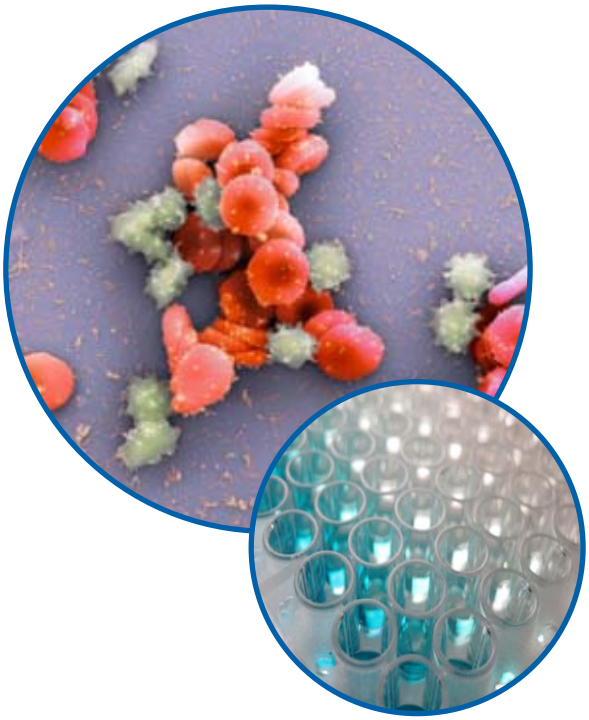

AdipoGen® LIFE SCIENCES

MANUAL

Progranulin (human) ELISA Kit

For research use only. Not for diagnostic use.

Version 3 (05-May-2015)

Cat. No. AG-45A-0018YEK-KI01

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1. Intended Use

The Progranulin (human) ELISA Kit is to be used for the *in vitro* quantitative determination of human progranulin in serum, plasma, urine, cell culture supernatant and cerebrospinal fluid (CSF; *R. Ghidoni et al.; Neurology 71, 1235 (2008)*). This ELISA Kit is for research use only.

2. Introduction

Progranulin (PGRN) also called epithelin precursor, proepithelin (PEPI), PC cell-derived growth factor (PCDGF), acrogranin, or paragranulin is a 593aa cysteine-rich protein of 68.5kDa, that is typically secreted in a highly glycosylated 88kDa form. As a result of proteolytic cleavage of PGRN by extracellular proteases such as elastase, neutrophil-derived protease, MMP-9, MMP-14 or ADAMTS7 a family of active 6kDa peptides (granulins (GRNs) A to G and paragranulin) are formed that each contain 10-12 highly conserved cysteine residues (1). The mRNA is expressed in many epithelial cells both *in vitro* and *in vivo*. He and Bateman (1999) demonstrated that overexpression of the progranulin gene in SW-13 adrenal carcinoma cells and MDCK nontransformed renal epithelia resulted in transfection-specific secretion of progranulin, acquired clonogenicity in semisolid agar, and increased mitosis in monolayer culture, whereas diminution of progranulin gene expression impaired growth of these cells (2). When applied to a cutaneous wound, progranulin increased the accumulation of neutrophils, macrophages, blood vessels, and fibroblasts in the wound. It acted directly on isolated dermal fibroblasts and endothelial cells to promote division, migration, and the formation of capillary-like tubule structures, concluding that progranulin is, therefore, probably a wound-related growth factor (3). PGRN appears to enhance survival and neurite outgrowth *in vitro* and *in vivo* (4). Neurons treated with PGRN displayed enhanced phosphorylation of the serine/threonine kinase Akt and the glycogen synthase kinase-3 beta (GSK-3β), a substrate of Akt, with subsequent inactivation of GSK-3β (5). Akt is a major component of pro-survival signalling pathways and regulates several functions including cell growth, apoptosis and survival among others. Mutations in PGRN have been found to be a common cause of familial frontotemporal lobar degeneration called FTLD (6). Since PGRN has neurotrophic properties and most mutations are predicted to result in a heterozygous loss of gene expression, PGRN deficiency so called hafloinsufficiency is thought to cause neurodegeneration in these patients. Reduced PGRN levels in serum, plasma, or CSF levels proved to be a valuable biomarker for early detection and diagnosis of PGRN mutation carriers in FTLD (7). PGRN has been also implicated in metabolism. Serum PGRN levels are increased in obese and type 2 diabetic patients (8). Using a murine HFD model obesity makes the adipose tissue-derived PGRN upregulated, which in turn induces IL-6, culminating in insulin resistance (9). It has been lately shown that PGRN is a biomarker for microalbuminuria in type 1 diabetes (10). Inflammation is closely related to metabolic dysfunctions. PGRN has been recently shown that it binds to TNFR1 and TNFR2, leading to attenuation of TNF-α signalling (11). Therefore, measurement of PGRN might give clues to both neurological and endocrinological inflammatory dysfunctions.

