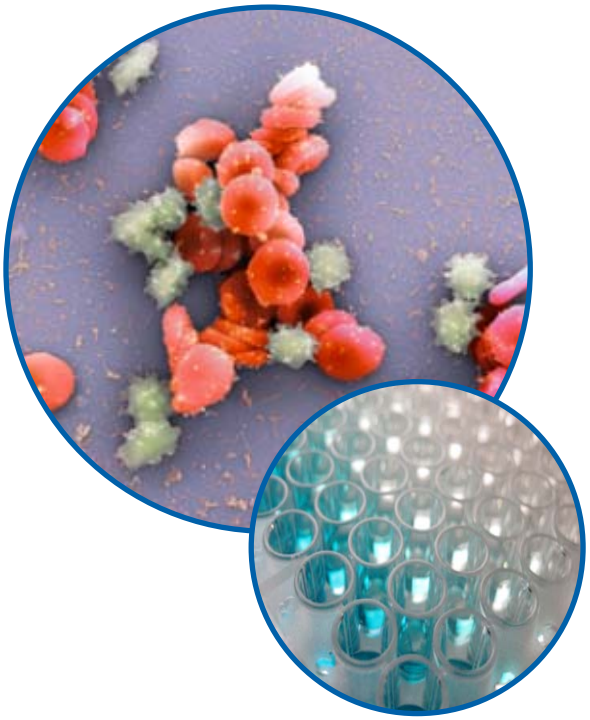

AdipoGen® LIFE SCIENCES

MANUAL

Adiponectin (rat) ELISA Kit

For research use only. Not for diagnostic use.

Version 3 (04-May-2015)

Cat. No. AG-45A-0005YEK-KI01

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1. Intended Use

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

2. Introduction

It is becoming clear that adipose tissue is not merely a storage place for excess energy unused but secretes a number of biologically active soluble factors collectively named adipocyto-kines, thereby regulating glucose and fatty acid metabolism. The gene encoding adiponectin was identified as an adipose tissue-specific gene by screening a subtractive adipocyte cDNA library (1). The gene product was independently identified as gelatin-binding protein-28 (GBP28) (2). The rat orthologue was isolated by several groups and designated adipocyte complement-related protein of 30kDa (Acrp30), adipocyte most abundant protein-1(APM1), or AdipoQ (3-4). Albeit differently being named it was unequivocally concluded that adiponectin is an adipocyte-specific protein and represents a major serum protein. The full length adiponectin in plasma exists as trimer, hexamer, and multimer whereas extremely low amount of globular domain itself also exists in plasma as trimer (5). Due to its adipocyte-specific expression it was thought that adipo-nectin could be responsible for regulation of adipocyte physiology. In many rodent models, it has been shown that infusion of adiponectin ameliorates hyperglycemia and hyperinsulinemia (6-8). One notable mechanism for decreasing blood sugar is that adiponectin inhibits hepatic glucose production by downregulating the key enzymes involved in gluconeogenesis such as phophoenolpyruvate carboxy-kinase and glucose-6-phosphatase (9). When skeletal muscle cells or a muscle cell line is treated with adiponectin, both glucose uptake and fatty acid oxidation are significantly upregulated (6-8). These studies suggests a unifying conclusion that adiponectin regulates both systemic and hepatic insulin resistance. Direct evidence was shown that administration of adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice (10). Therefore, measurement of serum adiponectin levels gives us important information on the role of adiponectin in regulation of glucose and/or lipid metabolism.

3. General References

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(2) Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma: Y. Nakano, et al.; J. Biochem. **20,** 803 (1996)

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(4) AdipoQ is a novel adipose-specific gene dysregulated in obesity: E. Hu, et al.; J. Biol. Chem. **271,** 10697 (1996)

(5) Oligomerization state-dependent activation of NF-kB signaling pathway by Acrp30: T.S. Taso, et al.; J. Biol. Chem. **277,** 29359 (2002)

(6) The adipocyte-secreted protein Acrp30 enhances hepatic insulin action: A.H. Berg, et al.; Nat. Med. **7,** 947 (2001)

