



## **Adiponectin (Mouse) Total, HMW ELISA**

For the quantitative determination of total and high molecular weight (HMW) adiponectin in mouse serum and plasma

For Research Use Only. Not For Use In Diagnostic Procedures.

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### **ALPCO Diagnostics**

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## INTENDED USE

The Adiponectin (Mouse) Total, HMW ELISA is designed for the quantitative determination of total and High Molecular Weight (HMW) adiponectin in mouse serum or plasma.

## INTRODUCTION

Adiponectin circulates as multiple isoforms<sup>1</sup> and is widely recognized as a key protein involved in insulin regulation and metabolic syndrome. Of the different multimers, the high molecular weight (HMW) form of adiponectin activates adenosine monophosphate activated protein kinase phosphorylation most effectively<sup>1</sup>, and HMW levels, or the ratio of HMW to total adiponectin, are more meaningful than the total adiponectin level for predicting insulin resistance and the development of metabolic syndrome<sup>2</sup>. Thiazolidinediones<sup>3</sup>, angiotensin type 1 receptor blockers<sup>4</sup>, and fibrates<sup>5,6</sup>, together with the activation of PPAR $\gamma$  or PPAR $\alpha$ , are reported to increase the secretion of adiponectin and have received considerable attention for the treatment of metabolic syndrome. Thiazolidinediones act primarily to increase the secretion of HMW adiponectin<sup>7</sup>. The identification of additional adiponectin-enhancing therapeutics would be beneficial for researchers, especially in the early stages of animal studies. Recently, an ELISA system was developed for the selective quantification of human adiponectin oligomers using protease pretreatment<sup>8</sup>; this kit reflects a similar system for simultaneous detection of mouse HMW and total adiponectin<sup>9</sup>.

## PRINCIPLE OF THE ASSAY

In the Adiponectin (Mouse) Total, HMW ELISA, both total and HMW adiponectin can be measured independently on the same plate. Samples are pretreated with or without protease, diluted, and then assayed for adiponectin as described below.

The principle of the sample pretreatment procedure is outlined briefly here and explained in greater detail in the ASSAY PROCEDURE.

- 1) To measure **total adiponectin**: Samples are not subjected to protease pretreatment but are diluted with Sample Pretreatment and Dilution Buffers; measurement in the ELISA quantifies the amount of all multimers of adiponectin without any modification to their respective structures. Total adiponectin includes HMW (12-mer and 18-mer), Mid-Molecular Weight (MMW) (hexamer); and Low Molecular Weight (LMW) (trimer + albumin-bound trimer).
- 2) To measure **HMW adiponectin**: Samples are pretreated with the protease that specifically digests MMW and LMW. Samples are then diluted with Sample Pretreatment and Dilution Buffers which stop the protease reaction. The remaining HMW adiponectin is then assayed in the ELISA.

The ELISA utilizes an antibody “sandwich” comprised of an anti-mouse adiponectin monoclonal antibody (MoAb) and an anti-mouse adiponectin polyclonal antibody (PoAb).

The microplate wells have been coated with anti-mouse adiponectin MoAb, and adiponectin in the Calibrators and pretreated samples is captured by the antibody during the first incubation. Washing removes all unbound material, and a biotin-conjugated PoAb is added, which binds to immobilized adiponectin in the wells. After the second incubation and subsequent washes, HRP-labeled streptavidin is added. Following a third incubation and subsequent wash step, O-phenylenediamine (OPD) is added as substrate. The colorimetric reaction is terminated with the addition of diluted H<sub>2</sub>SO<sub>4</sub>. The intensity of

the color development, i.e., absorbance value, is proportional to the adiponectin concentration in the sample.

## KIT COMPONENTS

Allow all reagents to reach room temperature before use.

