

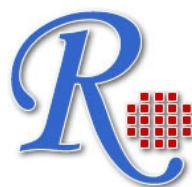
# **RayBio<sup>®</sup> Human/Mouse/Rat ANP Enzyme Immunoassay Kit**

**Please Read the Manual Carefully  
Before Starting your Experiment**

**User Manual 3.2  
(Revised April 1, 2013)**

**RayBio<sup>®</sup> ANP Enzyme  
Immunoassay Kit Protocol**

(Cat#: EIA-ANP-1)



**RayBiotech, Inc.**

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**RayBio® Human/Mouse/Rat ANP Enzyme  
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## I. INTRODUCTION

Atrial natriuretic peptide (ANP) is a 28-amino acid peptide hormone secreted by cardiac myocytes of the atrium. ANP plays an important role in the homeostatic regulation of body water, sodium, potassium and fat, by acting to reduce the water, sodium and adipose loads on the circulatory system, thus reducing blood pressure.

ANP peptide contains a 17-amino acid ring which is formed by a disulfide bond between two cysteine residues at positions 7 and 23. ANP is closely related to BNP (brain natriuretic peptide) and CNP (C-type natriuretic peptide), which all share the same amino acid ring.

The mechanism of ANP-induced vasodilatation is through binding to a specific set of ANP receptors. Receptor-agonist binding causes a reduction in blood volume and therefore a reduction in cardiac output and systemic blood pressure. Lipolysis is increased and renal sodium reabsorption is decreased. The overall effect of ANP on the body is to counter increases in blood pressure and volume caused by the renin-angiotensin system.

In addition to its vasodilatation effect, ANP also serves as a adipokine. Studies have shown that ANP increases the release of free fatty acids from adipose tissue, activates adipocyte plasma membrane NPR-A receptors, and increases intracellular cGMP levels that induce the phosphorylation of a hormone-sensitive lipase and perilipin A.

