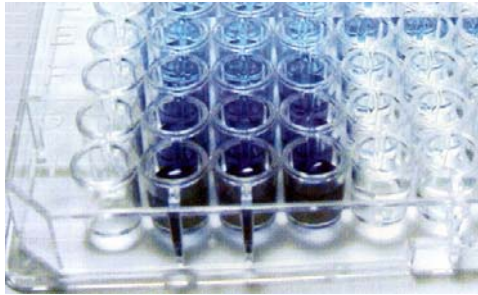


Rat Adiponectin ELISA Kit

Cat. No. A0434EK



Instruction Manual
Version 2.0.2

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES



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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 adipocytokines, 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 adiponectin 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 phosphoenolpyruvate 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 suggest 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.

Assay Principles

This kit is an enzyme-linked immunosorbent assay (ELISA) for quantitative determination of adiponectin in rat serum, plasma or cell culture supernatants.

Polyclonal antibody specific for rat adiponectin has been pre-coated onto 96 well microplate. Standards and samples are pipetted into the wells and any adiponectin present is bound by immobilized antibody. Bound adiponectin is captured by anti-rat adiponectin monoclonal antibody. HRP conjugated mouse IgG is added. After washing, a substrate solution is added. The colors develop in proportion to the bound adiponectin quantity. The color development is stopped and the intensity of color is measured.

Kit Components

- 1) Antibody coated 96-well plate, 12 X 8-well strips
- 2) 5x Wash concentrate, 100 ml
- 3) 5x Diluent, 50 ml
- 4) Secondary antibody, 12 ml
- 5) 100x Detector, 150 μ l
- 6) Standard, recombinant rat adiponectin expressed by HEK 293 cells, 1 vial, lyophilized
- 7) QC sample = a positive control of rat serum adiponectin, 1 vial, lyophilized (For actual concentrations of QC sample, see the 'Certificate of analysis' enclosed.)
- 8) Substrate I, 6 ml
- 9) Substrate II, 6 ml
- 10) Stop solution, 12 ml

Reagents Description

Antibody coated 96-well plate, 12 x 8-well strips, with absorbed polyclonal antibody against rat adiponectin
5x Wash concentrate, buffered detergent solution, supplied as a 5x concentrate
5x Diluent, for sample and reagent dilution
1x Secondary antibody, monoclonal antibody against rat adiponectin
100x detector, HRP conjugated mouse IgG
Standard, 48.0 ng, 1 vial each, recombinant rat adiponectin
QC sample, 1 vial each, rat serum
Substrate I and II, chromogenic reagents
Stop solution, 1M H₃PO₄

Storage of Reagents

Reagent must be stored at 2-8°C when not in use. Reagents must be brought to room temperature before use. Do not expose reagents to temperatures greater than 25°C. Diluted wash solution may be stored at room temperature for up to one month.