Reagent	Composition	Amount	Preparation
Wash Buffer Concentrate	Phosphate buffer (pH 7.2)	1 x 100 ml	10X
Sample Pretreatment Buffer	Borate buffer (pH 11.0)	1 x 70 ml	Ready to use
Dilution Buffer	Phosphate buffer (pH 7.2) containing BSA	1 x 100 ml	Ready to use
MoAb Coated Wells	Rat anti-mouse adiponectin monoclonal antibody coated plate	1 plate	Ready to use
Calibrator Stock Solution	Recombinant mouse adiponectin	1 x 1 ml	8 ng/ml*
Biotin-Conjugated PoAb	Biotin-conjugated rabbit anti-mouse adiponectin polyclonal antibody	1 x 6 ml	Ready to use
Enzyme-Labeled Streptavidin	HRP-labeled streptavidin	1 x 6 ml	Ready to use
Substrate	O-phenylenediamine (OPD)	2 vials	Lyophilized
Substrate Buffer	H <sub>2</sub> O <sub>2</sub> in citrate buffer (pH 5.0)	1 x 15 ml	Ready to use
Stop Reagent	7.7 % H <sub>2</sub> SO <sub>4</sub>	1 x 15 ml	Ready to use
Protease Concentrate	Protease	1 x 1 ml	10X
Protease Buffer	Tris buffer (pH 8.0)	1 x 15 ml	Ready to use

\*Approximate concentration; see vial for actual concentration.

## MATERIALS REQUIRED BUT NOT INCLUDED

- Microfuge tubes (1 ml)
- Precision pipettes with disposable tips capable of dispensing 10 µl, 100 µl, 500 µl
- Repeating or multichannel pipette
- Volumetric container
- Volumetric pipettes
- Distilled (deionized) water
- Incubator or water bath (37 °C)
- Microplate washer or wash bottle
- Microplate reader with 492 and optional 600/700 nm filter

## REAGENT PREPARATION AND STORAGE

***All components are stable at 2-10°C until the expiration date of the kit unless otherwise indicated.***

### Wash Buffer

Dilute Wash Buffer Concentrate with 900 ml of distilled water. Working Wash Buffer is stable until the expiration date of the kit when stored at 2-10°C.

### MoAb Coated Wells

Unused strips should be returned to the laminate bag, sealed and stored at 2-10°C.

### Working Calibrator

- Create a standard curve by serial dilution as indicated in the table below.
- The remaining undiluted Calibrator should be stored at 2-10°C.
- Diluted Calibrator is not stable and should not be stored.

Standard Number	Quantity of Standard	Quantity of Dilution Buffer	Final Concentration (ng/ml)
1	200 µl of Calibrator stock	0	*8.0
2	200 µl of Standard 1	200 µl	4.0
3	200 µl of Standard 2	200 µl	2.0
4	200 µl of Standard 3	200 µl	1.0
5	200 µl of Standard 4	200 µl	0.5
6	200 µl of Standard 5	200 µl	0.25
7	200 µl of Standard 6	200 µl	0.125
8	0	200 µl	0

**\*Approximate concentration; see vial for actual concentration.**

### Substrate

Just prior to use, reconstitute the Substrate (lyophilized) by adding 6 ml of Substrate Buffer to the Substrate vial. The Substrate Solution should be used immediately after reconstitution, and the remaining solution should be discarded.

### Protease Solution

- Just prior to use, dilute the Protease Concentrate 1:10 with Protease Buffer. For example, dilute 1 ml Protease Concentrate with 9 ml Protease Buffer.
- Diluted Protease Concentrate is not stable and should not be stored.

## SAMPLE COLLECTION

Serum or plasma samples can be used with the assay. Because the measurement of HMW adiponectin requires samples be pretreated with a protease, it is recommended that serine protease inhibitors, such as aprotinin, not be used when collecting samples.

## ASSAY PROCEDURE

### Pretreatment of samples

All performance characteristics for this assay were established using 10 µl of sample. Lower sample volumes have been evaluated and the recommended starting sample volume is between 5-10 µl. See Sample Volume Adjustment (section 7) for supporting data.

- **Option 1: To measure total adiponectin:**

To 10 µl of serum, add 100 µl of the Protease Buffer and 700 µl of Sample Pretreatment Buffer and stir thoroughly (1: 81 dilution).

