DATA SHEET

Mouse/Rat Adiponectin ELISA Kit
User Manual

UM-100201
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See List of Components for Storage Conditions
FOR RESEARCH USE ONLY

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Notice to Purchaser

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I. Introduction and Protocol Overview

Obesity, and obesity-related disorders, are reaching alarming proportions in the US, and are on the increase in Europe and Asia. A deeper understanding of the molecular and cellular dynamics of such disorders, and their subsequent amelioration, will have a far-reaching impact on the quality of life of millions of people worldwide.

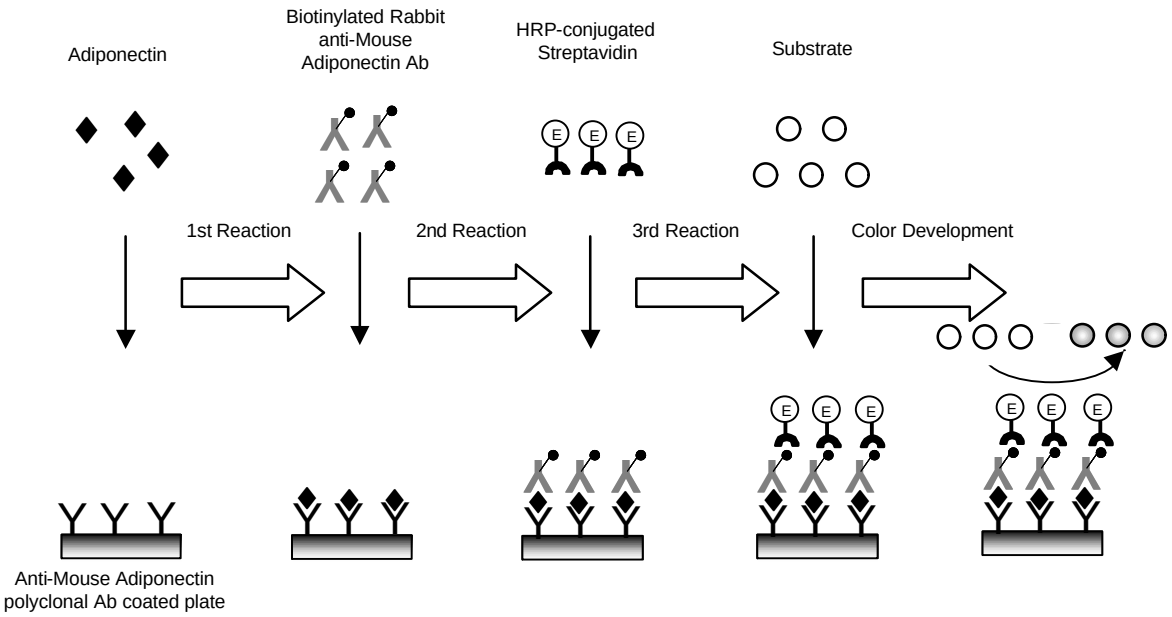
Adipocytes (fat cells) express a variety of proteins that function in the homeostatic control of glucose and lipid metabolism. Insulin regulates the translocation and secretion of many of these proteins in response to changes in energy balance. Adipocyte complement-related protein of 30 kDa (Acrp30), now known as adiponectin, is a protein whose secretion from adipocytes is enhanced by insulin stimulation.

It has been suggested that the development of non-insulin dependent (Type II) diabetes may involve dysregulation of adiponectin secretion (1). In support of the link between obesity and Type II diabetes, it has been shown that decreased expression of adiponectin correlates with insulin resistance (2,3), and that adiponectin appears to be a potent insulin enhancer linking adipose tissue and whole-body glucose metabolism (4).

The B-Bridge **Mouse/Rat Adiponectin ELISA Kit** is designed to measure the concentration of mouse/rat adiponectin from mouse/rat serum, adipocytes, or conditioned medium.

