Denosumab (Prolia®) ELISA

SKU: HUMB00036

Enzyme immunoassay for the quantitative determination of denosumab (Prolia®) in serum and plasma

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Required Volume (µl)	10
Total Time (min)	70
Sample	Serum, plasma
Sample Number	96
Detection Limit (ng/mL)	3
Spike Recovery (%)	Between 85-115
Shelf Life (year)	1

Intended Use

Enzyme immunoassay for the quantitative determination of denosumab (Prolia®) in serum and plasma. Elisa Genie denosumab ELISA has been especially developed for the quantitative analysis of denosumab in serum and plasma samples between the Cmin and Cmax range of concentrations indicated in the pharmacokinetics section of prospectus.

Summary and Explanation

According to the prospectus; denosumab (Prolia®) is indicated for the treatment of osteoporosis in postmenopausal women to reduce the risk of vertebral, non-vertebral and hip fractures and to increase bone mass in men with osteoporosis at increased risk of fracture. PROLIA is reimbursed for women and men aged 70 or over with a Bone Mineral Density (BMD) T-score of -2.5 or less.

PROLIA has a mechanism of action unlike other osteoporosis treatments. Administered every six months as a subcutaneous injection, PROLIA is the only therapy that targets RANK Ligand, an essential regulator of osteoclasts (the cells that break down bone). Through highly specific inhibition of RANK ligand, PROLIA decreases bone resorption, and improves bone density at all measured skeletal sites.

PROLIA is a twice-yearly injection under the skin, usually administered by a general practitioner or practice nurse, and works by targeting the cells that break down bone (osteoclasts) thereby making bone less susceptible to osteoporosis-related fractures.

Prolia binds to RANKL, a transmembrane or soluble protein essential for the formation, function, and survival of osteoclasts, the cells responsible for bone resorption. Prolia prevents RANKL from activating its receptor, RANK, on the surface of osteoclasts and their precursors. Prevention of the RANKL/RANK interaction inhibits osteoclast formation, function, and survival, thereby decreasing bone resorption and increasing bone mass and strength in both cortical and trabecular bone.

In clinical studies, treatment with 60 mg of Prolia resulted in reduction in the bone resorption marker serum type 1 C-telopeptide (CTX) by approximately 85% by 3 days, with maximal reductions occurring by 1 month.

CTX levels were below the limit of assay quantitation (0.049 ng/mL) in 39-68% of subjects 1-3 months after dosing of Prolia. At the end of each dosing interval, CTX reductions were partially attenuated from a maximal reduction of ≥ 87% to ≥ 45% (range: 45% to 80%), as serum denosumab levels diminished, reflecting the reversibility of the effects of Prolia on bone remodelling. These effects were sustained with continued treatment. Upon reinitiation, the degree of inhibition of CTX by Prolia was similar to that observed in patients initiating Prolia treatment.

Consistent with the physiological coupling of bone formation and resorption in skeletal remodelling, subsequent reductions in bone formation markers (i.e., osteocalcin and procollagen type 1 N-terminal peptide [PlNP]) were observed starting 1 month after the first dose of Prolia. After discontinuation of Prolia therapy, markers of bone resorption increased to levels 40-60% above pre-treatment values but returned to baseline levels within 12 months.

In a study conducted in healthy male and female volunteers (n = 73, age range: 18 to 64 years) following a single subcutaneously administered Prolia dose of 60 mg after fasting (at least for 12 hours), the mean maximum denosumab concentration (Cmax) was 6.75 mcg/mL (standard deviation [SD] = 1.89 mcg/mL). The median time to maximum denosumab concentration (Tmax) was 10 days (range: 3 to 21 days). After Cmax, serum denosumab concentrations declined over a period of 4 to 5 months with a mean half-life of 25.4 days (SD = 8.5 days; n=46). The mean area-under-the-concentration-time curve up to 16 weeks (AUC0-16 weeks) of denosumab was 316 mcg·day/mL (SD = 101 mcg·day/mL).

No accumulation or change in denosumab pharmacokinetics with time was observed upon multiple dosing of 60 mg subcutaneously administered once every 6 months.

