B-Bridge International, Inc.

Human Adiponectin ELISA Kit
User Manual

UM-100101
Published 01 June 2002
Catalog# K1001-1

See List of Components for Storage Conditions
FOR RESEARCH USE ONLY

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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.

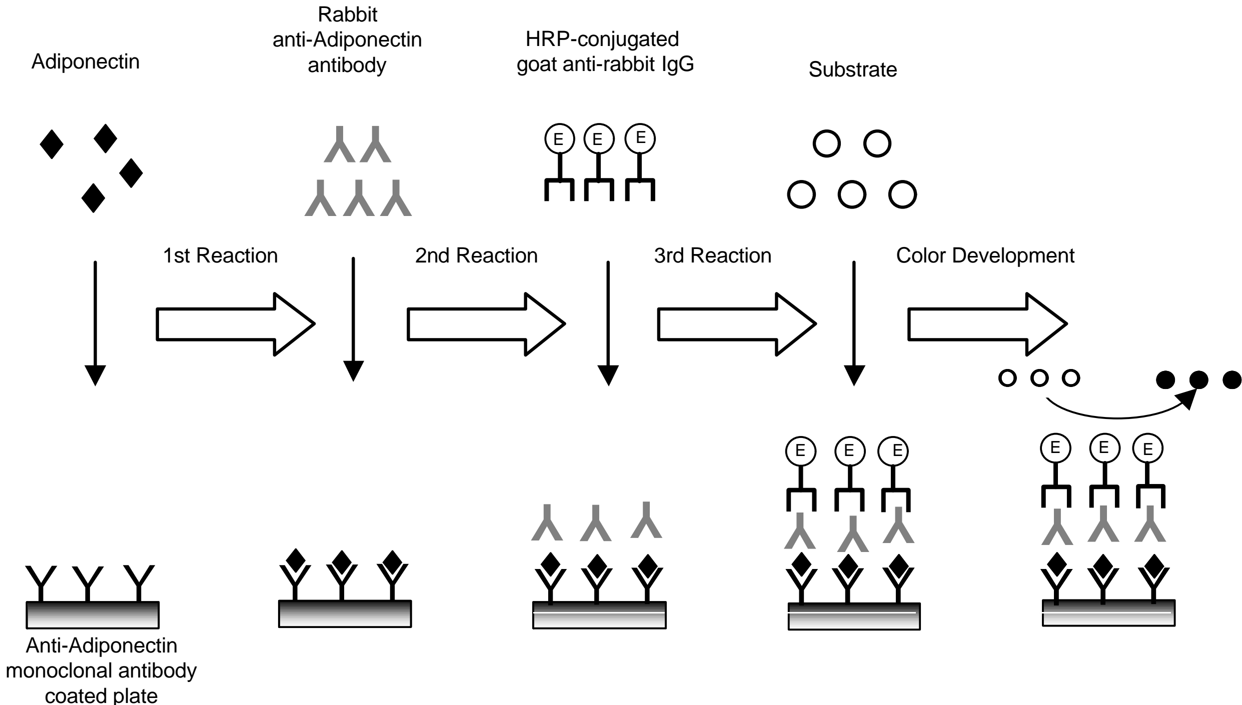
Adipocytes (fat cells) express a variety of proteins that function in the homeostatic control of glucose and lipid metabolism. Insulin regulates the translocation and secretion of many of these proteins in response to changes in energy balance. Adipocyte complement-related protein of 30 kDa (Acrp30), 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 **Human Adiponectin ELISA Kit** is designed to measure the concentration of human adiponectin from human serum/plasma, human adipocytes, or conditioned medium.

The principle of the assay is shown in Figure 1. Pre-treated samples and serially diluted standard (recombinant human 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-adiponectin monoclonal antibody immobilized in the well (1st Reaction). After washing, the secondary rabbit anti-adiponectin antibody is added to each well and allowed to incubate (2nd Reaction). The secondary rabbit anti-adiponectin polyclonal antibody will bind to the adiponectin trapped in the well in the 1st Reaction. After washing, a conjugate of horseradish peroxidase and goat anti-rabbit IgG is added to each well and allowed to incubate (3rd Reaction). The detection antibody will recognize and bind to the 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**.

