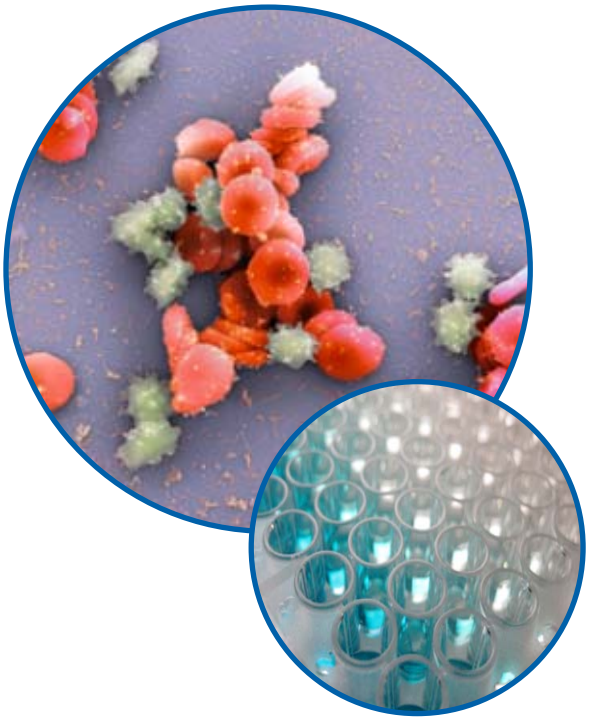


AdipoGen® LIFE SCIENCES

MANUAL

Progranulin (rat) ELISA Kit

For research use only. Not for diagnostic use.

Version 2 (04-May-2015)

Cat. No. AG-45A-0043YEK-KI01

Table of Contents

1. Intended Use

The Progranulin (rat) ELISA Kit is to be used for the *in vitro* quantitative determination of rat progranulin in serum and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

The granulins are a family of cysteine-rich polypeptides, some of which have growth modulatory activity. The widespread occurrence of granulin mRNA in cells from the hematopoietic system and in epithelia implies important functions in these tissues. All 4 known human granulin-like peptides are encoded in a single precursor, progranulin, which has a highly conserved 12-cysteine backbone defining a consensus sequence that is repeated 7 times (1). Progranulin is a 593-amino acid glycoprotein, the mRNA of which is expressed in many epithelial cells both in vitro and in vivo. He and Bateman 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). They proposed that the rate of growth of some epithelia is proportional to the level of intrinsic progranulin gene expression, and that elevated progranulin gene expression confers a transformed phenotype on epithelial cells including anchorage independence in vitro and growth as tumors in nude mice. They also found that in murine transcutaneous puncture wounds, progranulin mRNA is expressed in the inflammatory infiltrate and is highly induced in dermal fibroblasts and endothelia following injury. 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). Due to its tumor-promoting activity and multiple functions on hematopoietic cells and endothelial cells, progranulin can be a novel marker for oncology and hematology.

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.; Proc. Nat. Acad. Sci. **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. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of rat 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 rat 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	(20 µl)	(DET)
1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
1 vial rat progranulin Standard (lyophilized)	(8 ng)	(STD)
1 bottle TMB Substrate Solution	(12 ml)	(TMB)
1 bottle Stop Solution	(12 ml)	(STOP)
2 plates 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 1:1'000 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!*

- **Rat 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.

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

NOTE: *As a starting point, 1/1'000 dilution of serum is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

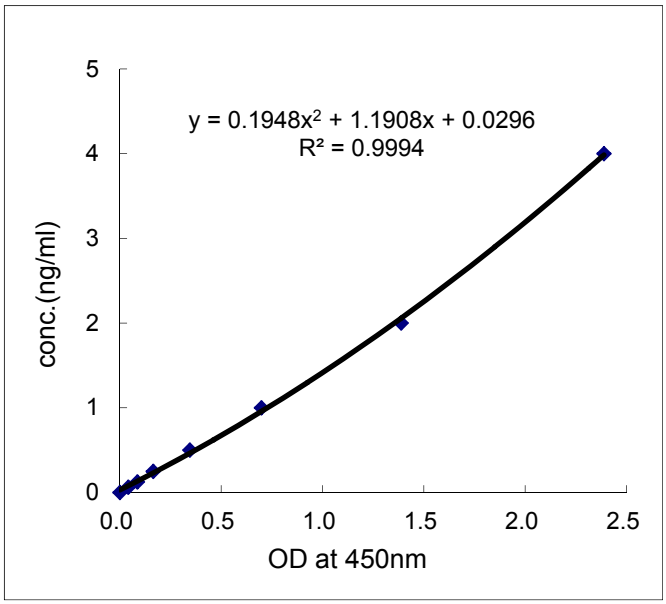
☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 16-well strips coated with progranulin antibody when opened can be stored at 4°C for up to 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 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 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 rat progranulin in the samples.