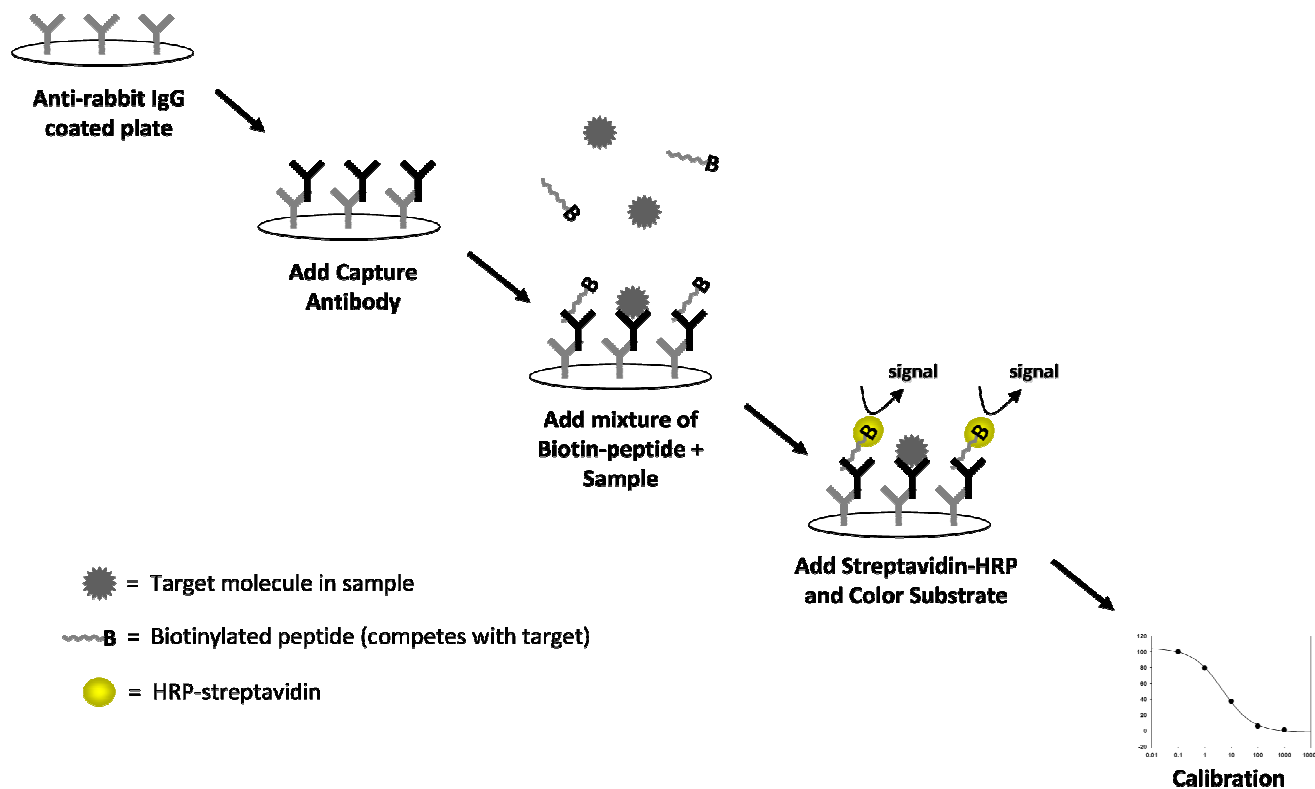
## II. GENERAL DESCRIPTION

The RayBio® ANP Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting ANP 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-ANP antibody, both biotinylated ANP peptide and peptide standard or targeted peptide in samples interacts competitively with the ANP antibody. Uncompeted (bound) biotinylated ANP 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 ANP peptide in the standard or samples. This is due to the competitive binding to ANP antibody between biotinylated ANP peptide and peptides in standard or samples. A standard curve of known concentration of ANP peptide can be established and the concentration of ANP peptide in the samples can be calculated accordingly.

This kit targets the common sequence of human, mouse and rat, and thus may be used to detect ANP expression in all these species with high specificity and sensitivity.

# Principle of Competitive EIA



### III. REAGENTS

1. ANP Microplate (Item A): 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml.
3. Lyophilized standard ANP peptide (Item C): 2 vials.
4. Lyophilized anti-ANP polyclonal antibody (Item N): 2 vials.
5. 1X Assay Diluent E (Item R): 2 vials, 25 ml/vial. Diluent for both standards and samples including serum or plasma, cell culture media or other sample types.
6. Lyophilized biotinylated ANP peptide (Item F): 2 vials.
7. HRP-Streptavidin concentrate (Item G): 600  $\mu$ l 50x concentrated HRP-conjugated Streptavidin.
8. Lyophilized positive control (Item M): 1 vial.
9. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'-tetramethylbenzidine (TMB) in buffered solution.
10. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
11. Assay Diagram (Item J).
12. User Manual (Item K).

### IV. STORAGE

- Standard, Biotinylated ANP peptide, and Positive Control should be stored at -20°C after arrival. **Avoid multiple freeze-thaws.**
- The remaining kit components may be stored at 4°C.
- Opened Microplate Wells and antibody (Item N) may be stored for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.
- If stored in this manner, RayBiotech warrants this kit for 6 months from the date of shipment.

## V. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450nm.
2. Precision pipettes to deliver 2 µl 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

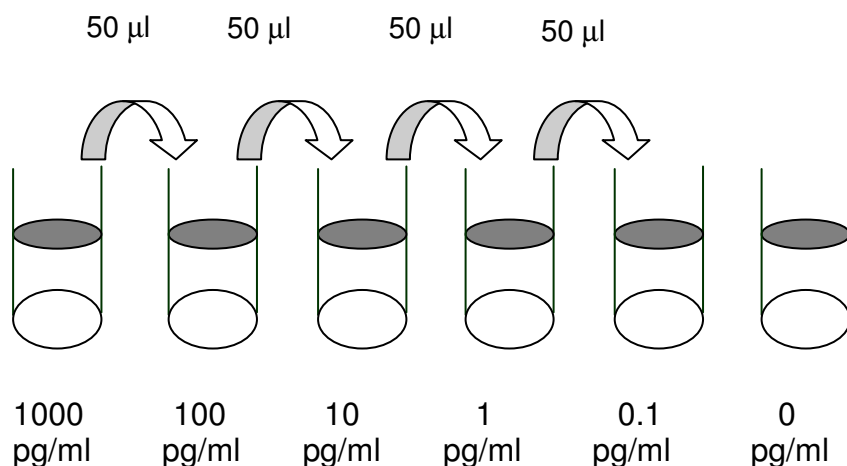
For sample and positive control dilutions, refer to steps 5, 6, 7 and 9 of Reagent Preparation.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.
2. Briefly centrifuge the ANP Antibody vial (Item N) and reconstitute with 5 µl of ddH<sub>2</sub>O before use. Add 50 µl of 1x Assay Diluent E into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.
3. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent E. This is your anti-ANP antibody working solution, which will be used in step 2 of the Assay Procedure.

