

# Instructions for Use

Version: 2.0.0

Revision date: 03 Oct 2023



## Denosumab ELISA Kit

**Catalog No:** abx395209

**Size:** 96T

**Range:** 78 ng/ml - 5000 ng/ml

**Sensitivity:** < 40.7 ng/ml

**Storage:** Store the 96-well plate, Standards, and Detection Reagent(s) at -20°C, and the rest of the kit components at 4°C.

**Application:** The quantitative detection of Denosumab in Human serum and plasma.

**Principle of the Assay:** This kit is based on competitive enzyme-linked immuno-sorbent assay technology. An antigen is pre-coated onto a 96-well plate. Standards, test samples, and biotin-conjugated reagent are added to the wells and incubated. A competitive inhibition reaction takes place between the biotin-labelled Denosumab and the unlabelled-Denosumab on the pre-coated antigen. The HRP-conjugated reagent is then added, and the whole plate is incubated. Unbound conjugates are removed using wash buffer at each stage. TMB substrate is used to quantify the HRP enzymatic reaction. After TMB substrate is added, only wells that contain sufficient Denosumab will produce a blue coloured product, which then changes to yellow after adding the acidic stop solution. The intensity of the color yellow is inversely proportional to the Denosumab amount bound on the plate. The OD is measured spectrophotometrically at 450 nm in a microplate reader, from which the concentration of Denosumab can be calculated.

### Kit Components

- Pre-coated 96-Well Microplate: 12 x 8
- Standard: 2 tubes
- Wash Buffer: (20X) 25 ml
- Standard Diluent Buffer: 25 ml
- Detection Reagent A: (100X) 60 µl
- Detection Reagent B: (100X) 120 µl
- Reagent Diluent: 25 ml
- TMB Substrate: 12 ml
- Stop Solution: 6 ml
- Plate Sealer: 3
- Hermetic Bag: 1

### Materials Required But Not Provided

- 37°C incubator
- Multi and single channel pipettes and sterile pipette tips
- Squirt bottle or automated microplate washer
- 1.5 ml tubes
- Distilled water
- Absorbent filter papers
- 100 ml and 1 liter graduated cylinders
- Microplate reader (wavelength: 450 nm)
- ELISA Shaker

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## Protocol

### A. Sample Preparation

Analyse immediately or store samples at 2-8°C (within 24 hrs). For long term storage, aliquot and store at -20°C or -80°C. Avoid multiple freeze-thaw cycles.

- **Serum:** Samples should be collected into a serum separator tube. Coagulate the serum by leaving the tube undisturbed in a vertical position overnight at 4°C or at room temperature for up to 1 hr. Centrifuge at approximately 1000 × g for 20 mins. If precipitate appears, centrifuge again. Assay immediately or aliquot and store at -20°C or -80°C.
- **Plasma:** Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 mins at 1000 × g, within 30 mins of collection. If precipitate appears, centrifuge again. Avoid hemolytic samples.

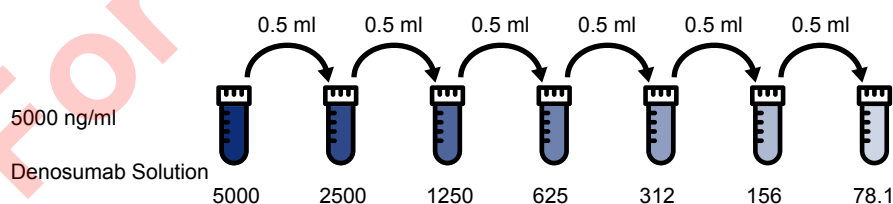
### Notes:

- Samples must be diluted so that the expected concentration falls within the kit's range. **Serum/Plasma samples require approximately a 10-fold dilution.**
- Always use non-pyrogenic, endotoxin-free tubes for blood collection.
- Fresh samples, or recently obtained samples, are recommended to prevent protein degradation and denaturation that may lead to erroneous results.
- NaN<sub>3</sub> cannot be used as a test sample preservative, since it inhibits HRP.
- If possible, prepare solid samples using sonication and/or homogenization, as lysis buffers may (on occasion) interfere with the kit's performance.
- If a sample is not indicated in the manuals applications, a preliminary experiment to determine the suitability of the kit will be required.

### B. Reagent Preparation

**Standard:** Prepare the Standard with 1 ml of Standard Diluent buffer to make the 5000 ng/ml Standard Solution. This is the highest standard. Allow the reconstituted standard to sit for 10 mins, with gentle agitation prior to carrying out the serial dilutions. Avoid foaming or bubbles. Label tubes in preparation for the serial dilutions - see the diagram below for reference. Aliquot 0.5 ml of the Standard Diluent Buffer into each tube (apart from the highest standard tube). Add 0.5 ml of the highest standard solution into the 1st tube and mix thoroughly. Transfer 0.5 ml from the 1st to 2nd tube, mix thoroughly, and so on.

