HUMAN ADIPONECTIN RIA KIT

125 TUBES (Cat. # HADP-61HK)

Millipore • 6 Research Park Drive • St. Charles, Missouri 63304 USA
Phone: 636-441-8400 • Toll Free U.S.: 866-441-8400 • Fax: 636-441-8050
techserve_dd@millipore.com • www.millipore.com/bmia

HUMAN ADIPONECTIN RIA KIT
125 TUBES (Cat. # HADP-61HK)

I. INTENDED USE

Adiponectin is a new member of an ever increasing family of adipocytokines and is also referred to as ACRP-30 (adipocyte compliment related protein-30), Adipo-Q, and APM-1 (adipose tissue most abundant gene transcript-1). Adiponectin is a predominant secretory protein from adipose tissue and circulates in micro-gram/mL quantities and has a structural homology with the type VIII collagen and hibernation specific protein, C1q.

In contrast to the majority of secreted proteins from adipose tissue, which are elevated in obesity, adiponectin appears to be either decreased or unaltered with degree of adiposity. More intriguingly, adiponectin seems to ameliorate the obesity related risk factors unlike other adipose tissue secretory proteins which contribute toward the health risks associated with obesity. Adiponectin also has an insulin sensitizing effect making it an excellent candidate in drug development for obesity and diabetes. Circulating adiponectin levels seem to be an excellent biochemical marker for improved insulin resistance in obese and diabetic states. ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A calibration or standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

The Millipore Adiponectin RIA assay utilizes ^{125}I-labeled Murine Adiponectin and a Multi-species Adiponectin Rabbit antiserum to determine the level of Adiponectin in serum, plasma or tissue culture media by the double antibody/PEG technique. The Adiponectin Standard is prepared using recombinant Human Adiponectin and can be used to determine the circulating levels of adiponectin in human serum/plasma samples.

III. REAGENTS SUPPLIED

Each kit is sufficient to run 125 tubes and contains the following reagents.

A. 10X Assay Buffer
100 mM Phosphate Buffer, pH 7.5 containing 0.08% Sodium Azide, 1% BSA
Quantity: 50 mL/vial
Preparation: Dilute the contents of the vial with 450 mL distilled or deionized water

B. Adiponectin Antibody
Adiponectin Antibody
Quantity: 13 mL/vial
Preparation: Ready to use

C. ^{125}I-Adiponectin
^{125}I-Adiponectin Label (specific activity 67.7 µCi/µg)
Lyophilized for stability. Freshly iodinated label contains < 3 µCi, (<111 kBq) calibrated to the 1st Monday of each month.
Quantity: 13.5 mL/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with 13.5 mL of 1X Assay Buffer. Allow to sit at room temperature for 30 minutes, with occasional gentle mixing.

NOTE: You will observe that the adiponectin tracer displays lower B_0 binding as it approaches its expiration date. This change does not alter the performance of the kit since the Quality Control Values remain within expected ranges throughout the tracer shelf life.

D. Human Adiponectin Standard
Purified Recombinant Adiponectin, 1 mL, lyophilized
Quantity: 1 mL / vial upon hydration
Preparation: Contents Lyophilized. Hydrate with 1 mL distilled or deionized water. The actual concentration of Human Adiponectin present in the vial will be lot dependent. Please refer to the analysis sheet for exact Human Adiponectin concentration present in a specific lot.

E. Quality Controls 1& 2
Purified Recombinant Adiponectin
Lyophilized for stability
Quantity: 1 mL/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with 1 mL distilled or deionized water.

F. Rabbit Carrier
30% Normal Rabbit Serum
Quantity: 2 mL/vial
Ready to use

G. Precipitating Reagent
Goat anti-Rabbit IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA,
0.08% Sodium Azide
Quantity: 130 mL/vial
Preparation: Ready to use; chill to 4°C

IV. STORAGE AND STABILITY

Prior to use, refrigerate all reagents between 2 and 8°C for short-term storage. For prolonged storage (>2 weeks), freeze at ≤ -20°C. Once the standards have been reconstituted, unused portions should be stored at ≤ -20°C. Avoid multiple freeze/thaw cycles. Refer to date on bottle for expiration when stored at ≤ -20°C. Do not mix reagents from different kits unless they have the same lot number.

