

Quantikine[®]

Human Pref-1/DLK-1/FA1 Immunoassay

Catalog Number DPRF10

For the quantitative determination of human Preadipocyte Factor-1/Delta-like Homolog 1/Fetal Antigen 1 (Pref-1/DLK-1/FA1) concentrations in cell culture supernates, 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

Preadipocyte Factor 1 (Pref-1) is best known as an inhibitor of adipocyte differentiation. The human cDNA encodes a 383 amino acid transmembrane protein that contains a signal peptide, an extracellular domain with six tandem epidermal growth factor (EGF)-like domains, a juxtamembrane region, a single pass transmembrane domain, and a short cytoplasmic tail (1 - 3). Pref-1 has also been identified as Delta-like Homolog 1(DLK-1), human adrenal specific cDNA pG2, and as a soluble, circulating glycoprotein called fetal antigen 1 (FA1) (1, 2, 4, 5). In rodents, Pref-1 has also been studied under the names of stromal-cell-derived protein 1 (SCP-1) and zona glomerulosa-specific factor (ZOG) (6, 7). Mouse and human Pref-1 share 86% amino acid identity and show significant homology to EGF-containing proteins of the Notch/Delta/Jagged family but do not contain residues required for EGF receptor binding (3). The gene for Pref-1 has been mapped to chromosome 14q32, and is paternally expressed (8, 9). It is reciprocally imprinted with a non-translated mRNA, GTL2, and this DLK-1/GTL2 domain shares many structural and regulatory features with the similarly imprinted IGF-II/H19 domain found on chromosome 7q11-13 (9).

Due to alternative splicing and proteolytic processing, multiple forms of Pref-1 protein have been identified. Four alternatively spliced forms produce cell-associated proteins; in addition to the full-length form, three shorter forms are the result of in-frame deletions in the juxtamembrane region. All four contain a proteolytic cleavage site located near the fourth EGF repeat, while the larger two isoforms contain an additional proteolytic site in the juxtamembrane region (10, 11). These proteolytic processing events release multiple soluble forms, however just cleavage products of the larger two, processed in the juxtamembrane region, release the active, soluble form (10, 11). Only this 38 kDa (in human) or 50 kDa (in mouse) diffusible form, containing the full complement of EGF domains is able to inhibit adipocyte differentiation (3, 11, 12). The enzyme responsible for conversion of membrane-bound Pref-1 to a biologically active soluble form is TACE (tumor necrosis factor α converting enzyme) (13). Furthermore, the circulating soluble form exhibits heterogeneous and complex glycosylation patterns in the C-terminal region (2, 6). However, receptor identity and downstream signaling characterization remains unknown (3).

The relative abundance of the different spliced or processed forms varies depending on the tissue or developmental stage examined (12, 14). In humans, Pref-1 is expressed embryonically in hepatocytes, glandular cells of the pancreas anlage, respiratory epithelial cells, mesodermal cells of the renal proximal tubules, adrenal cortex, fetal chondroblasts, skeletal myotubes, and cells of the testes and ovaries (15 - 17). However, Pref-1 expression decreases during fetal development and becomes restricted post-natally to preadipocytes, pancreatic islet cells, thymic stromal cells, somatotrophic cells of the pituitary and adrenal gland cells (18 - 23). In mouse 3T3-L1 preadipocytes, Pref-1 is released as a 50 kDa heterogenous protein that is indistinguishable from the form purified from amniotic fluid, known as FA1 (14). In addition, tumors with neuroendocrine features, such as neuroblastoma, pheochromocytoma, and some small cell lung carcinoma cell lines express Pref-1, suggesting a role in neuroendocrine function (1, 2). Furthermore, Pref-1 is found in serum and urine, is highly enriched in second trimester amniotic fluid, and shows a 10-fold increase in patients with renal failure (2, 24).

A variety of *in vitro* and *in vivo* experiments demonstrate that Pref-1 functions to inhibit adipocyte differentiation. While Pref-1 is expressed abundantly in preadipocytes, its expression is absent during adipocyte differentiation (18). In addition, constitutive overexpression of Pref-1 in 3T3-L1 preadipocytes blocks their conversion to adipocytes (18). Pref-1 null mice display growth retardation, skeletal malformation, obesity, and increased serum lipid metabolites: phenotypes that mimic the human mUPD14 obesity syndrome (25). As compared to their normal littermates, null mice exhibit lower weights at weaning and end up with accelerated weight gain and an increase in adipose tissue mass, due to enhancement of both adipocyte differentiation and fat cell maturation (25). Finally, transgenic mice with over-expression of Pref-1 in adipose tissue or in the liver show a marked decrease in adipose tissue mass and reduced expression of adipocytokines and adipocyte markers, such as SCD, C/EBP α , Fas, and Resistin (26).

