Quantikine® ELISA

Human Dkk-1 Immunoassay

Catalog Number DKK100B

For the quantitative determination of human Dickkopf related protein 1 (Dkk-1) concentrations in cell culture supernates, serum-free cell culture supernates, serum, and platelet-poor 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 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-free cell culture supernates, serum, and platelet-poor 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 this kit can be used to determine relative mass values of natural 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 human 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 diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Dkk-1 Microplate	898600	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Dkk-1.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Dkk-1 Standard	898602	2 vials of recombinant human Dkk-1 in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a fresh standard for each assay. Discard after use.
Human Dkk-1 Conjugate	898601	21 mL of a polyclonal antibody specific for human Dkk-1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-14	895180	12 mL of a buffered protein solution with preservatives.	
Calibrator Diluent RD5-18	895335	21 mL of a buffered protein solution with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* 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.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human Dkk-1 Controls (optional; R&D Systems®, Catalog # QC241).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates/Serum-Free Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge at 2-8 °C for 15 minutes at 1000 x g within 30 minutes of collection. For complete platelet removal, an additional centrifugation step of the separated plasma at 1500 x g for 10 minutes at 2-8 °C is recommended. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Dkk-1 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of Dkk-1, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory and Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

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

SAMPLE PREPARATION

Use polypropylene tubes.

Serum samples require a 20-fold dilution due to endogenous levels. A suggested 20-fold dilution is 10 μL of sample + 190 μL of Calibrator Diluent RD5-18.

REAGENT PREPARATION

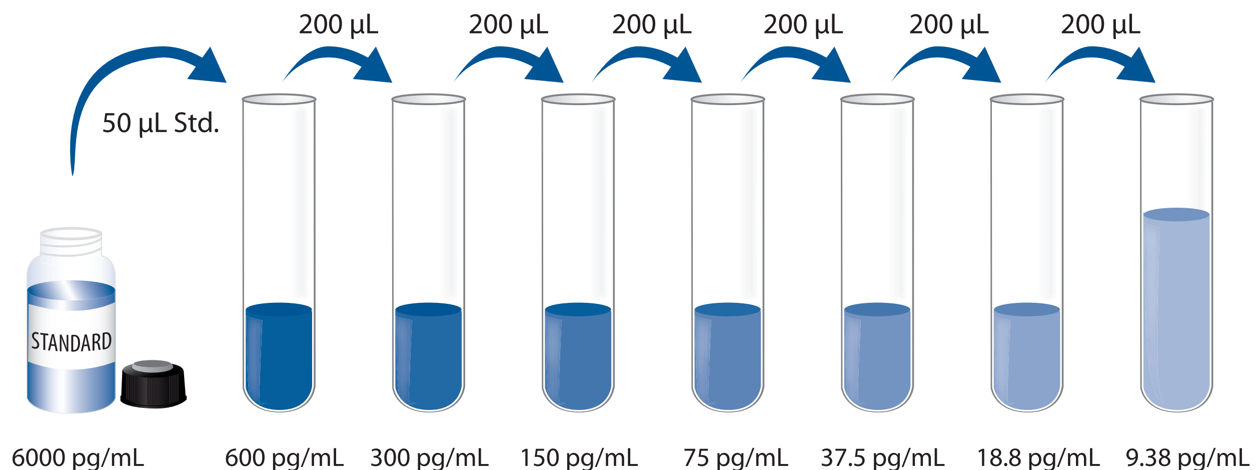
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 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 µL of the resultant mixture is required per well.

