KRIBIOLISA™ Anti-Denosumab (PROLIA™ / XGEVA™) ELISA

REF : KBI2026

Ver 2.3

RUO

Enzyme Immunoassay for the Quantitative Determination of Anti-Denosumab 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 KBI2026 Σ 96 tests

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Introduction:

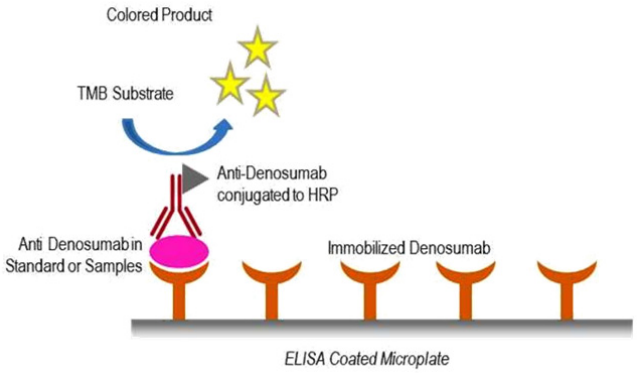
Denosumab (trade names Prolia and Xgeva) is a human monoclonal antibody for the treatment of osteoporosis, treatment-induced bone loss, metastases to bone, and giant cell tumor of bone. Denosumab is contraindicated in people with low blood calcium levels. The most common side effects are joint and muscle 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™ Anti- Denosumab ELISA is used as an analytical tool for quantitative determination of Anti-Denosumab in human serum and plasma.

Principle:

The method employs the sandwich immunoassay technique. Denosumab is pre-coated onto microwells. Samples or standards are pipetted into microwells and antibodies to Denosumab present in the sample are bound by the capture antibody. After washing microwells, HRP conjugate is pipetted and incubated. Free HRP conjugate will be removed by washing cycle. The ready to use substrate solution (TMB) is added to microwells and color develops directly proportionally to the amount of Anti- Denosumab present in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
Denosumab Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Denosumab monoclonal antibody.	1 x 96 wells
Anti- Denosumab Standard	Lyophilized Anti- Denosumab in a buffered protein base and preservative sodium azide < 0.01% - (lyophilized, concentrated 1 ug/ml)	2 vials
Denosumab:HRP Conjugate concentrated	Denosumab conjugated to Horseradish Peroxidase concentrated (1 mg/ml)	1 vial
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
(1X) Sample Diluent	Buffered protein base with preservative sodium azide < 0.01%	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with preservative sodium azide < 0.01% with 1:1000 dilution of human serum	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	0.73M Phosphoric 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. for 1:1000 (1 ul sample + 999 ul sample diluent) prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires the samples to be kept at -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 concentrated Standard lyophilized vial with 1 ml of Standard Diluent to obtain a concentration of 1 ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. This is the top standard. 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 provided in the Kit + 1 ml of Standard Diluent (1X)
1000 ng/ml	Standard No.6	1000 ul of reconstituted original Standard
750 ng/ml	Standard No.5	750 ul Standard No.6 + 250 ul Standard Diluent (1X)
500 ng/ml	Standard No.4	666.7 ul Standard No.5 + 333.3 ul Standard Diluent (1X)
250 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
125 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
62.5 ng/ml	Standard No.1	500 ul Standard No.2 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No.0	Only Standard Diluent (1X)

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

5. **Working Denosumab:HRP Conjugate – Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

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 Anti- Denosumab.
3. High Dose Hook Effect is due to excess of antibody for very high concentrations of Anti- Denosumab present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Anti- Denosumab concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
4. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Anti- Denosumab.
5. It is recommended that all Standards and Samples be assayed in duplicates.
6. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
7. 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.
8. The plates should be read within 30 minutes after adding the Stop Solution.
9. 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. Add **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 no soak time 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. Add **100 ul** of **working 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 no soak time 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:

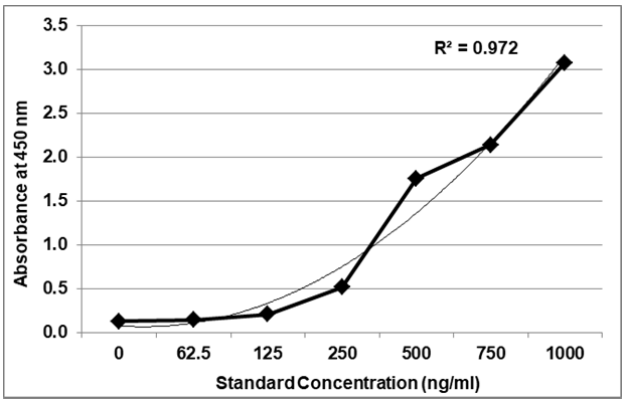
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Anti- 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 Anti-Denosumab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit 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 1000 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.138	0.116	0.127	60.1	--
62.5	0.148	0.139	0.143	71.3	114.1
125	0.227	0.184	0.206	106.4	85.1
250	0.534	0.495	0.514	220.5	88.2
500	1.786	1.729	1.757	560.8	112.2
750	2.099	2.178	2.139	674.9	90.0
1000	2.882	3.272	3.077	1044.0	104.4

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.