No.	Component	Quantity
1	**25X WASH SOLUTION**	1 Bottle (40mL)
2	**SAMPLE PRE-TREATMENT SOLUTION**	1 Bottle (10mL)
3	**5X SAMPLE DILUENT**	1 Bottle (50mL)
4	**PRIMARY ANTIBODY-COATED PLATE** One plate holds 12x8-well strips (96 wells), with adsorbed mouse anti-human adiponectin monoclonal antibody. Plate is provided in a resealable foil pouch with desiccant.	1 Plate
5	**ADIPONECTIN STANDARD** Recombinant human adiponectin (12.0 ng/mL)	1 Vial (2mL)
6	**SECONDARY ANTIBODY SOLUTION** Rabbit anti-human adiponectin polyclonal antibody	1 Bottle (12mL)
7	**DETECTION ANTIBODY CONCENTRATE** Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG	1 Vial (0.1mL)
8	**DETECTION ANTIBODY DILUENT**	1 Bottle (15mL)
9	**SUBSTRATE A**	1 Bottle (7.5mL)
10	**SUBSTRATE B**	1 Bottle (7.5mL)
11	**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)
- Heat block or equivalent
- 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 (6.0 ng/mL, 3.0 ng/mL, 1.5 ng/mL, 0.75 ng/mL, 0.375 ng/mL) by serially diluting the supplied adiponectin standard solution (12.0 ng/mL) with 1X Sample Diluent . Use undiluted adiponectin (12.0 ng/mL) and 1X Sample Diluent for 12.0 ng/mL and 0 ng/mL standard solutions, respectively.

4. Detection Antibody Solution
   Prepare the Detection Antibody Solution by adding one part Detection Antibody Concentrate to 200 parts Detection Antibody Diluent. Prepare only as much Detection Antibody Solution as needed.

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 Pre-Treatment

Human Serum or Plasma Samples

1. Allow all the reagents needed for sample pre-treatment, including serum or plasma (EDTA- or heparin-containing), to come to room temperature (20-30°C) prior to the start of the sample pre-treatment.

2. Mix 10 μL of serum or plasma samples with 90 μL of Sample Pre-treatment Solution and heat the mixture for 5 minutes at 100°C using a heat block. To heat the samples, use well-closed containers (1.5 mL or more in capacity) such as microtubes.

3. Add 900 μL of 1X Sample Diluent to each container after heating (1:100 diluted samples at final volume).

4. Transfer 20 μL of each diluted sample to a clean container and then add 1.0 mL of 1X Sample Diluent to the container (1:5100 dilution at final volume). Repeat for each sample.

Human Adipocyte Cellular Extracts or Conditioned Media From Human Adipocytes

1. Allow all the reagents needed for sample pre-treatment, including cellular extracts or conditioned media, to come to room temperature (20-30°C) prior to the start of the sample pre-treatment.

2. Prepare Diluted Sample Pre-treatment Solution by adding one part Sample Pre-treatment Solution to 4 parts deionized water or equivalent. Mix 10 μL of the adipocyte cellular extract or conditioned media with 90 μL of Diluted Sample Pre-treatment Solution, and heat the mixture for 5 minutes at 100°C using a heat block. To heat the samples, use well-closed containers (1.5 mL or more in capacity) such as microtubes.

3. Add 900 μL of 1X Sample Diluent to each container after heating (1:100 diluted samples at final volume).

Note: Make sure to eliminate precipitation in the Pre-Treatment Solution by vortexing or warming it up to 37°C before use.

VI. Human Adiponectin ELISA Protocol

1. Allow all reagents to come to room temperature (20-30°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) and immediately aspirate using a plate washer. If wells are washed manually, invert the plate(s) and gently tap on a clean absorbent towel.

4. Add 100 μL of adiponectin standard or pre-treated 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 20-30°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 Secondary Antibody Solution into each well.