Human Dkk-1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Dkk-1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 6000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD5-18 into the 600 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 600 pg/mL standard serves as the high standard. Calibrator Diluent RD5-18 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 standards, samples, and controls 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 50 µL of Assay Diluent RD1-14 to each well.
4. Add 50 µL 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 µL) 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 µL of the Human 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 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL 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 may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

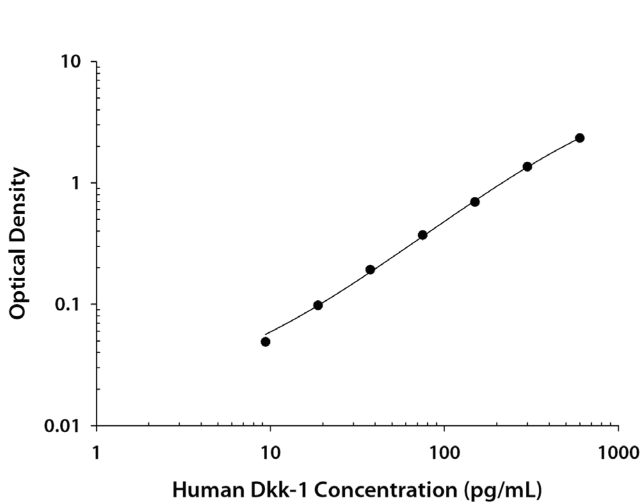
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human 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.

If 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	0.022 0.022	0.022	—
9.38	0.070 0.071	0.071	0.049
18.8	0.118 0.122	0.120	0.098
37.5	0.208 0.222	0.215	0.193
75	0.392 0.394	0.393	0.371
150	0.706 0.728	0.717	0.695
300	1.373 1.384	1.379	1.357
600	2.347 2.366	2.357	2.335

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 twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	75.6	187	360	75.6	192	372
Standard deviation	2.21	3.28	9.48	5.81	16.7	30.1
CV (%)	2.9	1.8	2.6	7.7	8.7	8.1

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	91	83-101%
Serum-free cell culture media (n=4)	82	77-90%
Platelet-poor EDTA plasma (n=4)	95	91-98%
Platelet-poor heparin plasma (n=4)	99	93-105%

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LINEARITY

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

		Cell culture media (n=4)	Serum-free cell culture supernates (n=4)	Serum (n=4)	Platelet-poor	
					EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	103	99	103	105	105
	Range (%)	100-105	97-101	99-107	104-105	102-108
1:4	Average % of Expected	103	99	103	111	111
	Range (%)	100-104	94-102	100-108	108-113	107-114
1:8	Average % of Expected	107	98	105	113	115
	Range (%)	101-111	94-101	100-109	109-116	111-117
1:16	Average % of Expected	113	96	106	112	117
	Range (%)	105-118	92-99	101-108	107-115	112-119

SENSITIVITY

Twenty-three assays were evaluated and the minimum detectable dose (MDD) of human Dkk-1 ranged from 0.217-0.948 pg/mL. The mean MDD was 0.419 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. 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/Platelet-poor Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human 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=37)	3566	2087-4740	728
Platelet-poor EDTA plasma (n=37)	305	124-737	117
Platelet-poor heparin plasma (n=37)	343	152-815	126

Cell Culture Supernates:

A431 human epidermoid carcinoma cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) until confluent. An aliquot of the cell conditioned media was taken off and centrifuged to remove any cells or debris. The cells were then washed with PBS before adding fresh media containing 10% FBS and cultured for an additional 24 hours. After the 24 hour incubation, cell conditioned media containing serum was taken off and centrifuged to remove any cells or debris. Aliquots of the cell culture supernates were removed and assayed for levels of human Dkk-1.

Condition	(pg/mL)
DMEM + 10% FBS	157
Confluent with serum	46,900
24 hour w/fresh media + serum	13,985

HT-29 human colon adenocarcinoma cells were cultured in McCoy's 5A media supplemented with 10% FBS until confluent. An aliquot of the cell conditioned media was taken off and centrifuged to remove any cells or debris. The cells were then washed with PBS before adding fresh media containing 10% FBS and cultured for an additional 24 hours. After the 24 hour incubation, cell conditioned media containing serum was taken off and centrifuged to remove any cells or debris. Aliquots of the cell culture supernates were removed and assayed for levels of human Dkk-1.

Condition	(pg/mL)
McCoy's 5A + 10% FBS	154
Confluent with serum	1665
24 hour w/fresh media + serum	1010

Serum-Free Cell Culture Supernates:
A431 human epidermoid carcinoma cells were cultured in DMEM media supplemented with 10% FBS until confluent, washed with PBS and changed to serum-free DMEM media for 24 hours prior to conditioned media harvest. An aliquot of the cell culture supernate was removed, assayed for human Dkk-1, and measured 2723 pg/mL.

