

Quantikine[®]

Mouse Dkk-1 Immunoassay

Catalog Number MKK100

For the quantitative determination of mouse Dickkopf related protein 1 (Dkk-1) concentrations in cell culture supernates, mouse serum, and plasma.

This package insert must be read in its entirety before using this product.

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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INTRODUCTION

Dickkopf related protein 1 (Dkk-1) is the founding member of the Dickkopf family of secreted proteins that includes Dkk-1, -2, -3, -4, and a related protein, Soggy (1, 2). Dickkopf, meaning 'big head' or 'stubborn', was discovered as an inducer of head formation in *Xenopus* (3). Dkk proteins contain two conserved cysteine-rich domains separated by a linker region (1 - 3). The C-terminal domain, which contains a colipase fold with a conserved pattern of ten cysteine residues, is necessary and sufficient for Wnt inhibition (4, 5). Mature mouse Dkk-1 is a 40 kDa glycosylated protein that shows 95%, 85%, 81%, and 83% amino acid (aa) sequence identity with rat, human, bovine, and rabbit Dkk-1, respectively. It also shows 42% and 38% aa sequence identity with mouse Dkk-2 and Dkk-4, respectively, with similarity mainly within the cysteine-rich domains.

Dkk-1 and Dkk-4 are well-documented antagonists of the canonical Wnt signaling pathway (1, 2). This pathway is activated by Wnt engagement of a receptor complex composed of the Frizzled proteins and one of two low-density lipoprotein receptor-related proteins, LRP-5 or LRP-6 (6). Dkk-1 antagonizes Wnt by forming ternary complexes of LRP-5/6 with Kremen-1 or Kremen-2 (7). Internalization of the Dkk-1/LRP-6/Kremen-2 complex downregulates Wnt signaling (6, 7). Dkk-1 has also been proposed to have Wnt-independent activity in some human cancer cell lines (8, 9). Dkk-1 is expressed throughout embryogenesis and antagonizes Wnt-7a during limb development, in developing neurons, keratinocytes, hair follicles, and the retina of the eye (10 - 14). Postnatal Dkk-1 is expressed mainly by osteoblasts and osteocytes (14).

The balance between Wnt signaling and Dkk-1 inhibition is critical for bone formation and homeostasis. Insufficient or excess Dkk-1 activity in bone results in increased or decreased bone density, respectively (14 - 16). High Dkk-1 expression has been shown and may be pathogenic in conditions where bone is eroded, such as rheumatoid arthritis, multiple myeloma, Paget's disease, and glucocorticoid-induced osteoporosis (17 - 22). Although the main phenotypes of experimental Dkk-1 deficiency are bone-related, it is important for regulating Wnt activity in other areas as well. Activity in the nervous system is indicated by the requirement of Dkk-1 expression for neural differentiation of mouse embryonic stem cells and for ischemic neuronal death (12, 23). Dkk-1 also regulates skin pigmentation and thickness by controlling Wnt signaling in melanocytes (13). Activation of Wnt by repression of Dkk-1 activity may be a factor in oncogenic transformation, for example, by the oncogene *c-myc* in mammary epithelial cell transformation or in human colon cancer (24, 25).

The Quantikine Mouse Dkk-1 immunoassay is a 4.5 hour solid-phase ELISA designed to measure Dkk-1 in cell culture supernates, mouse serum, and plasma. It contains NS0-expressed recombinant mouse Dkk-1 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant mouse Dkk-1. Results obtained using natural mouse Dkk-1 showed dose response curves that were parallel to the standard curves obtained using the Quantikine mouse kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse Dkk-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Dkk-1 has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any mouse Dkk-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse Dkk-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse Dkk-1 bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with the Calibrator Diluent and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins and other factors present in biological samples. Until all factors have been tested, however, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- For best results, pipette reagents and samples into the center of each well.
- The samples must be added to the plate within 15 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

MATERIALS PROVIDED

Mouse Dkk-1 Microplate (Part 893325) - One 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse Dkk-1.