The Quantikine Human Pref-1 Immunoassay is a 4.5 hour sandwich ELISA designed to measure human Pref-1 in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human Pref-1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Pref-1 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Pref-1 kit can be used to determine relative mass values for naturally occurring Pref-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Pref-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Pref-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for Pref-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 Pref-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, dilute the samples with 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.

REAGENTS

Pref-1 Microplate (Part 893250) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Pref-1.

Pref-1 Conjugate (Part 893251) - 21 mL of polyclonal antibody against Pref-1 conjugated to horseradish peroxidase with preservatives.

Pref-1 Standard (Part 893252) - 100 ng of recombinant human Pref-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 RD6-11 (Part 895489) - 21 mL of a buffered protein solution with preservatives.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of 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 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 RD6-11	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	
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.
- Human Pref-1 Controls (optional; available from R&D Systems).

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 approximately 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 heparin or EDTA 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.*

REAGENT PREPARATION

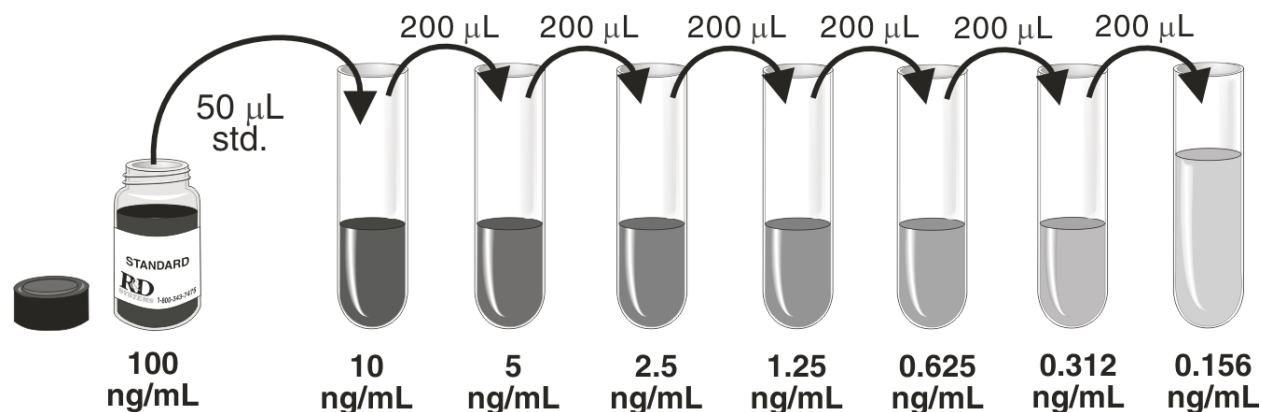
Bring all reagents to room temperature before use.

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.

Pref-1 Standard - Reconstitute the Pref-1 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 100 ng/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.

Pipette 450 μ L of Calibrator Diluent RD6-11 into the 10 ng/mL tube. Pipette 200 μ L of Calibrator Diluent RD6-11 into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 ng/mL standard serves as the high standard. The Calibrator Diluent serves as the zero standard (0 ng/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, reseal.
3. Add 100 μL of Assay Diluent RD1W 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. 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 Pref-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 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **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.

ASSAY PROCEDURE SUMMARY

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



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



3. Add 50 μL Standard, control, or sample to each well.
Incubate 2 hrs. at RT.



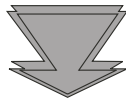
4. Aspirate and wash 4 times.



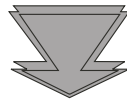
5. Add 200 μL Conjugate to each well.
Incubate 2 hrs. at RT.



6. Aspirate and wash 4 times.



7. Add 200 μL Substrate Solution to each well.
Incubate 30 min. **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

CALCULATION OF RESULTS

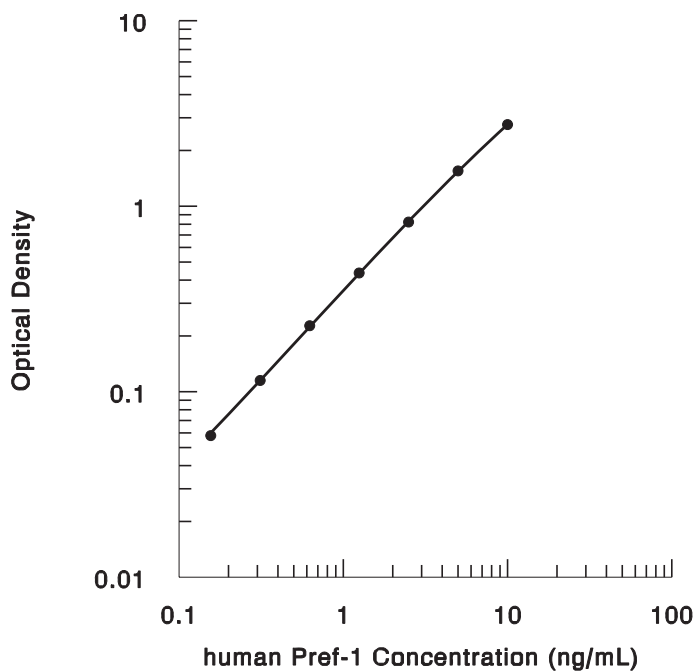
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 Pref-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.