HT-29 human colon adenocarcinoma cells were cultured in McCoy's 5A media supplemented with 10% FBS until confluent, washed with PBS and changed to serum-free McCoy's 5A media for 24 hours prior to conditioned media harvest. An aliquot of the cell culture supernate was removed, assayed for human Dkk-1, and measured 151 pg/mL.

HT1080 human fibrosarcoma cells were cultured in MEM media supplemented with 10% FBS until 70% confluent, then changed to serum-free RPMI media for 48 hours prior to conditioned media harvest. An aliquot of the cell culture supernate was removed and assayed for human Dkk-1. No detectable levels were observed.

PC-3 human prostate cancer cells were cultured in RPMI media supplemented with 10% FBS until confluent, then changed to serum-free RPMI media for 72 hours prior to conditioned media harvest. An aliquot of the cell culture supernate was removed, assayed for human Dkk-1, and measured 138,816 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant 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:

Cerberus
Dkk-2
Dkk-3
Dkk-4
LRP-5
LRP-6
Kremen-1
Kremen-2
Soggy-1
WIF-1

Recombinant mouse:

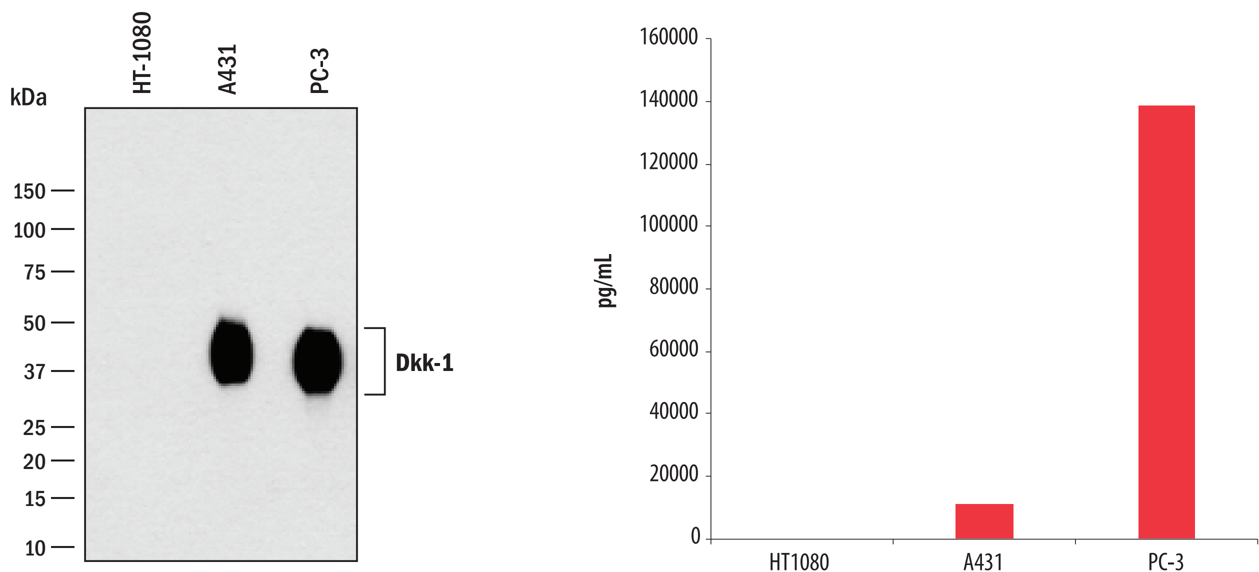
Cerberus

Recombinant human LRP-5 cross-reacts approximately 0.02% in this assay.

Recombinant mouse Dkk-1 (aa 1-272) cross-reacts approximately 1.0% in this assay.

Recombinant mouse Dkk-1 (aa 1-144) cross-reacts approximately 1.5% in this assay.

Recombinant rat Dkk-1 cross-reacts approximately 17% in this assay.