(7) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity: T. Yamauchi, et al.; Nat. Med. **7,** 941 (2001)

(8) Adiponectin stimulates utilization and fatty-acid oxidation by activating AMP-activated protein kinase: T. Yamauchi, et al.; Nat. Med. **8,** 1 (2002)

(9) Endogenous glucose production is inhibited by the adipose-derived protein Acrp30: T.P. Combs, et al.; J. Clin. Invest. **108,** 1875 (2001)

(10) Globular adiponectin protected ob/ob mice from diabetes and apoE-deficien mice from atherosclerosis: T. Yamauchi, et al.; J. Biol. Chem. **278,** 2461 (2003)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of rat adiponectin in biological fluids. A polyclonal antibody specific for adiponectin 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, adiponectin is recognized by the addition of a monoclonal antibody specific for adiponectin (Detection Antibody). After removal of excess monoclonal antibody, HRP conjugated anti-mouse 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 adiponectin 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 Adiponectin 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-mouse IgG)	(150 µl)	(HRP 100X)
1 vial rat Adiponectin Standard (lyophilized)	(48 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 1:1'000 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 100X (HRP Conjugated anti-mouse 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.*

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

  - This reconstitution produces a stock solution of 48 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) (**48 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:

    24 , 12 , 6 , 3 , 1.5 , 0.75 , 0.375 and 0 ng/ml.

Dilute further for the standard curve:

To obtain	Add	Into
24 ng/ml	300 µl of adiponectin (48 ng/ml)	300 µl of ELISA Buffer 1X
12 ng/ml	300 µl of adiponectin (24 ng/ml)	300 µl of ELISA Buffer 1X
6 ng/ml	300 µl of adiponectin (12 ng/ml)	300 µl of ELISA Buffer 1X
3 ng/ml	300 µl of adiponectin (6 ng/ml)	300 µl of ELISA Buffer 1X
1.5 ng/ml	300 µl of adiponectin (3 ng/ml)	300 µl of ELISA Buffer 1X
0.75 ng/ml	300 µl of adiponectin (1.5 ng/ml)	300 µl of ELISA Buffer 1X
0.375 ng/ml	300 µl of adiponectin (0.75 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.

Serum, Plasma 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 or plasma are 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 adiponectin 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, plasma 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-mouse 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 20 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 **(STOP)** 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 adiponectin concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the adiponectin 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 adiponectin in the samples.

10. Typical Data

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

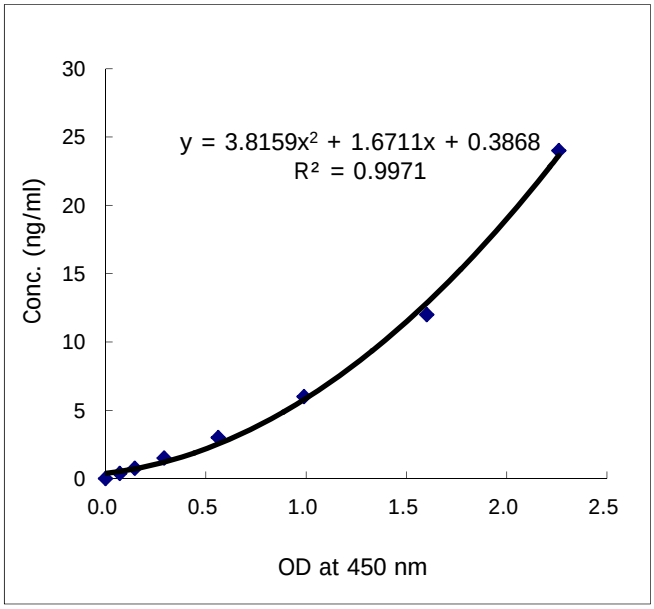


Figure: Standard curve

Standard rAdiponectin (ng/ml)	Optical Density (mean)
24	2.260
12	1.602
6	0.989
3	0.561
1.5	0.292
0.75	0.146
0.375	0.071
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of adiponectin that can be detected by this assay is 50 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: 0.375 ng/ml – 24 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant rat adiponectin. It does not cross-react with mouse adiponectin, human adiponectin, rat Nampt, rat resistin, rat RELM-α, rat leptin, human TNF-α.