3. General References

(1) Isolation and sequence of the granulin precursor cDNA from human bone marrow reveals tandem cysteine-rich granulin domains: V. Bhandari, et al.; PNAS **89,** 1715 (1992)

(2) Progranulin gene expression regulates epithelial cell growth and promotes tumor growth in vivo: Z. He, et al.; Cancer Res. **59,** 3222 (1999)

(3) Progranulin is a mediator of the wound response: Z. He, et al.: Nature Med. **9,** 225 (2003)

(4) Progranulin functions as a neurotrophic factor to regulate neurite outgrowth and enhance neuronal survival: P. Van Damme, et al.; J. Cell Biol. **181,** 37 (2008)

(5) Progranulin promotes neurite outgrowth and neuronal differentiation by regulating GSK-3β: X. Gao, et al.; Protein Cell **1,** 552 (2010)

(6) Optimal Plasma Progranulin Cutoff Value for Predicting Null Progranulin Mutations in Neurodegenerative Diseases: A Multicenter Italian Study: R. Ghidoni, et al.; Neurodegener. Dis. **9,** 121 (2012)

(7) Losing protein in the brain: The case of progranulin: R. Ghidoni, et al.; Brain Res. **1476,** 172 (2012)

(8) Serum progranulin concentrations may be associated with macrophage infiltration into omental adipose tissue: B.S. Youn, et al.; Diabetes **58,** 627 (2009)

(9) PGRN is a Key Adipokine Mediating High Fat Diet-Induced Insulin Resistance and Obesity through IL-6 in Adipose Tissue: T. Matsubara, et al.; Cell Metab. **15,** 38 (2012)

(10) Novel urinary protein biomarkers predicting the development of microalbuminuria and renal function decline in type 1 diabetes: D. Schlatzer, et al.; Diabetes Care **35,** 549 (2012)

(11) The growth factor progranulin binds to TNF receptors and is therapeutic against inflammatory arthritis in mice: W. Tang, et al.; Science **332,** 478 (2011)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human progranulin in biological fluids. A polyclonal antibody specific for progranulin 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, progranulin is recognized by the addition of a biotinylated polyclonal antibody specific for progranulin (Detection Antibody). After removal of excess biotinylated antibody, HRP labeled streptavidin (STREP-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 progranulin 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 progranulin 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	(30 µl)	(DET)
1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
1 vial human progranulin Standard (lyophilized)	(8 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 1:1000 in ELISA Buffer 1X
  (10 µl DET + 10 ml ELISA Buffer 1X).
  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
  - Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

  NOTE: *The diluted STREP-HRP is not stable and cannot be stored!*

- **Human Progranulin Standard (STD)** has to be reconstituted with 1 ml of deionized water.
  - This reconstitution produces a stock solution of 8 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) (**8 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:
    4 , 2 , 1 , 0.5 , 0.25 , 0.125 , 0.063 and 0 ng/ml.