8. Cover plate(s) securely with a (new, clean) plate sealer and incubate at 20-30°C for 60 minutes.

9. Repeat the wash procedure described in step 6.

Prepare Detection Antibody Solution according to ***Reagent Preparation*** *(Step 4).*

10. Dispense 100 μL of Detection Antibody Solution into each well.

11. Cover plate(s) securely with a plate sealer and incubate at 20-30°C for 60 minutes.

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

12. Repeat the wash procedure described in step 6.

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

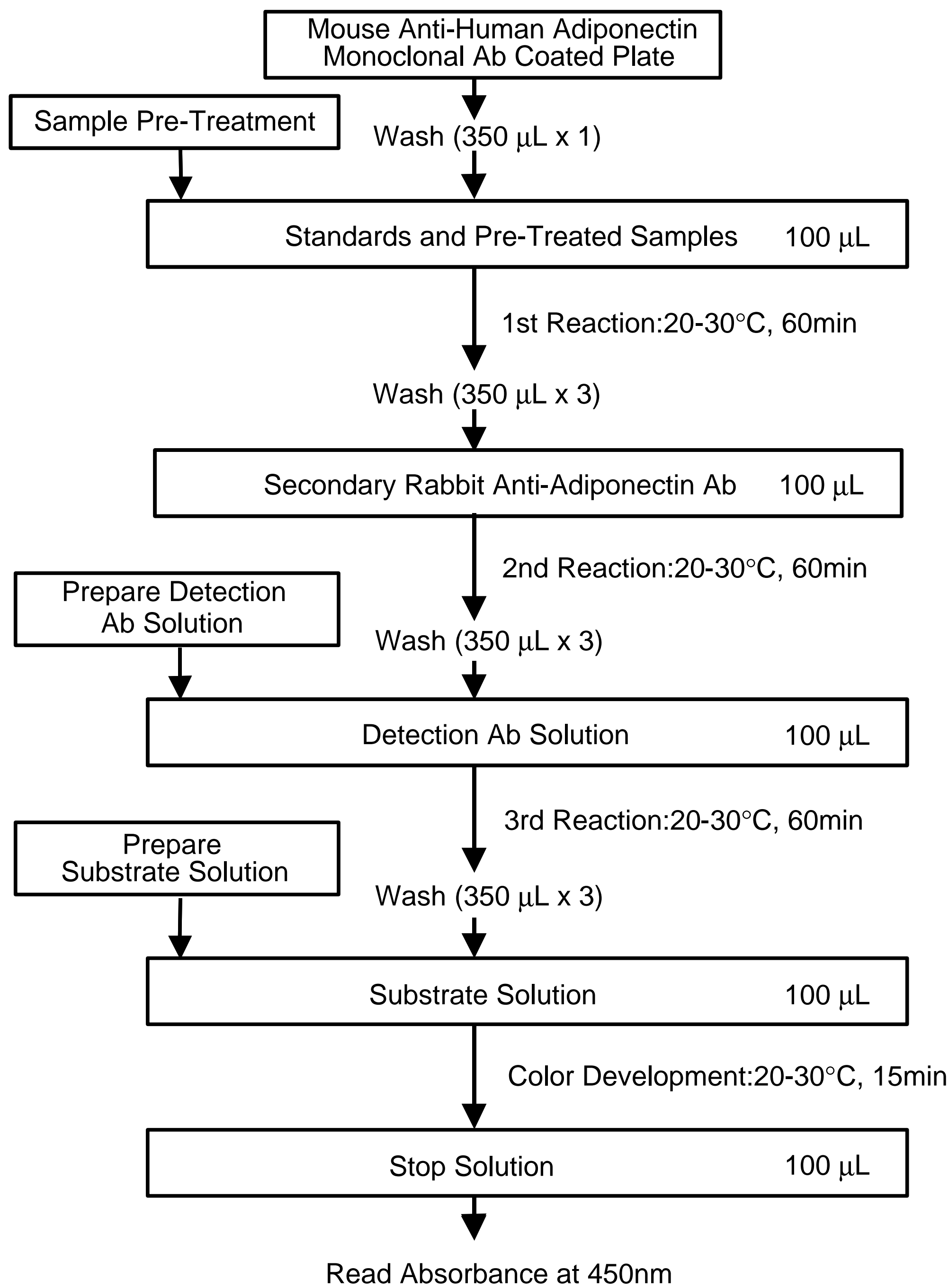
14. Incubate the plate at 20-30°C for 15 minutes.