D. Intra-assay precision:

Eight samples of known concentrations of rat adiponectin were assayed in replicates 12 times to test precision within an assay.

Samples	Means (µg/ml)	SD	CV (%)	n
1	7.70	0.18	2.29	12
2	6.04	0.37	6.07	12
3	10.10	0.50	4.96	12
4	11.61	0.75	6.48	12
5	11.89	0.26	2.19	12
6	4.41	0.33	7.53	12
7	6.29	0.22	3.43	12
8	4.67	0.25	5.40	12

E. Inter-assay precision:

Eight samples of known concentrations of rat adiponectin were assayed in 8 separate assays to test precision between assays.

Samples	Means (µg/ml)	SD	CV (%)	n
1	5.45	0.30	5.55	8
2	8.04	0.49	6.08	8
3	4.20	0.11	2.60	8
4	7.78	0.34	4.42	8
5	9.04	0.48	5.35	8
6	7.80	0.28	3.60	8
7	3.25	0.10	3.21	8
8	2.49	0.20	8.10	8

F. Recovery:

When samples (serum or plasma) are spiked with known concentrations of rat adiponectin, the recovery averages 95% (range from 87% to 105%).

Samples	Average recovery (%)	Range (%)
1	97.6	96.3-99.7
2	95.1	87.4-104.2
3	97.7	94.0-102.9
4	96.8	90.7-100
5	95.1	91.8-97.2
6	93.7	88.2-96.6
7	94.0	90.8-97.0
8	93.5	91.8-96.8

G. Linearity:

Different rat serum samples containing adiponectin were diluted several fold (1/1,000 to 1/2,000) and the measured recoveries ranged from 90% to 103%.

Samples	Sample Dilution	Expected (µg/ml)	Observed (µg/ml)	% of Expected
1	1 : 1,000	12.9	12.9	100
	1 : 1,200	10.8	11.0	102.3
	1 : 1,500	8.6	8.5	98.6
	1 : 1,700	7.6	7.7	100.6
	1 : 2,000	6.5	5.8	90.0
2	1 : 1,000	6.2	6.2	100
	1 : 1,200	5.2	5.2	100.1
	1 : 1,500	4.1	4.1	99.5
	1 : 1,700	3.6	3.6	98.9
	1 : 2,000	3.1	3.0	96.9

H. Expected values:

Adiponectin levels range in plasma and serum from **3 to >7 µg/ml** (from normal rats).

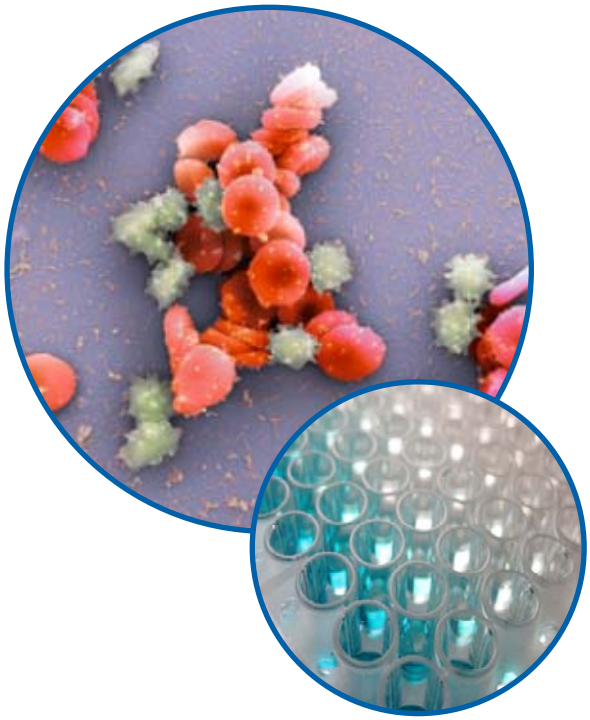
12. Technical Hints and Limitations

- 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



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For more References please visit www.adipogen.com!

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