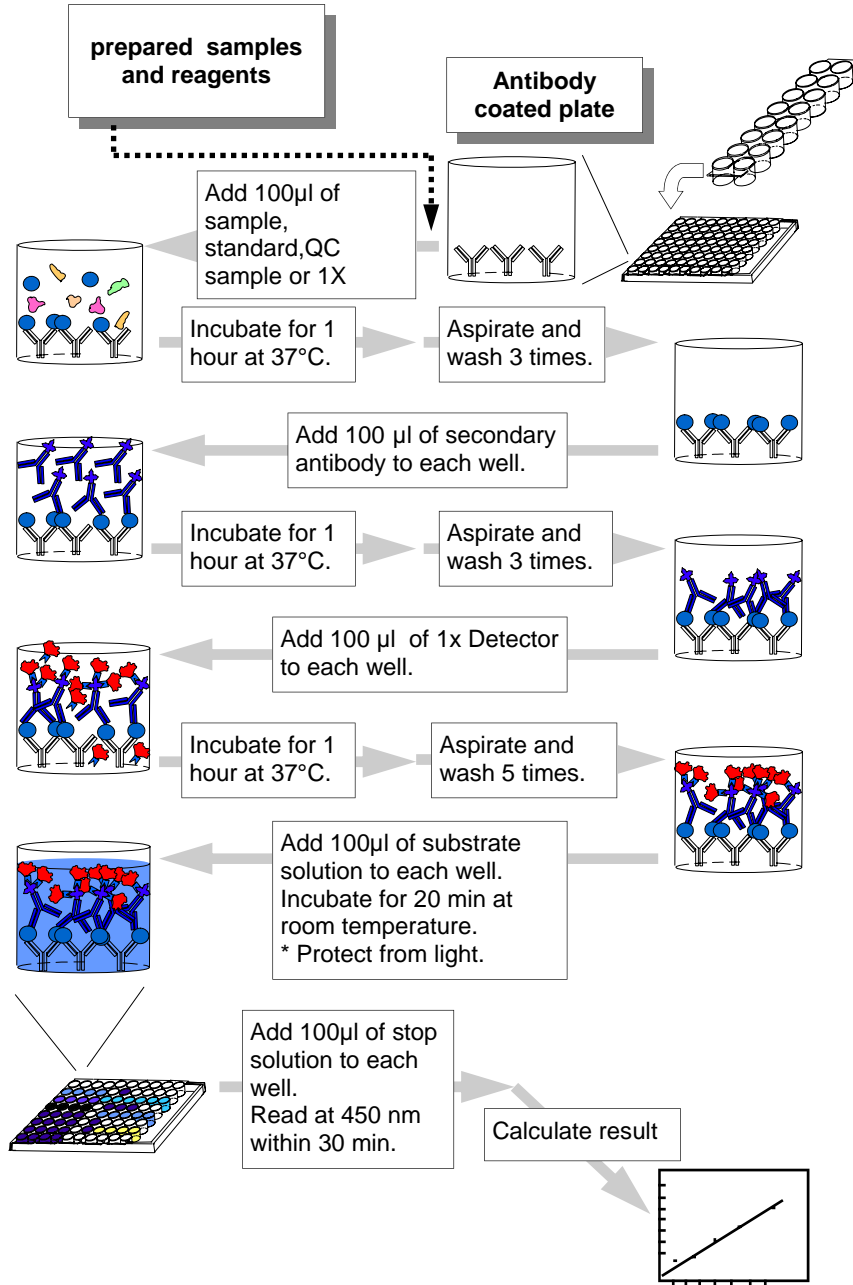
Materials Required but not Supplied

Precision single and multi-channel pipettes
Disposable pipette tips
Microtubes or equivalent for preparing dilutions
Disposable plastic containers for preparing working detector antibody and substrate
Reagent reservoirs
Microwell or microstrip plate reader 450 nm
Deionized water

Serum Collection and Storage

Blood samples for measurement of serum adiponectin are collected in microtubes and all tubes are centrifuged at 4°C for collection of serum. These are stored at -80°C until analyses

Flow Chart of Assay Procedure



Assay Procedure

1) Preparation of Working Reagents

1. Allow all samples and kit components to equilibrate to room temperature (20-25°C).
2. Plan the plate configuration and create a plate map. Calculate the amount of working reagents to use (See table below). It is recommended that standards and samples be run in duplicate.
3. Prepare **1X Wash Solution**. Dilute 5X Wash Concentrate 1:5 with deionized water (1 part 5X Wash Concentrate with 4 parts deionized water). The diluted 1X Wash Solution is stable for one month at room temperature.
4. Prepare **1X Diluent**. Dilute 5X Diluent 1:5 with deionized water (1 part 5X Diluent with 4 parts deionized water).
5. Prepare **1X Detector**. Dilute 100X Detector 1:100 with 1X Diluent (1 part 100X Detector with 99 parts 1X Diluent). Use the 1X Detector within one hour of preparation.
6. Freshly prepare just before use the **Substrate Solution** by adding one part Substrate I to one part Substrate II.

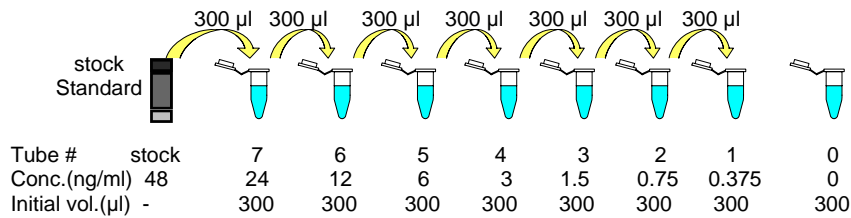
The amount of working reagents needed for 1 well				
Working reagents	Total volume needed	Stock solution added	Dilution solution added	Note
1X Wash Solution	2.8 ml	0.56 ml of 5X Wash Concentrate	2.24 ml of ddH ₂ O	Stable for 1 month at RT
1X Diluent	2 ml	0.4 ml of 5X Diluent	1.6 ml of ddH ₂ O	in the case of 10 µl plasma
1X Detector	110 µl	1.1 µl of 100X Detector	108.9 µl of 1X Diluent	Use within 1 hr.
Substrate Solution	110 µl	55 µl of Substrate I	55 µl of Substrate II	Freshly prepared just before use

7. Prepare working aliquots of the Standard as follows :
When opening the lyophilized Standard, remove cap gently as

the lyophilizate may have become dislodged during shipping. Add 1 ml of deionized water to the Standard vial to make a stock concentration of 48 ng/ml. Mix well.

A recommended dilution scheme is as follows :

- Label 8 microcentrifuge tubes #0-7. Add 300 µl of the 1X Diluent to the microcentrifuge tubes # 0-7.
- Add 300 µl of the stock Standard solution to tube #7 and vortex. This is Standard tube # 7 with a concentration of 24 ng/ml.
- Standards # 6 to 1 are then prepared by performing a 1:2 dilution of the preceding standard. Do not add any standard to the tube # 0.