- **Option 2: To measure HMW adiponectin:**

To 10 µl of serum, add 100 µl of the prepared Protease Solution and incubate for 20 min at 37°C. Add 700 µl of the Sample Pretreatment Buffer and stir thoroughly (1: 81 dilution).

\*Pretreated samples are stable at room temperature for at least 1 hr.

### Dilution of pretreated samples

To 1.0 ml of Dilution Buffer, add 10 µl of a pretreated sample obtained in Options 1 and/or 2 (1: 101 dilution; **final dilution: 1: 8181**).

\* Diluted pretreated samples are stable at room temperature for at least 1 hr.

### Assay Method

1. Take the necessary number of strips out of the laminate bag, add 50 µl each of the working Calibrators and diluted samples to each test well, and incubate the covered plate for 60 min at 20-30°C.
2. Remove contents from wells, add 350-400 µl of working Wash Buffer to each well, and thoroughly remove the droplets. Repeat this cycle twice.
3. Add 50 µl of the Biotin conjugated-PoAb to each washed well and incubate the covered plate for 60 min at 20-30°C.
4. Wash 3 times as in Step 2.
5. Add 50 µl of the Enzyme-labeled Streptavidin to each washed well and incubate the covered plate for 30 min at 20-30°C.
6. Wash 3 times as in Step 2.
7. Add 50 µl of the working Substrate Solution to each washed well, and incubate the covered plate for 10 min at 20-30°C. Then add 50 µl of the Stop Reagent to each test well.
8. The absorbance of each well should be measured between 10 – 30 minutes following the addition of the Stop Solution using a plate reader set at a wavelength of 492 nm, and a reference wavelength of 600-700 nm, if desired.

### Calculation

Calculate the absorbance by subtracting the absorbance of the Zero Standard from those of other Standards and diluted samples. Plot the absorbance of the standards against the standard concentrations on loglog or semi-log graph paper. Draw a smooth curve through these points to construct the calibration curve. Read the concentrations for the diluted samples from the calibration curve. Calculate the concentration for the diluted samples by multiplying by the dilution factor (**1:8,181**).

Alternatively, a quadratic or cubic spline curvefit can be used

- **Total adiponectin** concentration is calculated from samples pretreated in Option 1.
- **HMW adiponectin** concentration is calculated from samples pretreated in Option 2.

## PROCEDURAL NOTES

1. Mouse serum and plasma (EDTA is recommended) are appropriate sample types.
2. Selective measurements of the same serum or plasma specimens should be in the same plate. Even in cases of the same lot, do not calculate between different plates.
3. A calibration curve must be run with each assay. Standards and samples should be assayed in duplicate.
4. When the concentration of adiponectin in a sample exceeds the calibration curve range, further dilute pretreated samples with Dilution Buffer, and assay again.
5. The specified reaction time and temperature should be observed strictly. This is especially important when working with the Protease Solution.
6. Each kit can be divided and used a maximum of two times. The remaining reagents should be stored as directed in the package insert and used before the expiration date.
7. Pretreated samples can be maintained at room temperature for at least 1 hr prior to the addition of Dilution Buffer, if necessary. Diluted pretreated samples can also be maintained at room temperature for at least 1 hr. However, adiponectin concentrations do decline slightly over time, so the pretreatment and subsequent dilution steps should be performed as quickly as possible, as should loading of the plate.
8. Remove the droplets completely after each wash step.
9. Do not allow the wells to dry out or to be damaged during the wash procedure.
10. Avoid carrying out this procedure in direct sunlight.

## WARNINGS AND PRECAUTIONS

1. Samples and reagents should be handled carefully as though capable of transmitting infection.
2. Stop Reagent (7.7% H<sub>2</sub>SO<sub>4</sub>) is poisonous and can cause severe burns. In case of eye contact, rinse immediately with plenty of water, and seek medical advice. In case of contact with skin or clothing, rinse immediately with plenty of water.