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

VI. Human 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

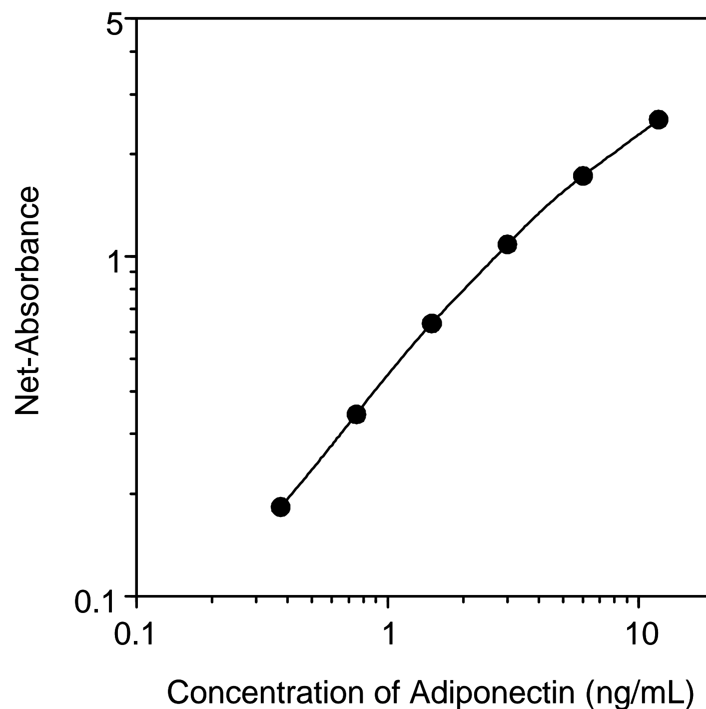
1. Subtract the mean absorbance value of the 0 ng/mL blank from each mean absorbance value of the standard series and samples tested (Net Absorbance).

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 (x5100 for serum or plasma samples and x100 for adipocyte cellular extracts or conditioned medium) to obtain the correct result for undiluted samples.

Figure 3. Typical Standard Curve



VII. 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 20-30°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 1 year 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 is the effect of freezing/thawing the standard and samples?

No significant effect was observed when adiponectin standards, untreated samples, pre-treated samples and diluted samples were frozen and thawed five times (Figure 4).

VIII. Troubleshooting Guide and FAQs *continued*

Figure 4. Effects of Freeze/Thaw

Std (ng/mL)	$OD_{450nm-650nm}$		
	NF	F/T x3	F/T x5
12.000	2.235	2.220	2.181
6.000	1.495	1.429	1.385
3.000	0.902	0.848	0.839
1.500	0.487	0.472	0.460
0.750	0.266	0.262	0.253
0.375	0.153	0.154	0.148
0.000	0.025	0.025	0.025

Untreated	Adiponectin (ng/mL)		
	NF	F/T x3	F/T x5
No.31	1.308	1.171	1.234
No.34	0.606	0.619	0.566
No.35	3.453	3.561	3.439

Diluted x5100	Adiponectin (ng/mL)		
	NF	F/T x3	F/T x5
No.31	1.308	1.326	1.346
No.34	0.606	0.691	0.708
No.35	3.453	3.703	3.664

Diluted x100	Adiponectin (ng/mL)		
	NF	F/T x3	F/T x5
No.31	0.882	0.889	0.866
No.34	0.508	0.505	0.519

NF = Not Frozen
F/T = Freeze/Thaw

4. Does the method of separation of serum/plasma affect the measurement of adiponectin?

There is no significant difference in measurements of adiponectin from separated serum samples. However, neither whole blood nor whole blood treated with citrate can be used.

Samples from three healthy individuals were taken and serum/plasma were treated by several different methods, results shown below (Figure 5).

