Product Manual

Dkk-1 (human), ELISA kit

Catalog #: ADI-900-151

96 Well Enzyme-Linked Immunsorbent Assay Kit

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Product Manual

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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

Check our website for additional protocols, technical notes, MSDS and FAQs.

INTRODUCTION

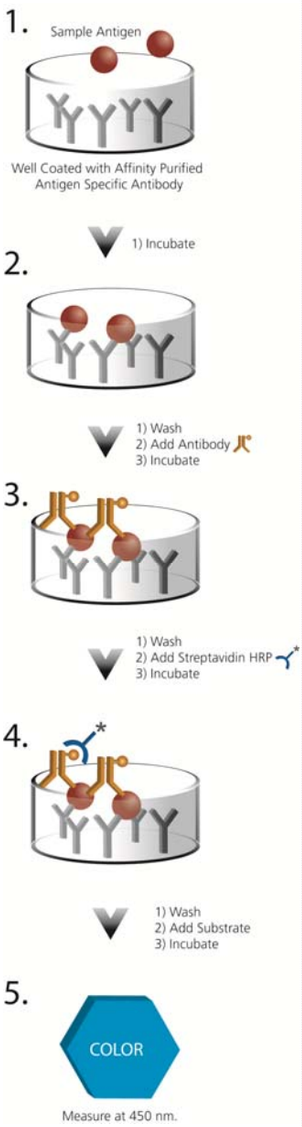
The Dkk-1 (human), EIA kit is a complete kit for the quantitative determination of Dkk-1 in plasma, serum, and culture fluids. Please read the complete kit insert before performing this assay.

Dkk-1 (Dickkopf or Dickkopf-1) is a secreted inhibitor of the Wnt/canonical signaling pathway which mediates a variety of cellular processes. Wnt proteins are extracellular signaling molecules that initiate activation of the canonical pathway by binding low-density lipoprotein receptor-related protein 5&6 (LRP5/6) coreceptors, triggering a cascade of events that stabilize β-catenin[1]. Wnts mediate osteoblast development and cell-to-cell interactions during embryogenesis, and are also responsible for regulating bone mass in adults during normal bone remodeling processes. Wnts are also found to be expressed during the stationary phase of the cell cycle and are thought to control expansion of bone marrow stromal cells preventing overpopulation in marrow[2].

There are at least two families of secreted inhibitors of Wnt signaling: the secreted frizzled-related protein family and the Dickkopf family[3]. The Dkk-1 protein consists of 266 amino acids including a 31 amino acid N-terminal signal peptide. Dkk-1 has a predicted molecular weight of 26kD and an apparent molecular weight of 35kD by SDS-PAGE and western blot analysis. Dkk-1 disrupts the canonical pathway by binding to the LRP5/6 coreceptors, preventing interaction with Wnt and facilitating degradation of β-catenin, which ultimately results in osteolytic breakdown of bone tissue.

Wnt signaling dysregulation has been implicated in cancer of the colon and stomach[4]. Gonzalez-Sancho reported that the Wnt/β-catenin pathway is downregulated by the induction of Dkk-1 expression, a mechanism that is lost in colon cancer, suggesting that Dkk-1 acts as a tumor suppressor gene in this cancer type[5]. Dkk-1 proteins are expressed during log phase of the cell cycle, suggesting that dysregulated Dkk-1 could be responsible for the growth of some malignancies. The Dkk-1 gene has also been determined to be a target of p53, as Cappuccio suggests it is a necessary requirement for the execution of cell death[6]. Tian reports that elevated Dkk-1 levels in bone marrow, plasma, and peripheral blood from patients with multiple myeloma correlated with the gene-expression patterns of Dkk-1, and were associated with the presence of focal bone lesions[7]. A recent study reported serum levels of Dkk-1 in patients with multiple myeloma before autologous stem cell transplantation[8]. The range of Dkk-1 serum levels in this group of patients was 6.4-234.8 ng/ml with a mean of

63.6 ng/ml and a standard deviation of 77.3. Hall et al. suggest that Dkk-1 may act like a switch on prostate cancer cell activity, causing cells to change from osetoblastic to a highly osteolytic cell line[9].



