Human Adiponectin

96-Well Plate

Cat. # EZHADP-61K

HUMAN ADIPONECTIN ELISA KIT
96-Well Plate (Cat. # EZHADP-61K)

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I. INTENDED USE

This Human Adiponectin (ACRP30) ELISA kit is used for the non-radioactive 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. One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for research purpose only.***

II. PRINCIPLES OF PROCEDURE

This assay is a Sandwich ELISA based, sequentially, on: 1) concurrent capture of Human Adiponectin molecules from samples to the wells of a microtiter plate coated with a monoclonal anti-human adiponectin antibodies, and binding of a second biotinylated monoclonal anti-human antibody to the captured molecules , 2) washing of unbound materials from samples, 3) binding of streptavidin-horseradish peroxidase conjugate to the immobilized biotinylated antibodies, 4) washing of excess of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm – 590nm after acidification of formed products. Since the increase in absorbance is 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.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

A. Human Adiponectin ELISA Plate
Coated with Mouse anti-Human Adiponectin Antibodies
Quantity: 1 plate
Preparation: Ready to Use
Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C.

B. Adhesive Plate Sealer
Quantity: 1 sheet
Preparation: Ready to Use

C. 10X HRP Wash Buffer Concentrate
10X concentrate of 50 mM Tris Buffered Saline containing Tween-20
Quantity: 2 bottles containing 50 mL each
Preparation: Dilute 1:10 with distilled or deionized water

D. Human Adiponectin Standard
Purified Recombinant Human Adiponectin, lyophilized.
Quantity: 100 ng
Preparation: Contents Lyophilized. Reconstitute with 0.5 mL distilled or deionized water to obtain 200 ng/mL.

E. Human Adiponectin Quality Controls 1 and 2
One vial each, lyophilized, containing diluted human serum at two different levels of Adiponectin.
Quantity: 0.5mL/bottle upon hydration
Preparation: Contents Lyophilized. Reconstitute each vial with 0.5mL distilled or deionized water

F. 10X Assay Buffer (Sample Diluent)
100mM Phosphate buffer, pH 7.5, containing 0.08% Sodium Azide, 1% BSA
Quantity: 50 mL
Preparation: Dilute 1:10 with distilled or deionized water to make 1X Assay Buffer

Note: Use 1X Assay Buffer to dilute samples (Section VIII, SAMPLE PREPARATION) and Standard Curve (Section IX, STANDARD AND QUALITY CONTROLS PREPARATION)

G. Assay Buffer A (Assay Running Buffer)
0.05M Phosphosaline containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA
Quantity: 7 mL
Preparation: Ready to Use

III. REAGENTS SUPPLIED (continued)

H. Human Adiponectin Detection Antibody

Pre-titered Biotinylated Mouse anti-Human Adiponectin Antibody
Quantity: 3 mL
Preparation: Ready to Use

I. Enzyme Solution

Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer
Quantity: 12 mL
Preparation: Ready to Use

J. Substrate (Light sensitive, avoid unnecessary exposure to light)

3, 3', 5, 5'-tetramethylbenzidine in buffer
Quantity: 12 mL
Preparation: Ready to Use.

K. Stop Solution (Caution: Corrosive Solution)

0.3 M HCl
Quantity: 12 mL
Preparation: Ready to Use

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8°C. For longer storage (> 2 weeks), freeze diluted Wash Buffer, Assay Buffer, and reconstituted Standards and Controls at ≤ –20°C. Minimize repeated freeze and thaw of the Adiponectin Standards and Quality Controls. Unused microtiter strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

A. Sodium Azide

Sodium Azide has been added to certain reagents as a preservative. Although the concentrations are low, 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.

B. Hydrochloric Acid

Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and Pipette Tips: 10 µL - 20 µL or 20 µL - 100 µL
2. Multi-Channel Pipettes and Pipette Tips: 5 µL ~ 50 µL and 50 µL ~ 300 µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum samples, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.