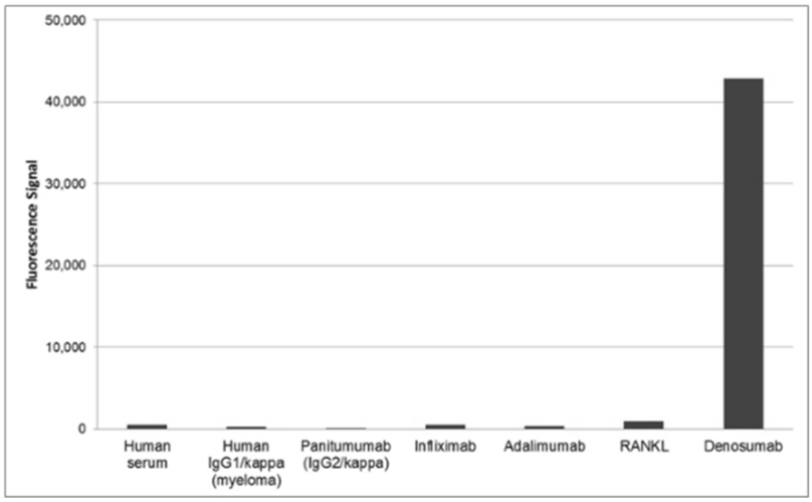
Sensitivity:
Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was 58 ng/ml.

Limit Of Quantification:
It is defined as the lowest detectable concentration that can be determined with an acceptable repeatability and the LOQ was found to be 60 ng/ml.

Specificity:
The capture antibody used in the kit demonstrates 100% cross reactivity to Alvotech's Denosumab biosimilar and the therapeutic reference innovator product. The antibody is a fully human IgG2 monoclonal antibody specific to receptor activator of nuclear factor kappa-B ligand (RANKL) expressed in mammalian cells.

The standard / calibrator used is a paratope specific anti-idiotypic antibody that specifically recognizes the free human monoclonal antibody denosumab. The antibody does not recognize free RANKL (receptor activator of nuclear factor kappa-B ligand) or denosumab in complex with human RANKL. This ensures a high degree of accuracy as an ADA standard.

Cross Reactivity:



The standard is highly specific to Denosumab and does not bind to or cross react with Human IgG1kappa, Pantiumumab, Infliximab, Adalimumab and RANKL ensuring accurate interpretation of the unknown concentrations when extrapolated on the graph.

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 (62.5 ng/ml), medium (250 ng/ml) and high (1000 ng/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	<12%	<14%
Medium	<8%	<10%
High	<8%	<10%

Lot to Lot Variation:

Standard Concentration (ng/ml)	Lot A OD450	Lot B OD450	Lot C OD450	Mean OD450	% STD DEV	CV	% CV
0	0.085	0.094	0.086	0.088	---	---	---
62.5	0.128	0.153	0.131	0.137	1.1	0.10	10.0
125	0.297	0.299	0.215	0.270	3.9	0.18	17.8
250	0.636	0.775	0.643	0.685	6.4	0.11	11.5
500	1.756	1.377	1.659	1.597	16.1	0.12	12.3
750	2.301	2.396	2.313	2.337	4.2	0.02	2.2
1000	3.024	3.020	3.055	3.033	1.6	0.01	0.6

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.

References:

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BL Langdahl, CS Teglbjærg, PR Ho… - The Journal of …, 2015 - academic.oup.com

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SCHEMATIC ASSAY PROCEDURE

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

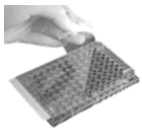
Components (2-8ºC) → 30 mins RT Before Use → 

Thaw at Room Temperature (18-24ºC) → USE NOW

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

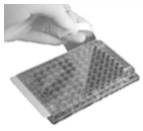
3.  Pipette **100 ul prepared Standards** / **diluted Samples** into the respective wells.

4. Cover plate  and incubate for 

at 37°C.

5.  Aspirate and wash wells 4 times with **Wash Buffer (1X) (no soak time).**

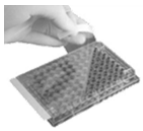
6.  Pipette **100 ul working Denosumab:HRP** into each well.

7. Cover plate  and incubate for 

at 37°C

8.  Aspirate and wash wells 4 times with **Wash Buffer (1X). (no soak time)**.

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.

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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This Limited Warranty states the entire obligation of Krishgen Biosystems with respect to the Products. If any part of this Limited Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.

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SYMBOLS KEY

MTP	**Denosumab Coated Microtiter Plate (12 x 8 wells)**
STD	**Anti-Denosumab Standard**
HRP CONJ	**Denosumab:HRP conjugate concentrated**
DETN DIL	**Detection Diluent**
1X SAMP DIL	**(1X) Sample Diluent**
1X STD DIL	**(1X) Standard Diluent**
20X WASH BUF	**(20X) Wash Buffer**
SUB TMB	**TMB Substrate**
SOLN STOP	**Stop Solution**
	**Consult Instructions for Use**
REF	**Catalog Number**
	**Expiration Date**
	**Storage Temperature**