HUMAN HIGH MOLECULAR WEIGHT (HMW) ADIPONECTIN ELISA KIT
96-Well Plate (Cat. # EZHMWA-64K)

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HUMAN HIGH MOLECULAR WEIGHT (HMW) ADIPONECTIN ELISA KIT
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I. INTENDED USE

This Human High Molecular Weight (HMW) Adiponectin ELISA kit is used for the non-radioactive quantification of Human HMW Adiponectin in serum, plasma, and adipocyte extracts or culture media samples with a simple sample pretreatment. Sample treatment specifically removes Hexameric and Trimeric Adiponectin in samples and allows for specific measurement of HMW Adiponectin only. The antibody pair does not recognize other human adipokines tested. This Human HMW Adiponectin kit is sufficient to measure 37 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) capture of Adiponectin molecules from samples to the wells of a microtiter plate coated with a monoclonal anti-adiponectin antibodies, 2) washing of unbound materials from samples, 3) binding of a second biotinylated polyclonal anti-adiponectin antibody to the captured molecules , 4) washing of unbound materials from samples, 5) binding of streptavidin-horseradish peroxidase conjugate to the immobilized biotinylated antibodies, 6) washing of excess of free enzyme conjugates, and 7) 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 – 590 nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured Human HMW 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 HMW Adiponectin.

III. REAGENTS SUPPLIED

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

A. Human HMW Adiponectin ELISA Plate (Cat # EP64)
Coated with Monoclonal Anti-Adiponectin Antibodies
Quantity: 1 Strip 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: 2 sheets
Preparation: Ready to Use

C. 10X HRP Wash Buffer Concentrate (Cat # EWB-HRP)
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 HMW Adiponectin Standard (Cat# E8064-K)
Adiponectin Calibrator, lyophilized.
Quantity: 0.5 mL upon hydration.
Preparation: Reconstitute with 0.5 mL distilled or deionized water. Refer to analysis sheet for exact concentration value.

E. Human HMW Adiponectin Digestion Controls 1 and 2 (Cat # EDC64)
One vial each containing serum at two different levels of Adiponectin.
Quantity: 0.25 mL/bottle, Lyophilized
Preparation: Reconstitute with 0.25 mL distilled or deionized water. Digestion required before use. See Section VIII for digestion procedure.

F. Sample Digestion Solution (Cat # ESDS)
Quantity: 600 µL
Preparation: Ready to use

G. Sample Digestion Buffer (Cat # EDGB - For the Digesting of Samples Only)
Quantity: 8 mL
Preparation: ready to use

III. REAGENTS SUPPLIED (continued)

H. Sample Dilution Buffer (10X) (Cat # ESDB - For Sample Dilution Only)
Quantity: 1mL
Preparation: Dilute 1:10 with 1X Assay Buffer (#AB-10XP)

I. 10X Assay Buffer (AB-10XP - For Diluting Sample Dilution Buffer Only)
Quantity: 50mL (10X)
Preparation: Dilute 1:10 with distilled or deionized water

J. Assay Running Buffer (Cat # EARB-4 - For Use in Running Assay and Diluting Standards Only)
0.05M Phosphosaline containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA
Quantity: 13 mL
Preparation: Ready to Use

K. Sample Preparation Plates (Cat # E-PLATE)
Quantity: Two 96-well solid plates with 2 plate sealers
Preparation: Ready to Use

L. Human HMW Adiponectin Detection Antibody (Cat # E1064)
Pre-titered Biotinylated Polyclonal anti-Adiponectin Antibody
Quantity: 12 mL
Preparation: Ready to Use

M. Enzyme Solution (Cat # EHRP)
Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer
Quantity: 12 mL
Preparation: Ready to Use

N. Substrate (Cat # ESS-TMB - Light sensitive, avoid unnecessary exposure to light)
3, 3', 5, 5'-tetramethylbenzidine in buffer
Quantity: 12 mL
Preparation: Ready to Use.