**Note:** Do not vortex the standard during reconstitution, as this will destabilize the protein. Once the standard has been reconstituted, it should be used within 15 mins. It is not recommended to reuse the reconstituted standard.



**Wash Buffer:** Dilute the concentrated Wash buffer 20-fold (1/20) with distilled water (i.e. add 20 ml of concentrated wash buffer into 380 ml of distilled water). If crystals have formed in the concentrated Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.

**Detection Reagent A Working Solution Preparation:** Prepare no more than 1 hr before the experiment.

1. Calculate the total volume of working solution required.
2. Dilute Detection Reagent A 100-fold with Reagent Diluent, and mix thoroughly. Pipette with a slow, smooth action to reduce volume errors.

**Detection Reagent B Working Solution Preparation:** Prepare no more than 15 mins before the experiment.

1. Calculate the total volume of working solution required.

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2. Dilute Detection Reagent B 100-fold with Reagent Diluent, and mix thoroughly. Pipette with a slow, smooth action to reduce volume errors.

## C. Assay Protocol

Prepare all standards, samples and reagents as directed above. Equilibrate the kit components and samples to room temperature prior to use. It is recommended to measure in duplicate, and to plot a standard curve for each test.

1. Set standard, test sample and control (zero) wells on the pre-coated plate respectively, and record their positions. *Add the solution to the bottom of each well without touching the side walls. Pipette the standards and samples up and down to mix before adding to the wells. Avoid foaming or bubbles.*
2. Prepare standard, sample and control (zero) tubes and label accordingly.
3. Aliquot 50 µl of the diluted standards into the standard tubes.
4. Aliquot 50 µl of appropriately diluted sample into the test sample tubes.
5. Aliquot 50 µl of Detection Reagent A Working Solution to all tubes and mix thoroughly.
6. Aliquot 100 µl of the premixed solutions to the Standard, Control (zero) and Sample wells respectively. Gently tap the plate to mix, or use a microplate shaker. Seal the plate and incubate for 2 hours at 37°C
7. Remove the cover and discard the solution. Wash the plate 3 times with 1X Wash Buffer. *Fill each well completely with Wash buffer (300 µl) using a multi-channel Pipette or autowasher (1-2 mins soaking period is recommended). Complete removal of liquid at each step is essential for good performance. After the final wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean absorbent paper towels.*
8. Aliquot 100 µl of Detection Reagent B working solution to each well. Seal the plate and incubate for 30 mins at 37°C.
9. Remove the cover, discard the solution and repeat the wash process as described above, 5 times.
10. Aliquot 100 µl of TMB Substrate into each well. Cover the plate with the plate sealer. Gently tap the plate to mix thoroughly. Incubate at 37°C for 10-20 minutes. The incubation time is for reference only, the optimal time should be determined by end user. Avoid exposure to light.
11. Aliquot 50 µl of Stop Solution into each well. It is important that the Stop Solution is mixed quickly and uniformly throughout the microplate to inactivate the enzyme completely.
12. Ensure that there are no fingerprints or water on the bottom of the plate, and that the fluid in the wells is free of bubbles. Measure the OD at 450 nm immediately.

This assay is competitive, therefore there is an inverse correlation between the concentration of the sample and the OD measured. Create a graph with the log of the standard concentration (Y) and OD measured (X). Apply a best fit trendline through the standard points. Use this graph to calculate sample concentrations based on their OD values. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

### Precautions:

- Before using the kit, centrifuge the tubes to bring down the contents trapped in the lid.
- Do not leave the wells uncovered for extended periods between incubations. The addition of reagents for each step should not exceed 10 mins.
- Ensure that the plate is properly sealed or covered during the incubation steps, and that the time and temperature are controlled.
- Do not reuse pipette tips and tubes.
- Do not use expired components, or components from a different kit.
- The TMB substrate should be used under sterile conditions, and light exposure should be minimised. Unused substrate should be colorless, or a very light yellow in appearance. Do not discard any residual solution back into the vial.
- Please note that this kit is optimised for detection of native samples, rather than recombinant proteins or synthetic chemicals. We are unable to guarantee detection of recombinant proteins, as they may have different sequences or tertiary structures to the native protein.

### Precision:

Intra-assay Precision (Precision within an assay): 3 samples with low, medium and high levels of Denosumab were tested 20 times on one plate, respectively.

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Inter-assay Precision (Precision between assays): 3 samples with low, medium and high levels of Denosumab were tested on 3 different plates, 8 replicates in each plate.

CV (%) = (Standard Deviation / Mean) × 100

Intra-Assay: CV < 20%

Inter-Assay: CV < 20%

For Reference Only