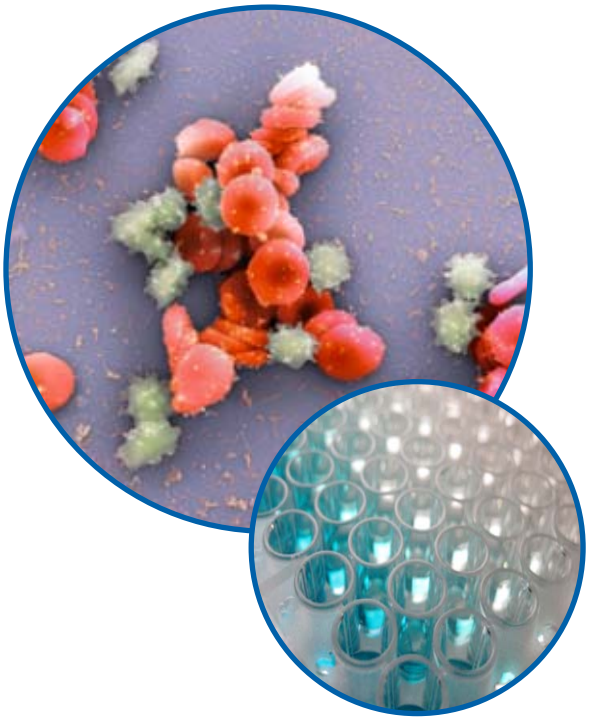


AdipoGen® LIFE SCIENCES

MANUAL

DLK1, Soluble (human) ELISA Kit

For research use only. Not for diagnostic use.

Version 3 (4-May-2015)

Cat. No. AG-45A-0032YEK-KI01

Table of Contents

1. Intended Use

The DLK1, Soluble (human) ELISA Kit is to be used for the *in vitro* quantitative determination of human DLK1 in serum and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

A mouse complementary DNA encoding a regulator of adipocyte differentiation called preadipocyte factor-1 (Pref-1), a novel member of the epidermal growth factor (EGF)-like family of proteins, was isolated, which is synthesized as a transmembrane protein with 6 tandem EGF-like repeats. It has been shown that Pref-1 exists as multiple splicing variants, yielding to different truncated proteins. While Pref-1 mRNA was abundant in preadipocytes, its expression was completely abolished during differentiation of cultured preadipocytes to adipocytes (1). Pref-1 was also independently cloned and called DLK1 (Delta-like) because of its homology to the Drosophila neurogenic protein Delta, which is involved in neural differentiation (2). A circulating form of fetal antigen-1 (FA1) from human amniotic fluid during the second trimester of pregnancy was isolated and turned out to be Pref-1 or DLK1. They reported that FA1 is identical to Pref-1 and DLK (3). Dlk1 (Pref-1) is well known to be a maternally inherited gene located 80 kb apart on mouse chromosome 12 (4). A soluble DLK1 (Pref-1) harboring the entire extracellular domain preceding the juxtamembrane part of DLK1 (Pref-1) has been shown to be able to inhibit adipogenesis of 3T3L1 cell line or MEF (5-6). This observation was corroborated by a DLK1 (Pref-1) transgenic model by showing that they had a substantial decrease in total fat pad weight. Adipose tissue from the transgenic mice showed reduced expression of adipocyte markers and adipocyte-secreted factors, whereas the preadipocyte marker DLK1 (Pref-1) was increased. DLK1 (Pref-1) transgenic mice with a substantial loss of adipose tissue exhibited hypertriglyceridemia, impaired glucose tolerance, and decreased insulin sensitivity (7). DLK1 (Pref-1) has been shown to inhibit adipogenesis by activation of Erk, a MAPK kinase, followed by upregulation of Sox9, which in turn physically binds to the 5' upstream region of C/EBP a gene, thereby inhibiting the gene transcription (6). Thus, serum or plasma levels of soluble DLK1 (Pref-1), which could be a decoy ligand, may negatively or positively affect adipogenesis.

