Instruction Manual for
Human Adiponectin Immunoassay kit

Catalog Number: *hAd-HKU-003*

For Research Use Only, Not For use in Diagnostic Procedures

Manufactured by the HBHA center, University of Hong Kong

INTRODUCTION

Adiponectin, also known as apM1, Acrp30, GBP28 and adipoQ, is a circulating hormone predominantly produced from adipose tissue[1]. Many pharmacological studies demonstrated this protein possess potent anti-diabetic, anti-atherogenic and anti-inflammatory functions. Supplement of adiponectin protein can decrease blood glucose [2], improve insulin sensitivity [3], alleviate fatty liver [4] and prevent atherosclerosis [5]. The protein is posttranslationally modified by hydroxylation and glycosylation [6], and forms three different oligomeric complexes in the circulation [7].

Many clinical studies demonstrated that plasma adiponectin is a useful biomarker for metabolic syndrome, nonalcoholic steatohepatitis and certain type of cancers [1]. Decreased circulating levels of plasma adiponectin ('hypoadiponectinaemia') are associated with increased body mass index (BMI), decreased insulin sensitivity, less favourable plasma lipid profiles, increased levels of inflammatory markers and increased risk for the development of type 2 diabetes, hypertension, and coronary heart diseases. Low adiponectin concentrations were found to be predictive of a future reduction in insulin sensitivity and cardiovascular disorders. Administration of the anti-diabetic drugs thiazolidinediones (TZDs) raises circulating adiponectin levels [8]. In addition, low plasma adiponectin levels are also associated with nonalcoholic steatohepatitis (NASH) and certain types of cancers.

PRINCIPLE OF THE PROCEDURE

This assay is a sandwich ELISA using the monoclonal antibodies against human adiponectin. The immunoplate is pre-coated with Anti-Human Adiponectin Capture monoclonal antibody and the nonspecific binding sites are blocked. The Human Adiponectin in the samples or in the standards can bind to the capture antibody immobilized in the wells. After washing procedure, horseradish peroxidase-labeled anti-Human Adiponectin Detection Antibody which can bind to the Human Adiponectin trapped in the wells is added. After washing of excess of free enzyme conjugates, the substrate 3,3' 5,5' tetramethylbenzidine (TMB) is added. The enzyme-substrate reaction is terminated by the addition of a stop solution. The intensity of the color is measured spectrophotometrically by the absorbance at 450 nm after acidification of formed products. Since the increases in absorbance are directly proportional to the amount of captured Human Adiponectin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Human Adiponectin. The detailed description for establishment and validation of this assay was published in J.Bio.Chem (*2005, 280:18073-18080*).

INTENDED USE

This Human Adiponectin ELISA kit is designed for quantification of Human Adiponectin in serum, plasma, and adipocyte extracts or cell culture media samples. This kit specifically measures native Human Adiponectin and has no cross reactivity to Mouse Adiponectin.

REAGENTS SUPPLIED

Each kit is sufficient for one 96-well plate and contains the following components:

1. Microtiter Strips (96 wells): coated with anti-human adiponectin monoclonal antibody, sealed
2. Wash Buffer (5x): 40 ml
3. Assay Buffer (10x): 20 ml
4. Detection Antibody (100x): monoclonal antibody against human adiponectin conjugated with horseradish peroxidase, 0.12 ml
5. Human Adiponectin Standard: 100 ng of recombinant human adiponectin, lyophilized
6. Quality controls: High and Low, lyophilized
7. Substrate A: 6 ml
8. Substrate B: 6 ml
9. Stop Solution: 6 ml
10. Plate covers x 2.

ADDITIONAL MATERIALS REQUIRED, BUT NOT PROVIDED

A. Pipettes and Pipette Tips
B. 96-well plate or manual strip washer
C. Buffer and Reagent Reservoirs
D. Paper towels or absorbent paper
E. Plate reader capable of reading absorbency at 450-650 nm
F. Distilled water or deionized water

STORAGE AND STABILITY of REAGENTS

The kit should be stored at 2-8^{o}C upon receipt, and should be equilibrated to room temperature before assay.
Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.
Remove any unused strips from the Human Adiponectin microplate, return them to the foil pouch and keep at 4°C.