## STORAGE OF REAGENTS

The kit reagents should be stored at 2-10°C. **DO NOT FREEZE.**

## EXPIRATION DATE

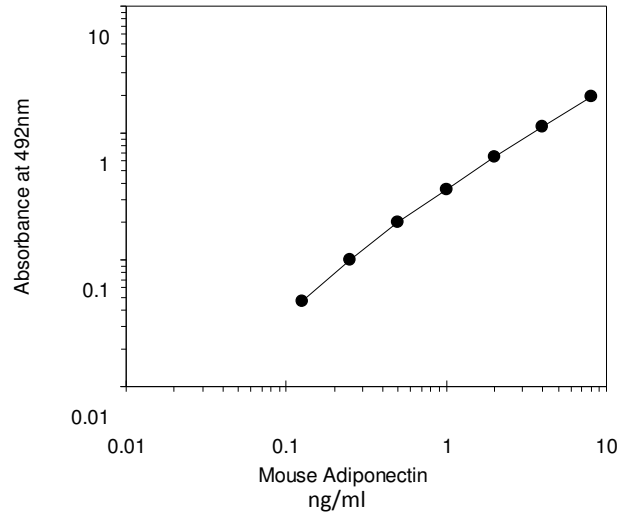
Indicated on the package.

## REFERENCES

- 1) Hada Y, et al. *Biochem Biophys Res Commun* 2007; 356:487–93.
- 2) Hara K, et al. *Diabetes Care* 2006; 29:1357–62.
- 3) Tsuchida A, et al. *Diabetes*. 2005; 54:3358–70.
- 4) Clasen R, et al. *Hypertension*. 2005; 46:137–43.
- 5) Nakano S, et al. *Am J Physiol Endocrinol Metab*. 2007; 292:E1213–22.
- 6) Hiuge A, et al. *Arterioscler Thromb Vasc Biol* 2007; 27:635–41.
- 7) Bodles AM, et al. *Am J Physiol Endocrinol Metab* 2006; 291:E1100–5.
- 8) Ebinuma H, et al. *Clin Chim Acta* 2006; 372:47–53.
- 9) Ebinuma H, et al. *Clin Chim Acta* 2009; 401:181-3.

## Performance Characteristics

### 1. Typical Standard Curve



### 2. Sensitivity

The sensitivity was determined by calculating the mean  $\pm$  3 standard deviations for 20 replicates of the Zero Standard. The sensitivity of the assay is 0.032 ng/ml.

### 3. Reproducibility

#### Intra-assay variation

The within run precision is expressed as the percentage coefficient of variation (CV%). This was determined based on the mean and standard deviation of 5 replicates of a sample run in a single assay. The table below shows the results of 2 unique samples.

	Mouse serum (1)		Mouse serum (2)	
	Total Adiponectin	HMW Adiponectin	Total Adiponectin	HMW Adiponectin
Mean ( $\mu\text{g}/\text{ml}$ )	24.4	4.98	30.6	11.8
SD	0.82	0.21	0.83	0.24
CV (%)	3.3	4.1	2.7	2.0

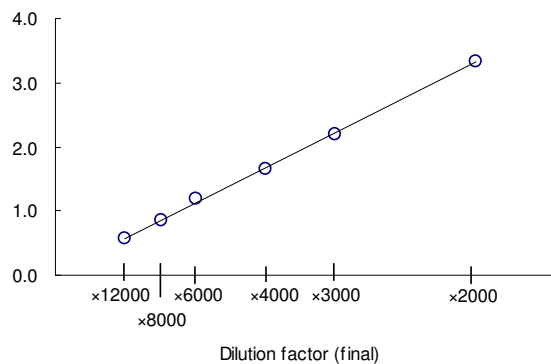
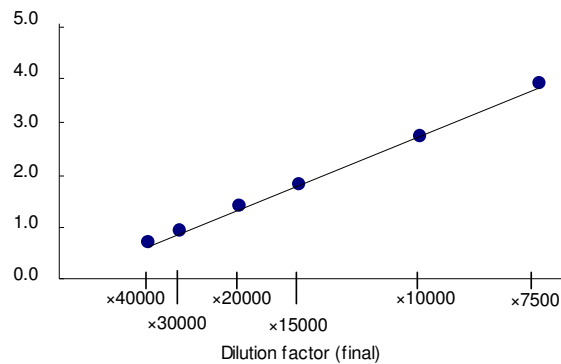
### Inter-assay variation