3. General References

(1) Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation: C.M. Smas, et al.; Cell **73,** 725 (1993)

(2) dlk, a putative mammalian homeotic gene differentially expressed in small cell lung carcinoma and neuroendocrine tumor cell line: J. Laborda, et al.; J. Biol. Chem. **268,** 3817 (1993)

(3) Protein structure of fetal antigen 1 (FA1): a novel circulating human epidermal-growth-factor-like protein expressed in neuroendocrine tumors and its relation to the gene products of dlk and pG2: C.H. Jensen, et al.; Europ. J. Biochem. **225,** 83 (1994)

(4) Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlkl-Gtl2 imprinted cluster on mouse chromosome 12: S.P. Lin, et al.; Nature Genet. **35,** 97 (2003)

(5) Pref-1 (preadipocyte factor 1) activates the MEK/extracellular signal-regulated kinase pathway to inhibit adipocyte differentiation: K.A. Kim, et al.; Mol. Cel. Biol. **27,** 2294 (2007)

(6) Pref-1 regulates mesenchymal cell commitment and differentiation through Sox9: Y. Wang, et al.; Cell Metab. **9,** 287 (2009)

(7) Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor-1 (Pref-1): K. Lee, et al.; J. Clin. Invest. **111,** 453 (2003)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human DLK1 in biological fluids. A monoclonal antibody specific for DLK1 has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, DLK1 is recognized by the addition of a purified polyclonal antibody specific for DLK1 (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-IgG (HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of DLK1 in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use.
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

1 plate coated with human DLK1 Antibody	(6 x 16-well strips)	
2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
1 vial Detection Antibody	(20 µl)	(DET)
1 vial HRP 100X (HRP Conjugated anti-guinea pig IgG)	(150 µl)	(HRP 100X)
1 vial human DLK1 Standard (lyophilized)	(60 ng)	(STD)
1 bottle TMB Substrate Solution	(12 ml)	(TMB)
1 bottle Stop Solution	(12 ml)	(STOP)
2 plate sealers (plastic film)		
2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450 nm
- Calibrated precision single and multi-channel pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 50 ml Wash Buffer 10X + 450 ml water) to obtain Wash Buffer 1X.

- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 20 ml ELISA Buffer 10X + 180 ml water) to obtain ELISA Buffer 1X.

- **Detection Antibody (DET)** has to be diluted to 1:2000 in ELISA Buffer 1X
  (5 µl DET + 10 ml ELISA Buffer 1X).
  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 100 µl in 10 ml of ELISA Buffer 1X (1:100).
  NOTE: *The diluted HRP is used within one hour of preparation.*

- **Human DLK1 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 60 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C.*

  - Dilute the standard protein concentrate (STD) (**60 ng/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.

  - Suggested standard points are:

    30 , 15 , 7.5 , 3.75 , 1.875 , 0.938 , 0.469 and 0 ng/ml.

Dilute further for the standard curve:

To obtain	Add	Into
30 ng/ml	300 µl of DLK1 (60 ng/ml)	300 µl of ELISA Buffer 1X
15 ng/ml	300 µl of DLK1 (30 ng/ml)	300 µl of ELISA Buffer 1X
7.5 ng/ml	300 µl of DLK1 (15 ng/ml)	300 µl of ELISA Buffer 1X
3.75 ng/ml	300 µl of DLK1 (7.5 ng/ml)	300 µl of ELISA Buffer 1X
1.875 ng/ml	300 µl of DLK1 (3.75 ng/ml)	300 µl of ELISA Buffer 1X
0.938 ng/ml	300 µl of DLK1 (1.875 ng/ml)	300 µl of ELISA Buffer 1X
0.469 ng/ml	300 µl of DLK1 (0.938 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample Collection, storage and dilution

Serum : Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Serum has to be used undiluted. Samples containing visible precipitates must be clarified before use.

NOTE: *If sample values fall outside the detection range of the assay, a lower or higher dilution in ELISA Buffer 1X may be required!*

8.3. Assay Procedure (Checklist)

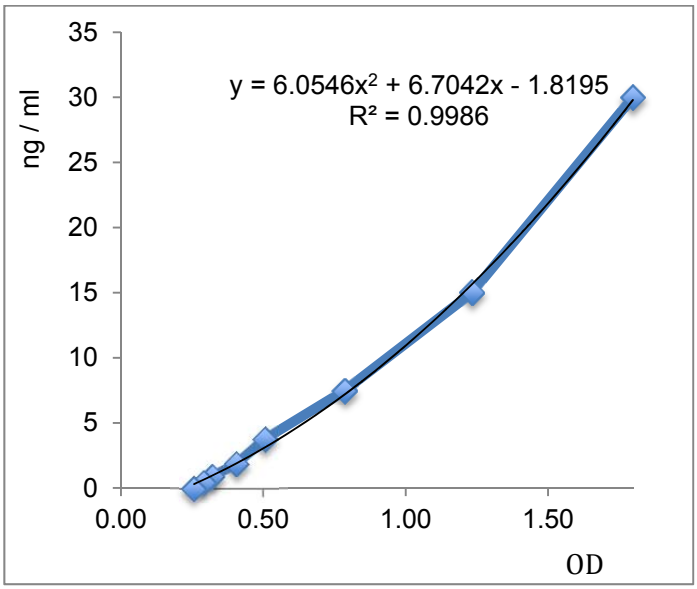
☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 16-well strips coated with DLK1 antibody when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted serum or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples).**
☐	3. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the Detection Antibody **(DET) (see 8.1. Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Conjugated anti-rabbit IgG) **(HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB Substrate Solution **(TMB)**.
☐	12. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 10 minutes**.
☐	13. Stop the reaction by adding 100 µl of Stop Solution **(STOP)**. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
	! CAUTION: CORROSIVE SOLUTION!
☐	14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

