

Quantikine[®]

Human Dkk-1 Immunoassay

Catalog Number DKK100

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

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

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2010/02/19

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

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INTRODUCTION

Dickkopf related protein 1 (Dkk-1) is the first identified 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 human Dkk-1 is a 40 kDa glycosylated protein that shows 86%, 87%, 91%, and 90% amino acid (aa) sequence identity with mouse, rat, bovine, and rabbit Dkk-1, respectively. It also shows 42% and 36% aa identity with human 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, LRP5 or LRP6 (6). Dkk-1 antagonizes Wnt by forming ternary complexes of LRP5/6 with Kremen1 or Kremen2 (7). Internalization of the Dkk-1/LRP6/Krm2 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).

Postnatally, 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).

Dkk-1 is also expressed in platelets. Release of platelet Dkk-1 occurs during activation of platelets, including clotting during collection of serum samples. Thus, caution is recommended when interpreting serum Dkk-1 values. Patients with atherosclerosis appear to release more Dkk-1 as a result of platelet activation than normal controls. Dkk-1 has been hypothesized to play a role in platelet-mediated endothelial cell activation leading to plaque formation (26, 27).

The Quantikine Human Dkk-1 immunoassay is a 4.5 hour solid phase ELISA designed to measure human Dkk-1 in cell culture supernates, serum, and plasma. It contains Sf 21-expressed recombinant human Dkk-1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Dkk-1 showed dose-response curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Human Dkk-1 kit can be used to determine relative mass values of naturally occurring human Dkk-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Dkk-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Dkk-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for 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 and color develops in proportion to the amount of Dkk-1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

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 appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, 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 in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Dkk-1 Microplate (Part 893314) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Dkk-1.

Dkk-1 Conjugate (Part 893315) - 21.5 mL of a goat polyclonal antibody against Dkk-1 conjugated to horseradish peroxidase with preservatives.

Dkk-1 Standard (Part 893316) - 20 ng of recombinant human Dkk-1 in a buffered protein solution with preservatives; lyophilized.

Assay Diluent RD1W (Part 895117) - 11 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5-24 (Part 895325) - 21 mL of a buffered protein solution with preservatives.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of a buffered surfactant with preservatives.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895032) - 6 mL of a 2 N sulfuric acid.

Plate Covers - 4 adhesive strips.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1W	
	Calibrator Diluent RD5-24	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Aliquot and store for up to 1 month at $\leq -20^{\circ}$ C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
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.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Human Dkk-1 Controls (optional; available from R&D Systems).
- **Polypropylene** test tubes for dilution.

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

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 - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 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 15 minutes at 1000 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

Use polypropylene tubes.

Cell culture supernate samples require at least a 2-fold dilution. A suggested 2-fold dilution is 200 μ L of sample + 200 μ L of Calibrator Diluent RD5-24 (1:6).

Plasma samples require a 4-fold dilution. A suggested 4-fold dilution is 100 μ L of sample + 300 μ L of Calibrator Diluent RD5-24 (1:2).

Serum samples require an 8-fold dilution. A suggested 8-fold dilution is 50 μ L of sample + 350 μ L of Calibrator Diluent RD5-24 (1:2).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

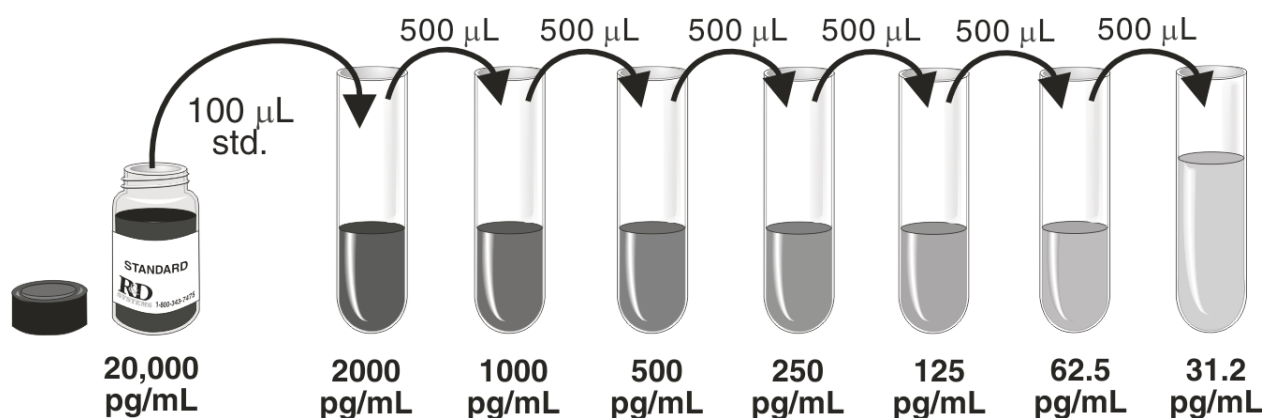
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μ L of the resultant mixture is required per well.