PRINCIPLE

1. Samples and standards are added to wells coated with a monoclonal antibody specific for Dkk-1. The plate is then incubated.
2. The plate is washed, leaving only bound Dkk-1 on the plate. A yellow solution of biotinylated polyclonal antibody to Dkk-1 is then added. This binds the Dkk-1 captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A blue solution of HRP conjugate is added to each well, binding to the Dkk-1 polyclonal. The plate is again in-cubated.
4. The plate is washed to remove excess HRP conjugate. TMB Substrate solution is added. The substrate generates a blue color when catalyzed by the HRP.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of Dkk-1 in the sample.

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

The standard should be handled with care due to the known and unknown effects of the antigen.

Activity of conjugate is affected by concentrations of chelators > 10mM (such as EDTA and EGTA).

Protect substrate from prolonged exposure to light.

Stop solution is caustic. Keep tightly capped.

MATERIALS SUPPLIED

1. **Assay Buffer 10 Concentrate (10X)**
   27ml, Catalog No. 80-0648
   Tris buffered saline containing detergents.

2. **human Dkk-1 Standard**
   250pg/vial, Catalog No. 80-1465
   Two vials containing 250 pg of recombinant human Dkk-1.

3. **Dkk-1 Clear Microtiter Plate**
   One Plate of 96 Wells, Catalog No. 80-1603
   A plate of break-apart strips coated with a mouse monoclonal antibody specific to Dkk-1.

4. **Wash Buffer Concentrate**
   100ml, Catalog No. 80-1287
   Tris buffered saline containing detergents.

5. **Dkk-1 EIA Antibody**
   10ml, Catalog No. 80-1461

6. **human Dkk-1 EIA Conjugate**
   10ml, Catalog No. 80-1463
   A blue solution of streptavidin conjugated to horseradish peroxidase.

7. **TMB Substrate**
   10ml, Catalog No. 80-0350
   A solution of 3,3’,5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide.

8. **Stop Solution 2**
   10ml, Catalog No. 80-0377
   A 1N solution of hydrochloric acid in water.

9. **Dkk-1 Assay Layout Sheet**
   1 each, Catalog No. 30-0230

10. **Plate Sealer**
    3 each, Catalog No. 30-0012
    A yellow solution of biotinylated polyclonal antibody to Dkk-1.

Reagents require separate storage conditions.

STORAGE

All components of this kit, except the standard, are stable at 4°C until the kit's expiration date. **The standard should be stored at or below -20ºC upon receipt.**

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent.
3. Protease Inhibitor Cocktail (PIC), Sigma #P8340 or equivalent.
4. Precision pipets for volumes between 5µl and 1,000µl.
5. Repeater pipet for dispensing 100µl.
6. Disposable beakers for diluting buffer concentrates.
7. Graduated cylinders.
8. A microplate shaker.
9. Lint-free paper for blotting.
10. Microplate reader capable of reading at 450 nm.
11. Graph paper for plotting the standard curve.

Bring all reagents to room temperature for at least 30 minutes prior to opening.

If inhibitors other than those recommended are usede, the end user is responsible for assay validation. In some cases, certain protease inhibitor cocktails may cause performance differences.

Plastic tubes must be used for standard preparation.

REAGENT PREPARATION

1. Wash Buffer

Prepare the wash buffer by diluting 50 ml of the supplied Wash Buffer Concentrate with 950 ml of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Assay Buffer

