DRG® Adiponectin (mouse) Elisa (EIA-4172)

Revised 13 Dec. 2005 **For Veterinary Use Only**

I. INTENDED USE

This Mouse Adiponectin (ACRP30) ELISA kit is used for the non-radioactive quantification of Mouse Adiponectin in serum, plasma, and adipocyte extracts or cell culture media samples. This kit has 100% cross reactivity to Mouse Adiponectin. There is no binding to Mouse Adiponectin globular domain, or to Rat 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 Mouse Adiponectin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of anti- mouse adiponectin monoclonal antibodies, and binding of a second biotinylated anti- mouse polyclonal antibody to the captured molecules, 2) wash away of unbound materials from samples, 3) conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away 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 absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured Mouse 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 Mouse Adiponectin.

III. REAGENTS SUPPLIED

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

A. Mouse Adiponectin ELISA Plate
Coated with Rat Monoclonal anti-Mouse 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 50mM Tris Buffered Saline containing Tween-20.
Quantity: Two bottles containing 50 ml each
Preparation: Dilute 1:10 with distilled or deionized water.

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

E. Quality Controls 1 and 2

Purified Recombinant Mouse Adiponectin in Assay Buffer, lyophilized.
Quantity: 1ml/vial upon hydration
Preparation: Contents Lyophilized. Reconstitute with 1 ml distilled or deionized water.

F. 10X Assay Buffer

Quantity: 50 ml
Preparation: Dilute 1:10 with distilled or deionized water to make 1X assay buffer
(0.05M Phosphosaline containing 0.025M EDTA, 0.08% Sodium Azide, 1% BSA).
Note: Use 1X Assay Buffer to dilute samples (Section VIII, SAMPLE PREPARATION)

G. Mouse Adiponectin Detection Antibody

Pre-titered Biotinylated Goat anti-Mouse Adiponectin Polyclonal Antibody
Quantity: 3.0 ml
Preparation: Ready to Use

H. Enzyme Solution

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

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

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

J. 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, Adiponectin Standards, Controls and reconstituted Standards and Controls at < –20°C. Minimize repeated freeze and thaw of the Adiponectin Standards and Quality Controls. 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

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.

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

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

   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.

2. 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.
3. 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.
4. 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 1:1000 in 1X Assay Buffer (See Section III, F). Cellular extract and culture media dilutions will vary.

a. Make Dilution A by adding 10 µl sample to 990 µl Assay Buffer and vortex.

b. Make Dilution B by adding 100 µl of Dilution A to 900 µl Assay Buffer and vortexing. Use Dilution B (1:1000) for the assay procedure.

IX. STANDARD AND QUALITY CONTROLS PREPARATION

A. Mouse Adiponectin Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using an Eppendorf pipette, reconstitute the Mouse Adiponectin Standard with 1.0 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 mix well.

2. Label seven tubes 1, 2, 3, 4, 5, 6, and 7. Add 0.5 ml Assay Buffer to each of the seven tubes. Prepare serial dilutions by adding 0.5 ml of the reconstituted standard to tube 1, mix well and transfer 0.5 ml of tube 1 to tube 2, mix well and transfer 0.5 ml of tube 2 to tube 3, mix well and transfer 0.5 ml of tube 3 to tube 4, mix well and transfer 0.5 ml of tube 4 to tube 5, mix well and transfer 0.5 ml of tube 5 to tube 6, mix well and transfer 0.5 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**
	1 ml	0	X (Refer to analysis sheet for exact concentration)

Tube #	**Volume of Assay Buffer to Add**	**Volume of Standard to Add**	**Standard Concentration ng/ml**
1	0.5 ml	0.5 ml of reconstituted standard	X/2
2	0.5 ml	0.5 ml of tube 1	X/4
3	0.5 ml	0.5 ml of tube 2	X/8
4	0.5 ml	0.5 ml of tube 3	X/16
5	0.5 ml	0.5 ml of tube 4	X/32

6	0.5 ml	0.5 ml of tube 5	X/64
7	0.5 ml	0.5 ml of tube 6	X/128

B. Mouse Adiponectin Quality Control 1 and 2 Preparation

1. Use care in opening the lyophilized Quality Control vials. Using an Eppendorf pipette, reconstitute each of the Mouse Adiponectin Quality Control 1 and Quality Control 2 with 1 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.

Note: Since the standard concentration may vary from lot to lot, please make sure to change the standard concentration in the template while reading absorbance in the spectrophotometer.