2. Promptly centrifuge the clotted blood at 2,000 to 3,000xg for 15 minutes at 4 ± 2°C.

3. Transfer and store serum samples in separate tubes. Date and identify each sample.

4. Use freshly prepared serum or aliquot and store samples at ≤ –20°C for later use. For long-term storage, keep at -70 °C. Avoid freeze/thaw cycles.

5. To prepare plasma samples, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.

6. If heparin is to be used as an anticoagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

7. Avoid using samples with gross hemolysis or lipemia.

VIII. SAMPLE PREPARATION

1. Allow all the reagents to come to room temperature.

2. Dilute serum or plasma samples 1:500 in 1X Assay Buffer (Sample Diluent). Cellular extract and culture media dilutions will vary.

3. Make Dilution A with 10 µL sample to 990 µL of 1X Assay Buffer (Sample Diluent) and mix well.

4. Make Dilution B by adding 100 µL of Dilution A to 400 µL of 1X Assay Buffer (Sample Diluent) and mixing well. Use Dilution B (1:500) for the assay procedure.

IX. STANDARD AND QUALITY CONTROLS PREPARATION

A. Human Adiponectin Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human Adiponectin Standard with 0.5 mL distilled or deionized water into the glass vial to give a 200 ng/mL concentration of Standard. Invert and mix gently, let sit for 5 minutes then vortex gently.

2. Label seven tubes 100, 50, 25, 12.5, 6.25, 3.125, 1.56 ng/mL. Add 0.2 mL Assay Buffer (Sample Diluent) to each of the seven tubes. Prepare serial dilutions by adding 0.2 mL of the 200 ng/mL reconstituted standard to the 100 ng/mL tube, mix well and transfer 0.2 mL of the 100 ng/mL reconstituted standard to the 50 ng/mL tube, mix well and transfer 0.2 mL of the 50 ng/mL Standard to the 25 ng/mL tube, mix well and transfer 0.2 mL of the 25 ng/mL Standard to the 12.5 ng/mL tube, mix well and transfer 0.2 mL of the 12.5 ng/mL Standard to the 6.25 ng/mL tube, mix well and transfer 0.2 mL of the 6.25 ng/mL Standard to the 3.125 ng/mL tube, mix well and transfer 0.2 mL of the 3.125 ng/mL Standard to the 1.56 ng/mL tube 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
200	0.5 mL	0

Standard Concentration ng/mL	Volume of Assay Buffer (Samples Diluent) to Add	Volume of Standard to Add
100	0.2 mL	0.2 mL of 200 ng/mL
50	0.2 mL	0.2 mL of 100 ng/mL
25	0.2 mL	0.2 mL of 50 ng/mL
12.5	0.2 mL	0.2 mL of 25 ng/mL
6.25	0.2 mL	0.2 mL of 12.5 ng/mL
3.125	0.2 mL	0.2 mL of 6.25 ng/mL
1.56	0.2 mL	0.2 mL of 3.125 ng/mL

B. Human Adiponectin Quality Control 1 and 2 Preparation

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 0.5 mL distilled or deionized water into the glass vials. Invert and mix gently, let sit for 5 minutes then mix well.

X. ASSAY PROCEDURE

Pre-warm all reagents to room temperature prior to setting up the assay.

1. Dilute the 10X Wash Buffer concentrate 10 fold by mixing the entire content of each bottle of Wash Buffer with 450 mL deionized water (dilute both bottles with 900 mL deionized water).

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and wash each well 3 times with 300 µl of diluted Wash Buffer per wash. Decant wash buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. **Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.**

3. Add 60 µL **Assay Buffer A** to all wells.

4. Add in duplicate 20 µL **Assay Buffer A** to blank wells.

5. Add in duplicate 20 µL Human Adiponectin Standards in the order of ascending concentration to the appropriate wells. Add in duplicate 20 µL QC1 and 20 µL QC2 to the appropriate wells. Add sequentially 20 µL of the unknown samples in duplicate to the remaining wells.

6. Add 20 µL Detection Antibody to all wells. **For best result all additions should be completed within one hour.** Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400 to 500 rpm.

7. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.

8. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

9. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

10. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.