Mouse Dkk-1 Conjugate (Part 893326) - 12 mL of a polyclonal antibody specific for mouse Dkk-1 conjugated to horseradish peroxidase with preservatives.

Mouse Dkk-1 Standard (Part 893327) - 2 vials (8 ng/vial) of recombinant mouse Dkk-1 in a buffered protein base with preservatives; lyophilized.

Mouse Dkk-1 Control (Part 893328) - 2 vials of recombinant mouse Dkk-1 in a buffered protein base with preservatives; lyophilized. The concentration range of mouse Dkk-1 after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

Assay Diluent RD1-21 (Part 895215) - 12 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5-26 Concentrate (Part 895525) - 21 mL of diluted buffered protein solution with preservatives.

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of diluted hydrochloric acid.

Plate Covers (Part 640197) - 4 adhesive strips.

STORAGE

| Unopened Kit | Store at 2 - 8° C. Do not use past kit expiration date. | |
|--------------------------------------|---|--|
| Opened/ Reconstituted Reagents | Mouse Dkk-1 Conjugate | May be stored for up to 1 month at 2 - 8° C.* |
| | Diluted Wash Buffer | |
| | Stop Solution | |
| | Calibrator Diluent RD5-26 | |
| | Assay Diluent RD1-21 | |
| | Unmixed Color Reagent A | |
| | Unmixed Color Reagent B | |
| | Mouse Dkk-1 Standard (4000 pg/mL) | Discard after use. Use a fresh Standard and Control for each assay. |
| | Mouse Dkk-1 Control | |
| | Microplate Wells | Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.* |

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 1000 mL graduated cylinders.
- **Polypropylene tubes for dilution.**

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging. Centrifuge for 20 minutes at approximately 2000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at approximately 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Mouse serum and plasma samples require a 10-fold dilution into Calibrator Diluent RD5-26 (1X). A suggested 10-fold dilution is 20 μ L of sample + 180 μ L of Calibrator Diluent RD5-26 (1X).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse Dkk-1 Control - Reconstitute the Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

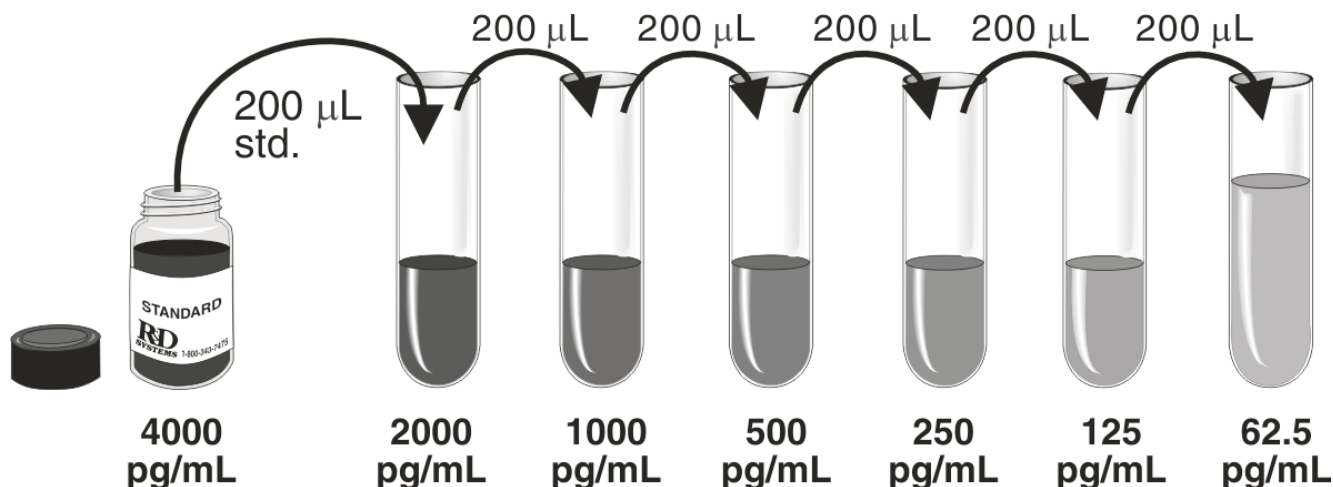
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

Calibrator Diluent RD5-26 (1X) - Dilute 20 mL of Calibrator Diluent RD5-26 Concentrate into 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (1X).