Dilute further for the standard curve:

To obtain	Add	Into
4 ng/ml	300 µl of progranulin (8 ng/ml)	300 µl of ELISA Buffer 1X
2 ng/ml	300 µl of progranulin (4 ng/ml)	300 µl of ELISA Buffer 1X
1 ng/ml	300 µl of progranulin (2 ng/ml)	300 µl of ELISA Buffer 1X
0.5 ng/ml	300 µl of progranulin (1 ng/ml)	300 µl of ELISA Buffer 1X
0.25 ng/ml	300 µl of progranulin (0.5 ng/ml)	300 µl of ELISA Buffer 1X
0.125 ng/ml	300 µl of progranulin (0.25 ng/ml)	300 µl of ELISA Buffer 1X
0.063 ng/ml	300 µl of progranulin (0.125 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.

Plasma : Collect plasma using heparin, EDTA, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -20°C for later use. Avoid repeated freeze/ thaw cycles.

Urine : Aseptically collect the urine of the day, voided directly into a sterile container. Assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze/thaw cycles.

Serum, Plasma, Urine, Cell Culture Supernatant or CSF have to be diluted in ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/200 dilution of serum or plasma and 1/20 dilution of urine are recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required! For CSF a starting dilution of 1/4-1/5 is recommended based on literature references using this ELISA Kit.**

** R. Ghidoni, et al.; Neurology **71,** 1235 (2008) | P. Steinacker, et al.; PLOs ONE **6,** e23600 (2011)*

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 progranulin 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 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 Labeled Streptavidin **(STREP-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 progranulin concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the progranulin 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 progranulin in the samples.

10. Typical Data

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

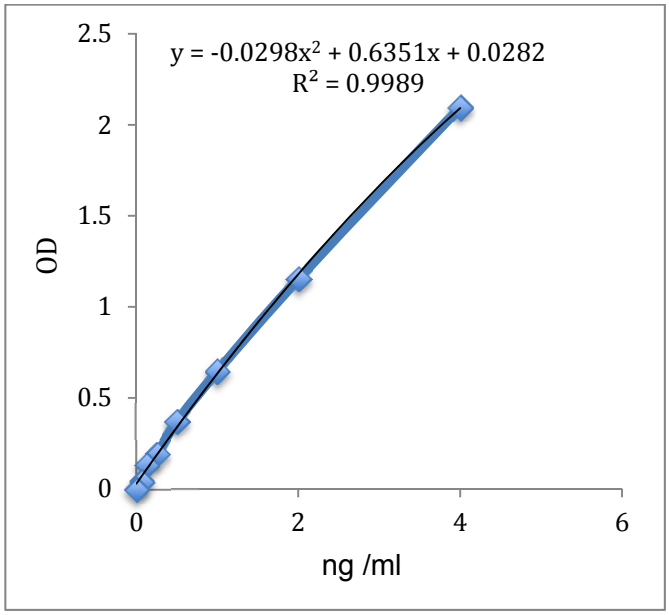


Figure: Standard curve

Standard hProgranulin (ng/ml)	Optical Density (mean)
4	2.097
2	1.1545
1	0.6445
0.5	0.3695
0.25	0.1905
0.125	0.134
0.0625	0.0415
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of progranulin that can be detected by this assay is 32 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: 0.063 ng/ml – 4 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human progranulin. It does not cross-react with human RBP4, human adiponectin, human Nampt, human leptin, human RELM-β, human ANGPTL6, human FABP4, human TNF-α, human IL-33, human GPX3, human resistin, human clusterin, human vaspin, human PAI1, human ANGPTL3, mouse Nampt, rat Nampt.

D. Intra-assay precision:

Six serum samples of known concentrations of human progranulin were assayed in replicates 8 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	129.31	4.90	3.79	8
2	137.48	7.75	5.63	8
3	128.25	8.89	6.93	8
4	248.28	15.70	6.32	8
5	144.99	7.36	5.08	8
6	95.54	2.88	3.02	8

Six urine samples of known concentrations of human progranulin were assayed in replicates 8 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	14.96	0.76	5.10	8
2	7.10	0.46	6.44	8
3	15.98	0.63	3.93	8
4	17.64	0.93	5.27	8
5	9.84	0.52	5.27	8
6	12.70	0.77	6.07	8