11. Wash wells 5 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

X. ASSAY PROCEDURE (continued)

12. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 5 to 20 minutes. Blue color should be formed in wells of Adiponectin standards with intensity proportional to increasing concentrations of Adiponectin.

Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

13. Remove sealer and add 100 µL Stop Solution **[CAUTION: CORROSIVE SOLUTION]** and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450 nm and 590nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units. The absorbance of highest Adiponectin standard should be approximately 2.2-2.8, or not to exceed the capability of the plate reader used.

Assay Procedure for Human Adiponectin ELISA kit (Cat. # EZHADP-61K)

	Step 1	Step 2	Step 3-4	Step 5	Step 6	Step 6-8	Step 9	Step 10-11	Step 12	Step 12	Step 13	Step 13
Well #	Dilute each bottle of 10X Wash Buffer with 450mL Deionized Water.	Wash plate 3X with 300 µl Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels	Assay Buffer A	Standards/Controls/	Detection Ab	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µl Wash Buffer	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 5X with 300 µl Wash Buffer	Substrate	Seal, Agitate, Incubate 5 - 20 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			80 µl		20 µl		100 µl		100 µl		100 µl	
C1, D1			60 µl	20 µl of 1.56 ng/mL Standard								
E1, F1			60 µl	20 µl of 3.125 ng/mL Standard								
G1, H1			60 µl	20 µl of 6.25 ng/mL Standard								
A2, B2			60 µl	20 µl of 12.5 ng/mL Standard								
C2, D2			60 µl	20 µl of 25 ng/mL Standard								
E2, F2			60 µl	20 µl of 50 ng/mL Standard								
G2, H2			60 µl	20 µl of 100 ng/mL Standard								
A3, B3			60 µl	20 µl of QC I								
C3, D3			60 µl	20 µl of QC II								
E3, F3			60 µl	20 µl of Sample								
G3, H3			60 µl	20 µl of Sample								
A4, B4 ↓			60 µl	20 µl of Sample								

XI. MICROTITER PLATE ARRANGEMENT

Human Adiponectin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	12.5 ng/mL	QC 1									
B	Blank	12.5 ng/mL	QC 1									
C	1.56 ng/mL	25 ng/mL	QC 2									
D	1.56 ng/mL	25 ng/mL	QC 2									
E	3.125 ng/mL	50 ng/mL	Sample 1									
F	3.125 ng/mL	50 ng/mL	Sample 1									
G	6.25 ng/mL	100 ng/mL	Sample 2									
H	6.25 ng/mL	100 ng/mL	Sample 2									

