

RayBio[®]
Human/Mouse/Rat Adiponectin
Enzyme Immunoassay Kit

User Manual 1.1

RayBio[®] Adiponectin
Enzyme Immunoassay Kit Protocol

(Cat#: EIA-ACRP-1)



RayBiotech, Inc.

**We Provide You With Excellent
Protein Array System and Service**

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Immunoassay Kit Protocol**

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I. INTRODUCTION

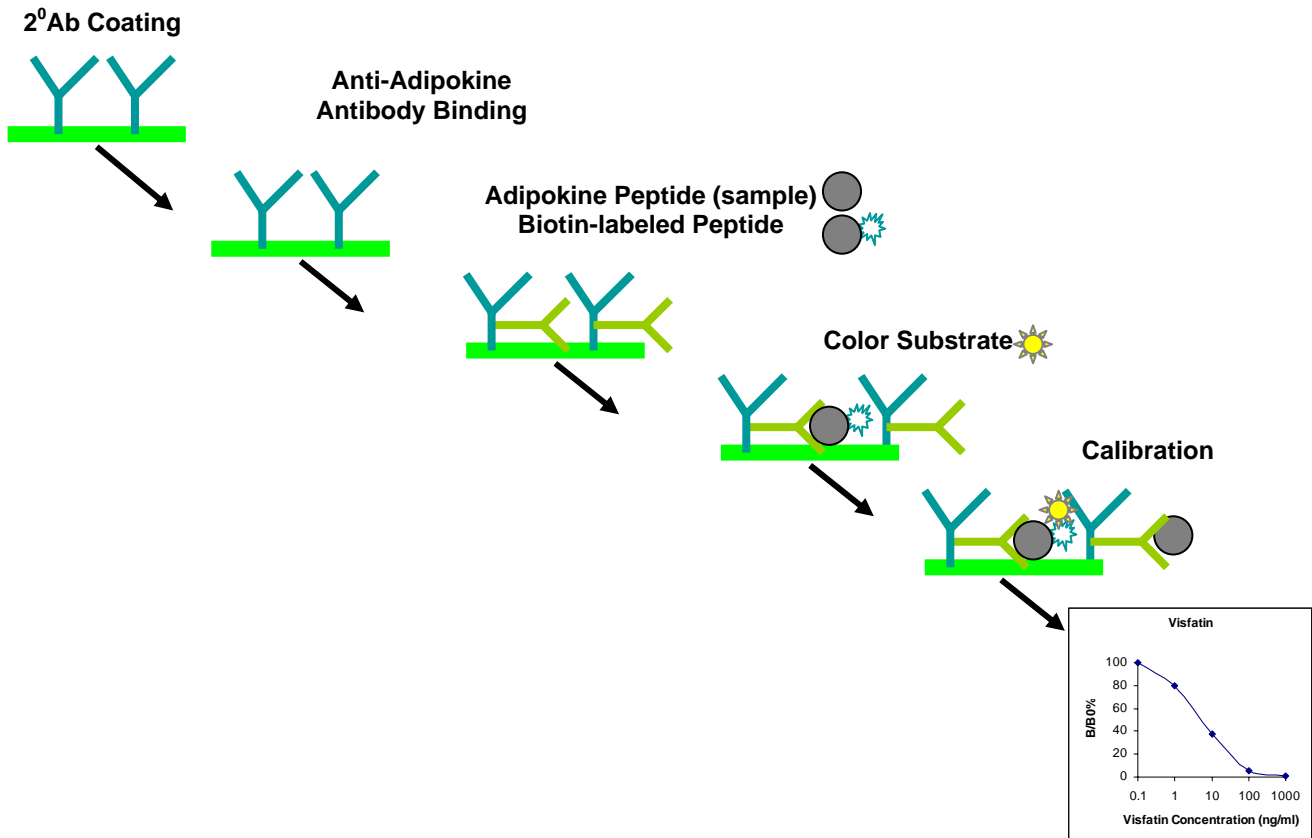
Adiponectin (Acrp30) is a 244- amino acid peptide that is expressed and secreted exclusively by adipose tissue. It is abundant in plasma, accounting for approximately 0.01% of all plasma protein at around 5-10 $\mu\text{g/mL}$. Adiponectin is a peptide hormone that plays a role in a number of metabolic processes, including the glucose regulation and lipid homeostasis. As a potent insulin enhancer, Adiponectin links adipose tissue and whole-body glucose metabolism. Levels of Adiponectin are decreased in obesity and it has been observed that Adiponectin levels are also reduced in patients with coronary artery disease. Adiponectin, like leptin, may exert its weight reduction effects via the brain. These two peptide hormones perform complementary actions with additive effects.