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 Microtiter Assay Plate from the foil pouch 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, use a gentle wash program for all washing steps described in this protocol.**

3. Add 60µl Assay Buffer to all wells.

4. Add in duplicate 20µl Assay Buffer to the blank wells.

5. Add in duplicate 20 µl Mouse Adiponectin Standards in the order of ascending concentration to the appropriate wells. Add in duplicate 20 µlQC1 and 20 µlQC2 to the appropriate wells. Add sequentially 20µl 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 5 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.

12. Add 100 µl of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for 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 usual recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time. One can monitor color development using 370nm filter, if available on the spectrophotometer. When the absorbance is between 1.2 and 1.8 at 370 nm, the stop solution can be added to terminate the color development.

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 Mouse Adiponectin ELISA kit

	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	Standards/ Controls/ Samples	Detection Ab	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 5X 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 12 - 18 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, A2			80 µl		20 µl		100 µl		100 µl		100 µl	
A3, A4			60 µl	20 µl of Tube 7								
A5, A6			60 µl	20 µl of Tube 6								
A7, A8			60 µl	20 µl of Tube 5								
A9, A10			60 µl	20 µl of Tube 4								
A11, A12			60 µl	20 µl of Tube 3								
B1, B2			60 µl	20 µl of Tube 2								
B3, B4			60 µl	20 µl of Tube 1								
B5, B6			60 µl	20 µl of QC I								
B7, B8			60 µl	20 µl of QC II								
B9, B10			60 µl	20 µl of Sample								
B11, B12			60 µl	20 µl of Sample								
C1, C2 ↓			60 µl	20 µl of Sample								

XI. MICROTITER PLATE ARRANGEMENT

Mouse Adiponectin ELISA

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

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 1000 dilution factor.

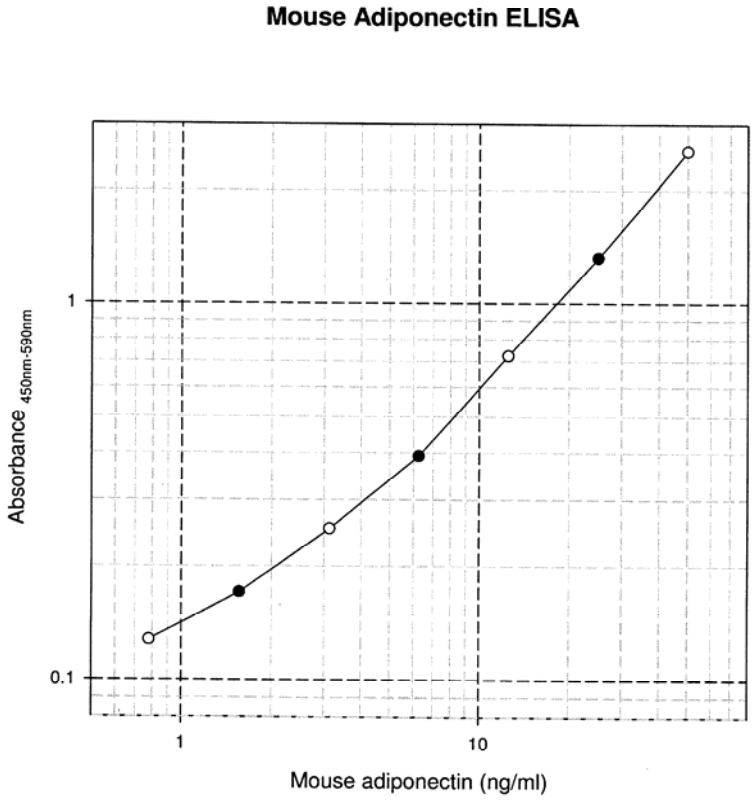
[**Note:** When sample volumes assayed differ from 20 m l, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 µl of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 µl, compensate the volume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

XIII. INTERPRETATION

A. Acceptance Criteria

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 0.5ng/ml mouse Adiponectin (20 µl sample size).
4. The approximate dynamic range of this assay is 1 - 50 ng/ml mouse Adiponectin (20 µl sample size). Any result greater than 50 ng/ml in a 20 µl sample should be diluted using 1X assay buffer, and the assay repeated until the results fall within range.

XIV. STANDARD CURVE



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

XV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Adiponectin that can be detected by this assay is 0.5 ng/ml when using a 20 µl sample size.

