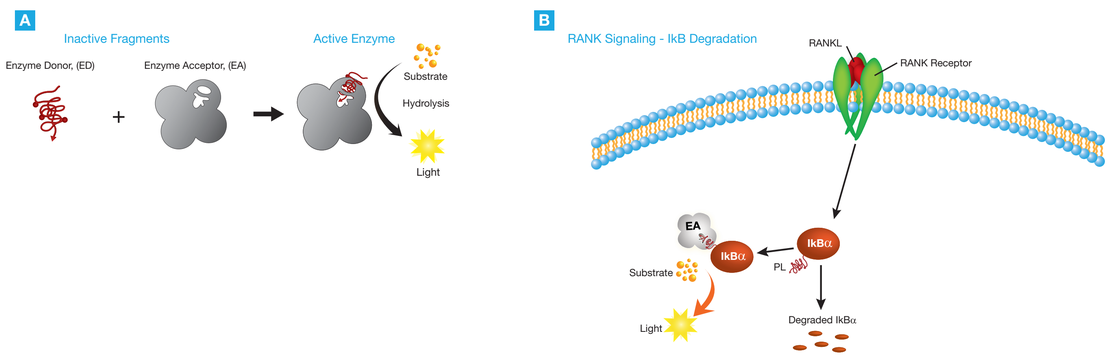
Abstract

Denosumab (Prolia®) is a fully human sequence derived monoclonal antibody used for the treatment of osteoporosis in menopausal women via inhibition of the RANK (receptor activator of nuclear factor-kappa B) pathway. Companies developing a biosimilar are required to demonstrate that the proposed biosimilar is “highly similar” to the innovator material, using a mechanism-of-action (MOA)-based assay.

The DiscoverX PathHunter® bioassay for Denosumab uses a U2 OS cell line expressing RANK and an IκB reporter protein that is tagged with one fragment of a split β-galactosidase system. When the tagged IκB is exposed to the other half of the split β-galactosidase protein, active β-galactosidase is formed which hydrolyses the substrate and produces a chemiluminescent signal. In the assay, RANKL binds to RANK on the cell surface resulting in NF-κB signalling and IκB degradation, leading to a decrease in chemiluminescent signal. Denosumab inhibits RANKL-based activation of RANK, leading to an increase in chemiluminescence. The increase in chemiluminescence is directly proportional to the functional activity of denosumab.

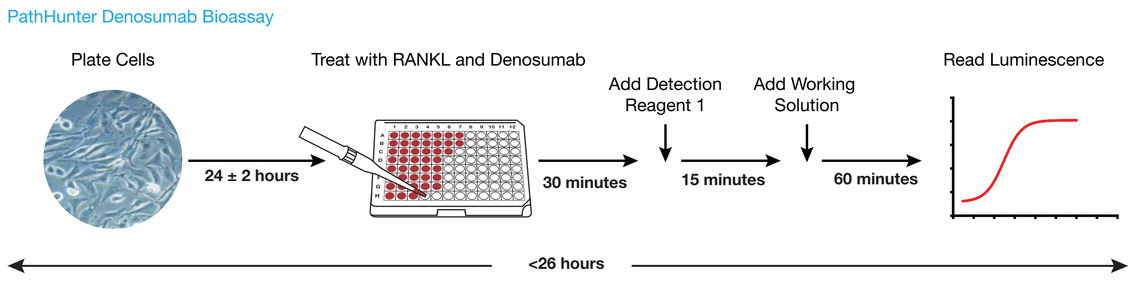
The data presented in this poster was obtained from an assay qualification performed at Sartorius Stedim BioOutsource. During the qualification study, data was obtained to demonstrate that the assay is accurate and precise over a linear range of 50% to 150% of reference standard. The assay also showed specificity for denosumab. The methodology is therefore deemed fit for the purposes of evaluating the functional comparability and potency of denosumab biosimilar and innovator material. The benefits of this commercially available assay are that it is easy-to-use, highly reproducible, and saves months of assay development time, translating into overall speed and cost savings in a biosimilar development program.