10. Typical Data

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



Standard rProgranulin (ng/ml)	Optical Density (mean)
4	2.389
2	1.388
1	0.699
0.5	0.346
0.25	0.165
0.125	0.087
0.063	0.042
0	0

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of progranulin that can be detected by this assay is 40 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 rat progranulin. It does not cross-react with human progranulin, human granulin C, human granulin F, rat RBP4, rat adiponectin, rat Nampt, rat ANGPTL4, rat lipocalin-2, rat resistin, mouse RBP4, mouse adiponectin, human RBP4, human adiponectin.

Mouse progranulin shows weakly 10% cross-reactivity in this assay.

D. Intra-assay precision:

Six samples of known concentrations of rat progranulin were assayed in replicates 6 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	239.71	15.18	6.33	6
2	335.92	26.10	7.77	6
3	362.92	21.80	6.01	6
4	380.62	12.98	3.41	6
5	356.61	9.20	2.58	6
6	398.11	29.00	7.28	6

E. Inter-assay precision:

Six samples of known concentrations of rat progranulin were assayed in 6 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	321.75	17.99	5.59	6
2	348.19	17.86	5.13	6
3	362.21	21.30	5.88	6
4	348.74	14.25	4.09	6
5	378.63	23.61	6.24	6
6	392.77	25.18	6.41	6

F. Recovery:

When samples (serum) are spiked with known concentrations of rat progranulin, the recovery averages 98% (range from 88% to 105%).

Samples	Average recovery (%)	Range (%)
1	99.74	95-105
2	100.26	95-105
3	92.51	88-100
4	99.64	95-105

G. Linearity:

Different rat serum samples containing progranulin were diluted several fold (1/800 to 1/1,200) and the measured recoveries ranged from 95% to 107%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1 : 800	374.53	374.53	100
	1 : 1,000	299.63	299.86	100.08
	1 : 1,200	249.69	251.95	100.91
2	1 : 800	313.19	313.19	100
	1 : 1,000	250.55	252.59	100.82
	1 : 1,600	208.79	204.76	98.07
3	1 : 800	369.32	369.32	100
	1 : 1,000	295.46	311.09	105.29
	1 : 1,600	246.21	253.78	103.07

H. Expected values:

Progranulin levels range in rat serum samples from **200 to 500 ng/ml**.

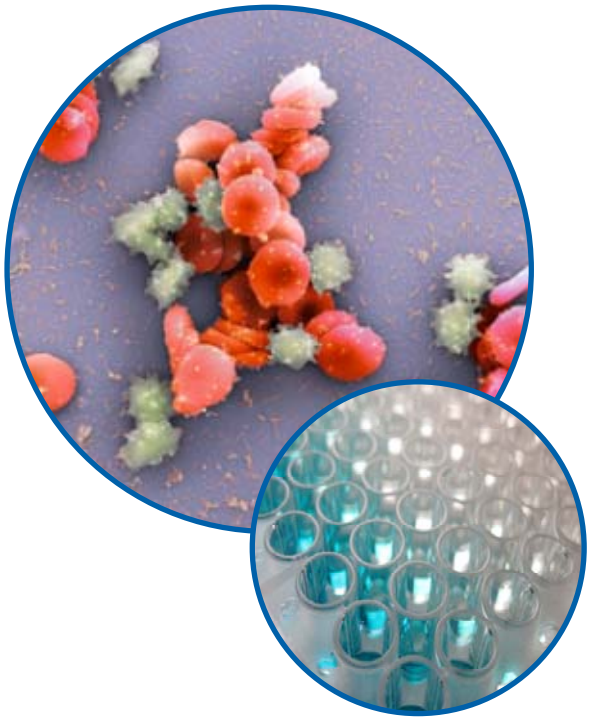
12. Technical Hints and Limitations

- 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.

14. Notes



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