Prolia pharmacokinetics were not affected by the formation of binding antibodies. A population pharmacokinetic analysis was performed to evaluate the effects of demographic characteristics. This analysis

showed no notable differences in pharmacokinetics with age (in postmenopausal women), race, or body weight (36 to 140 kg).

The safety of Prolia in the treatment of postmenopausal osteoporosis was assessed in a 3- year, randomized, double-blind, placebo-controlled, multinational study of 7808 postmenopausal women aged 60 to 91 years. A total of 3876 women were exposed to placebo and 3886 women were exposed to Prolia administered subcutaneously once every 6 months as a single 60 mg dose. All women were instructed to take at least 1000 mg of calcium and 400 IU of vitamin D supplementation per day.

The incidence of all-cause mortality was 2.3% (n = 90) in the placebo group and 1.8% (n = 70) in the Prolia group. The incidence of nonfatal serious adverse events was 24.2% in the placebo group and 25.0% in the Prolia group. The percentage of patients who withdrew from the study due to adverse events was 2.1% and 2.4% for the placebo and Prolia groups, respectively.

Hypocalcemia
Decreases in serum calcium levels to less than 8.5 mg/dL were reported in 0.4% women in the placebo group and 1.7% women in the Prolia group at the month 1 visit. The nadir in serum calcium level occurs at approximately day 10 after Prolia dosing in subjects with normal renal function. In clinical studies, subjects with impaired renal function were more likely to have greater reductions in serum calcium levels compared to subjects with normal renal function.

In a study of 55 patients with varying degrees of renal function, serum calcium levels < 7.5 mg/dL or symptomatic hypocalcemia were observed in 5 subjects. These included no subjects in the normal renal function group, 10% of subjects in the CrCL 50 to 80 mL/min group, 29% of subjects in the CrCL < 30 mL/min group, and 29% of subjects in the hemodialysis group. These subjects did not receive calcium and vitamin D supplementation. In a study of 4,550 postmenopausal women with osteoporosis, the mean change from baseline in serum calcium level 10 days after Prolia dosing was -5.5% in subjects with creatinine clearance < 30 mL/min vs. -3.1% in subjects with CrCL > 30 mL/min.

Immunogenicity
Denosumab is a human monoclonal antibody. As with all therapeutic proteins, there is potential for immunogenicity. Using an electrochemiluminescent bridging immunoassay, less than 1% (55 out of 8113) of patients treated with Prolia for up to 5 years tested positive for binding antibodies (including pre-existing, transient, and developing antibodies). None of the patients tested positive for neutralizing antibodies, as was assessed using a chemiluminescent cell-based in vitro biological assay. No evidence of altered pharmacokinetic profile, toxicity profile, or clinical response was associated with binding antibody development.

The incidence of antibody formation is highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of a positive antibody (including neutralizing antibody) test result may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of antibodies to denosumab with the incidence of antibodies to other products may be misleading.

Test Principle

Solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. Standards and samples (serum or plasma) are incubated in the microtitre plate coated with the reactant specific for denosumab (Prolia®). Following incubation wells are washed and then horse radish peroxidase (HRP) is added and binds to Denosumab. After incubation, the wells are washed, and the bound enzymatic activity is detected by addition of chromogen-substrate. The colour developed is proportional to the amount of denosumab (Prolia®) in the sample or standard. Results of samples can be determined directly using the standard curve.

Warnings and Precautions

1. For professional use only.

2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information please refer to the local distributor.

3. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.

4. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.

5. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.

6. Chemicals and prepared or used reagents must be treated as hazardous waste according the national biohazard safety guidelines or regulations.

7. Avoid contact with Stop solution. It may cause skin irritations and burns.

8. All reagents of this kit containing human serum (i.e. standards) have been tested and were found negative for HIV I/II, HBsAg and HCV. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

9. Some reagents contain sodium azide (NaN3) as preservatives. In case of contact with eyes or skin, flush immediately with water. NaN3 may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with large volume of water to avoid azide build-up.