XII. CALCULATIONS

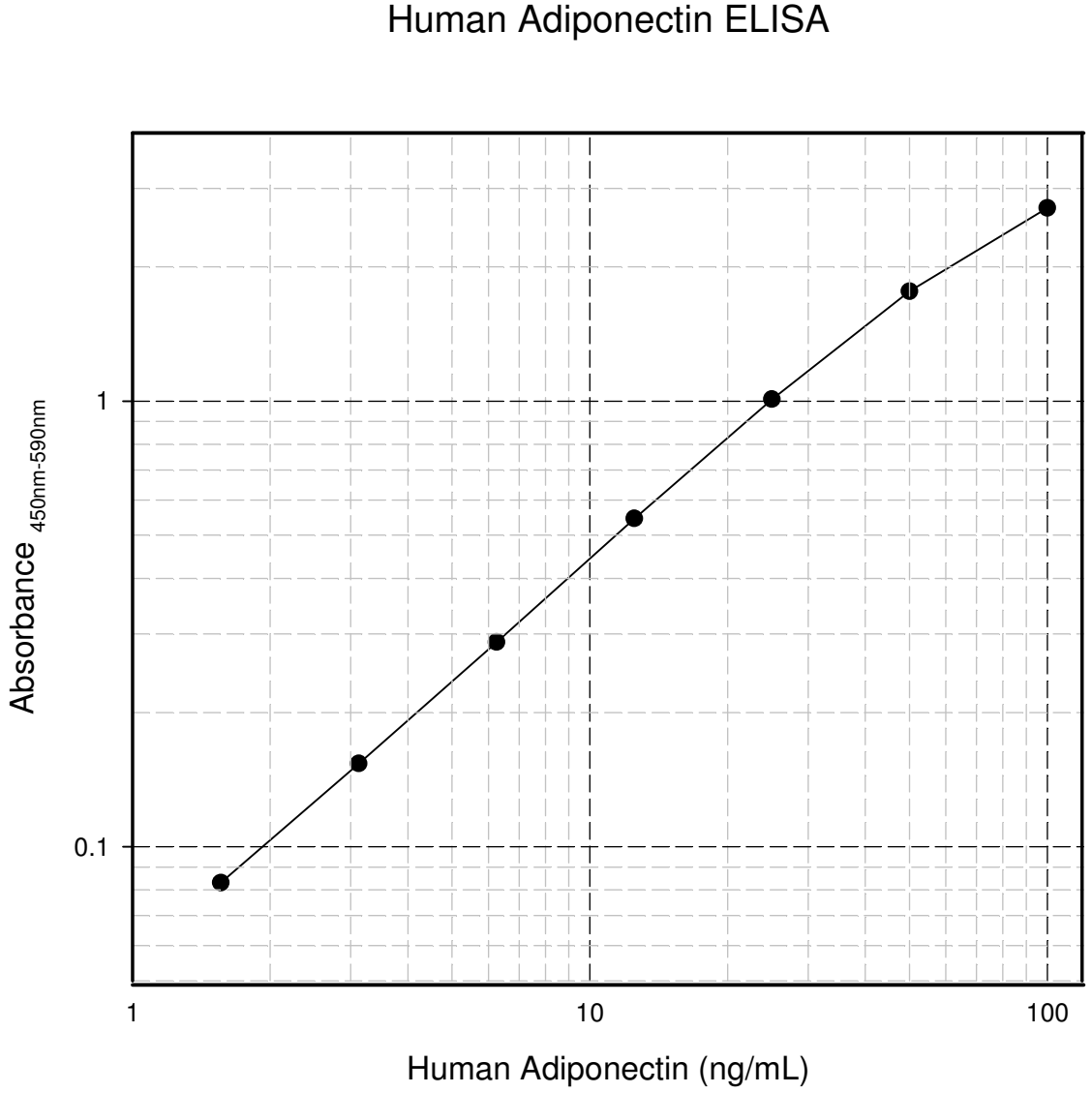
The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function. Final results should be multiplied by a 500 dilution factor.

Note: When sample volumes assayed differ from 20 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 µL, compensate the volume deficit with assay buffer (sample diluent).

XIII. INTERPRETATION

1. The assay 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 a supervisor.
2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.
3. The limit of sensitivity of this assay is 0.78ng/mL Human Adiponectin (20 µL sample size).
4. The appropriate range of this assay is 1.56 ng/mL to 100 ng/mL Human Adiponectin (20 µL sample size). Any result greater than 100 ng/mL in a 20 µL sample should be diluted using assay diluent, and the assay repeated until the results fall within range.

XIV. STANDARD CURVE



Typical Standard Curve, not to be used to calculate data.

XV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Adiponectin that can be detected by this assay is 0.78 ng/mL when using a 20 µL sample size.

B. Specificity

The antibody pair used in this assay is specific to Human Adiponectin and does not significantly cross-react with mouse Adiponectin and other cytokine or hormone molecules tested, as shown in the following table.