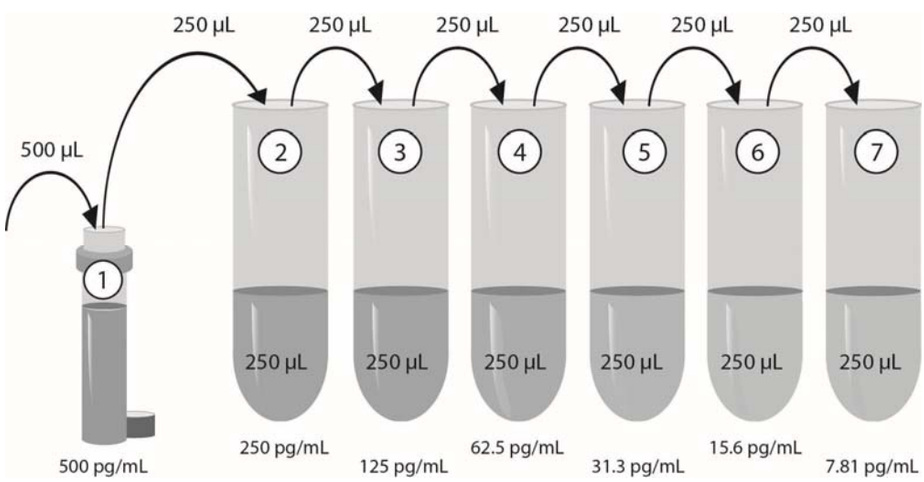
Prepare the assay buffer by diluting 5ml of the supplied Assay Buffer 10 Concentrate with 45ml of deionized water. This can be stored at 4°C until the kit expiration, or for 3 months, whichever is earlier.

3. PIC and PMSF Addition

Immediately prior to use, PIC and PMSF must be added to the assay buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/ml PIC, or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM.

Inhibitors must be freshly added to the assay buffer to ensure optimal integrity of the standards and samples. Each inhibitor treated buffer should incubate for 5-10 minutes at room temperature before it is used. Buffers treated with inhibitors should be used within 1 hour of preparation.

4. human Dkk-1 Standards



Add 500µl of assay buffer plus inhibitors to the lyophilized human Dkk-1 standard vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial Standard #1. Label six 12 x 75 mm polypropylene tubes #2 through #7. Pipet 250µl of the assay buffer plus inhibitors into tubes #2 through #7. Add 250µl of reconstituted Standard #1 to tube #2 and vortex thoroughly. Add 250µl of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #7.

Diluted standards should be used within 30 minutes of preparation.

The concentration of Dkk-1 in tubes is labeled above.

SAMPLE HANDLING

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

Human culture fluids, serum, EDTA plasma, and heparin plasma are suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Dkk-1 concentrations were measured in serum, EDTA plasma, and culture media. Dkk-1 was spiked into the samples and diluted with assay buffer plus inhibitors, then assayed in the kit.

Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/ thaw cycles.

Sample	Recommended Dilution	% Recovery
Human plasma EDTA	≥1:8	109%
MEM tissue culture media	≥1:3	116%
Human serum	≥1:8	109%

Add PIC/PMSF to buffers prior to preparing samples.

Culture media supplemented with 10% fetal bovine serum (FBS) requires a minimum dilution of 1:3 in the assay buffer plus inhibitors. This kit also recognizes bovine Dkk-1.* It is recommended that users evaluate each lot of FBS used for the levels of endogenouse bovine Dkk-1 present. The user has the option to either dilute the culture fluids to eliminate the endogenouse bovine Dkk-1 or subtract the concentration of bovine Dkk-1 contributing to the total Dkk-1 detected.

*Bovine Dkk-1 has 90% sequence identity to human Dkk-1. Cross-reactivity to bovine Dkk-1 is observed.

Sample Values

The following human samples were diluted to the minimum recommended dilution and tested in the assay for presence of Dkk-1.

			Concentration (pg/ml)		
Sample Type	Pathology	# Samples	Average	Median	Range
Serum	None	12	1130	891	295-2351
Plasma	None	10	1442	1348	799-2229
Serum	Colon Cancer	10	6354	6513	3137-9791
Plasma	Colon Cancer	10	2633	2354	1026-4604

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards, controls and samples should be run in duplicate.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the reagent to the side of the wells to avoid possible contamination.