Building a Biologically Relevant Bioassay for Denosumab

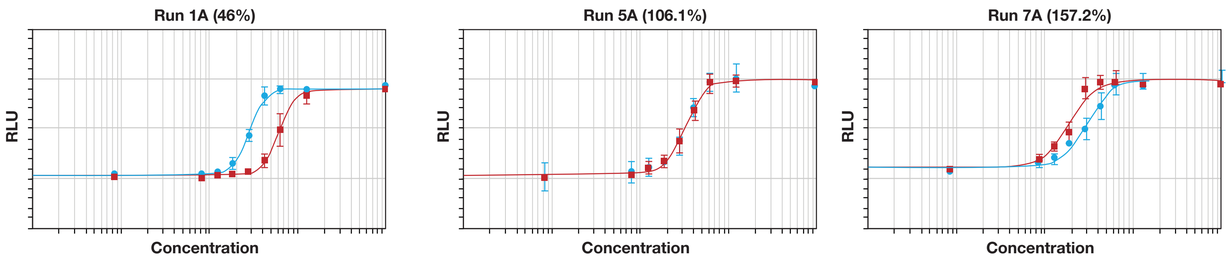


A. DiscoverX’s proprietary PathHunter Enzyme Fragment Complementation (EFC) technology consists of the β-galactosidase (β-gal) enzyme, split into two inactive components, the enzyme donor peptide (ED) and enzyme acceptor (EA). When brought together in close proximity, ED complements with EA forming active β-gal. The active enzyme hydrolyzes the substrate generating chemiluminescent light, providing a highly amplified signal and thus an assay of high sensitivity. B. Denosumab inhibits maturation of osteoclasts by binding to and inhibiting RANK Ligand (RANKL), preventing it from activating its receptor, RANK. The DiscoverX PathHunter bioassay for Denosumab uses a U20S cell line expressing RANK and an IκB reporter protein that is tagged with PL, a high-affinity variant of ED. When the tagged IκB is exposed to the other half of the split β-galactosidase protein, active β-galactosidase is formed which hydrolyses the substrate and produces a chemiluminescent signal. In the assay, RANKL binds to RANK on the cell surface resulting in NF-κB signalling and IκB degradation, resulting in a decrease in chemiluminescent signal. Denosumab inhibits RANKL-based activation of RANK, leading to an increase in chemiluminescence. The increase in chemiluminescence is directly proportional to the functional activity of denosumab.

Get Rapid Results with an Easy-to-Use Protocol



Relative Potency of Denosumab from 50% to 150%



Reference Standard (Blue)
Test Material (Red)

To determine the linearity of the assay, samples were measured at 50%, 100%, and 150% of the reference standard (100%). Data is shown for a single analyst performing the assay at the same location, on different days for each experiment. As shown in the panels above, the upper and lower asymptotes of the reference standard and sample curves are both fully defined, and the samples are parallel to the reference standard, allowing accurate relative potency measurements to be reported. The calculated relative potency values obtained are shown in parenthesis at the top of each graph: all relative potencies are within 8% of the expected value.

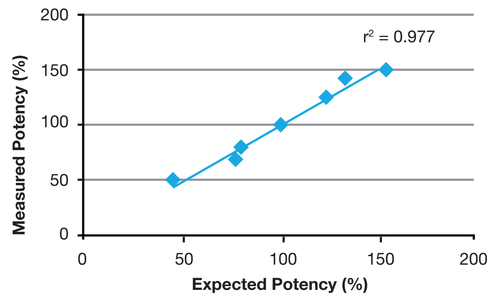
High Accuracy & Precision of Assay Through Multiple Analysts, Locations, and Days

Days	Analyst	Location	Expected Potency %	Measured Potency %	Mean Potency %	Recovery %	RSD %
1	X	A	50	46	46.6	93.2	7.2
2	X	B		50.2			
3	Y	B		43.6			
4	Y	A	100	100.5	101.6	101.6	4.0
5	X	A		106.1			
6	Y	B		98.2			
7	Y	A	150	160.1	155.2	103.5	4.0
8	Y	B		148.3			
9	X	A		157.3			

Nine different assays were performed on different days, by two analysts and in two separate buildings. The analysts tested samples from 50% to 150% relative potency compared to the reference sample (100%). Measured relative potencies are indicated on the table above. The assay has a maximum inaccuracy within 13% for the 50% sample, and 85% of all the samples are within 10% of the expected result. The intermediate precision of the assay is 7.2% RSD.