ng/mL	O.D.	Average	Corrected
0	0.020 0.020 0.077	0.020	—
0.156	0.079 0.131	0.078	0.058
0.312	0.139 0.242	0.135	0.115
0.625	0.251 0.442	0.247	0.227
1.25	0.471 0.838	0.457	0.437
2.5	0.845 1.549	0.842	0.822
5	1.590 2.747	1.570	1.550
10	2.807	2.777	2.757

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 (ng/mL)	1.74	3.44	6.33	1.67	3.37	6.19
Standard deviation	0.075	0.125	0.194	0.118	0.186	0.371
CV (%)	4.3	3.6	3.1	7.1	5.5	6.0

RECOVERY

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

Sample	Average % Recovery	Range
Cell culture media (n=4)	107	102 - 113%
Serum (n=4)	99	91 - 107%
EDTA plasma (n=4)	96	86 - 101%
Heparin plasma (n=4)	95	89 - 99%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Pref-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 (n=4)	Heparin plasma (n=4)	EDTA plasma (n=4)
1:2	Average % of Expected	111	102	103	102
	Range (%)	109 - 113	97 - 108	99 - 105	97 - 104
1:4	Average % of Expected	105	100	105	101
	Range (%)	99 - 108	98 - 109	101 - 108	98 - 104
1:8	Average % of Expected	103	99	105	101
	Range (%)	98 - 107	92 - 106	101 - 107	94 - 105
1:16	Average % of Expected	97	97	103	100
	Range (%)	93 - 100	93 - 99	99 - 106	96 - 102

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of Pref-1 ranged from 0.005 - 0.034 ng/mL. The mean MDD was 0.012 ng/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 human Pref-1 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of Pref-1 in this assay.

Sample Type	Mean (ng/mL)	Standard Deviation (ng/mL)	Range (ng/mL)
Serum (n=36)	0.508	0.193	0.182 - 0.941
EDTA plasma (n=35)	0.502	0.187	0.231 - 0.908
Heparin plasma (n=35)	0.451	0.149	0.215 - 0.806

Cell Culture Supernates - Human peripheral blood lymphocytes (1×10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf serum, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 μ g/mL PHA. An aliquot of the cell culture supernate was removed and assayed for levels of natural Pref-1. No detectable levels were observed.

SPECIFICITY

This assay recognizes recombinant and natural human Pref-1. The factors listed below were prepared at 100 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range recombinant human Pref-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Adiponectin/Acrp30
Amphiregulin
CD40 Ligand
Complement Factor D
CRP
Cryptic
CTACK
EGF
EGF R
Epiregulin
ErbB2
ErbB3
ErbB4
HB-EGF
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IL-10
IL-11
IL-12
IL-12 p40
Leptin
MCP-1
MDC
Midkine
Notch-3
Resistin
Serpine A1
Serpine A3
Serpine A4
Serpine A5
Serpine B6
Serpine B8
Serpine B9
Serpine C1
Serpine D1
Serpine E1/PAI-1
Serpine E2
Serpine F2
Serpine I1
Serpine I2
TECK
TGF- α
TNF- α
TNF- β
Tpo
TRAIL
TRANCE

Recombinant mouse:

Adiponectin/Acrp30
Agrp
Amphiregulin
Betacellulin
CRP
EGF
Eotaxin
Epigen
Epiregulin
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-11
IL-12
IL-12 p40
IL-13
JE/MCP-1
Leptin
MCP-3
MIP-1 γ
MCP-5
Notch-3
OPG
RANTES
SCF
Serpine E1/PAI-1
TNF- α
Tpo
TRANCE

Recombinant rat:

Agrp
CRP
EGF
IL-2
IL-4
IL-6
IL-10
Leptin
Notch-1
Notch-2
TNF- α

Recombinant porcine:

IL-1 α
IL-1 β
IL-2
IL-4
IL-6
IL-8
IL-10
TNF- α

Mouse Pref-1 cross-reacts 0.9% in this assay.

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PLATE LAYOUT

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

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								