Figure 5. Effects of Separation Method

Serum		Plasma		
Coagulated	Coagulated + Sep'n Gel	Heparin	Citrate	EDTA $(Na)_2$
3.234	3.422	3.136	2.766	3.304
1.941	2.157	2.036	1.539	1.744
1.275	1.289	1.206	0.990	1.127

(Adiponectin:ng/mL)

VIII. Troubleshooting Guide and FAQs *continued*

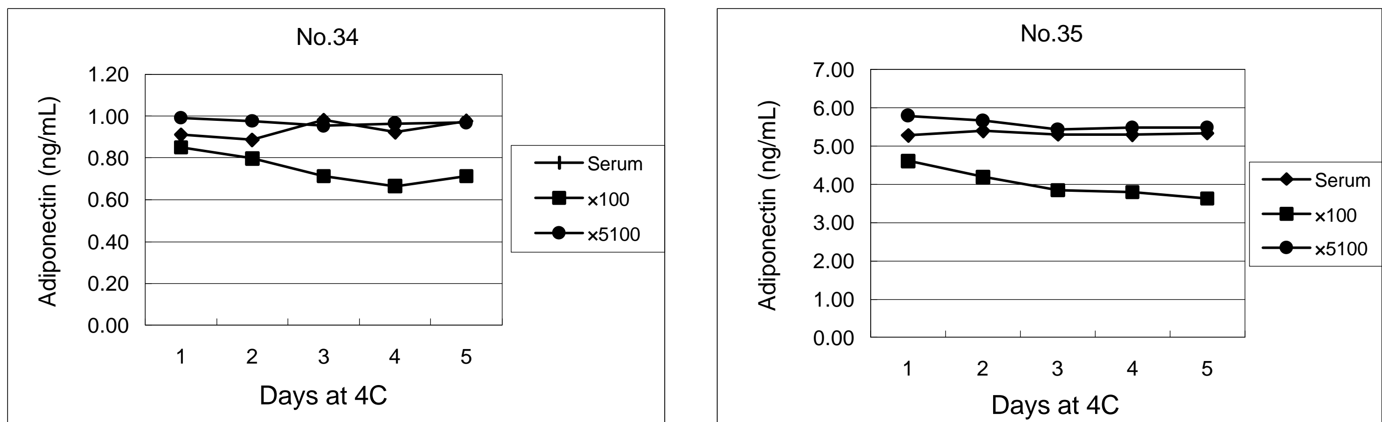
5. At what temperature should samples be stored (both untreated and pre-treated)?

Samples should be stored at -70°C.

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

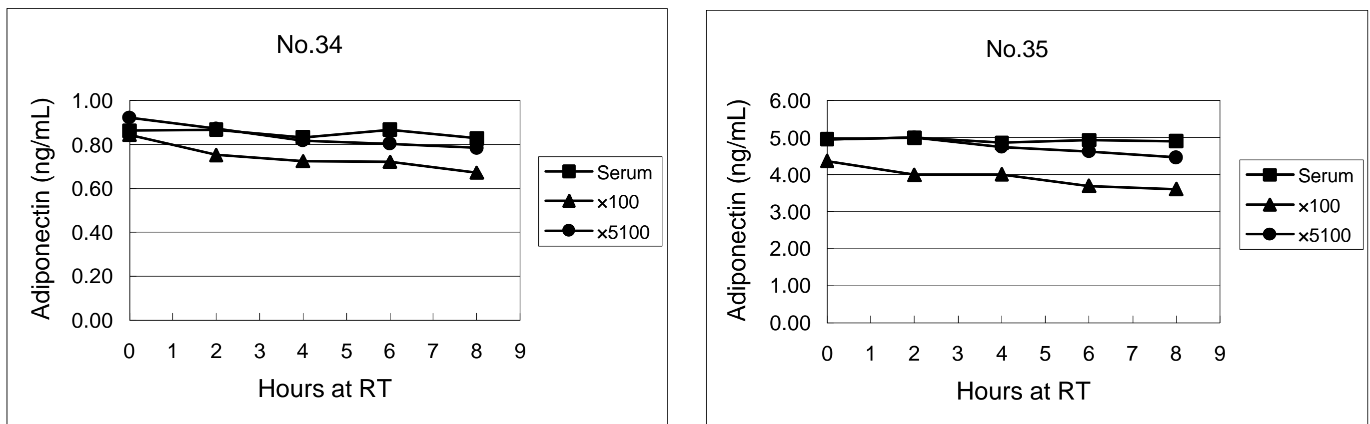
Untreated serum and pre-treated x5100 diluted serum can be stored at 4°C for up to 7 days. Pre-treated x100 diluted serum, however, cannot be stored refrigerated without a significant decrease in detectable adiponectin (Figure 6).