O. Stop Solution (Cat # ET-TMB - 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 except for the Sample Digestion Solution (cat # ESDS), Sample Dilution Buffer (cat # ESDB), and Sample Digestion Buffer (cat # EDGB) which must be stored at ≤ -20°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 HMW Adiponectin Standards and Digestion 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 ~ 50 µL and 50 ~ 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
9. 37°C Incubator

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 3,000 x g 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 AND DIGESTION CONTROL 1 AND 2 PREPARATION

Preparation for Digestion Control 1 and Control 2
Use care in opening the lyophilized Digestion Control 1 and Control 2. Using a pipette, reconstitute the Digestion Control 1 and 2 with 0.25 mL distilled or deionized water into the glass vial. Invert and mix gently, let sit for 5 minutes then vortex gently.

IMPORTANT NOTE: SAMPLE AND DIGESTION CONTROL 1 AND 2 PREPARATION SHOULD BE PERFORMED JUST PRIOR TO SET UP OF THE ASSAY.

TO AVOID HIGH CVs: CAREFUL ATTENTION SHOULD BE PAID TO ENSURE SAMPLES ARE SHAKEN VIGOROUSLY.

1. Add 170 µL of Sample Digestion Buffer **Cat # EDGB** (Not to be confused with Sample Digestion Solution) to the appropriate sample well of one of the solid Sample Preparation Plates Cat # E-PLATE for each sample to be digested.

   NOTE: There are sufficient sample preparation reagents to process 37 samples and digestion control 1 and 2, which can then be run in duplicate in the assay.

VIII. SAMPLE AND DIGESTION CONTROL 1 AND 2 PREPARATION (continued)

2. Add 20 µL of each sample or digestion control to the appropriate wells.

3. Seal plate and shake for **5 minutes** on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 700 rpm, or as vigorously as possible without splashing or cross-contaminating.

4. Remove plate from shaker and add 10 µL of Sample Digestion Solution **Cat # ESDS** (Mix Sample Digestion Solution well before adding to wells) to each sample or digestion control well (Samples are now 1:10).

5. Seal plate and shake for **5 minutes** on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 700 rpm, or as vigorously as possible without splashing or cross-contaminating.

6. Place plate carefully in 37°C incubator for 2 hours (Prepare Sample Dilution Buffer while incubating - Step 7).

7. Prepare 1X Sample Dilution Buffer **Cat # ESDB**: Dilute the 10X Assay Buffer **Cat # AB-10XP** concentrate 10 fold by mixing the entire content of the bottle of 10X Assay Buffer **Cat # AB-10XP** with 450 mL deionized water. Use this **1X** Assay Buffer to dilute the 10X Sample Dilution Buffer **Cat # ESDB** 10 fold by adding 900 µl of Sample Dilution Buffer **Cat # ESDB** to 8.1 mL of **1X** Assay Buffer Cat # **AB-10XP**. Mix well.

8. After the Step 6 - 2 hour incubation, remove plate from 37°C incubator. Carefully remove plate sealer and shake plate for **5 minutes** on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 700 rpm, or as vigorously as possible without splashing or cross-contaminating.

9. Add 190 µL of Prepared 1X Sample Dilution Buffer (prepared in Step 7) to the appropriate wells of the 2nd – unused- solid Sample Preparation Plate Cat # E-PLATE.

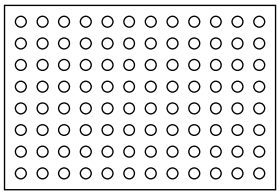
10. Add 10 µL of each digested sample or digestion control to corresponding wells of the 2nd Sample preparation plate containing the 1X Sample Dilution Buffer (Samples and digestion controls are now 1:200). Seal and shake plate for **5 minutes** on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 700 rpm, or as vigorously as possible without splashing or cross-contaminating.

11. Proceed with Assay. Samples and digestion controls should be used immediately.
    NOTE: 1:10 Digested samples and digestion controls may be sealed and kept at -20C for future use. 1:200 Diluted Samples and Controls should be discarded after immediate use.

