KRIBIOLISA™ Denosumab (PROLIA™) (free and partially bound drug) ELISA

REF : KBI1026-2

Ver 2.0

RUO

Enzyme Immunoassay for the Quantitative Determination of Denosumab (free and partially bound complex) in human serum and plasma.

RUO	**For Research Use Only**	**REF**	**Catalog Number**
	**Store At**	**LOT**	**Batch Code**
	**Manufactured By**		**Biological Risk**
	**Expiry Date**		**Consult Operating Instructions**

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REF KBI1026-2 Σ 96 tests

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Introduction:

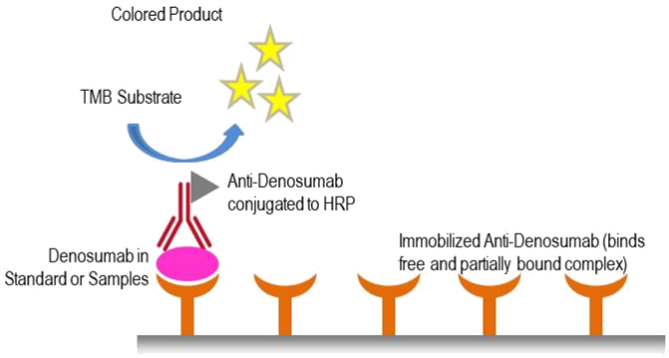
Denosumab (trade names Prolia™ and Xgeva™) is a human monoclonal antibody for the treatment of osteoporosis, treatment-induced bone loss, metastases to bone, and giant cell tumor of bone. Denosumab is contraindicated in people with low blood calcium levels. The most common side effects are joint and muscle pain in the arms or legs. Denosumab is a RANKL inhibitor, which works by preventing the development of osteoclasts which are cells that break down bone (bone resorption).

Intended Use:

The KRIBIOLISA™ Denosumab (PROLIA) ELISA is used as an analytical tool for quantitative determination of Denosumab (PROLIA) in human serum and plasma. Denosumab is a fully human monoclonal antibody that binds the cytokine RANKL (receptor activator of NFκB ligand). The capture antibody in the KRIBIOLISA™ Denosumab ELISA kit inhibits the binding of Denosumab to RANKL leading to the kit estimating only the free and partially bound Denosumab in samples.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Antibodies to Denosumab free drug are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Denosumab present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated anti-Denosumab antibody is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Denosumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
Anti-Denosumab Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Denosumab free drug monoclonal antibody.	1 x 96 wells
Denosumab Standard	Recombinant Denosumab in a buffered protein base with preservative sodium azide (lyophilized, 1 ug/ml)	2 vials
Anti-Denosumab:HRP Conjugate	Anti-Denosumab conjugated to Horseradish Peroxidase with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
(1X) Sample Diluent	Buffered protein base with preservative thiomersol < 0.01%	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with 1:1000 dilution human serum and preservative sodium azide < 0.01%	10 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersol < 0.01%. May turn yellow over time.	25 ml
TMB Substrate	Stabilized Chromogen	12 ml
Stop Solution	2N Sulfuric Acid	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul
3. Deionized (DI) water

4. Wash bottle or automated microplate washer
5. Graph paper or software for data analysis
6. Timer
7. Absorbent Paper

Handling/Storage:

1. All reagents should be stored at 2ºC to 8ºC for stability.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.

Sample Preparation and Storage:

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20°C.

Preparation Before Use:

Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

Test Sample Preparation - Samples have to be diluted 1:1000 (v/v), e.g. 1 µl sample + 999ul (1X) Sample diluent prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires - 20°C.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. To make Wash Buffer (1X); dilute 25 ml of 20X Wash Buffer in 475 ml of DI water.
4. **Standards Preparation**: Reconstitute the lyophilized standard in 1 ml Standard diluent to get a concentration of 1 ug/ml. Dilute 640 ul of original **Standard (1 ug/ml)** with 360 ul of Standard Diluent to generate a **640 ng/ml Standard Solution**. Prepare further **Standards** by diluting the Standard Solution as per the below table. Use the Standard Diluent as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Dilution Particulars
1 ug/ml	Lyophilized Standard	Lyophilized standard + Reconstitute in 1 ml Standard Diluent
640 ng/ml	Standard No.7	640 ul Reconstituted Standard (1 ug/ml) + 360 ul Standard Diluent
320 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent
160 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent
80 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent
40 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent
20 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent
10 ng/ml	Standard No.1	500 ul Standard No.2 + 500 ul Standard Diluent
0 ng/ml	Standard No.0	Only Standard Diluent

Use the Standards immediately upon reconstitution. Discard balance standard after use. Do not store them for further experiments.