Calibrator Diluent RD5-24 (1:6) (*for use with cell culture supernate samples*) - To prepare Calibrator Diluent RD5-24 (1:6), add 2 mL of Calibrator Diluent RD5-24 to 10 mL of deionized or distilled water.

Calibrator Diluent RD5-24 (1:2) (*for use with serum/plasma samples*) - To prepare Calibrator Diluent RD5-24 (1:2), add 6 mL of Calibrator Diluent RD5-24 to 6 mL of deionized or distilled water.

Dkk-1 Standard - Reconstitute the Dkk-1 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 30 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 μ L of Calibrator Diluent RD5-24 (1:6) (*for cell culture supernate samples*) or Calibrator Diluent RD5-24 (1:2) (*for serum/plasma samples*) into the 2000 pg/mL tube. Pipette 500 μ L of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent 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, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, 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 100 μ L of Assay Diluent RD1W to each well.
4. Add 100 μ L of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four 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 200 μ L of the Dkk-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 μ L of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, 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.

*Samples require dilution. See Sample Preparation section.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and Standards as instructed.



2. Add 100 μL Assay Diluent RD1W to each well.



3. Add 100 μL Standard, control, or sample* to each well. Incubate 2 hours on the shaker at RT.



4. Aspirate and wash 4 times.



5. Add 200 μL Conjugate to each well. Incubate 2 hours on the shaker at RT.



6. Aspirate and wash 4 times.



7. Add 200 μL Substrate Solution to each well. Incubate 30 minutes **on the benchtop. Protect from light.**



8. Add 50 μL Stop Solution to each well. Read at 450 nm within 30 min.
 λ correction 540 or 570 nm

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

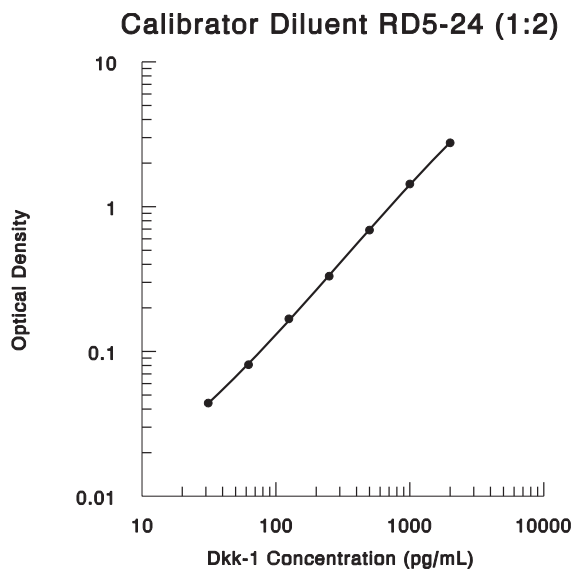
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 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.

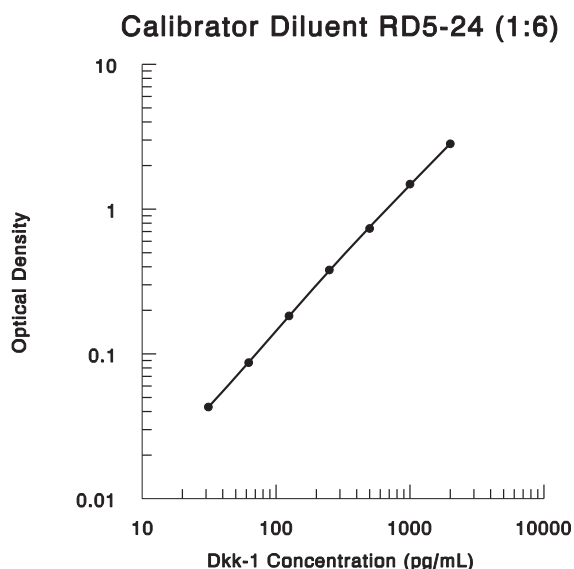
Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

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	0.059	0.059	—
31.2	0.103	0.103	0.044
62.5	0.140	0.140	0.081
125	0.225	0.227	0.168
250	0.386	0.390	0.331
500	0.736	0.748	0.689
1000	1.446	1.494	1.435
2000	2.786	2.820	2.761



pg/mL	O.D.	Average	Corrected
0	0.042	0.045	—
31.2	0.087	0.088	0.043
62.5	0.130	0.132	0.087
125	0.227	0.228	0.183
250	0.424	0.425	0.380
500	0.761	0.780	0.735
1000	1.523	1.533	1.488
2000	2.852	2.871	2.826

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.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

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 separate assays to assess inter-assay precision.