The principle of the assay is shown in Figure 1. Samples and serially diluted standard (recombinant mouse adiponectin) solutions are added to an appropriate number of wells of the microtiter plate and incubated. Adiponectin in the sample will be bound by the primary anti-mouse adiponectin polyclonal antibody immobilized in the well (1st Reaction). After washing, the biotinylated secondary rabbit anti-mouse adiponectin antibody is added to each well and allowed to incubate (2nd Reaction). The biotinylated secondary rabbit anti-mouse adiponectin polyclonal antibody will bind to the adiponectin trapped in the well in the 1st Reaction. After washing, a conjugate of horseradish peroxidase (HRP) and streptavidin is added to each well and allowed to incubate (3rd Reaction). The HRP-conjugated streptavidin will recognize and bind to biotinylated rabbit anti-adiponectin antibody trapped in the well in the 2nd Reaction. After washing, the colorimetric substrate for the enzyme is added to all wells and incubated. The color development is terminated by the addition of a stop solution. The intensity of the color is measured at 450 nm. The concentrations of the samples tested are calculated using the absorbance values of the adiponectin standard solutions assayed at the same time.

Figure 1. Assay Principle



II. List of Components

- Store all components at 2-8°C. **DO NOT FREEZE**.

1	**25X WASH SOLUTION**	1 Bottle (40mL)
2	**5X SAMPLE DILUENT**	1 Bottle (50mL)
3	**PRIMARY ANTIBODY-COATED PLATE** One plate holds 12x8-well strips (96 wells), with adsorbed rabbit anti-mouse adiponectin polyclonal antibody. Plate is provided in a resealable foil pouch with desiccant.	1 Plate
4	**ADIPONECTIN STANDARD** Recombinant mouse adiponectin (8.0 ng/mL)	1 Vial (2mL)
5	**BIOTINYALED SECONDARY ANTIBODY SOLUTION** Biotinylated rabbit anti-mouse adiponectin polyclonal antibody	1 Bottle (12mL)
6	**HRP-CONJUGATED STREPTAVIDIN** Horseradish peroxidase (HRP)-conjugated streptavidin	1 Vial (0.1mL)
7	**STREPTAVIDIN DILUENT**	1 Bottle (15mL)
8	**SUBSTRATE A**	1 Bottle (7.5mL)
9	**SUBSTRATE B**	1 Bottle (7.5mL)
10	**STOP SOLUTION**	1 Bottle (15mL)
	PLATE SEALERS Six sealers per package	1 Package

MSDS forms are available on our website—please visit www.b-bridge.com

III. Additional Materials Required

The following materials are required, but not supplied:

- Graduated cylinder
- Micropipettor(s) and disposable pipette tips
- Null strips for 96-well plate
- 96-well plate or manual strip washer
- Paper towels or absorbent paper
- Plate reader capable of measuring absorbance at a wavelength of 450nm (reference filter at 650 nm, optional)
- Well-closed containers such as microtubes (1.5 mL or more in capacity)

IV. Reagent Preparation and Storage

1. 1X Wash Solution
   Prepare 1X Wash Solution by mixing all of the 25X Wash Solution (40mL) with 960 mL of deionized water or equivalent. If crystals are observed in the 25X Wash Solution bottle, warm the bottle in a 37°C water bath until the crystals disappear. After preparation, store 1X Wash Solution at 2-8°C.

2. 1X Sample Diluent
   Prepare 1X Sample Diluent by mixing all of the 5X Sample Diluent (50mL) with 200mL of deionized water or equivalent. After preparation, store 1X Sample Diluent at 2-8°C.

3. Adiponectin Standard Solution
   Prepare each Adiponectin Standard (4.0 ng/mL, 2.0 ng/mL, 1.0 ng/mL, 0.5 ng/mL, 0.25 ng/mL) by serially diluting the supplied adiponectin standard solution (8.0 ng/mL) with 1X Sample Diluent . Use undiluted adiponectin (8.0 ng/mL) and 1X Sample Diluent for 8.0 ng/mL and 0 ng/mL standard solutions, respectively.