The between run precision is expressed as the percentage coefficient of variation (CV%). This was determined based on the mean and standard deviation across 5 assays of duplicate measurements of a single sample. The table below shows the results of 2 unique samples.

	Mouse serum (1)		Mouse serum (2)	
	Total Adiponectin	HMW Adiponectin	Total Adiponectin	HMW Adiponectin
Mean ( $\mu\text{g}/\text{ml}$ )	21.2	4.6	28.7	11.3
	0.7	0.2	1.2	0.2
CV (%)	3.1	3.3	4.0	1.9

### 4. Dilution Linearity

The linearity of the assay was determined by preparing dilutions of a pretreated mouse serum sample. The expected values were compared to the obtained values to determine a percent recovery. The range of recovery was 98.3-101.3% for total adiponectin (serial dilutions of  $\times 7,500$ - $40,000$ ) and 99.1-106.5% for HMW adiponectin (serial dilutions of  $\times 2,000$ - $12,000$ ).



Total and HMW adiponectin levels in serial dilution of mice sera (n = 5)

	x 40,000	x 30,000	x 20,000	x 15,000	x 10,000	x 7,500
Total Adiponectin (µg/ml)	28.2	27.9	28.4	27.9	28.0	28.8
Mean Recovery (%)	99.2	98.4	99.9	98.3	98.7	101.3

	x 12,000	x 8,000	x 6,000	x 4,000	x 3,000	x 2,000
HMW Adiponectin (µg/ml)	6.68	6.57	6.65	7.06	6.76	6.77
Mean Recovery (%)	100.8	99.1	100.3	106.5	102.0	102.1

### 5. Spike and Recovery

Varying amounts of purified HMW adiponectin from mouse serum were added to a mouse serum sample and assayed in the ELISA.

	HMW Adiponectin Added (µg/ml)	Observed (µg/ml)	Expected (µg/ml)	Recovery O/E (%)
Total Adiponectin	0	20.4	-	-
	1.02	21.2	21.4	99
	2.04	22.5	22.5	100
	4.08	25.0	24.5	102
	8.16	30.5	28.6	107
HMW Adiponectin (Protease treatment)	0	4.33	-	-
	1.02	5.20	5.35	97
	2.04	6.13	6.37	96
	4.08	8.31	8.41	99
	8.16	12.23	12.49	98

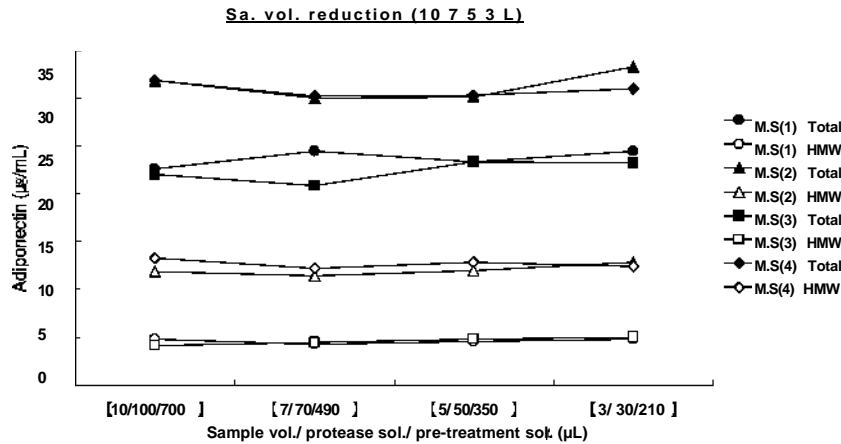
### 6. Comparison of Serum vs. Plasma Samples

Serum and EDTA, heparin, and citrate plasma samples were obtained from C57Bl/6 mice (n = 6) and assayed for total and HMW adiponectin.