Analyte	Max. Conc.	Crossreactivity
Human Amylin	10ng/mL	n.d.
Human C-Peptide	10ng/mL	n.d.
Human GLP-1	10ng/mL	n.d.
Human Insulin	10ng/mL	n.d.
Human Leptin	100ng/mL	n.d.
Human Resistin	20µg/mL	n.d.
Mouse Resistin	2µg/mL	n.d.
Mouse Adiponectin	20µg/mL	n.d.
Human Cytokines:		
1. IL-1ß	10ng/mL	n.d.
2. IL-2	10ng/mL	n.d.
3. IL-4	10ng/mL	n.d.
4. IL-5	10ng/mL	n.d.
5. IL-6	10ng/mL	n.d.
6. IL-7	10ng/mL	n.d.
7. IL-8	10ng/mL	n.d.
8. IL-10	10ng/mL	n.d.
9. IL-12	10ng/mL	n.d.
10.IL-13	10ng/mL	n.d.
11. IFN- γ	10ng/mL	n.d.
12.TNF- α	200ng/mL	n.d.
13.GMCSF	10ng/mL	n.d.

n.d. = not detectable

XV. ASSAY CHARACTERISTICS (continued)

C. Precision

Within and Between Assay Variation

Sample No.	Mean Adiponectin Levels (ng/mL)	Within% CV	Between% CV
1	17.73	7.4	8.4
2	29.13	0.9	2.4
3	39.1	1.8	6.2

The assay variations of Millipore Human Adiponectin ELISA Kits were studied on three human serum samples with varying concentrations of endogenous Adiponectin. The mean within variation was calculated from results of six 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.

D. Recovery

Spike & Recovery of Human Adiponectin in Serum

Sample No.	Adiponectin Added ng/mL	Expected ng/mL	Observed ng/mL	% of Recovery
1	0	17.5	17.5	100
	20	37.5	34.5	92
	50	67.5	64.2	95
	100	117.5	119.9	102
2	0	58.2	58.2	100
	20	78.2	78.4	100
	50	108.2	108.1	100
	100	158.2	154.5	98
3	0	42.3	42.3	100
	20	62.3	59.3	95
	50	92.3	85	92
	100	142.3	137.6	97

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%.