VIII. SAMPLE AND DIGESTION CONTROL 1 AND 2 PREPARATION (continued)

Sample and Digestion Control Preparation Flow Chart:

All sample and digestion control shaking should be performed at 700 rpm or as vigorously as possible without splashing or cross-contaminating.



Sample Preparation Plate #1

Add 170 µl Sample Digestion Buffer Cat # EDGB to each sample well and Digestion Control well of Sample Preparation Plate #1

↓

Add 20µl each sample or Digestion Control to appropriate wells. Seal Plate. Shake 5 min.

↓

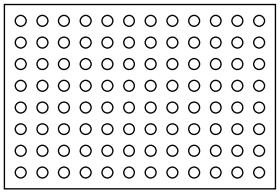
Add 10µl Sample Digestion Solution Cat # ESDS to each sample well and Digestion Control well. Seal Plate. Shake 5 min.

↓

Incubate Plate at 37C for 2hr.

↓

Remove Plate from 37C, remove seal and shake 5 min.



Sample Preparation Plate #2

Add 190 µl of Prepared Sample Dilution Buffer Cat # ESDB (Diluted 10 fold with Prepared 1X Assay Buffer Cat # AB-10XP) to each appropriate Sample well and Digestion Control well of Sample Preparation Plate #2.

↓

Add 10 µl of digested samples and digested Controls from Sample Preparation Plate #1 to corresponding wells of Sample Preparation Plate #2. Seal Plate. Shake 5 min.

↓

Samples are now ready for Assay Procedure

NOTE: If the proper plate shaker is not available to ensure low CVs or the normal high throughput system is not needed, the following alternative protocol can be used to prepare samples.

IX. ALTERNATIVE SAMPLE AND DIGESTION CONTROL 1 AND 2 PREPARATION USING POLYPROPYLENE MICROCENTRIFUGE TUBES

Preparation for Digestion Control 1 and Control 2

Use care in opening the lyophilized Digestion Control 1 and Control 2. Using a pipette, reconstitute the Digestion Control 1 and 2 with 0.25ml distilled or deionized water into the glass vial. Invert and mix gently, let sit for 5 minutes, then vortex gently.

IMPORTANT NOTE: SAMPE AND DIGESTION CONTROL 1 AND 2 PREPARATION SHOULD BE PERFORMED JUST PRIOR TO SET UP OF THE ASSAY.

IX. ALTERNATIVE SAMPLE AND DIGESTION CONTROL 1 AND 2 PREPARATION USING POLYPROPYLENE MICROCENTRIFUGE TUBES (continued)

TO AVOID HIGH CVs: CAREFUL ATTENTION SHOULD BE PAID TO ENSURE SAMPLES ARE VORTEXED WELL.

1. Add 170 µL of Sample Digestion Buffer **Cat # EDGB** (Not to be confused with Sample Digestion Solution) to the each microcentrifuge tube for each sample to be digested.

NOTE: There are sufficient sample preparation reagents to process 37 samples and digestion control 1 and 2, which can then be run in duplicate in the assay.

2. Add 20 µL of each sample or digestion control to the appropriate tubes and vortex well.

3. Add 10 µL of Sample Digestion Solution **Cat # ESDS** (Mix Sample Digestion Solution well before adding to tubes) to each sample or digestion control tube and vortex well (Samples are now 1:10).

4. Place tubes in rack and place in 37°C incubator for 2 hours (Prepare Sample Dilution Buffer while incubating - Step 5).

5. Prepare 1X Sample Dilution Buffer **Cat # ESDB**: Dilute the 10X Assay Buffer **Cat # AB-10XP** concentrate 10 fold by mixing the entire content of the bottle of 10X Assay Buffer **Cat # AB-10XP** with 450 mL deionized water. Use this **1X** Assay Buffer to dilute the 10X Sample Dilution Buffer **Cat # ESDB** 10 fold by adding 900 µL of Sample Dilution Buffer **Cat #ESDB** to 8.1 mL of **1X** Assay Buffer Cat # **AB-10XP**. Mix well.