II. GENERAL DESCRIPTION

The RayBio® Adiponectin Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Adiponectin peptide based on the principle of Competitive Enzyme Immunoassay.

The microplate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-Adiponectin antibody, both biotinylated Adiponectin peptide and peptide standard or targeted peptide in samples interacts competitively with the Adiponectin antibody. Uncompeted (bound) biotinylated Adiponectin peptide then interacts with Streptavidin-horseradish peroxidase (SA-HRP) which catalyzes a color development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide-SA-HRP complex and inversely proportional to the amount of Adiponectin peptide in the standard or samples. This is due to the competitive binding to Adiponectin antibody between biotinylated Adiponectin peptide and peptides in standard or samples. A standard curve of known concentration of Adiponectin peptide can be established and the concentration of Adiponectin peptide in the samples can be calculated accordingly.

HOW DOES EIA WORK?



III. REAGENTS

1. Adiponectin Microplate (Item A): 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml
3. Standard Adiponectin Peptide (Item C): 2 vials
4. Anti-Adiponectin polyclonal antibody (Item N): 2 vials
5. Assay Diluent A (Item D): 30 ml, contains 0.09% sodium azide as preservative. For Standard/Sample (serum/plasma) diluent.
6. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (cell culture medium/urine) diluent.
7. Biotinylated Adiponectin peptide, (Item F): 2 vials

8. HRP-Streptavidin concentrate (Item G): 8 μ l 4,000x concentrated HRP-conjugated Streptavidin.
9. Positive control (Item M): 1 vial
10. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'-tetramethylbenzidine (TMB) in buffered solution.
11. Stop Solution (Item I): 8 ml of 2 M sulfuric acid.
12. Assay Diagram (Item J).
13. User Manual (Item K)

IV. STORAGE

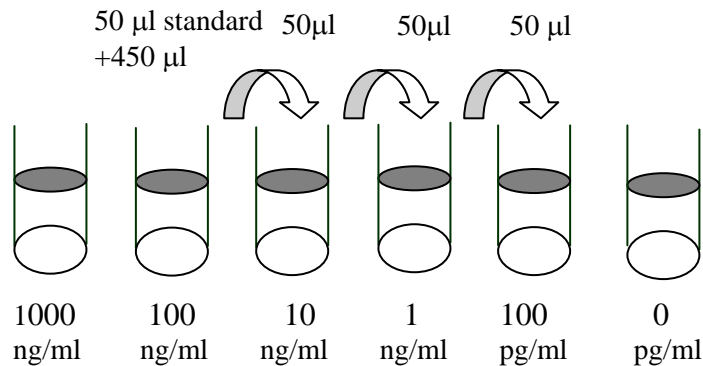
The kit may be stored for up to 6 months at -20°C from the date of shipment. Standard, Biotinylated Adiponectin peptide, and positive control should be stored at -20°C or -80°C (recommended at -80°C) after arrival. Opened Microplate Wells and antibody may be stored for up to 1 month at 2° to 8°C . Return unused wells to the pouch containing desiccant pack, reseal along entire edge. Avoid multiple freeze-thaws for Standard, Biotinylated Adiponectin peptide and positive control.

V. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Precision pipettes to deliver 2 μ l to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1 liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions.
9. Orbital shaker
10. Aluminum foil
11. Saran Wrap

VI. REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Briefly centrifuge the Anti-Adiponectin Antibody vial (Item N) before use. Add 50 µl of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently. The Anti-Adiponectin antibody concentrate should be diluted 100-fold with 1x Assay Diluent B. This is your anti-Adiponectin antibody working solution, which will be used in step 2 of Part VII Assay Procedure.
3. Briefly centrifuge the vial of Biotinylated Adiponectin (Item F) before use. Add 10 µl of biotinylated Adiponectin (Item F) to 5 ml of Assay Diluent A (if using serum/plasma samples) or 1X Assay Diluent B (if using cell culture medium/urine samples). Pipette up and down to mix gently. The final concentration of biotinylated Adiponectin will be 20 ng/ml. This solution will be used in step 4 of Part VI Reagent Preparation, which will be used as the standard diluent. For sample and positive control dilutions, refer to steps 5 and step 6 of Part VI Reagent Preparation.
4. Preparation of standard: Briefly centrifuge standard Adiponectin vial (Item C). In a separate tube, pipette 10 µl of standard Adiponectin Peptide (Item C) into 990 µl of biotinylated Adiponectin solution (prepared in step 3 above) to prepare a 1000 ng/ml standard. Pipette up and down to mix gently. Pipette 50 µl of 1000 ng/ml Adiponectin standard into a tube with 450 µl of biotinylated Adiponectin solution. This will be your stock standard solution (100 ng/ml Adiponectin, 20 ng/ml biotinylated Adiponectin). Pipet 450 µl of biotinylated Adiponectin solution into 6 tubes. Use the stock standard to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Biotinylated Adiponectin serves as the zero standard (0 pg/ml), or total binding.



5. Sample dilution: If your samples need to be diluted, use Assay Diluent A + biotinylated Adiponectin for serum/plasma samples. For cell culture medium and urine samples, use 1X Assay Diluent B + biotinylated Adiponectin as the diluent. It is very important to make sure the final concentration of the biotinylated Adiponectin is 20 ng/ml in all diluted samples.

For example: For a 4-fold dilution of sample: First make a 1:10 dilution of 10 µg/ml biotinylated Adiponectin peptide (Item F) by adding 2 µl of Item F to 18 µl of appropriate Assay Diluent; pipette up and down to mix gently. In a separate tube, pipette 146 µl of appropriate Assay Diluent, 4 µl of 1:10 biotinylated Adiponectin, and 50 µl of your biological sample; mix gently.

6. Positive control dilution: briefly centrifuge the positive control vial (Item M). To the tube of Item M, add 101 µl Assay Diluent B (Item E) and 4 µl of 10-fold diluted biotinylated Adiponectin peptide (item F).
7. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.
8. If the 20X Wash Concentrate (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.

9. Briefly spin the HRP-Streptavidin concentrate vial (Item G) before use. HRP-Streptavidin concentrate should be diluted 4,000-fold with 1X Assay Diluent B.

For example: For 10000 fold Dilution of HRP- Streptavidin solution, Briefly spin the vial (Item G) and pipette up and down to mix gently . Add 2 μ l of HRP-Streptavidin concentrate into a tube with 198 μ l 1X Assay Diluent B to prepare a 100-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix thoroughly and then pipette 100 μ l of prepared 100-fold diluted solution into a tube with 10 ml 1x Assay Diluent B to prepare a final 10,000 fold diluted HRP-Streptavidin solution.

VII. ASSAY PROCEDURE:

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 μ l anti-Adiponectin antibody (see Reagent Preparation step 2) to each well and incubate for 1.5 hours.
3. Discard the solution and wash wells 5 times with 1x Wash Solution (200 μ l each).
4. Add 100 μ l of each standard (see Reagent Preparation step 4), positive control (see Reagent Preparation step 6) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or overnight at 4°C.
5. Discard the solution and wash 4 times with 1x Wash Solution (200 μ l each).