	Sample (n = 6)	Mean Adiponectin (µg/ml)	Mean Plasma/Serum (%)
Total Adiponectin	Serum	35.0	-
	EDTA plasma	33.1	94
	Heparin plasma	30.0	86
	Citrate plasma	30.3	86
HMW Adiponectin (Protease treatment)	Serum	9.13	-
	EDTA plasma	9.01	99
	Heparin plasma	8.21	90
	Citrate plasma	7.51	82

## 7. Sample Volume Adjustment

Volumes of sample, Protease Solution and Sample Pretreatment Buffer were reduced proportionally; pretreated samples were diluted with Dilution Buffer and assayed for total and HMW adiponectin (final dilution x 8,181).



Note: The recommended starting sample volume in this kit is between 5 and 10 µL.

## 8. Sample Stability

### a. Effects of Freeze/Thaw (F/T)

		Recovery (%)			
Mouse serum (1)	(µg/ml)	F/T (1X)	F/T (2X)	F/T (3X)	F/T (4X)
Total Adiponectin	20.9	92	102	100	96
HWM Adiponectin	4.65	97	99	98	102

		Recovery (%)			
Mouse serum (2)	(µg/ml)	F/T (1X)	F/T (2X)	F/T (3X)	F/T (4X)
Total Adiponectin	28.2	102	105	104	111
HWM Adiponectin	12.0	99	96	92	99

### b. Stability of Frozen Samples Over Time

Serum samples were stable for at least 10 months (M) when stored at -30°C.

		Recovery (%)				
Control serum	(µg/ml)	2M	4M	6M	8M	10M
Total Adiponectin	28.7	95	98	85	92	89
HWM Adiponectin	11.3	96	100	90	92	91
(%) HMW/Total	39	101	102	105	100	102

### c. Stability of Pretreated Samples

Serum samples (n = 8) pretreated with Protease Solution were tested for stability at room temperature.

Sample (n = 8)	Mean (µg/ml)	Recovery (%) at 25°C		
		1hr	2hr	4hr
Total Adiponectin	27.1	96	94	93
HWM Adiponectin	7.84	95	90	86

Note: Adiponectin levels in pretreated samples tend to decrease with time. Therefore, the subsequent dilution step should be performed as soon as possible after pretreatment.

### d. Stability of Pretreated, Diluted Samples

Serum samples (n = 8) pretreated with Protease Solution and diluted with Dilution Buffer as indicated in the Assay Procedure were tested for stability at room temperature.

Sample (n = 8)	Mean (µg/ml)	Recovery (%) at 25°C		
		1hr	2hr	5hr
Total Adiponectin	27.3	98	96	96
HWM Adiponectin	8.57	97	93	91

Note: Adiponectin levels in pretreated, diluted samples tend to decrease with time. Therefore, the samples should be applied to the plate as soon as possible after dilution.