4. HRP-Conjugated Streptavidin Solution
   Prepare the HRP-Conjugated Streptavidin by mixing 60 uL of HRP-Conjugated Streptavidin and 12mL of Streptavidin Diluent. Prepare only as much as needed immediately before the third reaction.

5. Substrate Solution
   Prepare the Substrate Solution by adding one part Substrate A to one part Substrate B. Prepare only as much Substrate Solution as needed. **Return Substrate A to 2-8°C immediately after the necessary volume is removed.**

Note: Do not mix reagents from different kits unless they have the same lot number.

V. Sample Preparation

Mouse Serum Samples

1. Allow all the reagents to come to room temperature (22-28°C) prior to the start of the sample preparation.

2. Mix 10 µL of serum samples with 1.0 mL of 1X Sample Diluent (1:101 diluted samples at final volume).

3. Transfer 10 µL of each diluted sample to a clean container and then add 1.0 mL of 1X Sample Diluent to the container (1:10,201 dilution at final volume). Repeat for each sample.

Rat Serum Samples

1. Allow all the reagents to come to room temperature (22-28°C) prior to the start of the sample preparation.

2. Mix 10 µL of serum samples with 1.0 mL of 1X Sample Diluent (1:101 diluted samples at final volume).

3. Transfer 100 µL of each diluted sample to a clean container and then add 1.0 mL of 1X Sample Diluent to the container (1:1,111 dilution at final volume). Repeat for each sample.

Adipocyte Cellular Extracts or Conditioned Media From Adipocytes

1. Allow all the reagents to come to room temperature (22-28°C) prior to the start of the sample preparation.

2. Mix 10 µL of adipocyte cellular extract or conditioned medium samples with 1.0 mL of 1X Sample Diluent (1:101 diluted samples at final volume).

3. Transfer 50 µL of each diluted sample to a clean container and then add 1.0 mL of 1X Sample Diluent to the container (1:2,121 dilution at final volume). Repeat for each sample.

VI. Mouse/Rat Adiponectin ELISA Protocol

1. Allow all reagents to come to room temperature (22-28°C) prior to the start of the assay.

Prepare 1X Wash Solution, 1X Sample Diluent, and Adiponectin Standards according to ***Reagent Preparation*** *(Steps 1, 2, and 3).*

2. Remove Primary Antibody-Coated Plate from its foil pouch. Remove any unneeded strips from the plate frame, reseal them in the foil pouch, and return the foil pouch to 2-8°C. If a 96-well plate washer is used, the plate frame should be completely filled with wells by adding as many null strips as necessary. Identify well position(s) for each sample on a data sheet or plate map.

3. Fill the wells with 1X Wash Solution (~350 µL/well) and immediately aspirate using a plate washer. Invert the plate(s) and gently tap on a clean absorbent towel.

4. Add 100 µL of adiponectin standard or diluted sample to the appropriate number of antibody-coated wells. Every plate must include the standard series to properly correlate the sample readings.

5. Cover plate(s) securely with a plate sealer and incubate at 22-28°C for 60 minutes.

6. Wash the plate(s) as follows:
   a. At the end of the incubation, carefully remove the plate sealer, avoiding splashing, and discard appropriately.
   b. Completely aspirate the liquid from the wells using a plate washer.
   c. Fill each well with 1X Wash Solution (~350 µL/well) and immediately aspirate. Avoid Wash Solution overflow.
   d. Repeat Step 6c two more times for a total of three washes.
   e. Invert the plate(s) and gently tap on a clean absorbent towel.

7. Dispense 100 µL of the Biotinylated Secondary Antibody Solution into each well.

8. Cover plate(s) securely with a (new, clean) plate sealer and incubate at 22-28°C for 60 minutes.

9. Repeat the wash procedure described in step 6.

Prepare HRP-Conjugated Streptavidin Solution according to ***Reagent Preparation*** *(Step 4)*.