Storage and Stability

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The strips of microtiter plate is stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

Specimen Collection and Storage

Serum, Plasma (EDTA, Heparin) *

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	-20°C	Keep away from heat or direct sun light Avoid repeated freeze-thaw cycles
Stability:	2 d	6 mon	

*. Denosumab (Prolia®) infusion camouflages/masks the presence of antibody to denosumab in serum/plasma samples. Therefore, blood sampling time is critical for detection of denosumab. Elisa Genie Laboratories propose to obtain blood sample just before the infusion of denosumab (Prolia®) or at least 2 weeks after the infusion of denosumab (Prolia®).

Materials Supplied

1 x 12 x 8	MTP	**Microtiter Plate** Break apart strips. Microtiter plate with 12 rows each of 8 wells coated with reactant.
8 x 0.3 mL	STND A-F HIGH CNTRL LOW CNTRL	**Denosumab Standards A-F, High Level Control, Low Level Control** 300, 100, 30, 10, 3 and 0 ng/mL. Ready to use. Used for construction of the standard curve. Contains denosumab (Prolia®), human serum, stabilizer and <0.1% NaN_3
1 x 50 mL	ASSAY BUF	**Assay Buffer** Blue coloured. Ready to use. Contains animal serum, proteins and <0.1% NaN_3
1 x 12 mL	HRP CONJ	**Peroxidase Conjugate** Red coloured. Ready to use. Contains horse radish peroxidase (HRP) and stabilizers.
1 x 12 mL	TMB SUBS	**TMB Substrate Solution** Ready to use. Contains TMB
1 x 12 mL	TMB STOP	**TMB Stop Solution** Ready to use. 1N HCl.
1 x 50 mL	WASHBUF CONC	**Wash Buffer, Concentrate (20x)** Contains Buffer with Tween 20.
2 x 1	ADH FILM	**Adhesive Film** For covering of Microtiter Plate during incubation.

Materials Required but not Supplied

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 µL.
2. Calibrated measures.
3. Tubes (1 mL) for sample dilution.
4. Wash bottle, automated or semi-automated microtiter plate washing system.
5. Microtiter plate reader capable of reading absorbance at 450/650 nm.
6. ddH_2O or deionised water, paper towels, pipette tips and timer.

Procedure Notes

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pre-treatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.

2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.

3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.

4. Use a pipetting scheme to verify an appropriate plate layout.

5. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel micropipette for pipetting of solutions in all wells.

6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.

7. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

Pre-Test Setup Instructions

1. Preparation of Components

Dilute/ dissolve	Component	with	Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer*	Up to 200 mL	ddH_2O	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	2 w

***.** Prepare Wash Buffer before starting assay procedure.

2. Dilution of Samples

Sample	To be diluted	With	Relation	Remarks
Serum/ Plasma	1:50	Assay Buffer	1:50	For dilution at 1:50; 10µl Sample + 490µl Assay Buffer

Patient samples with a concentration of denosumab above the measuring range are to be rated as > "Highest Standard (Standard A)". The result must not be extrapolated. The patient sample in question should be further diluted with Assay Buffer and retested.

Test Procedure

1	Pipette 100 µL of each ready-to use Standards, High Level Control, Low Level Control or diluted Serum Samples into the respective wells of microtiter plate. Wells A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1: Standard F G1: High Level Control H1: Low Level Control A2 and on: Sample (serum/plasma)
2	Cover the plate with adhesive foil. Incubate 30 min at room temperature (18- 25°C).
3	Remove adhesive foil. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
4	Pipette 100 µL of ready-to use Peroxidase into each well.
5	Cover the plate with adhesive foil. Incubate 30 min at room temperature (18- 25°C).
6	Remove adhesive foil. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
7	Pipette 100 µL of TMB Substrate Solution into each well
8	Incubate 10 min (without adhesive foil.) at room temperature (18-25°C) in the dark.
9	Stop the substrate reaction by adding 100 µL of Stop Solution into each well. Briefly mix contents by gently shaking the plate. Colour changes from blue to yellow.
10	Measure optical density with a photometer at 450/650 nm within 30 min after pipetting of the Stop Solution.

Quality Control

The test results are only valid only if the test has been performed following the instructions. Moreover, the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All standards must be found within the acceptable ranges as stated above and/or label. If the criteria are not met, the run is not valid and should be repeated. In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

Calculation & Interpretation of Results

1. Using the standards (300; 100; 30; 10; 3; 0 ng/mL) disregarding zero standard, construct a standard curve by plotting the OD450/650 nm for each of 4 standards on the vertical (Y-axis) axis versus the corresponding denosumab concentration on the horizontal (X-axis) axis, thus creating a standard curve by 4 points obtained.