Serum/Plasma Assay

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	212	599	1358	243	620	1371
Standard deviation	8.85	24.5	44.4	18.4	37.4	63.1
CV (%)	4.2	4.1	3.3	7.6	6.0	4.6

Cell Culture Supernate Assay

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	223	600	1353	221	577	1287
Standard deviation	9.38	15.3	36.5	16.9	35.0	69.6
CV (%)	4.2	2.6	2.7	7.7	6.1	5.4

RECOVERY

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

Sample	Average % Recovery	Range
Cell culture media* (n=4)	103	97 - 109%
Serum* (n=4)	99	85 - 115%
EDTA plasma* (n=4)	94	86 - 102%
Heparin plasma* (n=4)	98	92 - 106%

*Samples were diluted prior to assay as directed by the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of natural Dkk-1 were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture samples* (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	99	102	104	101
	Range (%)	92 - 103	99 - 104	96 - 113	99 - 103
1:4	Average % of Expected	103	107	109	99
	Range (%)	97 - 109	103 - 110	105 - 114	92 - 107
1:8	Average % of Expected	108	109	110	100
	Range (%)	101 - 111	106 - 111	108 - 112	93 - 109
1:16	Average % of Expected	109	104	107	101
	Range (%)	105 - 112	97 - 109	102 - 112	92 - 113

*Samples were diluted prior to assay as directed by the Sample Preparation section.

SENSITIVITY

One hundred seventy-nine assays were evaluated and the minimum detectable dose (MDD) of Dkk-1 ranged from 0.94 - 15.6 pg/mL. The mean MDD was 4.23 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 *Sf 21*-expressed recombinant human Dkk-1 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of Dkk-1 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum* (n=36)	2513	1357 - 5240	727
EDTA plasma* (n=36)	630	172 - 1488	334
Heparin plasma* (n=36)	424	151 - 865	171

*Samples were diluted prior to assay as directed by the Sample Preparation section.

Cell Culture Supernates -

Human peripheral blood leukocytes were cultured in DMEM supplemented with 5% fetal calf serum, 5 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 μ g/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed, assayed for levels of natural Dkk-1, and measured below the low standard, 31.2 pg/mL.

Human prostate adenocarcinoma (PC3) cells were cultured in Ham's F-12K supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The cells were cultured unstimulated or stimulated with 10 ng/mL TNF- α . Aliquots of the cell culture supernates were removed and assayed for levels of natural Dkk-1.

Condition	Observed Value (pg/mL)
Unstimulated	151,051
Stimulated	128,905

Human umbilical vein endothelial (HUVEC) cells were cultured in EGM-2 media and grown to 90% confluence. An aliquot of the cell culture supernate was removed, assayed for levels of natural Dkk-1, and measured 5320 pg/mL.

Human epidermoid carcinoma (A431) cells were cultured in DMEM media supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, assayed for levels of natural Dkk-1, and measured 37,750 pg/mL.

Human epithelial keratinocytes (NHEK) were cultured in KGM-2 media (KBM + supplement) with 2 mM L-glutamine. The cells were split before confluence to avoid attachment growth inhibition. An aliquot of the cell culture supernate was removed, assayed for levels of natural Dkk-1, and measured 510 pg/mL.

SPECIFICITY

This assay recognizes oligomeric forms of recombinant and natural human Dkk-1. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Dkk-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

β -Catenin
Cerberus
Dkk-3
Dkk-4
Kremen-1
Kremen-2
LRP-6
Soggy-1
WIF-1

Recombinant mouse:

Soggy-1

Recombinant mouse Dkk-1 shows approximately 8.2% cross-reactivity in this assay.

Recombinant rat Dkk-1 shows approximately 34.4% cross-reactivity in this assay.

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PLATE LAYOUT

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

1											
2											
3											
4											
5											
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7											
8											
9											
10											
11											
12											
	A	B	C	D	E	F	G	H			