PREPARATION OF REAGENTS

A. 1X Assay buffer. Prepare 1X Assay buffer by mixing all of the 10X Assay Buffer (20 mL) with 180 mL of distilled water or deionized water. This assay buffer will be used to reconstitute some components in this kit. If precipitates are observed in the 10x assay buffer bottle, warm the bottle in a 37°C water bath until the precipitates disappear. After preparation, store 1X Assay buffer at 2-8°C for up to 3 months.
B. 1X Wash buffer. Prepare 1X Wash buffer by mixing all of the 5X Wash Buffer (40 mL) with 160 mL of distilled water or deionized water. After preparation, store 1X Wash buffer at 2-8°C for up to 3 months

C. 1X Detection antibody solution. Dilute the 100X Detection Antibody with 1X Assay buffer, 100 ul of the 1X Detection antibody solution is required per well. Prepare only as much 1X Detection antibody solution as needed. Return the 100X Detection Antibody to 2-8°C immediately after the necessary volume is removed.
D. Substrate solution. Substrate A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 ul of the mixture is required per well. Prepare only as much Substrate solution as needed. Return Substrate A & B to 2-8°C immediately after the necessary volume is removed.

PREPARATION OF HUMAN ADIPONECTIN STANDRADS, QUALITY CONTROLS AND SAMPLES

Human adiponectin standard: Reconstitute the Human Adiponectin Standard with 500 µl of deionized or distilled water. This reconstitution produces a stock solution of 200 ng/ml. Allow the standard sit for 10 minutes with gentle agitation prior to make dilutions. Prepare standard as follows:

Standard volume	*Assay Buffer*	*Concentration*
Stock	-------	200 ng/ml
200 µl of stock	200 µl	100 ng/ml
200 µl of std. 100 ng/ml	200 µl	50 ng/ml
200 µl of std. 50 ng/ml	200 µl	25 ng/ml
200 µl of std. 25 ng/ml	200 µl	12.5 ng/ml
200 µl of std.12.5 ng/ml	200 µl	6.25 ng/ml
200 µl of std.6.25 ng/ml	200 µl	3.12 ng/ml
200 µl of std.3.12 ng/ml	200 µl	1.55 ng/ml

1X Assay buffer serves as the zero standard (0 ng/ml).

Human adiponectin controls. Reconstitute Human Adiponectin Control High & Low with 500 µl of distilled water or deionized water per vial. Allow at least 10 minutes for complete reconstitution and invert the vials several times to mix and vortex. Reconstituted quality controls are ready to use. Unused portions may be stored at -20ºC for up to one month.

Sample preparation

Serum and plasma samples generally require a 1000-fold dilution into 1x Assay buffer. A suggested 1000-fold dilution is achieved by creating a 50-fold dilution of 10 ul sample + 490 ul 1X Assay buffer, and further diluting 20-fold with 10 ul of the 50-fold diluted sample + 190 ul 1X Assay buffer. Cellular extract and culture media dilutions will vary and need to be optimized by the user, also use 1X Assay buffer to prepare these samples.

ASSAY PROCEDURE

1. Remove the Human Adiponectin Microplate from the foil pouch, remove excess microplate strips from the plate frame, return them to the foil pouch and reseal.
2. Add 100 µl of samples, quality controls or standards per well, cover with an adhesive strip and incubate for 1 hours at room temperature.
3. Aspirate and wash the wells 3-times with 1x washing solution (300 µl/well).
4. Add 100 µl of the 1X Detection antibody solution to each well, cover with an adhesive strip and incubate for 1 hour at room temperature.
5. Repeat the aspiration/wash as in step 3.
6. Add 100 µl of Substrate solution to each well. Incubate for 15 minutes at room temperature. Avoid placing the plate in direct light.
7. Add 50 µl of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
8. Determine the optical density of each well immediately, using a microplate reader set to 450 nm.