V. REAGENT PRECAUTIONS

A. Radioactive Materials

This radioactive material may be received, acquired, possessed and used only by research personnel or clinical laboratories for in vitro research tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of the U. S. Nuclear Regulatory Commission (NRC) or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

The following are suggested general rules for the safe use of radioactive material. The customer's Radiation Safety Officer (RSO) is ultimately responsible for the safe handling and use of radioactive material.

1. Wear appropriate personal devices at all times while in areas where radioactive materials are used or stored.

2. Wear laboratory coats, disposable gloves, and other protective clothing at all times.

3. Monitor hands, shoes, and clothing and immediate area surrounding the workstation for contamination after each procedure and before leaving the area.

4. Do not eat, drink, or smoke in any area where radioactive materials are stored or used.

5. Never pipette radioactive material by mouth.

6. Dispose of radioactive waste in accordance with NRC rules and regulations.

7. Avoid contaminating objects such as telephones, light switches, doorknobs, etc.

8. Use absorbent pads for containing and easily disposing of small amounts of contamination.

9. Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste. Inform Radiation Safety Officer.

B. Sodium Azide

Sodium Azide has been added to all reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Borosilicate glass tubes, 12 x 75 mm. (NOTE: Polypropylene or polystyrene tubes may be used if the investigator finds that the pellet formation is acceptably stable in their system.)
2. Borosilicate glass tubes, 12 x 100 mm, or equivalent for sample and standard dilutions
3. 10, 20, 100 and 1000 µL pipettes with disposable tips
4. 100 µL & 1.0 mL repeating dispenser
5. Refrigerated swing bucket centrifuge capable of developing 2,000 - 3,000 xg. (Use of fixed-angle buckets is not recommended.)
6. Absorbent paper
7. Vortex mixer
8. Refrigerator
9. Gamma Counter

VII. SPECIMEN COLLECTION AND STORAGE

1. Sample volumes of at least 5 µl of human serum or plasma can be used (see Sample Preparation, Section VIII. D). Sample volumes of 50 - 100 µL of Tissue Culture Media may also be used.

2. Specimens can be stored at 2-8°C if they will be tested within 24 hours of collection. For longer storage, specimens should be stored at ≤ -20°C. Avoid multiple freeze/thaw cycles.

3. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

For optimal results, accurate pipetting and adherence to the protocol are recommended.

A. Dilute the 10X Assay Buffer with 450 mL distilled or deionized water to prepare working concentration of 1X Assay Buffer.

B. Human Adiponectin Standard Preparation

1. Use care in opening the lyophilized standard vial. Using a pipette, reconstitute the Human Adiponectin Standard with 1 mL distilled or deionized water into the glass vial to give a concentration prescribed in the analysis sheet. Mix well.

3. Label eight glass tubes 1, 2, 3, 4, 5, 6, 7 and 8. Add 0.5 mL Assay Buffer to each of the eight tubes. Prepare serial dilutions by adding 0.5 mL of the reconstituted standard to Tube 1, mix well and transfer 0.5 mL of the Tube 1 Standard to Tube 2, mix well and transfer 0.5 mL of the Tube 2 Standard to Tube 3, mix well and transfer 0.5 mL of the Tube 3 Standard to Tube 4, mix well and transfer 0.5 mL of the Tube 4 Standard to Tube 5, mix well and transfer 0.5 mL of the Tube 5 Standard to Tube 6, mix well and transfer 0.5 mL of the Tube 6 Standard to Tube 7, mix well and transfer 0.5 mL of the Tube 7 Standard to Tube 8 and mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at ≤ -20°C. Avoid multiple freeze/thaw cycles.