10. Dispense 100 µL of HRP-Conjugated Streptavidin Solution into each well.

11. Cover plate(s) securely with a plate sealer and incubate at 22-28°C for 60 minutes.

12. Repeat the wash procedure described in step 6.

Prepare Substrate Solution according to ***Reagent Preparation*** *(Step 5).*

13. Dispense 100 µL of Substrate Solution into each well.

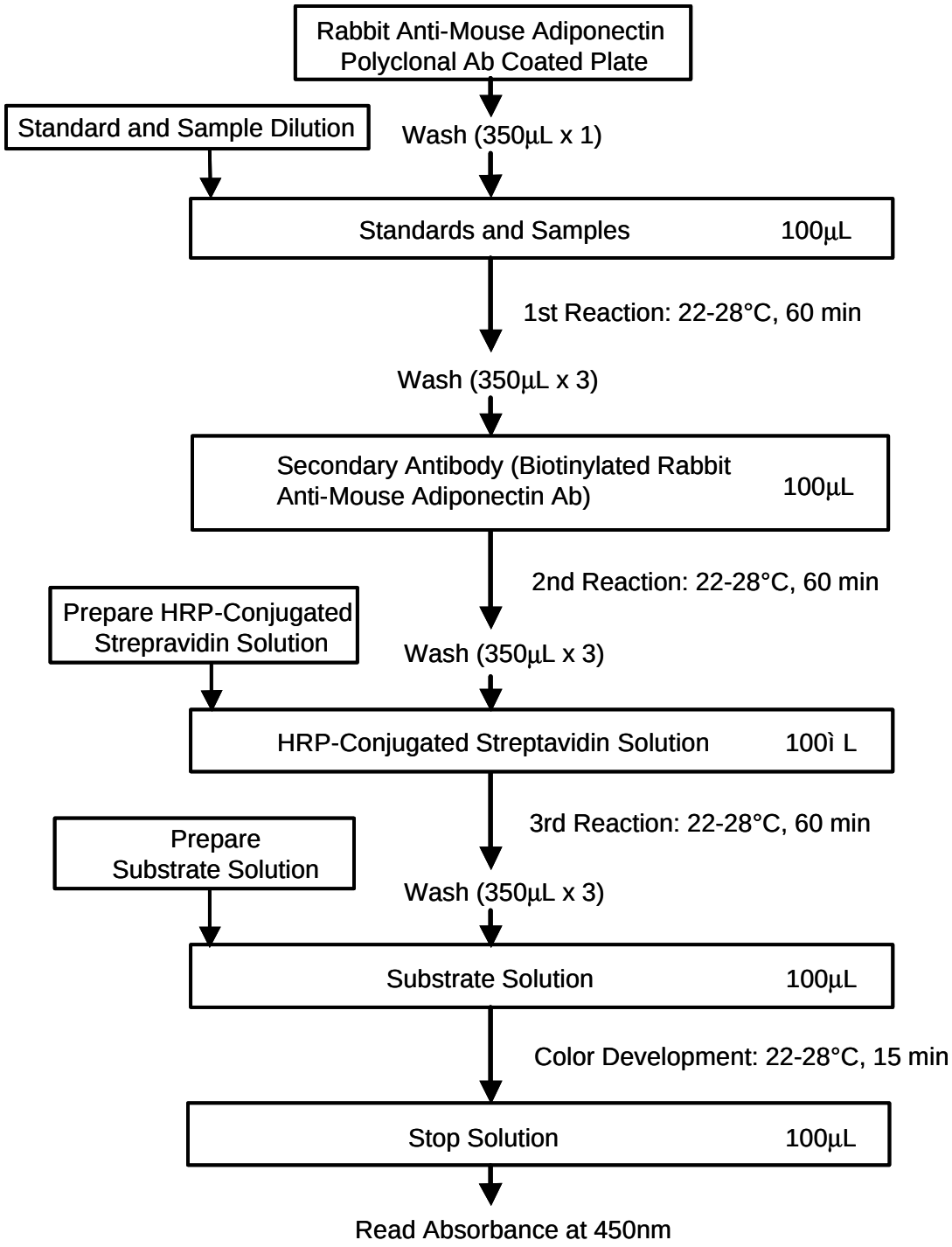
14. Incubate the plates at 22-28°C for 15 minutes.