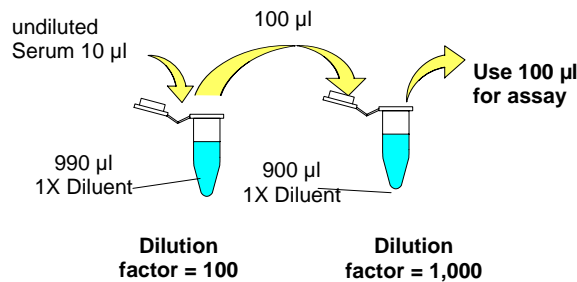
8. Reconstitute QC sample in 1 ml of deionized water.

2) Sample Preparation

Step 1. Dilute serum 1:100 with 1X Diluent (example, 10 µl serum plus 990 µl 1X Diluent; dilution factor=100) and mix well.

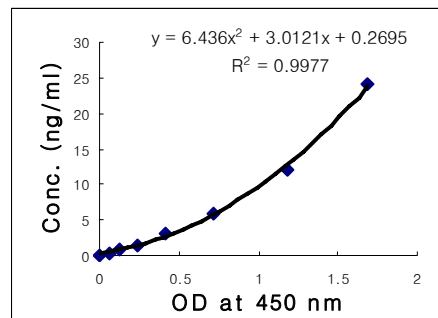
Step 2. Dilute the step 1 sample 1:10 with 1X Diluent (example, 100 µl of the step 1 sample plus 900 µl 1X Diluent, dilution factor=1,000)

* If samples fall the outside range of assay, a lower or higher dilution may be required.



3) Experiment procedure

1. Remove the appropriate number of microwell strips from the sealed foil pouch.
2. Pipette 100µl of standard 0 to 7, the reconstituted QC sample and diluted serum sample into the antibody-coated plate according to the plate configuration. Use a new pipette tip for each standard or sample.
3. Incubate at 37°C for 1 hour.
4. Remove the solution and wash 3 times with 250 µl of 1X Wash Solution to each well.
5. Add 100µl Secondary Antibody to each well.
6. Incubate at 37°C for 1 hour.
7. Remove the solution and wash 3 times with 250 µl of 1X Wash Solution to each well.
8. Add 100 µl 1X Detector to each well.
9. Incubate at 37°C for 1 hour.
10. Remove the solution and wash 5 times with 250 µl of 1X Wash Solution to each well.
11. Add 100 µl of the Substrate Solution to each well.
12. Incubate at room temperature for 20 min.
* Protect from light.
13. Using the multi-channel pipette, add 100 µl Stop Solution to each well.
14. Read at 450 nm.
15. Subtract the absorbance of the blank from the readings for each standard and sample.
16. Construct a standard curve by plotting the known concentrations (Y) of standard versus the absorbances (X) of standard. A measurable range is typically shown between 0.375 ng/ml and 24 ng/ml.
17. Calculate the adiponectin concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
18. The adiponectin concentrations calculated must be multiplied by dilution factor [see **2) Sample Preparation**] to obtain the concentrations of the undiluted samples (Dilution factor of lyophilized QC sample is 1000).



Performance Characteristics

1) Sensitivity

The limit of detection: 50 pg/ml

2) Precision

a. Intra-Assay (precision within an assay)

8 samples of known concentration were tested 12 times on one plate to assess intra-assay precision.

Sample No.	Average (µg/ml)	CV(%)
1	7.70	1.78
2	6.04	4.44
3	10.10	4.05
4	11.61	5.46
5	11.89	1.76
6	4.41	5.16
7	6.29	3.01
8	4.67	4.16

b. Inter-Assay (precision between assays)

8 samples of known concentration were tested 8 times on separated plate to assess inter-assay precision.

Sample No.	Average (µg/ml)	CV(%)
1	5.45	5.55
2	8.04	6.08
3	4.20	2.60
4	7.78	4.42
5	9.04	5.35
6	7.80	3.60
7	3.25	3.21
8	2.49	8.10

3) Recovery

The recovery of Adiponectin spiked to four different levels in six different rat serum samples throughout the range of assay was evaluated.

Sample No.	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

4) Specificity

a. Not cross reaction with human and mouse sera

b. Cross Reactivity with each protein

Analyte	Max. Conc. (ng/ml)	Cross Reactivity (%)
Rat Adiponectin	24	100
Mouse Adiponectin	100	N. R.
human Adiponectin	100	N. R.
Rat Visfatin	100	N. R.
Rat Resistin	100	N. R.
Rat RELM- α	100	N. R.
Rat Leptin	100	N. R.
Human TNF- α	100	N. R.

N. R : No Cross-Reactivity

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Troubleshooting Guide

Problem	Possible Cause	Solution
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 appropriate for the system.
	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 detector 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.
	Technique problem	Proper mixing of reagents and wash steps are critical.

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