

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

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

**User Manual 2.1  
(Revised July 5, 2011)**

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

(Cat#: EIA-BNP-1)



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

**Tel: (Toll Free) 1-888-494-8555 or 770-729-2992; Fax: 770-206-2393;  
Web: [www.raybiotech.com](http://www.raybiotech.com) Email: [info@raybiotech.com](mailto:info@raybiotech.com)**



**RayBiotech, Inc.**

---

**RayBio® Human/Mouse/Rat BNP Enzyme  
Immunoassay Kit Protocol**

**TABLE OF CONTENTS**

I.	Introduction.....	2
II.	General Description.....	3
III.	Reagents.....	4
IV.	Storage.....	5
V.	Additional Materials Required.....	5
VI.	Reagent Preparation.....	6
VII.	Assay Procedure.....	9
VIII.	Assay Procedure Summary.....	10
IX.	Calculation of Results.....	11
A.	Typical Data.....	11
B.	Sensitivity.....	12
C.	Detection Range.....	12
D.	Reproducibility.....	12
X.	Specificity.....	12
XI.	References.....	12
XII.	Troubleshooting Guide.....	14

## I. INTRODUCTION

Brain natriuretic peptide (BNP), (aka B-type natriuretic peptide), is a 32 amino acid polypeptide secreted by the ventricles of the heart in response to excessive stretching of myocytes in the ventricles. BNP was originally identified in extracts of porcine brain, but in humans it is produced mainly in the cardiac ventricles.

At the time of release, a co-secreted 76 amino acid N-terminal fragment (NT-proBNP) is also released with BNP.

BNP binds to and activates NPRA in a similar fashion to atrial natriuretic peptide (ANP) but with 10-fold lower affinity. The biological half-life of BNP, however, is twice as long as that of ANP. Both ANP and BNP have limited ability to bind and activate NPRB.

Physiologic actions of BNP include decrease in systemic vascular resistance and central venous pressure as well as an increase in natriuresis. Thus, the resulting effect of BNP is a decrease in cardiac output and a decrease in blood volume.

Tests showing elevated levels of BNP or NT-proBNP in blood are used as a diagnosis of heart failure and may be useful to establish prognosis in heart failure, as both markers are typically higher in patients with poorer outcome.