*NOTE: the following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure).*

4. Briefly centrifuge the vial of biotinylated ANP peptide (Item F) and reconstitute with 20 µl of ddH<sub>2</sub>O before use. Add 5 µl of Item F to 5 ml 1X Assay Diluent E. Pipette up and down to mix gently. *The final concentration of biotinylated ANP will be 10 pg/ml.* This solution will only be used as the diluent in step 5 of Reagent Preparation.
5. Preparation of Standards: Label 6 microtubes with the following concentrations: 1000 pg/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml, 0.1 pg/ml and 0 pg/ml. Pipette 450 µl of biotinylated ANP solution into each tube, except for the 1000 pg/ml (leave this one empty). *It is very important to make sure the concentration of biotinylated ANP is 10 pg/ml in all standards.*
  - a. Briefly centrifuge the vial of standard ANP peptide (Item C) and reconstitute with 10 µl of ddH<sub>2</sub>O. In the tube labeled 1000 pg/ml, pipette 8 µl of Item C and 792 µl of 10 pg/ml biotinylated ANP solution (prepared in step 4 above). This is your ANP stock solution (1000 pg/ml ANP, 10 pg/ml biotinylated ANP). Mix thoroughly. This solution serves as the first standard.
  - b. To make the 100 pg/ml standard, pipette 50 µl of ANP stock solution the tube labeled 100 pg/ml. Mix thoroughly.
  - c. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 µl of biotinylated ANP and 50 µl of the prior concentration until 0.1 pg/ml is reached. Mix each tube thoroughly before the next transfer.
  - d. The final tube (0 pg/ml ANP, 10 pg/ml biotinylated ANP) serves as the zero standard (or total binding).





6. Prepare a 10-fold dilution of Item F. To do this, add 2 µl of Item F to 18 µl of the 1X Assay Diluent E. This solution will be used in steps 7 and 9.
7. Positive Control Preparation: Briefly centrifuge the positive control vial and reconstitute with 100 µl of ddH<sub>2</sub>O before use (Item M). To the tube of Item M, add 101 µl 1x Assay Diluent E. Also add 2 µl of 10-fold diluted Item F (prepared in step 6) to the tube. This is a 2-fold dilution of the positive control. Mix thoroughly. The positive control is a cell culture medium sample with an expected signal between 10% and 30% of total binding (70-90% competition) if diluted as described above. It may be diluted further if desired, but be sure the final concentration of biotinylated ANP is 10 pg/ml.
8. If Item B (20X Wash Concentrate) 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. Sample Preparation: Use 1X Assay Diluent E + biotinylated ANP to dilute samples, including serum/plasma, cell culture medium and other sample types.

*It is very important to make sure the final concentration of the biotinylated ANP is 10 pg/ml in every sample. EXAMPLE: to make a 4-fold dilution of sample, mix together 2.5 µl of 10-fold diluted Item F (prepared in step 6), 185 µl of 1X Assay Diluent E, and 62.5 µl of your sample; mix gently. The total volume is 250 µl, enough for duplicate wells on the microplate.*

*Do not use Item F diluent from Step 5 for sample preparation. If you plan to use undiluted samples, you must still add biotinylated ANP to a final concentration of 10 pg/ml. EXAMPLE: Add 2.5 µl of 10-fold diluted Item F to 247.5 µl of sample. NOTE: Optimal sample dilution factors should be determined empirically, however you may contact technical support (888-494-8555; techsupport@raybiotech.com) to obtain recommended dilution ranges for serum or plasma.*

10. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use. The HRP-Streptavidin concentrate should be diluted 50-fold with 1X Assay Diluent E.

## **VII. ASSAY PROCEDURE:**

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl anti-ANP antibody (see Reagent Preparation step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycles/sec). You may also incubate overnight at 4 degrees C.
3. Discard the solution and wash wells 4 times with 1x Wash Buffer (200-300 µl each), Washing may be done with a multichannel pipette or an automated plate washer. Complete

removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of each standard (see Reagent Preparation step 5), positive control (see Reagent Preparation step 7) and sample (see Reagent Preparation step 9) into appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) or overnight at 4 °C.
5. Discard the solution and wash 4 times as directed in Step 3.
6. Add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation step 10) to each well. Incubate for 45 minutes with gentle shaking at room temperature. It is recommended that the incubation time should not be shorter or longer than 45 minutes.
7. Discard the solution and wash 4 times as directed in Step 3.
8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).
9. Add 50 µl of Stop Solution (Item I) to each well. Read absorbances at 450 nm immediately.

## VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.  
↓
2. Add 100 µl anti-ANP antibody to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.  
↓
3. Add 100 µl standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.  
↓
4. Add 100 µl prepared streptavidin solution. Incubate 45 minutes at room temperature.  
↓
5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.  
↓
6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately

## IX. CALCULATION OF RESULTS

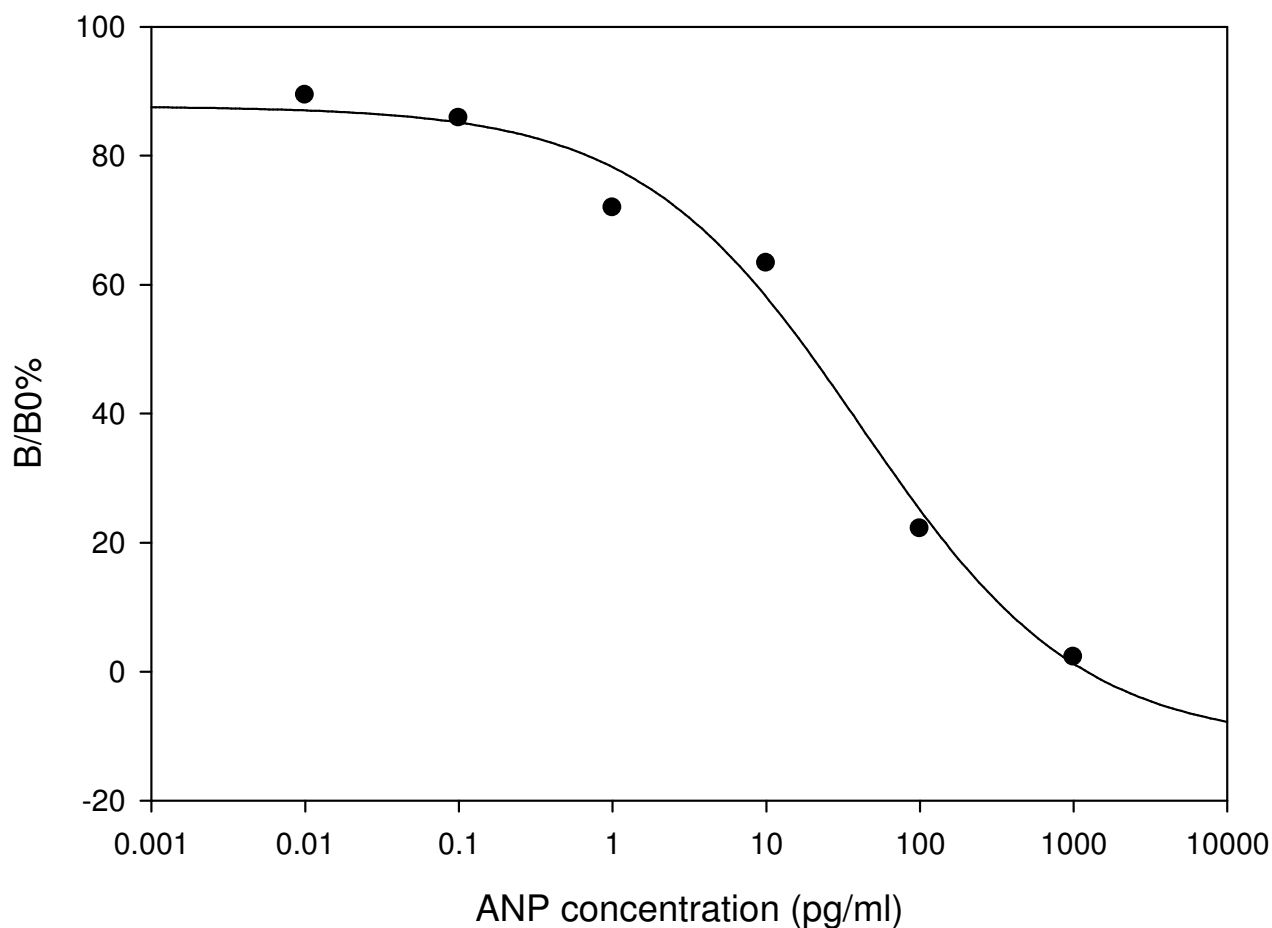
Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis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

Percentage absorbance =  $(B - \text{blank OD}) / (B_0 - \text{blank OD})$  where  
B = OD of sample or standard and  
B<sub>0</sub> = 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.

### ANP EIA



## B. SENSITIVITY

The minimum detectable concentration of ANP is 2.3 pg/ml.

## C. DETECTION RANGE

0.1-1,000 pg/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: Ghrelin, Nesfatin, Angiotensin II, NPY and APC.

## XI. REFERENCES

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## XII. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	1. Inaccurate pipetting 2. Improper standard dilution	1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution	1. Ensure sufficient incubation time; assay procedure step 2 change to over night 2. Check pipettes and ensure correct preparation
3. Large CV	1. Inaccurate pipetting	1. Check pipettes
4. High background	1. Plate is insufficiently washed 2. Contaminated wash buffer	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	1. Improper storage of the EIA kit 2. Stop solution	1. Store your standard at $\leq -20^{\circ}\text{C}$ after receipt of the kit. 2. Stop solution should be added to each well before measure

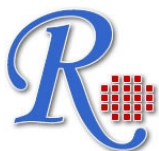
RayBio® EIA kits:

If you are interested in other EIA kits, please visit [www.raybiotech.com](http://www.raybiotech.com) for details.



**Notes:**

This product is for research use only.



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