15. Dispense 100 µL of Stop Solution into each well. The plate should be read immediately.

VI. Mouse/Rat Adiponectin ELISA Protocol *continued*

16. Read the plate at 450 nm using a plate reader. If using a dual filter instrument, the recommended reference wavelength is 650nm.

Figure 2. Flow Chart of Assay Procedure



VII. Calculation of Results

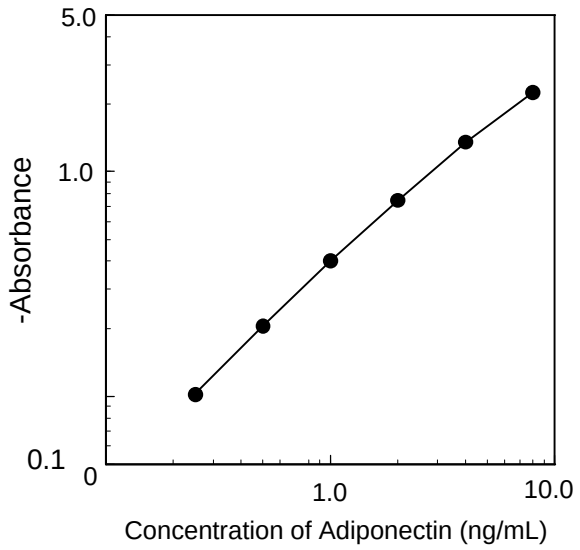
1. Subtract the mean absorbance value of the 0 ng/mL blank from each mean absorbance value of the standard series and samples tested (Net Absorbance).

2. Plot the concentrations of each standard and the calculated Net Absorbances on the X-axis and Y-axis, respectively. Fit an appropriate regression curve on the plots.

3. Determine the adiponectin concentrations of the samples by interpolation of the regression curve formula.

4. The adiponectin concentrations calculated must be multiplied by the appropriate dilution factor (x10,201 for mouse serum samples, x1,111 for rat serum samples, and x2,121 for adipocyte cellular extracts or conditioned medium) to obtain the correct result for undiluted samples.

Figure 3. Typical Standard Curve



VIII. Troubleshooting Guide and FAQs

Troubleshooting Guide

1. Lack of signal or weak signal in all wells
Possible explanations:

- Omission of a reagent or a step.
- Improper preparation or storage of a reagent.
- Assay performed before reagents were allowed to come to 22-28°C.
- Plate reader did not perform well.

2. High signal and background in all wells
Possible explanations:

- Improper or inadequate washing; be certain that all wash volumes and repetitions were correct.
- Improper dilution of detection antibody.
- Overdeveloping; decrease the incubation time before the Stop Solution is added.

3. High background in sample wells only
Possible explanations:

- Sample concentration was too high.
- Improper dilution of detection antibody.

4. Weak signal in sample wells only
Possible explanations:

- Sample concentration was too low.
- Improper dilution of detection antibody.

FAQs (Frequently Asked Questions)

1. What is the shelf life of this kit?

Currently, all components of this kit have a shelf life of 6 months when stored at 2-8°C. However, it is fully anticipated that this will be extended in the future. The expiration date appears on the top label of the product package.

2. Can I pool reagents?

Yes, as long as the reagents are from the same lot.

3. What kind of samples can be measured with this kit.

The kit can measure adiponectin in mouse and rat serum, adipocyte cell extract and conditioned medium. The kit contains mouse recombinant adiponectin as the standard, rat samples are measured as a mouse adiponectin equivalent.

VIII. Troubleshooting Guide and FAQs *continued*

4. What is the effect of freezing/thawing the standard and samples?

No significant effect was observed when adiponectin standards, untreated samples (mouse serum, rat serum, & conditioned medium*), diluted samples (mouse serum:x10,210, rat sample & conditioned medium:x1,111) were frozen and thawed five times (Figure 4).
*Conditioned medium was obtained from 3T3-L1 differentiated into adipocytes.