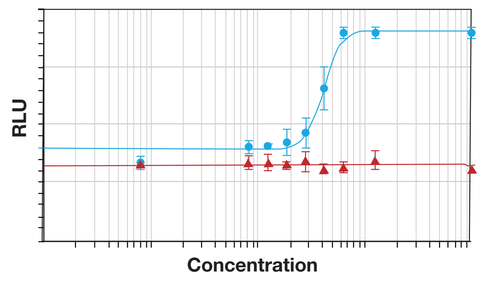
Qualification of Denosumab Bioassay

Expected Potency (%)	Measured Potency (%)	Recovery (%)
50	46	92
50	50.2	100.4
50	43.6	87.2
70	78.4	112
80	82	102.5
100	100.5	100.5
100	106.1	106.1
100	98.2	98.2
125	125.3	100.2
143	133.9	93.6
150	160.1	106.7
150	148.3	98.9
150	157.3	104.9



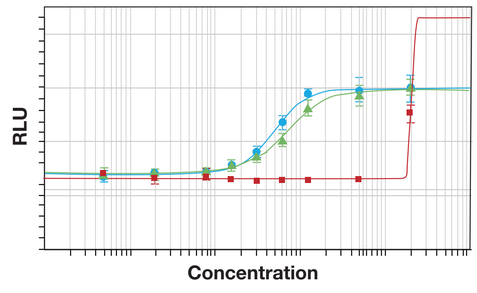
Here we have tested the PathHunter RANK-IκB cell line with Denosumab, demonstrating a robust response and a high level of reproducibility with multiple runs. The RANK-IκB cell line was tested with 7 different test concentrations ranging from 50% to 150%, compared to a 100% reference standard of denosumab, by two different operators in separate buildings, with each assay being run on a separate day. The measured relative potencies were plotted against the expected relative potencies with a high degree of accuracy and precision.

Denosumab Assay Has High Specificity



To assess the specificity of this assay, the cells were treated with either the reference molecule, Denosumab (in blue) , or a control monoclonal antibody (in red) that lacks RANKL-specific binding. The control molecule produces no dose–dependent response in the assay (red) whereas Denosumab produces a dose-response curve (blue) as expected, demonstrating specificity of the assay for the target drug.

PathHunter Denosumab Bioassay Detects Loss of Activity Due to Heat and pH Stress



Reference Standard (Blue)
94°C for 5 hours (Red)
0.01M Acetic Acid (Green)

The IκB reporter assay for Denosumab activity was able to detect stressed samples, and exhibits stability indicating properties. One sample was heat treated at 94ºC for 5 hours (shown in red) and the second sample shown in green was treated with acid (0.01M acetic acid for 5 minutes, then neutralised with 0.01M NaOH). The heat treated sample (in red) demonstrates a total loss in activity as would be expected from a denatured protein sample and the acid treated sample (in green) demonstrates reduced potency of the antibody in low pH, compared to the reference standard. Both these samples demonstrate that this assay can be used in stability studies for the therapeutic antibody.

Summary

A Fit-for-Purpose MOA-Based Bioassay for Denosumab

- MOA-based bioassay developed using an IκB reporter to measure RANK receptor activation , which generated results in 24-26 hours, with an easy-to-use protocol
- Assay was qualified at Sartorius Stedim BioOutsource, Glasgow UK
- Data demonstrates highly accurate and precise measures of relative potency over a linear range of 50% to 150% of reference standard
- The assay also showed specificity for denosumab, with additional capabilities to detect stressed samples
- The assay and method are deemed fit for the purposes of evaluating the functional comparability and potency of denosumab biosimilar and innovator materials

Benefits of PathHunter RANK- IκB Cell Line (DiscoverX Catalog Number 93-0994C3)

- Easy-to-use protocol enabling quick implementation in any lab & with any analyst
- Highly reproducible data reducing the number of failed tests
- Saves months of assay development time translating into overall cost savings in a biosimilar development program

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