Figure 6. Measurement of Adiponectin After Storage at 4°C



Untreated serum and pre-treated serum samples are stable for 8 hours at RT. Pre-treated x100 diluted serum samples cannot be stored at RT without a decrease in detectable Adiponectin (Figure 7). Therefore, samples should be diluted x5100 following pre-treatment and heating. Ideally, all samples should be stored at -70°C.

Figure 7. Measurement of Adiponectin After Storage at RT



VIII. Troubleshooting Guide and FAQs *continued*

7. What if I only heat the samples for 5 minutes in the pre-treatment incubation step?

As shown in Figure 8 below, samples were heated at 100°C in a heat block for 0 to 20 minutes, then the ELISA was performed and the samples were tested. For samples heated between 1 minute and 20 minutes, there was no significant difference in the adiponectin concentrations recorded.

Figure 8. Effects of Pre-Treatment Incubation Times

	Pre-Treatment Incubation Time (minutes)						
Sample	0	1	3	5	7	10	20
No.34	0.083	0.985	0.918	0.901	0.931	0.899	0.976
No.35	0.270	5.192	5.443	5.104	5.202	4.405	4.744

Adiponectin: ng/mL)

8. What temperature range can I use in the pre-treatment incubation step?

When samples were heated for 5 minutes at 5°C intervals from 80°C to 100°C, there was no significant difference in the adiponectin concentrations recorded (Figure 9).

Figure 9. Effects of Pre-Treatment Incubation Temperature

	Pre-Treatment Incubation Temp (°C)				
Sample	80	85	90	95	100
No.34	1.057	1.047	1.044	1.122	1.040
No.35	5.830	5.655	5.870	6.145	5.814

(Adiponectin: ng/mL)

9. How reproducible are the results?

Several experiments were performed to determine the reproducibility of data obtained using this kit. In one experiment, 8 control high and control low samples were assayed (i.e., 16 samples total on one plate, measured on a plate reader simultaneously), data shown in Figure 10 (first table). In the second table are the results of measuring single control high and low samples from the same ELISA 6 times consecutively (i.e., one sample of each measured on a plate reader 6 times in a row). The third table shows the results of eight assays (control high and low) run by four different people.

VIII. Troubleshooting Guide and FAQs *continued*

Figure 10. Reproducibility

8 Samples From Same Plate Measured Simultaneously

	test-1	test-2	test-3	test-4	test-5	test-6	test-7	test-8	mean	SD	CV (%)
Control High	2.648	2.722	2.746	2.715	2.510	2.831	2.759	2.783	2.714	0.098	3.62
Control Low	0.717	0.714	0.706	0.752	0.706	0.739	0.684	0.687	0.713	0.023	3.28

2 Samples Measured 6 Times Consecutively

	test-1	test-2	test-3	test-4	test-5	test-6	mean	SD	CV (%)
Control High	2.714	2.803	2.934	3.203	3.001	2.921	2.929	0.169	5.76
Control Low	0.713	0.699	0.776	0.737	0.786	0.731	0.740	0.034	4.64

8 Assays by 4 Different People

	test-1	test-2	test-3	test-4	mean	SD	CV (%)
Control High	2.829	3.016	2.872	3.001	2.930	0.093	3.18
Control Low	0.780	0.698	0.835	0.786	0.775	0.057	7.33

(Adiponectin: ng/mL)