6. After the Step 4 - 2 hour incubation, remove tubes from 37°C incubator and vortex well.

7. Add 190 µL of Prepared 1X Sample Dilution Buffer (prepared in Step 5) to a **second set of empty** polypropylene microcentrifuge tubes to correspond to each sample and digestion control.

8. Add 10 µL of each **digested sample or digestion control** to the new corresponding tubes (prepared in Step 7) containing the 1X Sample Dilution Buffer (Samples and digestion controls are now 1:200). Vortex well.

9. Proceed with Assay. Samples and digestion controls should be used immediately.

NOTE: 1:10 Digested samples and digestion controls may be sealed and kept at -20C for future use. 1:200 Diluted Samples and Controls should be discarded after immediate use.

X. STANDARD PREPARATION

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

2. Label seven tubes 1, 2, 3, 4, 5, 6 and 7. Add 0.25 mL Assay Running Buffer **(cat #EARB-4)** to each of the seven tubes. Prepare serial dilutions by adding 0.25 mL of the reconstituted standard to tube 1, mix well and transfer 0.25 mL of tube 1 to tube 2, mix well and transfer 0.25 mL of tube 2 to tube 3, mix well and transfer 0.25 mL of tube 3 to tube 4, mix well and transfer 0.25 mL of tube 4 to tube 5, mix well and transfer 0.25 mL of tube 5 to tube 6, mix well and transfer 0.25 mL of tube 6 to tube 7 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.

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

Tube #	Volume of Assay Running Buffer (#EARB-4) to Add	Volume of Standard to Add	Standard Concentration (ng/mL)
1	0.25 mL	0.25 mL of reconstituted standard	X/2
2	0.25 mL	0.25 mL of tube 1	X/4
3	0.25 mL	0.25 mL of tube 2	X/8
4	0.25 mL	0.25 mL of tube 3	X/16
5	0.25 mL	0.25 mL of tube 4	X/32
6	0.25 mL	0.25 mL of tube 5	X/64
7	0.25 mL	0.25 mL of tube 6	X/128

XI. 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 90 µL Assay Running Buffer **(cat # EARB-4)** to all wells.

4. Add in duplicate 10 µL Assay Running Buffer **(cat # EARB-4)** to blank wells.

5. Add in duplicate 10 µL Human HMW Adiponectin Standards in the order of ascending concentration to the appropriate wells. Add in duplicate 10 µL Digestion Control 1 and 10 µL Digestion Control 2 to the appropriate wells. Add sequentially 10 µL of the pretreated unknown samples in duplicate to the remaining wells.
   For best result all additions should be completed within 30 minutes.

6. 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 1X Wash Buffer, 300 µl per well per wash. Decant and tap firmly after each wash to remove residual buffer.

9. Add 100 µL Detection Antibody to all wells. Cover the plate with plate sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400 to 500 rpm.

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

XI. ASSAY PROCEDURE (continued)

11. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

12. 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.

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

14. Wash wells 3 times with 1X Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

15. Add 100 µL of Substrate (TMB) 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 the Human HMW Adiponectin standards with intensity proportional to increasing concentrations of Human HMW 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.

16. 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 590 nm 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 HMW Human Adiponectin ELISA kit (Cat. # EZHMWA -64K)