Figure 4. Effects of Freeze/Thaw

Standard	$OD_{450nm-650nm}$		
(ng/mL)	NF	F/T x3	F/T x5
8.000	2.626	2.511	2.451
4.000	1.677	1.577	1.565
2.000	0.927	0.916	0.856
1.000	0.490	0.491	0.443
0.500	0.279	0.257	0.255
0.250	0.156	0.158	0.151
0.000	0.035	0.036	0.036

	Adiponectin (ng/mL)		
Untreated	NF	F/T x3	F/T x5
Mouse Serum	0.514	0.566	0.568
Rat Serum	3.036	3.052	3.019
Condition'd medium	1.223	1.222	1.264

	Adiponectin (ng/mL)		
Diluted	NF	F/T x3	F/T x5
Mouse Serum (x10210)	0.588	0.530	0.565
Rat Serum (x1111)	3.051	2.973	3.124
Condition'd medium (x1111)	1.150	1.192	1.177

NF = Not Frozen
F/T = Freeze/Thaw

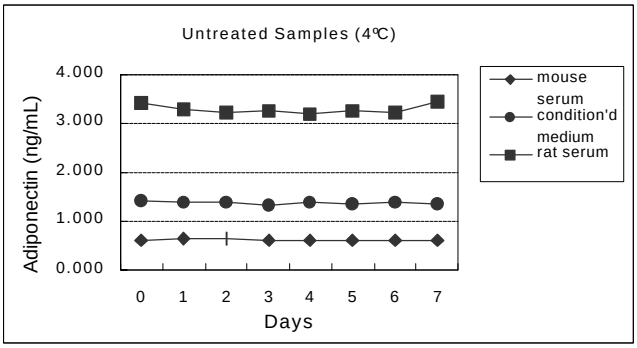
5. At what temperature should samples be stored (both untreated and diluted)?

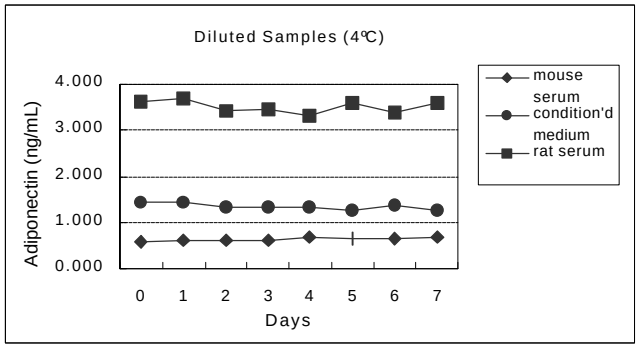
Samples should be stored at -70°C.

6. How stable are the samples at 4°C and at room temperature (25°C)?

Untreated and diluted samples (serum and conditioned medium) can be stored at 4°C for up to 7 days. However we recommend that you store the samples in a freezer (Figure 5).