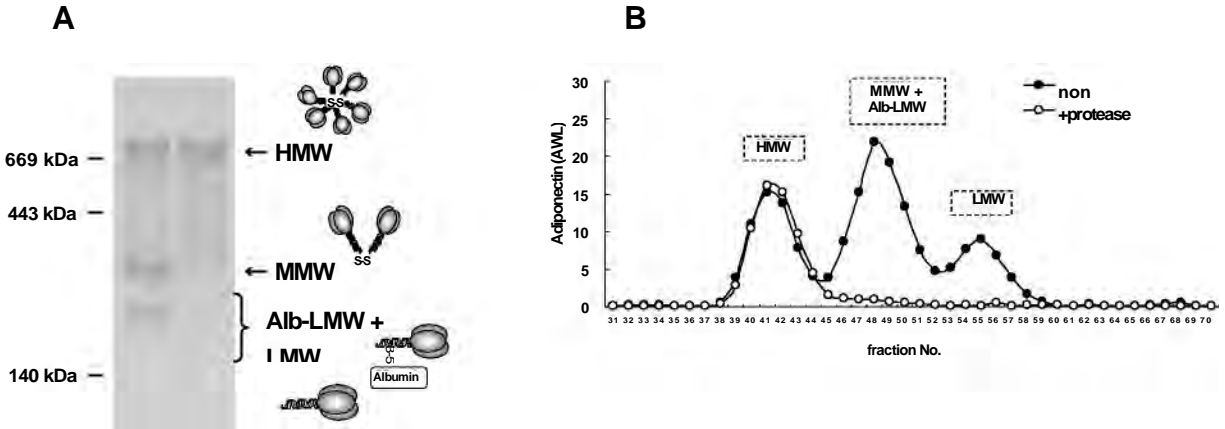
## 9. Interference of Hemoglobin

Varying amounts of extracted hemoglobin from a C57BL/6J mouse were added to mouse serum samples (n = 2) and assayed for total and HMW adiponectin.

	Hemoglobin Added (mg/dl)				
	100	250	500	750	1000
	% Recovery				
Total Adiponectin (mean= 21.5 µg/ml)	101	100	101	106	106
HMW Adiponectin (mean = 7.35 µg/ml)	94	95	102	96	97

## 10. Protease Specificity

Mouse serum, with or without protease pretreatment, was separated by native PAGE and analyzed by Western blotting using the polyclonal detection ab in the ELISA (A). Pretreated mouse serum was fractionated by gel filtration chromatography on a Superdex 200 column and detected by the ELISA (B). Only the HMW form of adiponectin was detected in the sample pretreated with the protease (A and B).<sup>9</sup>



## 11. Protease Digestion (Time)

Mouse serum samples (n = 6) were treated with Protease Solution for 0 to 30 minutes at 37°C and assayed for HMW adiponectin.

	Protease pretreatment (minutes)					
	0	10	15	20	25	30
HMW Adiponectin (mean, µg/ml)	30.7	9.8	9.1	9.0	8.8	8.9
SD (µg/ml)	10.4	3.9	3.7	3.6	3.5	3.6

There were no significant differences in adiponectin concentration between 15 minutes and 30 minutes.

## 12. Protease Digestion (Temperature)<sup>9</sup>

Mouse serum samples (n = 6) were treated with Protease Solution for 20 minutes at 33 - 41°C and assayed for HMW adiponectin.

	Protease pretreatment (temperature)				
	33°C	35°C	37°C	39°C	41°C
HMW adiponectin (mean, µg/ml)	10.0	9.8	9.1	8.5	7.7
SD (µg/ml)	3.8	3.6	3.0	3.1	2.8

There were no significant differences in the adiponectin concentration between 33 and 37°C, but the adiponectin concentration decreased significantly with digestion ≥ 39°C (p<0.05).

### 13. Correlation to Alternate Commercial Assay<sup>9</sup>

Total adiponectin was quantified in 34 mouse serum samples in the current ELISA and an alternate commercially available assay. Regression analysis yielded a line with the equation  $y = 1.12x + 2.82$  and a correlation coefficient of  $r = 0.972$ , where the current ELISA =  $y$  and the alternate ELISA kit =  $x$ . The mean ( $\pm$  SD) total and HMW adiponectin concentrations in these sera as quantified in the current ELISA were  $30.1 \pm 9.1$  mg/l (range 20.1 - 62.4) and  $6.6 \pm 2.9$  mg/l (range 3.6 - 13.6), respectively, with a HMW/total value of  $0.22 \pm 0.05$  (range 0.12 - 0.34).

### 14. Specificity

- The antibodies in the Adiponectin (Mouse) Total, HMW ELISA recognize native multimeric forms of adiponectin and have an extremely weak reactivity to the denatured dimeric or monomeric forms.
- No signal has been obtained when sera from the following species were measured in the ELISA: human, monkey, rat, rabbit, goat, sheep and pig.