CALCULATION

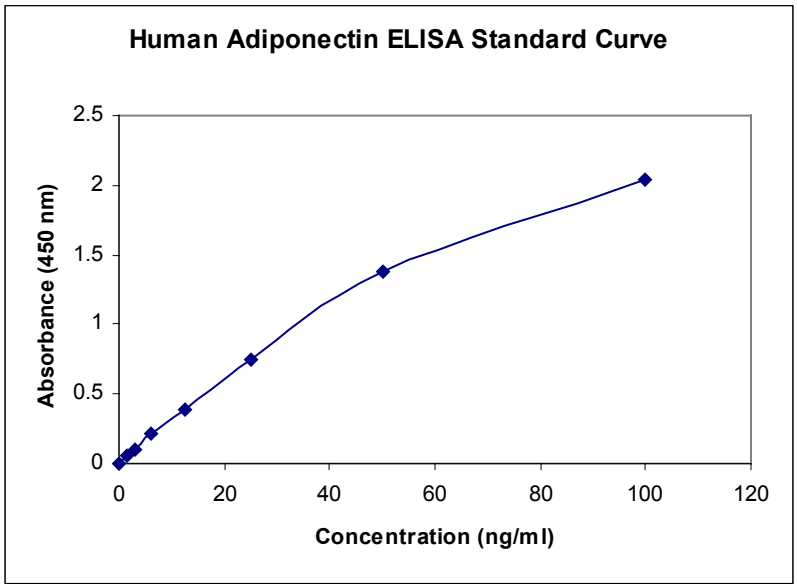
The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Adiponectin concentrations versus the

log of the O.D. and the best fit line can be determined by regression analysis. Final results should be multiplied by a dilution factor.

TYPICAL STANDARD CURVE

The following standard curve is provided for demonstration only. A standard curve should be generated for each set of sample assayed.

(ng/ml)	Absorbance (450 nm)	Corrected Absorbance
0	0.062	0
1.55	0.116	0.054
3.12	0.169	0.107
6.25	0.277	0.215
12.5	0.449	0.387
25	0.803	0.741
50	1.44	1.378
100	2.107	2.045



ASSAY CHARACTERISTICS

1. Sensitivity: The lowest level of Adiponectin that can be detected by this assay is 1.56 ng/ml.
B. Specificity: The antibody pair used in this assay is specific to Human Adiponectin and does not cross-react with mouse and rat Adiponectin, and other cytokine or hormone molecules tested, including human resistin, TNFα, ANGPTL4, insulin, leptin, IL6.
C. Precision: The assay variations of this ELISA kits were studied on four human serum samples with varying concentrations of endogenous Adiponectin. The mean within variation was calculated from results of five duplicate determinations in each assay of the indicated samples. The mean between variations of each sample was calculated from results of four separate assays with duplicate samples in each assay.

Sample No.	Mean Adiponectin Levels (μg/ml)	Within% CV	Between% CV
1	9.21	4.02	4.97
2	21.33	3.65	4.68
3	5.32	3.27	4.53
4	15.72	4.18	5.01

D. Recovery: Varying amounts of Human Adiponectin were added to three human serum samples and the Adiponectin content was determined in three separate assays. The % of recovery = observed Adiponectin concentrations/expected Adiponectin concentrations x 100%.

Sample No.	Average recovery	Range (%)
1	99.3	97-104
2	99.8	98-103
3	101.1	99-105
4	100.2	98-104

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ASSAY PROCEDURE SUMMARY

Add 100 µl Standard, control or sample to each well.
Incubate 1 hour at RT.
↓
Aspirate and wash 3 times.
↓
Add 100 µl Detection antibody to each well.
Incubate 1 hour at RT.
↓
Aspirate and wash 3 times.
↓
Add 100 µl Substrate solution to each well.
Incubate 10 minutes at RT.
↓
Add 50 µl Stop solution to each well.
Read at 450 nm within 15 minutes.
↓
Calculation