Prior to the addition of the antibody, conjugate and substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 100µl of the assay buffer plus inhibitors into the S0 (0pg/ml standard) wells.
2. Pipet 100µl of Standards #1 through #7 to the bottom of the appropriate wells.
3. Pipet 100µl of the samples to the bottom of the appropriate wells.
4. Seal the plate. Incubate for 1 hour on a plate shaker (~500rpm) at room temperature.
5. Empty the contents of the wells and wash by adding 400µl of wash buffer to every well. Repeat 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100µl of yellow antibody into each well except the blank.
7. Seal the plate. Incubate for 1 hour on a plate shaker (~500rpm) at room temperature.
8. Wash as above (Step 5).
9. Add 100µl of blue conjugate to each well except the blank.
10. Seal the plate. Incubate for 30 minutes on a plate shaker (~500rpm) at room temperature.
11. Wash as above (Step 5).
12. Pipet 100µl of substrate solution into each well.
13. Incubate for 30 minutes on a plate shaker (~500rpm) at room temperature.
14. Pipet 100µl of stop solution into each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of Dkk-1 in samples. We recommend that the data be handled by an immunoassay software package utilizing a 4-parameter logistic curve fitting program. If data reduction software is not readily available, the concentrations can be calculated as follows:

1. Calculate the average Net OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

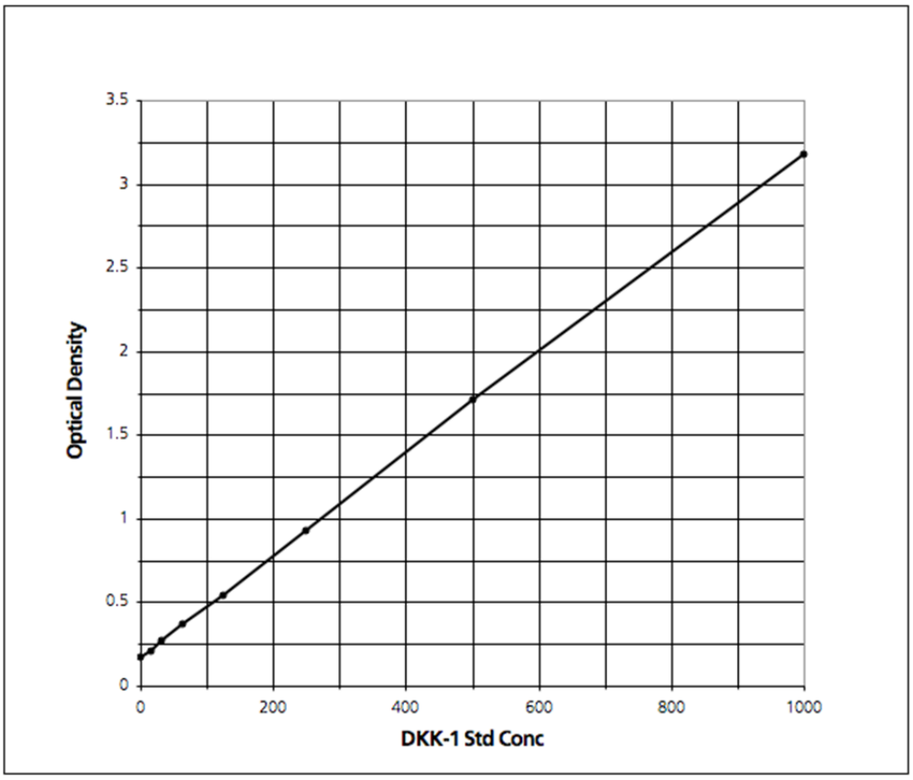
2. Using linear graph paper, plot the average Net OD for each standard versus Dkk-1 concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Net OD	Dkk-1 (pg/ml)
S0	0.168	
S1	3.178	**500**
S2	1.706	**250**
S3	0.930	**125**
S4	0.536	**62.5**
S5	0.366	**31.3**
S6	0.268	**15.6**
S7	0.207	**7.81**
Unknown 1	2.275	**345**
Unknown 2	0.373	**34.4**