Figure 5. Measurement of Adiponectin After Storage at 4°C

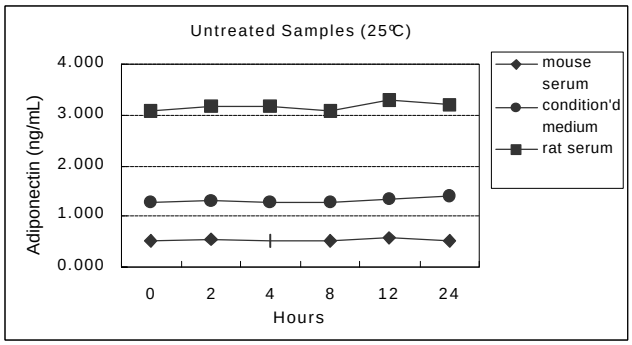


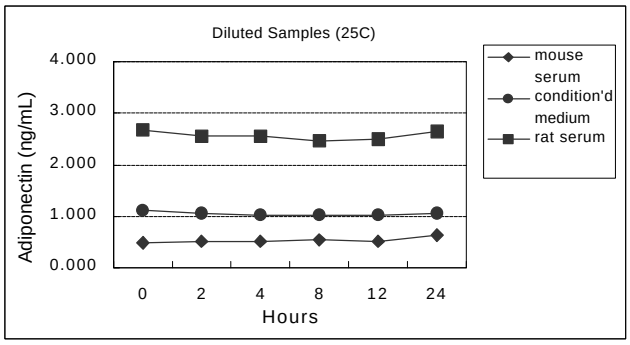


VIII. Troubleshooting Guide and FAQs *continued*

Untreated and diluted samples (serum and conditioned medium) are stable for 24 hours at RT. Ideally, all samples should be stored frozen. (Figure 6)

Figure 6. Measurement of Adiponectin After Storage at 25°C





7. How should I dilute high-value samples?

High-value samples should be diluted with Sample Diluent. Calculation of Adiponectin concentration should be adjusted for the dilution.

8. What temperature range can I use in the incubation steps?

Measurements are consistent between 22-28°C

Figure 7. Effects of incubation times

		Incubation Temperature		
		22	25	28
Absorbance ($OD_{450-650nm}$)	8 ng/mL	1.657 1.746	1.969 2.127	2.522 2.388
	4 ng/mL	0.992 1.094	1.248 1.264	1.504 1.386
	2 ng/mL	0.572 0.595	0.704 0.696	0.862 0.853
	1 ng/mL	0.313 0.327	0.395 0.371	0.486 0.456
	0.5 ng/mL	0.178 0.182	0.214 0.242	0.266 0.249
	0.25 ng/mL	0.104 0.109	0.131 0.140	0.159 0.155
	0 ng/mL	0.028 0.033	0.034 0.037	0.034 0.041
Adiponectin Level (ng/mL)	Mouse High	5.302	4.877	5.264
	Mouse Low	0.728	0.647	0.597
	Rat High	4.744	4.540	4.696
	Rat Low	0.556	0.507	0.470

VIII. Troubleshooting Guide and FAQs *continued*

9. How reproducible are the results?

Several experiments were performed to determine the reproducibility of data obtained using this kit. In one experiment, four kinds of samples were assayed (i.e., 32 samples total on one plate, measured on a plate reader simultaneously), data shown in Figure 8 (first table). In the second table are the results of measuring the 4 kinds of samples from the same ELISA 6 times consecutively (i.e., one sample of each measured on a plate reader 6 times in a row). CV was less than 10%. The third table shows the results of assays run by four different people. CV was less than 15%.

Figure 8. Reproducibility

Intra-measurement Reproducibility

	test-1	test-2	test-3	test-4	test-5	test-6	test-7	test-8	mean	CV %
Mouse High	5.124	5.187	5.325	5.362	5.050	5.367	5.096	5.105	5.202	2.5
Mouse Low	0.769	0.659	0.731	0.709	0.766	0.661	0.703	0.712	0.714	5.8
Rat High	4.523	4.987	4.510	4.387	4.497	4.880	4.378	4.609	4.596	4.8
Rat Low	0.530	0.534	0.524	0.601	0.511	0.604	0.521	0.559	0.548	6.6

(ng/mL)

Inter-measurement Reproducibility

	test-1	test-2	test-3	test-4	test-5	test-6	mean	CV %
Mouse High	5.214	5.772	4.963	4.888	5.436	4.791	5.177	7.2
Mouse Low	0.714	0.718	0.651	0.648	0.661	0.668	0.677	4.6
Rat High	4.596	5.305	4.301	3.944	5.635	4.433	4.702	13.6
Rat Low	0.548	0.540	0.499	0.462	0.561	0.508	0.520	7.1

(ng/mL)