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 μ L of the resultant mixture is required per well.

Mouse Dkk-1 Standard - Reconstitute the mouse Dkk-1 Standard with 2.0 mL of Calibrator Diluent RD5-26 (1X). Do not substitute other diluents. This reconstitution produces a stock solution of 4000 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 μ L of Calibrator Diluent RD5-26 (1X) into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The undiluted mouse Dkk-1 Standard serves as the high standard (4000 pg/mL). Calibrator Diluent RD5-26 (1X) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, control, and standards be assayed in duplicate.

1. Prepare all reagents, standard dilutions, control, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 μ L of Assay Diluent RD1-21 to each well.
4. Add 50 μ L of Standard, Control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 μ L) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 μ L of mouse Dkk-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 μ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 μ L of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Serum and plasma samples require dilution. See the Sample Preparation section.

PROCEDURE SUMMARY AND CHECKLIST

1. Bring all reagents to room temperature.
 Prepare reagents and samples as instructed.
 Return unused components to storage temperature as indicated in the instructions.
2. Add 50 μL Assay Diluent RD1-21 to each well.
3. Add 50 μL Standard, Control, or sample* to each well.
 Cover the plate and incubate for 2 hours at room temperature.
4. Aspirate and wash each well five times.
5. Add 100 μL Conjugate to each well.
 Cover the plate and incubate for 2 hours at room temperature.
6. Aspirate and wash each well five times.
7. Add 100 μL Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
8. Add 100 μL Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

*Serum and plasma samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

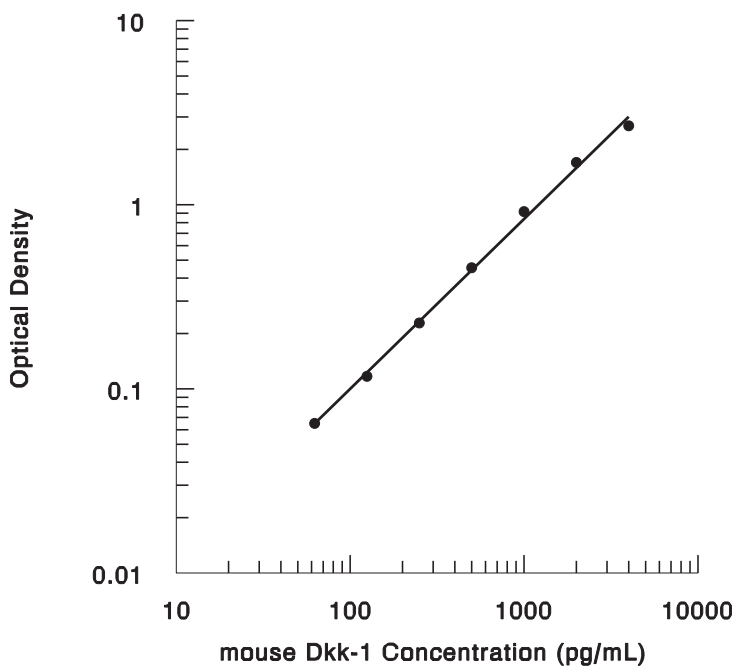
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse Dkk-1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

Because serum and plasma samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



| pg/mL | O.D. | Average | Corrected |
|-------|-------------------------|---------|-----------|
| 0 | 0.070 0.071 0.135 | 0.071 | — |
| 62.5 | 0.136 0.186 | 0.136 | 0.065 |
| 125 | 0.189 0.297 | 0.188 | 0.117 |
| 250 | 0.300 0.524 | 0.299 | 0.228 |
| 500 | 0.528 0.989 | 0.526 | 0.455 |
| 1000 | 0.989 1.750 | 0.989 | 0.918 |
| 2000 | 1.789 2.723 | 1.770 | 1.699 |
| 4000 | 2.791 | 2.757 | 2.686 |