E. Inter-assay precision:

Six serum samples of known concentrations of human progranulin were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	133.81	9.52	7.12	5
2	149.66	8.28	5.53	5
3	128.45	9.41	7.32	5
4	220.68	10.40	4.71	5
5	140.61	9.21	6.55	5
6	92.23	6.50	7.05	5

Six urine samples of known concentrations of human progranulin were assayed in 4 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	7.00	0.34	4.92	4
2	14.73	1.07	7.25	4
3	10.91	0.56	5.15	4
4	16.46	1.06	6.41	4
5	9.10	0.42	4.63	4
6	8.98	0.65	7.23	4

F. Recovery:

The recovery of progranulin spiked to three different levels in five different serum samples and four different urine samples throughout the range of assay was evaluated.

Samples		Average recovery (%)	Range (%)
Serum	**1**	97.16	91-103
	2	97.98	93-102
	3	97.69	95-101
	4	95.41	89-99
	5	96.73	91-100
Urine	**1**	100.76	95-105
	2	98.79	95-105
	3	97.42	92-102
	4	99.04	95-105

G. Linearity:

Different human serum samples containing progranulin were diluted several fold (1/200 to 1/800) and the measured recoveries ranged from 93% to 102%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1 : 200	156.13	156.13	100
	1 : 400	78.06	78.79	101
	1 : 800	39.03	39.78	102
2	1 : 200	239.69	239.69	100
	1 : 400	119.84	111.94	93
	1 : 800	59.92	57.27	96
3	1 : 200	165.84	165.84	100
	1 : 400	82.92	80.94	98
	1 : 800	41.46	40.26	97

Different human urine samples containing progranulin were diluted several fold (1/20 to 1/80) and the measured recoveries ranged from 97% to 106%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1 : 20	12.20	12.20	100
	1 : 40	6.10	6.46	105.88
	1 : 80	3.05	3.10	101.73
2	1 : 20	11.47	11.47	100
	1 : 40	5.74	5.58	97.37
	1 : 80	2.87	2.95	102.93
3	1 : 20	8.70	8.70	100
	1 : 40	4.35	4.45	102.27
	1 : 80	2.17	2.27	104.30

H. Expected values:

Progranulin levels range in plasma and serum from **100 to 250 ng/ml** (from healthy donors).

Progranulin levels range in urine from **5 to 20 ng/ml** (from healthy donors).

Progranulin levels in CSF are expected at **<10 ng/ml** based on literature references using this ELISA Kit.*

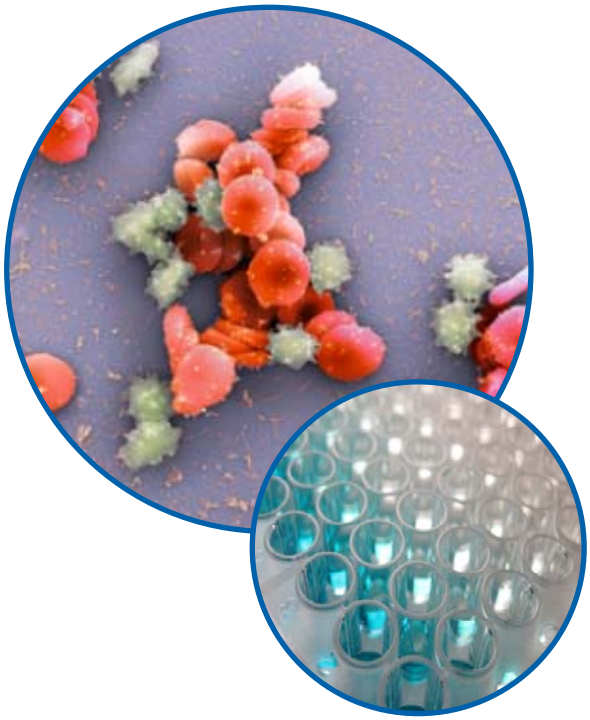
** R. Ghidoni, et al.; Neurology **71,** 1235 (2008) | P. Steinacker, et al.; PLOs ONE **6,** e23600 (2011)*

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 STREP-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.



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For more References please visit www.adipogen.com!

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