2. The concentration of the samples can be read directly from this standard curve. Using the absorbance value for each sample, determine the corresponding concentration of denosumab from the standard curve. Find the absorbance value on the Y-axis and extend a horizontal line to the curve. At the point of intersection, extend a vertical line to the X-axis and read the denosumab concentration for the unknown sample.

3. If computer data regation is going to be used, we recommend primarily "4 Parameter Logistic (4PL)" or secondly the "point-to-point calculation".

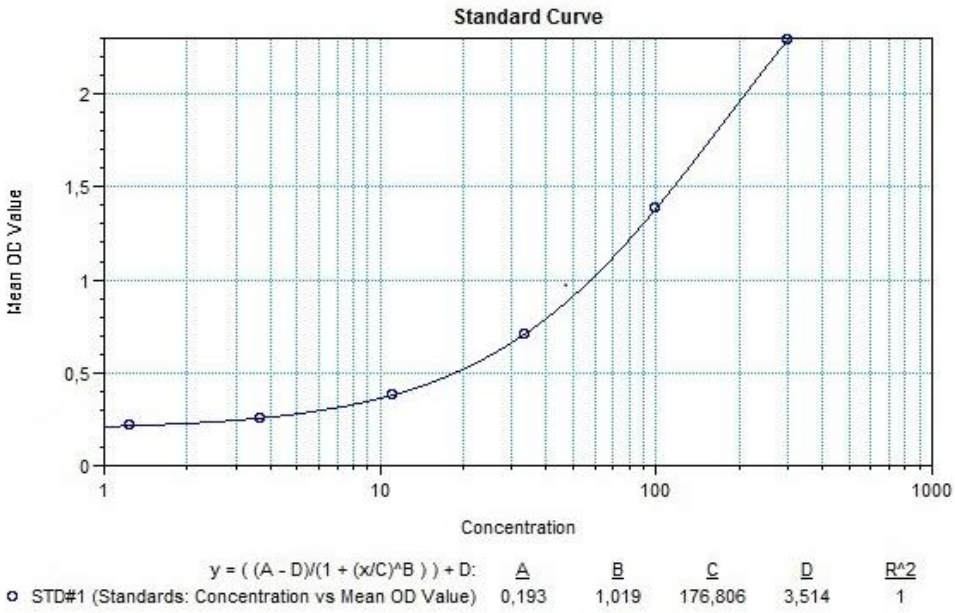
4. To obtain the exact values of the samples, the concentration determined from the standard-curve must be multiplied by the dilution factor (50x). Any sample reading greater than the highest standard should be further diluted appropriately with Assay Buffer and retested. Therefore, if the pre-diluted samples have been further diluted, the concentration determined from the standard curve must be multiplied by the further dilution factor.

E.g.; If the pre-diluted sample further diluted in a ratio of 1:10 then results should be multiplied by 500.

5. Automated method: Computer programs can also generally give a good fit.

Typical Calibration Curve

(Example. Do not use for calculation!)



Standard	Concentration (ng/mL)	Mean OD 450/650
A	300	2,291
B	100	1,383
C	30	0,709
D	10	0,376
E	3	0,255
F	0	0,035

Assay Characteristics

1. Specificity: There is no cross reaction with native serum immunoglobulins and tested monoclonal antibodies such as infliximab (Remicade®), adalimumab (Humira®), trastuzumab (Herceptin®) and bevacizumab (Avastin®)**.**

2. Sensitivity: The lowest detectable level that can be distinguished from the zero standard is 60 pg/mL.

3. Precision of Kit:

Intra-assay CV: <15% for denosumab range 300-3 ng/ml
Inter-assay CV: <15% for denosumab range 300-3 ng/ml

4. Recovery: Recovery rate was found to be between 85-115% with normal human serum samples with known concentrations.

Automation

Experiments have shown that the Elisa Genie Denosumab ELISA is also suitable to run on an automated ELISA processor.