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Denosumab (PROLIA). High Dose Hook Effect is due to excess of antibody for very high concentrations of Denosumab (PROLIA) present in the sample.
3. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Denosumab (PROLIA).
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay. All steps must be performed at 37°C

2. Pipette **100 ul** of **prepared Standards** or **diluted Samples** into the respective wells.

3. Cover the plate and incubate for 60 minutes at 37°C

4. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

5. Pipette without delay in the same order **100 ul** of **Anti-Denosumab:HRP Conjugate** into each well.

6. Cover the plate and incubate for 60 minutes at 37°C

7. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

8. Add **100 ul** of **TMB Substrate** in each well.

9. Incubate the plate at 37°C for 30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

10. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.

11. Read the absorbance at 450 nm with a microplate reader.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Denosumab concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve.

At the point of intersection, draw a vertical line to the X-axis and read the Denosumab (free drug) Concentration. If samples were diluted, multiply by the appropriate dilution factor.

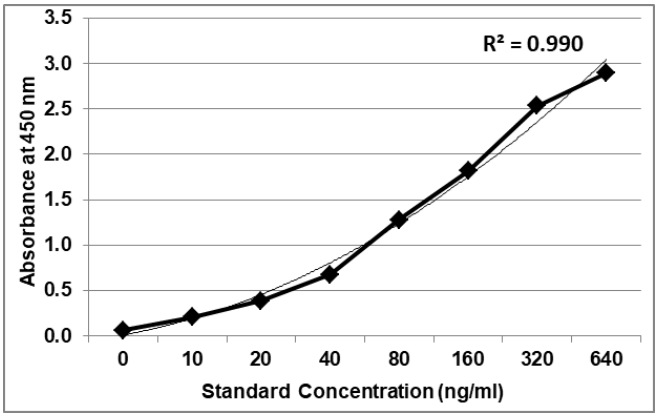
Software which is able to generate a cubic spline curve-fit or 4PL (2nd order polynomial) is best recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 640 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.056	0.065	0.060	0.1	--
10	0.186	0.226	0.206	9.8	97.9
20	0.417	0.360	0.388	20.5	102.5
40	0.682	0.670	0.676	38.3	95.6
80	1.246	1.310	1.278	84.9	106.2
160	1.834	1.800	1.817	149.7	93.6
320	2.569	2.504	2.536	340.7	106.5
640	2.862	2.931	2.897	619.5	96.8

Typical Graph



Abs = absorbance @450nm

Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Performance Characteristics of the Kit:

This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

Sensitivity:
Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was found to be less than 8 ng/ml
Limit of Quantification: The working range for a method is defined by the lower and upper limits of quantification (LLOQ and ULOQ, respectively). The LLOQ and ULOQ can be defined as the endpoints of an interval in which the %CV is 20%. LLOQ for the KRIBIOLISA Denosumab ELISA is 5 ng/ml and the ULOQ is 1280 ng/ml.

Specificity:
The capture antibody used in the kit has been validated to measure only the free and partially bound Denosumab complex as it inhibits the binding of Denosumab to RANKL. The standard used in the kit has been calibrated against commercially sourced Prolia Injection[1].

Dilutional Linearity:
Dilution linearity is performed to demonstrate that a sample with a spiked concentration above the ULOQ can be diluted to a concentration within the working range and still give a reliable result. Neat Sera was spiked with 1280 ug/ml of Denosumab. Serial Dilution from 1280 ug/ml to 640 ug/ml, 320 ug/ml, 160 ug/ml, 80 ug/ml, 40 ug/ml, 20 ug/ml, 10 ug/ml, 5 ug/ml, 2.5 ug/ml, 1.25 ug/ml was prepared with Standard Diluent. Then as indicated in IFU Assay Procedure, each sample was subsequently diluted 1:1000. These ug/ml diluted as ng/ml was then run.

Spiked Sample + 1:1000 diluted Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration (ng/ml)	% Interpolated Concentration against Actual Concentration	% STD Deviation	CV	% CV
0	0.064	0.057	0.060	1.5	--	0.4	0.07	7.4
1.25	0.065	0.065	0.065	1.9	155.6	0.0	0.00	0.4
2.5	0.081	0.096	0.088	3.9	154.1	1.1	0.12	12.4
5	0.118	0.102	0.110	5.6	112.2	1.2	0.11	10.7
10	0.107	0.119	0.113	5.8	58.5	0.8	0.07	7.5
20	0.220	0.257	0.238	15.8	79.2	2.7	0.11	11.1
40	0.504	0.596	0.550	47.0	117.5	6.5	0.12	11.8
80	0.803	0.808	0.806	87.7	109.7	0.4	0.00	0.5
160	1.012	0.949	0.981	133.8	83.6	4.5	0.05	4.5
320	1.149	1.447	1.298	362.2	113.2	21.1	0.16	16.2
640	1.247	1.495	1.371	523.0	81.7	17.6	0.13	12.8
1280	1.502	1.499	1.500	'---	'---	0.2	0.00	0.1