B. Specificity

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

Analyte	Max. Conc.	Cross Reactivity
Human Adiponectin	200ng/ml	< 2%
Mouse gAcrp	1000ng/ml	n.d.
Mouse Endocrines:		
Insulin	10nM	n.d.
Amylin	10nM	n.d.
Leptin	10nM	n.d.
Glucagon	10nM	n.d.
GLP-1	10nM	n.d.
Mouse Cytokines:		
IL-1ß	10nM	n.d.
IL-2	10nM	n.d.
IL-4	10nM	n.d.
IL-5	10nM	n.d.
IL-6	10nM	n.d.
IL-9	10nM	n.d.
IL-10	10nM	n.d.
IL-12	10nM	n.d.
IL-13	10nM	n.d.
IFN-γ	10nM	n.d.
TNF-α	10nM	n.d.
GMCSF	10nM	n.d.
MIP-1α	10nM	n.d.
KC	10nM	n.d.
RANTES	10nM	n.d.
MCP-1	10nM	n.d.

n.d. = not detectable

No cross reactivity to rat serum or plasma samples.

C. Precision

Within and Between Assay Variation

Sample No.	Mean Adiponectin Levels (ng/ml)	Within% CV	Between% CV
1	9.18	3.8	6.8
2	13.74	5.4	1.4
3	6.89	8.2	4.9
4	7.23	5.6	10.8

The assay variations of DRG Mouse Adiponectin ELISA kits were studied on four mouse 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 Mouse Adiponectin in Serum

Sample No.	Adiponectin Added (ng/ml)	Expected (ng/ml)	Observed (ng/ml)	% of Recovery
1	0	7.1	7.1	100
	5	12.1	12.6	104
	10	17.1	17	99
	20	27.1	26.7	99
2	0	7.2	7.2	100
	5	12.2	13.7	112

	10	17.5	17.8	103
	20	27.2	28.9	106
3	0	10.49	10.49	100
	5	15.49	15.76	102
	10	20.49	22.17	108
	20	30.49	31.07	102

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

E. Linearity

Effect of Serum Dilution

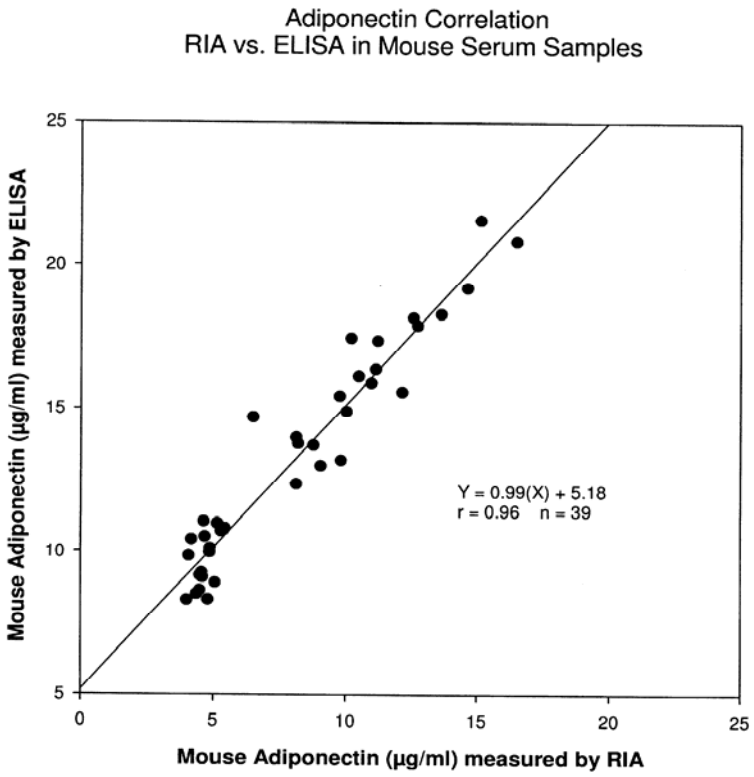
Sample No.	Volume Sampled (µl)	Expected (ng/ml)	Observed (ng/ml)	% Of Expected
1	20	14.3	14.3	100
	10	7.15	7.3	102
	5	3.6	4.1	114
	3.33	2.4	2.9	121
2	20	14.99	14.99	100
	10	7.49	7.23	96
	5	3.75	4.37	116
	3.33	2.5	2.81	112
3	20	14.43	14.43	100
	10	7.2	7.17	99
	5	3.61	4.16	115

	3.33	2.41	2.58	107

Three mouse 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, 4.0, and 6.0 representing 20 µl, 10 µl, 5 µl, and 3.3µl sample volumes assayed, respectively, were applied in the calculation of observed Adiponectin concentrations. % expected = observed/expected x 100%.

XVI. CORRELATION

XVI. CORRELATION GRAPH



GRAPH

Serum samples obtained from 39 mouse subjects were assayed for Adiponectin content using both DRG Mouse Adiponectin RIA Kit (Catalogue No. RIA-3766) and Mouse Adiponectin ELISA Kit (Catalogue No. EIA-4172). 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.

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 HRP Wash Buffer or 3) overexposure to light after substrate has been added.