XV. ASSAY CHARACTERISTICS (continued)

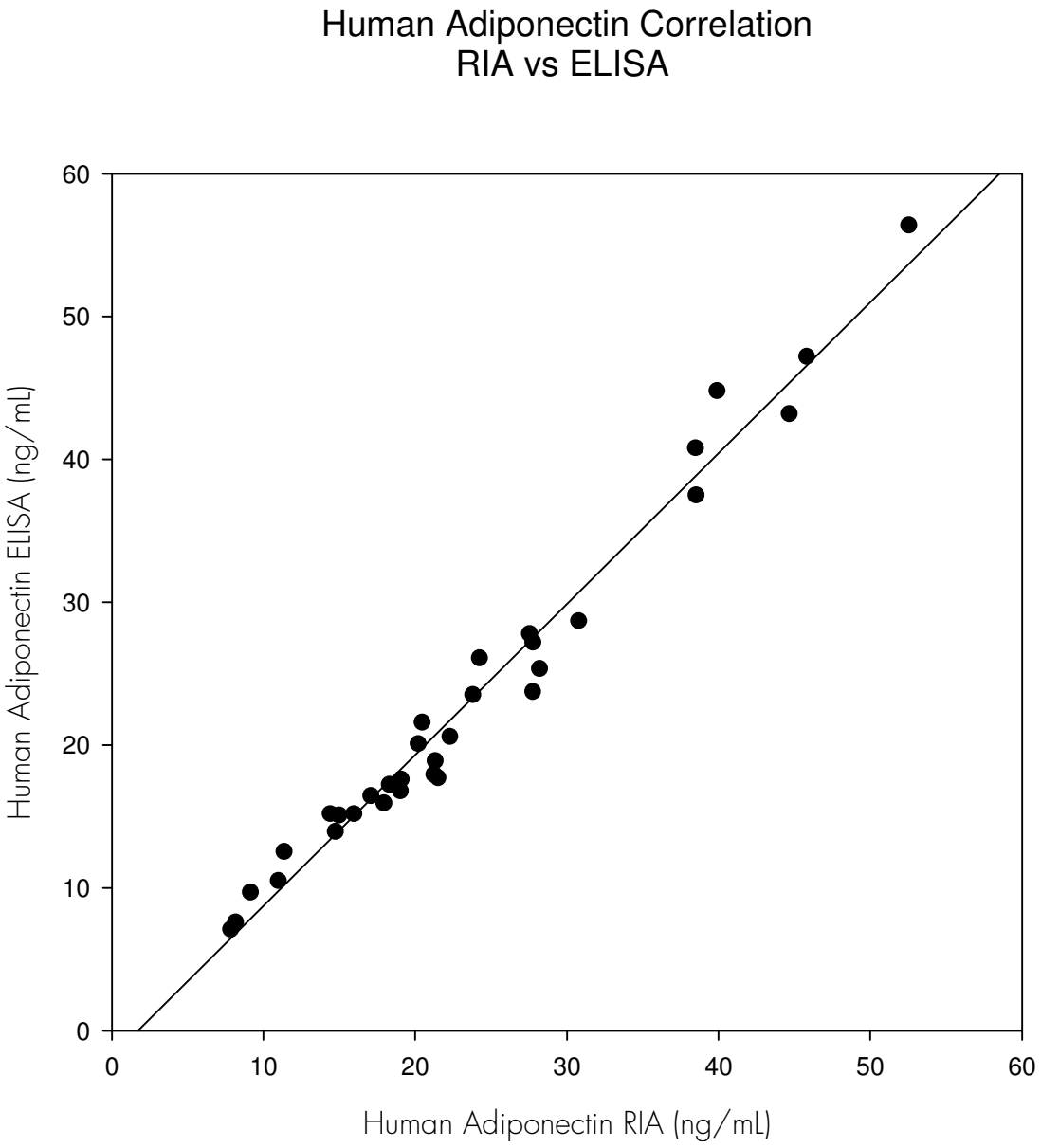
E. Linearity

Effect of Serum Dilution

Sample No.	Volume Sampled	Expected ng/mL	Observed ng/mL	% Of Expected
1	20µl	17.5	17.5	100
	10µl	8.75	8.2	94
	4µl	3.5	3	86
	2µl	1.75	1.5	86
2	20µl	58.2	58.2	100
	10µl	29.1	28	96
	4µl	11.64	10.5	90
	2µl	5.82	4.7	81
3	20µl	42.3	42.3	100
	10µl	21.15	20.2	96
	4µl	8.46	6.8	80
	2µl	4.23	3.6	85

Three human serum samples with the indicated sample volumes were assayed in three separate experiments. Required amounts of assay buffer were added to compensate for lost volumes below 20 µL. The resulting dilution factors of 1.0, 2.0, 5.0, and 10.0 representing 20 µL, 10 µL, 4 µL, and 2 µL sample volumes assayed, respectively, were applied in the calculation of observed Adiponectin concentrations.
% expected = observed/expected x 100%.

XVI. CORRELATION GRAPH



Serum samples obtained from 33 human subjects were assayed for Adiponectin content using both Millipore Human Adiponectin RIA Kit (Catalogue #HADP-61HK) and Human Adiponectin ELISA Kit (Catalogue EZHADP-61K). Correlation of the two kits is derived by linear regression analysis of paired results from each sample.

XVII. QUALITY CONTROLS

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.

XVIII. TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. Do not let the absorbency reading of the highest standard reach 3.0 units or higher after acidification.
9. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with Wash Buffer or 3) overexposure to light after substrate has been added.

XIX. REPLACEMENT REAGENTS

Reagents	**Cat. #**
Human Adiponectin ELISA Plate	EP61
10X HRP Wash Buffer Concentrate (50 mL)	EWB-HRP
Human Adiponectin Standards	E8061-K
Human Adiponectin Quality Controls 1 and 2	E6361-K
10X Assay Buffer (Sample Diluent)	AB-10XP
Assay Buffer A (Assay Running Buffer)	EAB1XU
Human Adiponectin Detection Antibody	E1061
Enzyme Solution	EHRP
Substrate	ESS-TMB
Stop Solution	ET-TMB

XX. 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

TELEPHONE ORDERS:
Toll Free US (800) MILLIPORE
FAX ORDERS: (636) 441-8050
MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

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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.