9. Calculation of Results

- Average the duplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding DLK1 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the DLK1 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation.
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of human DLK1 in the samples.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:



Standard hDLK1 (ng/ml)	Optical Density (mean)
30	1.80
15	1.23
7.5	0.79
3.75	0.50
1.875	0.44
0.938	0.32
0.469	0.29
0	0.26

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of DLK1 that can be detected by this assay is 336 pg/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.469 ng/ml – 30 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human DLK1. It does not cross-react with human DLL1, human DNER, human adiponectin, human progranulin, human ANGPTL3, human RBP4, human resistin, human vaspin, human GPX3, human Nampt, human IL-33, human clusterin, human leptin, human CTRP5, mouse Nampt, rat Nampt.

D. Intra-assay precision:

Seven samples of known concentrations of human DLK1 were assayed in replicates 5 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	1.09	0.04	4.07	5
2	1.47	0.1	6.70	5
3	1.63	0.09	5.36	5
4	0.79	0.03	4.43	5
5	1.77	0.07	3.74	5
6	1.76	0.09	5.27	5
7	0.79	0.04	5.44	5

E. Inter-assay precision:

Four samples of known concentrations of human DLK1 were assayed in 4 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	1.67	0.14	8.17	4
2	2.15	0.21	9.60	4
3	2.00	0.16	7.85	4
4	0.77	0.05	6.94	4

F. Recovery:

When samples (serum) are spiked with known concentrations of human DLK1, the recovery averages 92% (range from 85% to 105%).

Samples	Average recovery (%)	Range (%)
1	88.68	85-95
2	85.73	85-95
3	100.54	95-105

G. Linearity:

Different human serum samples containing DLK1 were diluted several fold (1 to 1/2) and the measured recoveries ranged from 98% to 106%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1	1.354	1.354	100
	1 : 2	0.677	0.665	98.3
2	1	0.707	0.707	100
	1 : 2	0.354	0.353	99.9
3	1	1.904	1.904	100
	1 : 2	0.952	1.004	105.4
4	1	0.839	0.839	100
	1 : 2	0.420	0.412	98.1

H. Expected values:

DLK1 levels range in serum from **0.4 to > 2.5 ng/ml** (from healthy donors).

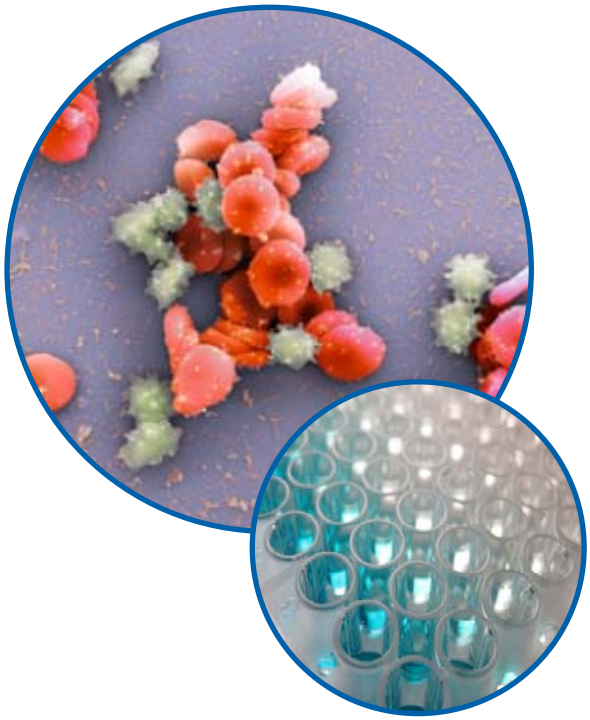
12. Technical Hints and Limitations

- It is recommended that all standards, controls and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution (TMB) protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution (STOP) should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes



Product Specific References:

1. G. Flehmig, et al.; PLos One **9,** e99785 (2014)

For more References please visit www.adipogen.com!

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