PERFORMANCE CHARACTERISTICS

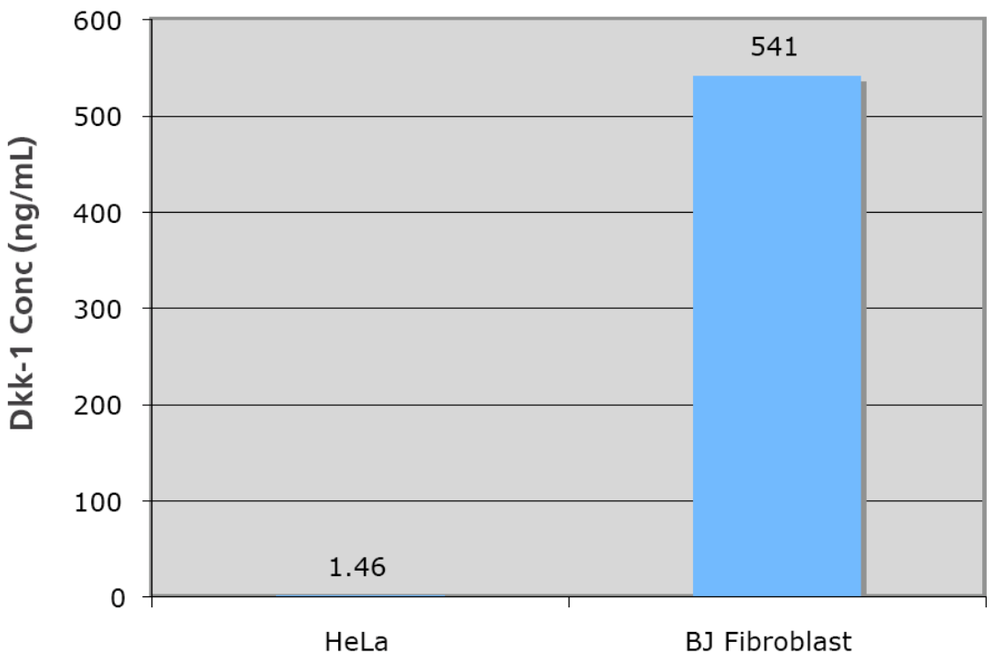
Specificity

The cross reactivities for a number of related compounds were determined by diluting cross reactants in the assay buffer at a concentration of 50,000pg/ml. These samples were then measured in the assay.

Compound	Cross Reactivity
human Dkk-1	100%
Dkk-4	<0.02%
GSK-3β	<0.02%
β-catenin	<0.02%
PIN 1	<0.02%

Cell Line Experiment

Dkk-1 is expected to be secreted from fibroblast and fibroblast-like cells. HeLa cells are known to be negative for Dkk-1. BJ fibroblast supernatants were serially diluted into assay buffer beginning at a 1:200 dilution. HeLa cell supernatants were serially diluted into assay buffer beginning at a 1:2 dilution. Supernatants came from comparable cell numbers.



Sensitivity

Sensitivity was calculated as the ratio of the mean OD plus 2 standard deviations of twen-ty-four replicates of the 0pg/ml standard to the mean of twenty-four replicates of the lowest standard, multiplied by the concentration of that standard (7.81pg/ml). This value was determined to be 0.979pg/ml.

Linearity

A buffer sample containing Dkk-1, at a concentration of 400pg/ml, was serially diluted 1:2 in the assay buffer and measured in the assay. The results are shown in the table below.

Dilution	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
Neat	---------	400.44pg/ml	-----
1:2	200.22pg/ml	218.88pg/ml	109.3%
1:4	100.11pg/ml	99.43pg/ml	99.3%
1:8	50.06pg/ml	52.34pg/ml	104.6%
1:16	25.03pg/ml	26.68pg/ml	106.6%
1:32	12.51pg/ml	13.07pg/ml	104.4%

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing Dkk-1 in a single assay.

pg/ml	%CV
296.3	7.4
86.2	6.7
35.3	3.7

Inter-assay precision was determined by measuring buffer controls of varying Dkk-1 concentrations in multiple assays over several days.

pg/ml	%CV
329.9	7.9
90.9	12.8
30.4	13.3

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