Standard Concentration ng/mL	Volume of Deionized Water to Add	Volume of Standard to Add
X (refer to analysis sheet for exact concentration)	1 mL	0

Tube #	Standard Concentration ng/mL	Volume of Assay Buffer to Add	Volume of Standard to Add
1	X / 2	0.5 mL	0.5 mL of reconstituted standard
2	X / 4	0.5 mL	0.5 mL of Tube 1
3	X / 8	0.5 mL	0.5 mL of Tube 2
4	X / 16	0.5 mL	0.5 mL of Tube 3
5	X / 32	0.5 mL	0.5 mL of Tube 4
6	X / 64	0.5 mL	0.5 mL of Tube 5
7	X / 128	0.5 mL	0.5 mL of Tube 6
8	X / 256	0.5 mL	0.5 mL of Tube 7

C. Human Adiponectin Quality Control 1 and 2 Preparation

1. Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Human Adiponectin Quality Control 1 and Quality Control 2 with 1 mL distilled or deionized water into the glass vials. Mix well.

VIII. ASSAY PROCEDURE (continued)

D. Sample Preparation

1. Human serum/plasma samples

   Circulating concentrations of Adiponectin in human sera are in µg/mL levels, whereas the assay range is 1 ~ 200 ng/mL. It is advisable to dilute the serum or plasma samples 1:500 prior to use in this Adiponectin. If the values do not fall within the standard curve range, then the dilution should be adjusted appropriately.

   RIA. Example to dilute human serum/plasma samples: Pipet 10 µL serum/plasma into 4990 µL Assay Buffer to prepare a 1:500 dilution.

   Note: When analyzing diluted samples, it is important to adjust the final result by the appropriate dilution factor to compensate for the dilution of sample.

2. Tissue Culture Medium

   50 – 100 µL tissue culture medium per tube is required for analysis. Since Adiponectin levels in tissue culture medium depend on incubation conditions, it is advisable to pilot test for appropriate volume and/or dilution before assaying all the samples.

   Use unconditioned tissue culture medium to determine the background or basal level.

E. Assay Set-Up, Day One

1. Pipet 300 µL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4), 200 µL to the Reference (Bo) tubes (5-6), and 100 µL to tubes 7 through the end of the assay.
2. Pipet 100 µL of Standards and Quality Controls in duplicate (see flow chart). For preparation, see Section VIII, Part B and C.
3. Pipet 100 µL of each diluted Sample in duplicate. Refer to Section IX for calculation modification of diluted samples.
4. Pipet 100 µL of ^{125}I-Adiponectin to all tubes. Important: For preparation, see Section III, Part C.
5. Pipet 100 µL of Adiponectin antibody to all tubes except Total Count Tubes (1-2) and NSB tubes (3-4).
6. Vortex, cover, and incubate overnight (20-24 hours) at room temperature (20-25°C).

F. Day Two

7 Add 10 µL of Rabbit Carrier to all tubes except Total Count Tubes (1-2).

8. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes except Total Count Tubes (1-2).
9. Vortex and incubate 20 minutes at 4°C.
10. Centrifuge, 4°C, all tubes [except Total Count Tubes (1-2)] for 20 minutes at 2,000-3,000 xg. NOTE: If less than 2,000 xg is used or if slipped pellets have been observed in previous runs, the time of centrifugation must be increased to obtain a firm pellet (e.g., 40 minutes). Multiple centrifuge runs within an assay must be consistent.

    Conversion of rpm to xg:

    $xg = (1.12 \times 10^{-5})\ (r)\ (rpm)^2$

    r = radial distance in cm (from axis of rotation to the bottom of the tube)

    rpm = rotational velocity of the rotor

11. Immediately decant the supernate of all tubes except Total Count tubes (1-2), drain tubes for at least 15-60 seconds (be consistent between racks), and blot excess liquid from lip of tubes. NOTE: Invert tubes only one time. Pellets are fragile and slipping may occur.
12. Count all tubes in a gamma counter for 1 minute. Calculate the ng/mL of Adiponectin in unknown samples using automated data reduction procedures (see Section IX).