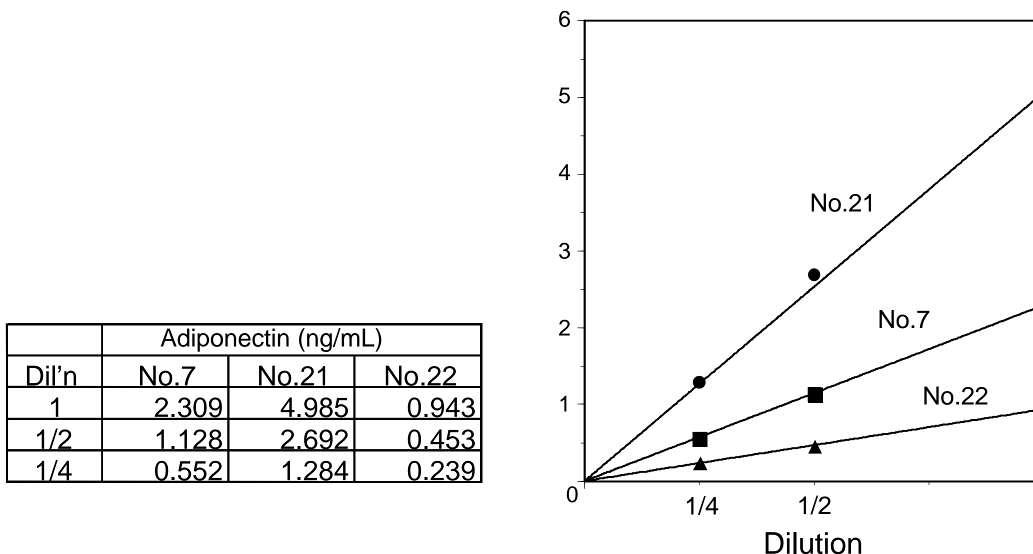
10. What is the range of adiponectin that can be detected by this kit?

We have established a minimum detectable limit as 23.4 pg/mL of adiponectin (unpublished data). The ELISA is linear within the range of 0.375 ng/mL to 12.0 ng/mL.

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

Three serum samples were pre-treated as described in the protocol, resulting in a final dilution of x5100 (labeled in the table below as dilution "1"). The samples were then further diluted x2 and x4. The data are linear (Figure 11).

Figure 11. Effects of Dilution



	Adiponectin (ng/mL)		
Dil'n	No.7	No.21	No.22
1	2.309	4.985	0.943
1/2	1.128	2.692	0.453
1/4	0.552	1.284	0.239

VIII. Troubleshooting Guide and FAQs *continued*

12. Will the mouse anti-human adiponectin monoclonal Ab detect adiponectin from other species?

Two experiments were conducted to examine the cross-reactivity of the anti-human monoclonal Ab. In the first, recombinant mouse adiponectin samples from a concentration of 0.313 to 320 ng/mL were assayed (see left table in Figure 12 below). In the second experiment, sera from different animals were pre-treated according to protocol and assayed (right table below). The results from the adiponectin standards run simultaneously are shown in the bottom table of Figure 12. There was no cross-reactivity observed.

Figure 12. Cross-Reactivity

Recombinant Mouse Adiponectin			
[Antigen] ng/mL	$OD_{450\text{-}650nm}$	Mean	Absorbance
320	0.023	0.023	0.000
	0.022		
160	0.022	0.022	0.000
	0.021		
80	0.021	0.022	0.000
	0.022		
40	0.023	0.022	0.000
	0.021		
20	0.022	0.022	0.000
	0.021		
10	0.023	0.022	0.000
	0.020		
5	0.023	0.022	0.000
	0.021		
2.5	0.023	0.022	0.000
	0.021		
1.25	0.022	0.023	0.000
	0.023		
0.625	0.020	0.021	0.000
	0.022		
0.313	0.021	0.021	0.000
	0.021		
0	0.024	0.023	–
	0.021		

Pre-Treated Sera From Different Species			
Species	$OD_{450\text{-}650nm}$	Mean	Absorbance
Mouse	0.025	0.027	0.000
	0.028		
Rat	0.028	0.033	0.003
	0.037		
Goat	0.032	0.035	0.005
	0.037		
Sheep	0.037	0.039	0.009
	0.04		
Porcine	0.021	0.024	0.000
	0.027		
Calf	0.028	0.032	0.002
	0.036		
FBS	0.025	0.029	0.000
	0.033		
(Blank)	0.026	0.030	–
	0.033		

Recombinant Human Adiponectin			
[Antigen] ng/mL	$OD_{450\text{-}650nm}$	Mean	Absorbance
12.000	2.562	2.547	2.524
	2.532		
6.000	1.763	1.746	1.723
	1.729		
3.000	1.070	1.105	1.082
	1.140		
1.500	0.647	0.657	0.634
	0.667		
0.750	0.370	0.365	0.342
	0.360		
0.375	0.212	0.206	0.183
	0.199		
0	0.024	0.023	–
	0.021		

IX. References

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