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in forty assays to assess inter-assay precision.

| Sample | Intra-assay Precision | | | Inter-assay Precision | | |
|--------------------|-----------------------|------|------|-----------------------|------|------|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 20 | 20 | 20 | 40 | 40 | 40 |
| Mean (pg/mL) | 230 | 478 | 1487 | 219 | 481 | 1492 |
| Standard deviation | 20.1 | 29.7 | 80.8 | 20.8 | 44.7 | 144 |
| CV (%) | 8.7 | 6.2 | 5.4 | 9.5 | 9.3 | 9.6 |

RECOVERY

The recovery of mouse Dkk-1 spiked to three levels throughout the range of the assay was evaluated.

| Sample Type | Average % Recovery | Range |
|-------------------------------|--------------------|------------|
| Cell culture supernates (n=4) | 99 | 88 - 107% |
| Mouse serum* (n=4) | 113 | 100 - 120% |
| Mouse heparin plasma* (n=4) | 113 | 104 - 119% |
| Mouse EDTA plasma* (n=4) | 109 | 93 - 117% |

*Mouse serum and plasma samples were diluted 20-fold prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing or spiked with high concentrations of mouse Dkk-1 in each matrix were diluted with Calibrator Diluent and assayed.

| | | Cell culture supernates (n=4) | Serum* (n=4) | Heparin plasma* (n=4) | EDTA plasma* (n=4) |
|------|-----------------------|-------------------------------|--------------|-----------------------|--------------------|
| 1:2 | Average % of Expected | 95 | 97 | 101 | 101 |
| | Range (%) | 93 - 99 | 93 - 104 | 95 - 109 | 95 - 107 |
| 1:4 | Average % of Expected | 91 | 96 | 101 | 101 |
| | Range (%) | 85 - 95 | 89 - 110 | 89 - 111 | 90 - 110 |
| 1:8 | Average % of Expected | 89 | 102 | 98 | 105 |
| | Range (%) | 81 - 97 | 94 - 116 | 87 - 108 | 93 - 114 |
| 1:16 | Average % of Expected | 88 | 98 | — | 100 |
| | Range (%) | 81 - 97 | 84 - 117 | — | 90 - 120 |

*Mouse serum and plasma samples were first diluted as described in the Sample Preparation section.

SENSITIVITY

Seventy-nine assays were evaluated and the minimum detectable dose (MDD) of mouse Dkk-1 ranged from 5.1 - 29.6 pg/mL. The mean MDD was 13.4 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant mouse Dkk-1 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for detectable levels of mouse Dkk-1 in this assay.

| | Mean (pg/mL) | Range (pg/mL) | Standard Deviation (pg/mL) |
|------------------------------|-----------------|------------------|-------------------------------|
| Mouse serum* (n=19) | 13,893 | 1610 - 27,564 | 7579 |
| Mouse heparin plasma* (n=19) | 4522 | 1446 - 9458 | 2549 |
| Mouse EDTA plasma* (n=19) | 10,069 | 1616 - 24,500 | 7394 |

*Samples were first diluted as described in the Sample Preparation section.

SPECIFICITY

This assay recognizes both recombinant and natural mouse Dkk-1. The factors listed below were prepared at 40 ng/mL in Calibrator Diluent RD5-26 (1X) and assayed for cross-reactivity. Preparations of the following factors at 40 ng/mL in a mid-range mouse Dkk-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

Dkk-2
Dkk-3
Dkk-4
Dkk-4 (R67S/R70S mutant)
Kremen-1
Kremen-2
LRP-6

Recombinant human Dkk-1 shows 26% cross-reactivity in this assay.

This assay has not been validated for rat samples, however, recombinant rat Dkk-1 shows 94% cross-reactivity.

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

| | | | | | | | | |
|----|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | |
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| 11 | | | | | | | | |
| 12 | | | | | | | | |
| | A | B | C | D | E | F | G | H |

NOTES