Assay Flow Chart

Day One						Day Two			
	Step 1	Step 2-3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9	Step 10-12
Tube #	Add Assay Buffer	Add Standard / Sample	Add ^{125}I-Adiponectin Tracer	Add Adiponectin Antibody	Vortex, Cover, and Incubate 20-24 hrs at RT	Add Rabbit Carrier	Add Precipitating Reagent	Vortex, and Incubate 20 min. at 4°C	Centrifuge for 20 min., Decant, and Count pellets
1,2	----	----	100 µl	----		----	----		
3,4	300 µl	----	100 µl	----		10 µl	1.0 mL		
5,6	200 µl	----	100 µl	100 µl		10 µl	1.0 mL		
7,8	100 µl	100 µl of Tube 8	100 µl	100 µl		10 µl	1.0 mL		
9,10	100 µl	100 µl of Tube 7	100 µl	100 µl		10 µl	1.0 mL		
11,12	100 µl	100 µl of Tube 6	100 µl	100 µl		10 µl	1.0 mL		
13,14	100 µl	100 µl of Tube 5	100 µl	100 µl		10 µl	1.0 mL		
15,16	100 µl	100 µl of Tube 4	100 µl	100 µl		10 µl	1.0 mL		
17,18	100 µl	100 µl of Tube 3	100 µl	100 µl		10 µl	1.0 mL		
19,20	100 µl	100 µl of Tube 2	100 µl	100 µl		10 µl	1.0 mL		
21,22	100 µl	100 µl of Tube 1	100 µl	100 µl		10 µl	1.0 mL		
23, 24	100 µl	100 µl of Reconst. Std.	100 µl	100 µl		10 µl	1.0 mL		
25, 26	100 µl	100 µl of QC-1	100 µl	100 µl		10 µl	1.0 mL		
27, 28	100 µl	100 µl of QC-2	100 µl	100 µl		10 µl	1.0 mL		
29, n	100 µl	100 µl of unknown	100 µl	100 µl		10 µl	1.0 mL		

IX. CALCULATIONS AND TRANSFORMATIONS

A. Explanation

The calculations for Adiponectin concentrations can be automatically performed by most gamma counters possessing data reduction capabilities or by independent treatment of the raw data using a commercially available software package.[2] Choose weighted 4-parameter or weighted log/logit for the mathematical treatment of the data.

NOTE: Be certain the procedure used subtracts the NSB counts from each average count, except Total Counts, prior to final data reduction.

B. Manual Calculation

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (Reference, Bo) (5-6), and all duplicate tubes for standards and samples to the end of the assay.

2. Subtract the average NSB counts from each average count (except for Total Counts). These counts are used in the following calculations.

3. Calculate the percentage of tracer bound
   (Total Binding Counts/Total Counts) X 100
   This should be 25-55%.

4. Calculate the percentage of total binding (%B/Bo) for each standard and sample
   %B/Bo = (Sample or Standard/Total Binding) X 100

5. Plot the %B/Bo for each standard on the y-axis and the known concentration of the standard on the x-axis using
   log-log graph paper.

6. Construct the reference curve by joining the points with a smooth curve.

7. Determine the B/Bo in the unknown samples by interpolation of the reference curve.

8. When analyzing Tissue Culture samples, the Adiponectin levels in the control media must be subtracted from the test samples to determine the actual Adiponectin level.

9. When analyzing human serum/plasma samples, the samples are diluted prior to analysis so the result must be multiplied by the appropriate dilution factor to obtain the correct final result.

X. INTERPRETATION

A. Acceptance Criteria

1. The run will be considered accepted when all Quality Control values fall within the calculated Quality Control Range; if any QC's fall outside the control range, review results with supervisor.
2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.
3. The limit of sensitivity for the Human Adiponectin assay is 1 ng/mL (100 µL sample size).
4. The limit of linearity for the Human Adiponectin assay is 200 ng/mL (100 µL sample size). Any result greater than 200 ng/mL should be repeated on dilution using Assay Buffer as a diluent.

XI. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Human Adiponectin that can be detected by this assay is 1 ng/mL when using a 100 µl sample size.

B. Performance

The following parameters of assay performance are expressed as Mean ± Standard Deviation.

ED_{80} = 3.8 ± 0.9 ng/mL
ED_{50} = 21.0 ± 5.7 ng/mL
ED_{20} = 128.0 ± 48.6 ng/mL

C. Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the Assay Buffer.