Serum-free conditioned media samples were analyzed by Western Blot and Quantikine® ELISA. The PC-3 sample was diluted 1:5 prior to analysis in the Western Blot, while other samples were run neat. The samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with the detection antibody used in this kit. The Western Blot shows a direct correlation with the ELISA value for these samples.

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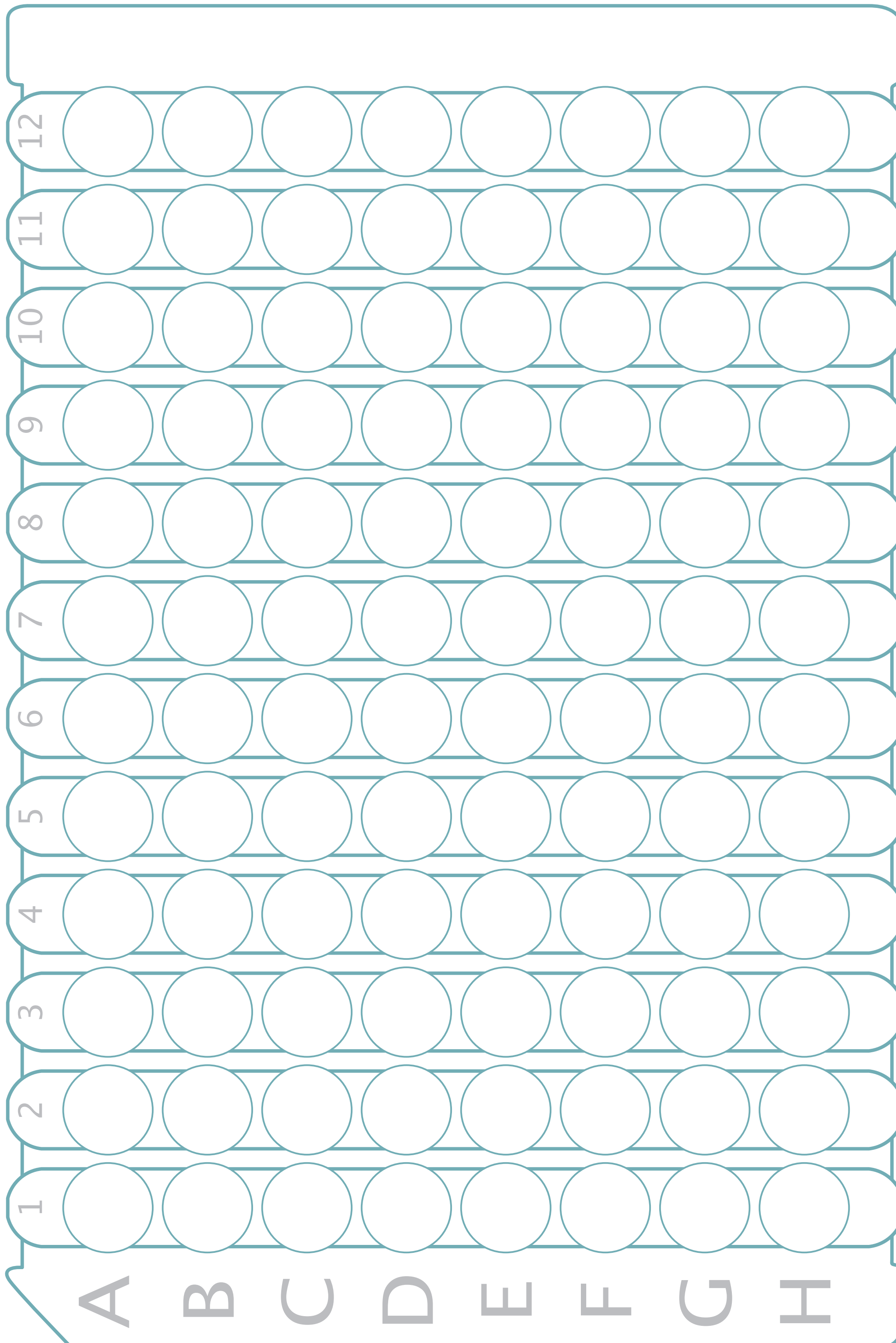
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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