	Step 1	Step 2	Step 3-4	Step 5	Step 6-7	Step 8	Step 9-10	Step 11	Step 12-13	Step 14	Step 14	Step 15	Step 15
Well #	**Dilute <u>each</u> bottle of 10X Wash Buffer with 450 mL Deionized Water.**	**Wash plate 3X with 300 µL 1X Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.**	Assay Running Buffer	Standards/ Controls/ Samples	**Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer.**	Detection Ab	**Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 3X with 300 µL Wash Buffer.**	Enzyme Solution	**Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 3X 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			100 µL	---------		100 µL		100 µL		100 µL		100 µL	
C1, D1			90 µL	10 µL of Tube 7									
E1, F1			90 µL	10 µL of Tube 6									
G1, H1			90 µL	10 µL of Tube 5									
A2, B2			90 µL	10 µL of Tube 4									
C2, D2			90 µL	10 µL of Tube 3									
E2, F2			90 µL	10 µL of Tube 2									
G2, H2			90 µL	10 µL of Tube 1									
A3, B3			90 µL	10 µL of reconstituted Standard									
C3, D3			90 µL	10 µL of Control 1									
E3, F3			90 µL	10 µL of Control 2									
G3, H3 ↓			90 µL	10 µL of Sample		↓		↓		↓		↓	

XII. MICROTITER PLATE ARRANGEMENT

Human HMW Adiponectin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 4	Reconstituted Standard	Sample 2								
B	Blank	Tube 4	Reconstituted Standard	Sample 2								
C	Tube 7	Tube 3	Control 1	Etc.								
D	Tube 7	Tube 3	Control 1	Etc.								
E	Tube 6	Tube 2	Control 2									
F	Tube 6	Tube 2	Control 2									
G	Tube 5	Tube 1	Sample 1									
H	Tube 5	Tube 1	Sample 1									

XIII. 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 dilution factor of 200.

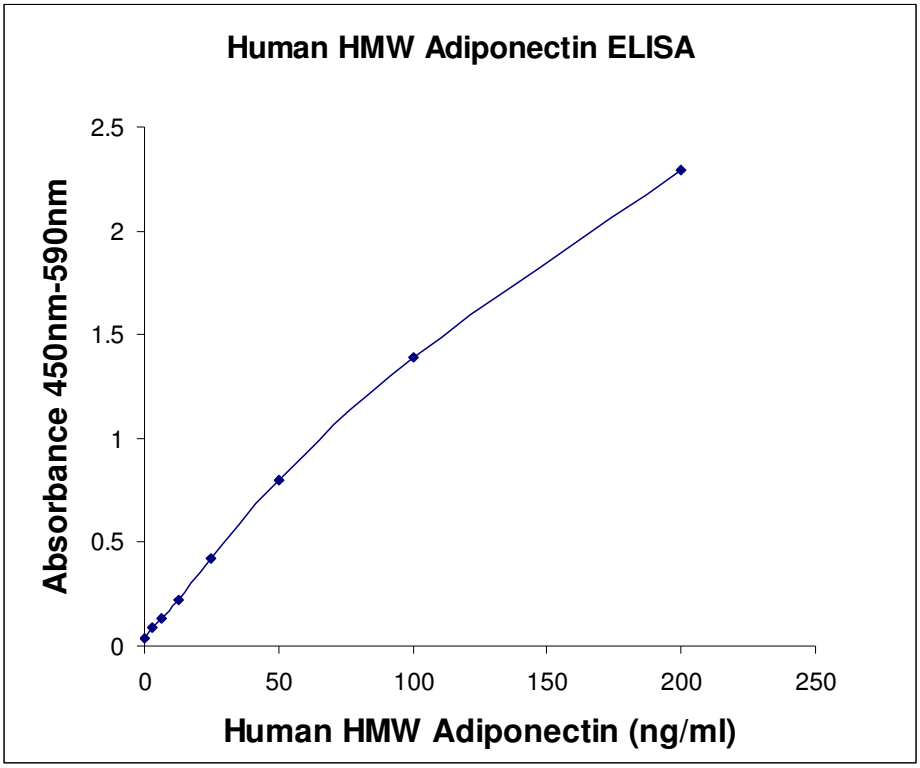
Note: When sample volumes assayed differ from 10 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 5 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 10 µL, compensate the volume deficit with Assay Running Buffer.