Original concentration (ug/ml)	Interpolated Concentration (ng/ml)	Dilution factor	Interpolated Final Concentration (ng/ml)	Interpolated Final Concentration (ug/ml)	% Recovery
0	1.5	1000.0	1509.6	1.5	--
1.25	1.9	1000.0	1945.1	1.9	155.6
2.5	3.9	1000.0	3851.4	3.9	154.1
5	5.6	1000.0	5608.1	5.6	112.2
10	5.8	1000.0	5845.4	5.8	58.5
20	15.8	1000.0	15844.2	15.8	79.2
40	47.0	1000.0	46992.1	47.0	117.5
80	87.7	1000.0	87736.2	87.7	109.7
160	133.8	1000.0	133821.2	133.8	83.6
320	362.2	1000.0	362167.2	362.2	113.2
640	523.0	1000.0	523010.5	523.0	81.7
1280	---	---	---	---	---

Precision:
Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (10ng/ml), medium (80ng/ml) and high (640ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

Pool	Intra Assay %CV	Inter Assay %CV
Low	<10%	<12%
Medium	<10%	<10%
High	<10-%	<10%

Safety Precautions:

- **This kit is For Research Use only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (< 0.1 % w/w) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials maybe derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
  - Do not smoke, eat or drink while handling kit material
  - Always use protective gloves
  - Never pipette material by mouth
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

[1]Prolia Injection is the registered trade mark of Amgen Inc.

References:

Denosumab versus zoledronic acid in patients previously treated with zoledronic acid
AD Anastasilakis, SA Polyzos, A Gkiomisi… - Osteoporosis …, 2015 - Springer

Population pharmacokinetic analysis of denosumab in patients with bone metastases from solid tumours
L Gibiansky, L Sutjandra, S Doshi, J Zheng… - Clinical …, 2012 - Springer

Prompt clinical and biochemical response to denosumab in a young adult patient with craniofacial fibrous dysplasia
C Eller-Vainicher, DS Rossi, G Guglielmi… - Clinical Cases in …, 2016 - ncbi.nlm.nih.gov

Effects of denosumab treatment on the expression of receptor activator of nuclear kappa-B ligand (RANKL) and TNF-receptor TNFRSF9 after total hip …
C Sköld, K Kultima, E Freyhult, A Larsson… - Osteoporosis …, 2022 - Springer

Population pharmacokinetic meta-analysis of denosumab in healthy subjects and postmenopausal women with osteopenia or osteoporosis
L Sutjandra, RD Rodriguez, S Doshi, M Ma… - Clinical …, 2011 - Springer

SCHEMATIC ASSAY PROCEDURE

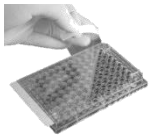
1. Remove all components, 30 minutes before adding into the assay plate.

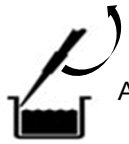
Components NOW (2-8ºC) → 30 mins RT Before Use →  → Thaw at Room Temperature (18-24ºC) → USE

2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

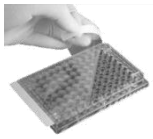
Kit Components → AVOID → COOL (2-8ºC) → THAW (22±4ºC) → THAW (22±4ºC) → COOL (2-8ºC) → AVOID → NO CHANGE IN RESULTS

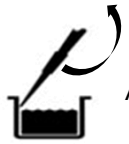
3.  Pipette **100 ul prepared Standards** / **diluted Samples** into each well.

4. Cover plate  and incubate for  at 37°C.

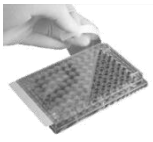
5.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**

6.  Pipette **100 ul Anti-Denosumab:HRP** into each well.

7. Cover plate  and incubate for  at 37°C.

8.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**

9.  Pipette **100 ul TMB Substrate** into each well.

10. Cover plate  and incubate for  at 37°C.

11.  Pipette **100 ul Stop Solution** into each well.

12. Read absorbance at 450nm with a  microplate reader within  of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml Denosumab (PROLIA) equivalent
1A 2A	zero std zero std			
1B 2B	10 ng/ml 10 ng/ml			
1C 2C	20 ng/ml 20 ng/ml			
1D 2D	40 ng/ml 40 ng/ml			
1E 2E	80 ng/ml 80 ng/ml			
1F 2F	160 ng/ml 160 ng/ml			
1G 2G	320 ng/ml 320 ng/ml			
1H 2H	640 ng/ml 640 ng/ml			
3A 4A	Sample			
3B 4B	Sample			

LIMITED WARRANTY

Krishgen Biosystems does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the Products; against defects in products or components not manufactured by Krishgen Biosystems, or against damages resulting from such non-Krishgen Biosystems made products or components. Krishgen Biosystems passes on to customer the warranty it received (if any) from the maker thereof of such non Krishgen made products or components. This warranty also does not apply to Products to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Krishgen Biosystems.

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