6. Add 100 μ l of prepared HRP-Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature.
7. Discard the solution and wash 5 times with 1x Wash Solution (200 μ l each).
8. Add 100 μ l of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark.
9. Add 50 μ l of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.



2. Add 100 μ l anti-Adiponectin antibody (1000X dilution) to each well. Incubate 1.5 hours at room temperature.



3. Add 100 μ l standard peptides or sample mixed with biotinylated Adiponectin peptide to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.



4. Add 100 μ l prepared Streptavidin solution. Incubate 45 minutes at room temperature.



5. Add 100 μ l TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.



6. Add 50 μ l Stop Solution to each well.
Read at 450 nm immediately

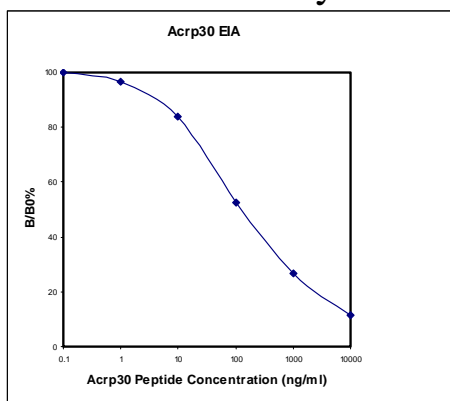
IX. CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit straight line through the standard points.

Percentage absorbance = $(B - \text{blank OD}) / (B_0 - \text{blank OD})$ where
B = OD of sample or standard and
 B_0 = OD of zero standard (total binding)

A. TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum detectable dose of Adiponectin is 2.47 ng/ml or 93.53 pM.

C. LINEARITY

1-100 ng/ml

D. REPRODUCIBILITY

Intra-Assay: CV<10%

Inter-Assay: CV<15%

X. SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested: Visfatin, Nesfatin, Ghrelin, CART, PYY, Angiotensin II, NPY and APC.

XI. RECOMMENDED DILUTION FACTORS (for most sera/plasma)

CYTOKINE	HUMAN		MOUSE		RAT
	Serum	Plasma	Serum	Plasma	Serum
Visfatin	4X-8X	4X-8X	4X-8X	4X-8X	2X-8X
Nesfatin	4X-8X	4X-8X	4X-8X	4X-8X	2X-4X
Ghrelin	4X-8X	2X	4X-8X	2X	2X
APC	4X	4X-8X	4X-8X	4X-8X	2X
Angiotensin II	4X	4X-8X	4X-8X	4X-8X	4X-8X
NPY	4X	2X	4X-8X	2X	2X
CART	4X-8X	4X-8X	2X-4X	2X	not tested

XII. REFERENCES

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3. Coppola A, Marfella R, Coppola L, Tagliamonte E, Fontana D, Liguori E, Cirillo T, Cafiero M, Natale S, Astarita C (2008). Effect of weight loss on coronary circulation and adiponectin levels in obese women. *Int. J. Cardiol.*. PMID 18378021.
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XII. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol style="list-style-type: none"> 1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution 	<ol style="list-style-type: none"> 1. Ensure sufficient incubation time; assay procedure step 2 change to over night 2. Check pipettes and ensure correct preparation
3. Large CV	<ol style="list-style-type: none"> 1. Inaccurate pipetting 	<ol style="list-style-type: none"> 1. Check pipettes
4. High background	<ol style="list-style-type: none"> 1. Plate is insufficiently washed 2. Contaminated wash buffer 	<ol style="list-style-type: none"> 1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed. 2. Make fresh wash buffer
5. Low sensitivity	<ol style="list-style-type: none"> 1. Improper storage of the EIA kit 2. Stop solution 	<ol style="list-style-type: none"> 1. Store your standard at $\leq -20^{\circ}\text{C}$ after reconstitution, others at 4°C. Keep substrate solution protected from light 2. Stop solution should be added to each well before measure

RayBio® EIA kits:

If you are interested in other EIA kits, please visit www.raybiotech.com for details.

Notes:

This product is for research use only.



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