XIV. INTERPRETATION

1. The assay will be considered accepted when all Digestion Control values fall within the calculated Digestion 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 0.5 ng/mL Human HMW Adiponectin (10 µL sample size).
4. The appropriate range of this assay is 1.56 ng/mL to 200 ng/mL Human HMW Adiponectin (10 µL sample size). Any result greater than 200 ng/mL in a 10 µL sample should be diluted using Sample Dilution Buffer, and the assay repeated until the results fall within range.

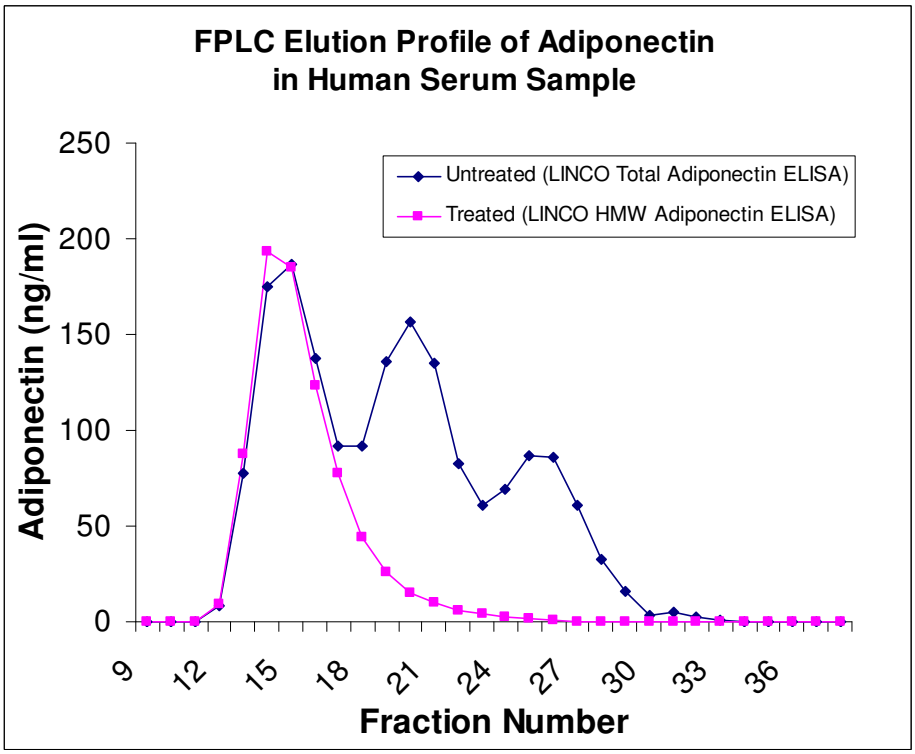
XV. HUMAN HMW ADIPONECTIN ELISA GRAPHS

A. STANDARD CURVE



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

B. FPLC PROFILE



Typical FPLC Profile of Human Serum Sample

XVI. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of HMW Adiponectin that can be detected by this assay is 0.5 ng/mL when using a 10 µL sample size.

B. Specificity

With simple sample pretreatment, this assay specifically measures HMW Adiponectin only and does not recognize Hexameric and Trimeric Adiponectin or other human adipokines.

C. Precision

Intra-Assay Variation

Sample No.	Mean HMW Adiponectin Levels (ng/mL)	Intra-Assay % CV
1	5.95	0.97
2	11.10	1.27
3	13.65	3.41
4	21.33	2.14
5	25.43	3.31
6	27.25	2.62
7	39.45	3.08
8	65.33	2.62

The intra-assay variations of Millipore Human HMW Adiponectin ELISA kits were studied on eight human serum samples with varying concentrations of endogenous HMW Adiponectin. The mean intra-assay variations were calculated from results of 4 duplicate determinations in each assay of the indicated samples.

Inter-Assay Variation

Sample No.	Mean HMW Adiponectin Levels (ng/mL)	Inter-Assay % CV
1	13.33	4.13
2	21.23	8.14
3	26.60	5.03
4	28.65	3.14
5	34.78	7.79
6	38.35	9.10
7	60.75	3.01
8	61.50	3.8

The inter-assay variations of Millipore Human HMW Adiponectin ELISA kits were studied on eight human serum samples with varying concentrations of endogenous HMW Adiponectin. The mean inter-assay variations of each sample were calculated from results of three separate assays with duplicate samples in each assay.