Mouse Adiponectin	400%
Human C1q	<0.01%

D. Precision

Within and Between Assay Variation

Sample	Sample Concentration (µg/mL)	Intra-assay Precision (%CV)	Interassay Precision (%CV)
1	1.5	3.59	9.25
2	3.0	6.21	6.90
3	7.5	1.78	9.25

Three human serum samples that target a Low (3 ng/mL), Middle (6 ng/mL), and High (15 ng/mL) concentration of Adiponectin were collected and pipetted into aliquots and frozen at ≤ -20°. Each set of samples was assayed to generate 5 reportable results (10 replicates) for each level (Low, Mid, High) repeated in six assays. Intra-assay Precision is reported as the %CV calculated from the mean of the 5 results from a single assay. Interassay Precision is reported as the %CV calculated from the mean value of the 30 results across the six assays

XI. ASSAY CHARACTERISTICS (continued)

E. Recovery

Spike & Recovery of Adiponectin in Human Serum

Human Serum (1:500 dilution)	Human Adiponectin Added (ng/mL)	Percent Recovery
1	5	117
	20	112
	100	99
2	5	114
	20	110
	100	101
3	5	116
	20	112
	100	103

Three human serum samples targeting a low concentration of Adiponectin were collected, diluted 1:500 in assay buffer, and pipetted into aliquots and frozen at ≤ -20°C. At the time of assay, 100 µL of 5, 20, and 100 ng/mL standards, targeting the ED(20), ED(50), and ED(80) of the assay, was prepared in assay buffer and pipetted into 100 µL of each of the three different diluted serum samples. Each set of samples was assayed to generate 3 reportable results (6 replicates) for each serum and repeated in six assays. Accuracy is reported as the mean of the percent recoveries calculated for each concentration for each sera across the six assays.

F. Linearity

Effect of Serum Dilution

Sample	Expected Concentration	Dilution	Percent Difference Between Dilution
1	7561 ng/mL	1:125	100%
		1:250	100%
		1:500	98%
		1:1000	93%
2	9937 ng/mL	1:125	100%
		1:250	118%
		1:500	104%
		1:1000	97%
3	13095 ng/mL	1:125	100%
		1:250	131%
		1:500	111%
		1:1000	103%

Samples for linearity were prepared by diluting three sera with known high levels of Adiponectin. The sera were diluted 1:125, 1:250, 1:500, and 1:1000 with assay buffer. Each set of samples was assayed to generate 3 reportable results (6 replicates) for each dilution in each sera and repeated in six assays. Because the reported results are much greater than the standard curve, the results are reported as the percent difference between the preceding dilutions.

XII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control (QC) specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for quality control decisions and is also responsible for making this system a written part of their laboratory manual. The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com/bmia.

XIII. REPLACEMENT REAGENTS

Reagents	Cat #
^{125}I-Adiponectin (<3 µCi, <111 kBq)	9060-HK
Human Adiponectin Standard (Lyophilized)	8061-K
Adiponectin Antibody (13 mL)	1060-HK
Rabbit Carrier (2 mL)	RC-HK
Precipitating Reagent (130 mL)	PR-81HK
10X Assay Buffer (50 mL)	AB-10XP
Quality Control 1 & 2 (Lyophilized)	6361-K

XIV. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size
   NOTE: Appropriate license from NRC (or equivalent) must be on file at Millipore before radioactive orders can be shipped.

TELEPHONE ORDERS:
Toll free US (866) 441-8400
(636) 441-8400

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:
To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

XIV. ORDERING INFORMATION (continued)

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.

XV. REFERENCES

1. Thorell, J.I. *Scand. J. Clin. Lab. Invest*. 31:187, 1973.

2. Feldman, H. and Rodbard, D. "Mathematical Theory of Radioimmunoassay," in: W.D. Odell and Doughaday, W.H. (Ed.), Principles of Competitive Protein-Binding Assays. Philadelphia: J.B. Leppincott Company; pp 158-203, 1971.

3. Westgard, J.O., et. al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem*. 27:493-501, 1981.