Inter-tester Reproducibility

	tester-1	tester-2	tester-3	tester-4	mean	CV %
Mouse High	5.214	5.144	5.610	5.072	5.260	4.6
Mouse Low	0.714	0.642	0.782	0.673	0.703	8.6
Rat High	4.596	4.181	4.765	4.448	4.498	5.5
Rat Low	0.548	0.465	0.507	0.473	0.498	7.6

(ng/mL)

10. What is the range of adiponectin that can be detected by this kit?

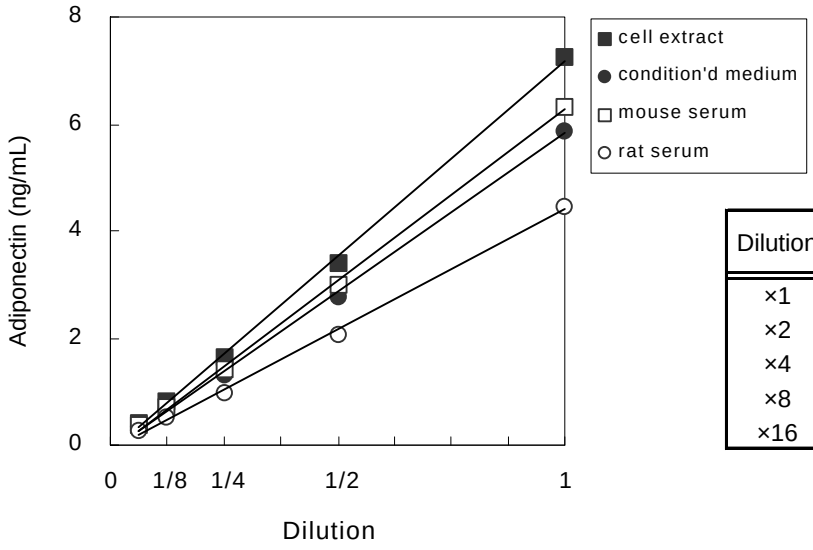
We have established a minimum detectable limit as 15.6 pg/mL of adiponectin (unpublished data). The ELISA is linear within the range of 0.25 ng/mL to 8.0 ng/mL.

11. What will the effect be if I dilute my samples beyond what is recommended?

The four samples were prepared as described in the protocol. The samples were then further diluted x2, x4, x8, and x16. The data are linear (Figure 9).

VIII. Troubleshooting Guide and FAQs *continued*

Figure 9. Effects of Dilution



	Adiponectin (ng/mL)			
Dilution	Cell Extract	Cond. Medium	Mouse	Rat
×1	7.257	5.866	6.332	4.454
×2	3.384	2.783	2.975	2.058
×4	1.627	1.314	1.415	0.961
×8	0.838	0.641	0.701	0.513
×16	0.415	0.322	0.357	0.256

12. Will this kit detect adiponectin from other species?

Serum samples (x1,111 diluted) from various species were tested with the kit (Figure 10). The results indicate detection levels are outside the kit's range (0.25 ng/mL - 8.0 ng/mL).

Figure 10. Cross-Reactivity

	$OD_{450-650nm}$	Mean	Net Absorbance
Sheep	0.091	0.087	0.034
	0.083		
Porcine	0.055	0.056	0.002
	0.056		
Calf	0.060	0.060	0.002
	0.059		
FBS	0.052	0.054	0.001
	0.055		
Chicken	0.059	0.058	0.000
	0.056		
(Blank)	0.058	0.055	-
	0.053		

IX. References

1. Nemet, D., et al. (2002) Relationships among adiponectin and other adipose cytokines, body composition, and fasting insulin in lower socioeconomic middle school children. American Physiological Society's (APS) Abstracts.

2. Yamauchi T., et al. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nature Medicine, Aug;7(8):941-6

3. Kubota, N., et al. (2002) Disruption of adiponectin causes insulin resistance and neointimal formation. J Biol Chem. May 24 (epub ahead of print)

4. Berg A.H., et al. (2001) The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nature Medicine, Aug;7(8):947-53