XVI. ASSAY CHARACTERISTICS (continued)

D. Recovery

Spike & Recovery of Human HMW Adiponectin in Serum

Sample No.	HMW Adiponectin Added ng/mL	Expected ng/mL	Observed ng/mL	% of Recovery
1	0	19.4	19.4	
	3.125	22.5	22.9	101.66
	25.0	44.4	49.1	110.59
	100.0	119.4	141	118.09
2	0	28.9	28.9	
	3.125	32.0	33.4	104.9
	25.0	53.9	58.3	108.16
	100.0	128.9	148.5	115.21
3	0	32.4	32.4	
	3.125	35.5	36.4	102.46
	25.0	57.4	60.4	105.23
	100.0	132.4	149.4	112.84
4	0	36.7	36.7	
	3.125	39.8	40.6	101.95
	25.0	61.7	67.2	108.91
	100.0	136.7	152.7	111.7
5	0	52.8	52.8	
	3.125	55.9	56	100.13
	25.0	77.8	81.5	104.76
	100.0	152.8	167.2	109.42
6	0	85.7	85.7	
	3.125	88.8	89.6	100.87
	25.0	110.7	116.5	105.24
	100.0	185.7	203	109.32

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

XVI. ASSAY CHARACTERISTICS (continued)

E. Linearity

Effect of Serum Dilution

Sample No.	Volume Sample	Expected ng/mL	Observed ng/mL	% Of Expected
1	10 µL	24.5	24.5	
	5 µL	12.25	13	106.12
	2.5 µL	6.13	6.3	102.86
	1.25 µL	3.06	3	97.96
2	10 µL	28.1	28.1	
	5 µL	14.05	14.6	103.91
	2.5 µL	7.03	7	99.64
	1.25 µL	3.51	3.5	99.64
3	10 µL	31.5	31.5	
	5 µL	15.75	15.5	98.41
	2.5 µL	7.88	8	101.59
	1.25 µL	3.94	4.1	104.13
4	10 µL	42.6	42.6	
	5 µL	21.3	22.1	103.76
	2.5 µL	10.65	10.5	98.59
	1.25 µL	5.33	5.5	103.29
5	10 µL	68.4	68.4	
	5 µL	34.2	32.5	95.03
	2.5 µL	17.1	17.3	101.17
	1.25 µL	8.55	8.9	104.09
6	10 µL	73.1	73.1	
	5 µL	36.55	36.7	100.41
	2.5 µL	18.28	18.8	102.87
	1.25 µL	9.14	9.6	105.06

Six 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 10 µL. The resulting dilution factors of 1.0, 2.0, 4.0, and 8.0 representing 10 µL, 5 µL, 2.5 µL, and 1.25 µL sample volumes assayed, respectively, were applied in the calculation of observed HMW Adiponectin concentrations.

The % expected = observed HMW Adiponectin concentrations/expected HMW Adiponectin concentrations x 100%.

XVII. DIGESTION CONTROLS

The ranges for Digestion 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.

XIV. REPLACEMENT REAGENTS

Reagents	Cat. #
Human HMW Adiponectin ELISA Plate	EP64
10X HRP Wash Buffer Concentrate	EWB-HRP
Human HMW Adiponectin Standard	E8064-K
Human HMW Adiponectin Digestion Controls 1 and 2	EDC64
10X Assay Buffer	AB-10XP
Assay Running Buffer	EARB-4
Sample Digestion Solution	ESDS
Sample Digestion Buffer	EDGB
Sample Dilution Buffer	ESDB
Human HMW Adiponectin Detection Antibody	E1064
Enzyme Solution	EHRP
Substrate	ESS-TMB
Stop Solution	ET-TMB
Sample Preparation Plates	E-PLATE

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 (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.

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 human 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.