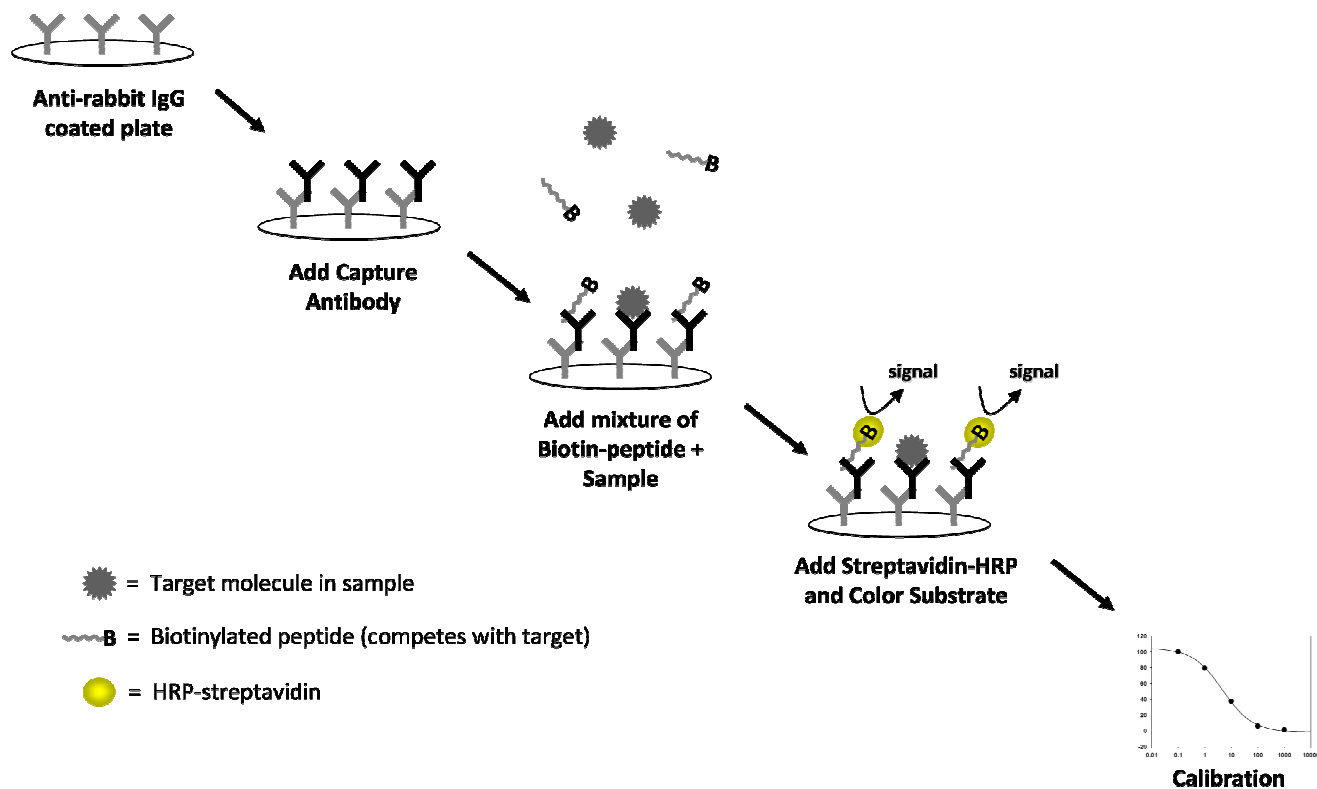
Both BNP and NT-proBNP have been approved as a marker for acute congestive heart failure (CHF). The plasma concentrations of both BNP are increased in patients with asymptomatic and symptomatic left ventricular dysfunction. There is no level of BNP that perfectly separates patients with and without heart failure.

## II. GENERAL DESCRIPTION

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

# Principle of Competitive EIA



### III. REAGENTS

1. BNP 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 BNP Peptide (Item C): 2 vials, 10 µl/vial
4. Anti-BNP polyclonal antibody (Item N): 2 vials, 5 µl/vial
5. Assay Diluent A (Item D): 30 ml, contains 0.09% sodium azide as preservative. Diluent for standards and serum or plasma samples.
6. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. Diluent for standards and cell culture media or other sample types.
7. Biotinylated BNP peptide, (Item F): 2 vials, 20 µl/vial
8. HRP-Streptavidin concentrate (Item G): 8 µl 5,000x concentrated HRP-conjugated Streptavidin.
9. Positive control (Item M): 1 vial, 100 µl
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

- Standard, Biotinylated BNP peptide, and Positive Control should be stored at -20 °C or -80 °C (recommended at -80 °C) after arrival. **Avoid multiple freeze-thaws.**
- The remaining kit components may be stored at -20 °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  $\mu$ 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

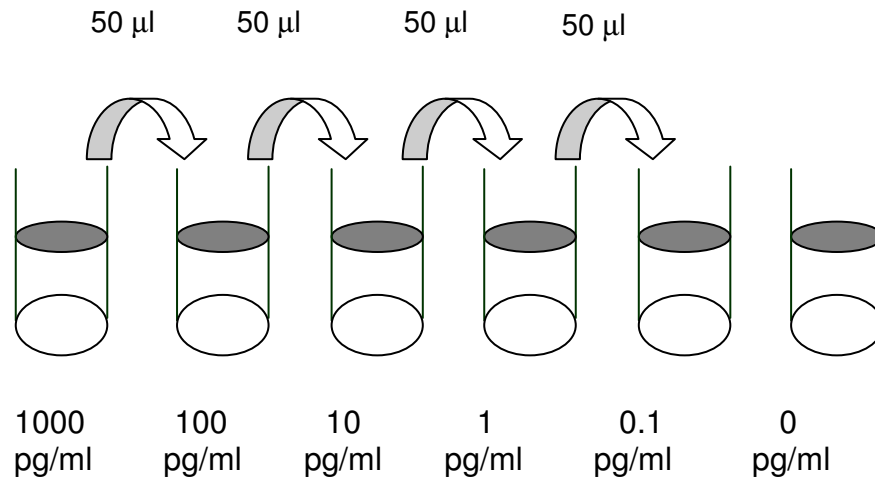
If testing plasma or serum samples, use Assay Diluent A to dilute Item F and Item C. If testing cell culture media or other sample types, use Assay Diluent B to dilute Item F and Item C. For sample and positive control dilutions, refer to steps 6, 7, 8 and 10 of Reagent Preparation.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.
2. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.
3. Briefly centrifuge the Anti-BNP Antibody vial (Item N) before use. Add 50  $\mu$ l of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.

4. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent B. This is your anti-BNP 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).*

5. Briefly centrifuge the vial of Biotinylated BNP (Item F) before use. Add 5 µl of Item F to 5 ml of the appropriate Assay Diluent. Pipette up and down to mix gently. *The final concentration of biotinylated BNP will be 10 pg/ml.* This solution will only be used as the diluent in step 6 of Reagent Preparation.
6. 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 BNP 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 BNP is 10 pg/ml in all standards.*
  - a. Briefly centrifuge the vial of BNP (Item C). In the tube labeled 1000 pg/ml, pipette 8 µl of Item C and 792 µl of 10 pg/ml biotinylated BNP solution (prepared in step 5 above). This is your BNP stock solution (1000 pg/ml BNP, 10 pg/ml biotinylated BNP). Mix thoroughly. This solution serves as the first standard.
  - b. To make the 100 pg/ml standard, pipette 50 µl of BNP 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 BNP 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 BNP, 10 pg/ml biotinylated BNP) serves as the zero standard (or total binding).



7. Prepare a 10-fold dilution of Item F. To do this, add 2 µl of Item F to 18 µl of the appropriate Assay Diluent. This solution will be used in steps 8 and 10.
8. Positive Control Preparation: briefly centrifuge the positive control vial (Item M). To the tube of Item M, add 101 µl 1x Assay Diluent B. Also add 2 µl of 10-fold diluted Item F (prepared in step 7) 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 BNP is 10 pg/ml.
9. 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.
10. Sample Preparation: Use Assay Diluent A + biotinylated BNP to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated BNP as the diluent. *It is very important to make sure the final*

*concentration of the biotinylated BNP 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 7), 185 µl of appropriate Assay Diluent, 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 BNP 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.*

11. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use. The HRP-Streptavidin concentrate should be diluted 5,000-fold with 1X Assay Diluent B.

*EXAMPLE: For 5,000-fold dilution of HRP-Streptavidin solution, briefly spin the vial (Item G) and pipette up and down to mix gently. Add 2 µl of HRP-Streptavidin concentrate into a tube with 198 µl 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 µl of prepared 100-fold diluted solution into a tube with 4.9 ml 1x Assay Diluent B to prepare a final 5,000-fold diluted HRP-Streptavidin solution.*






Note: Do not use Assay Diluent A for HRP-Streptavidin preparation in Step 11.

## 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  $\mu$ l anti-BNP antibody (see Reagent Preparation step 4) 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  $\mu$ l 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  $\mu$ l of each standard (see Reagent Preparation step 6), positive control (see Reagent Preparation step 8) and sample (see Reagent Preparation step 10) 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  $\mu$ l of prepared HRP-Streptavidin solution (see Reagent Preparation step 11) to each well. Incubate for 45 minutes with gentle shaking at room temperature. It is recommended that 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l anti-BNP antibody to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.  

3. Add 100  $\mu$ l standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.  

4. Add 100  $\mu$ l prepared streptavidin solution. Incubate 45 minutes at room temperature.  

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

6. Add 50  $\mu$ l Stop Solution to each well. Read at 450 nm immediately

## **IX. CALCULATION OF RESULTS**

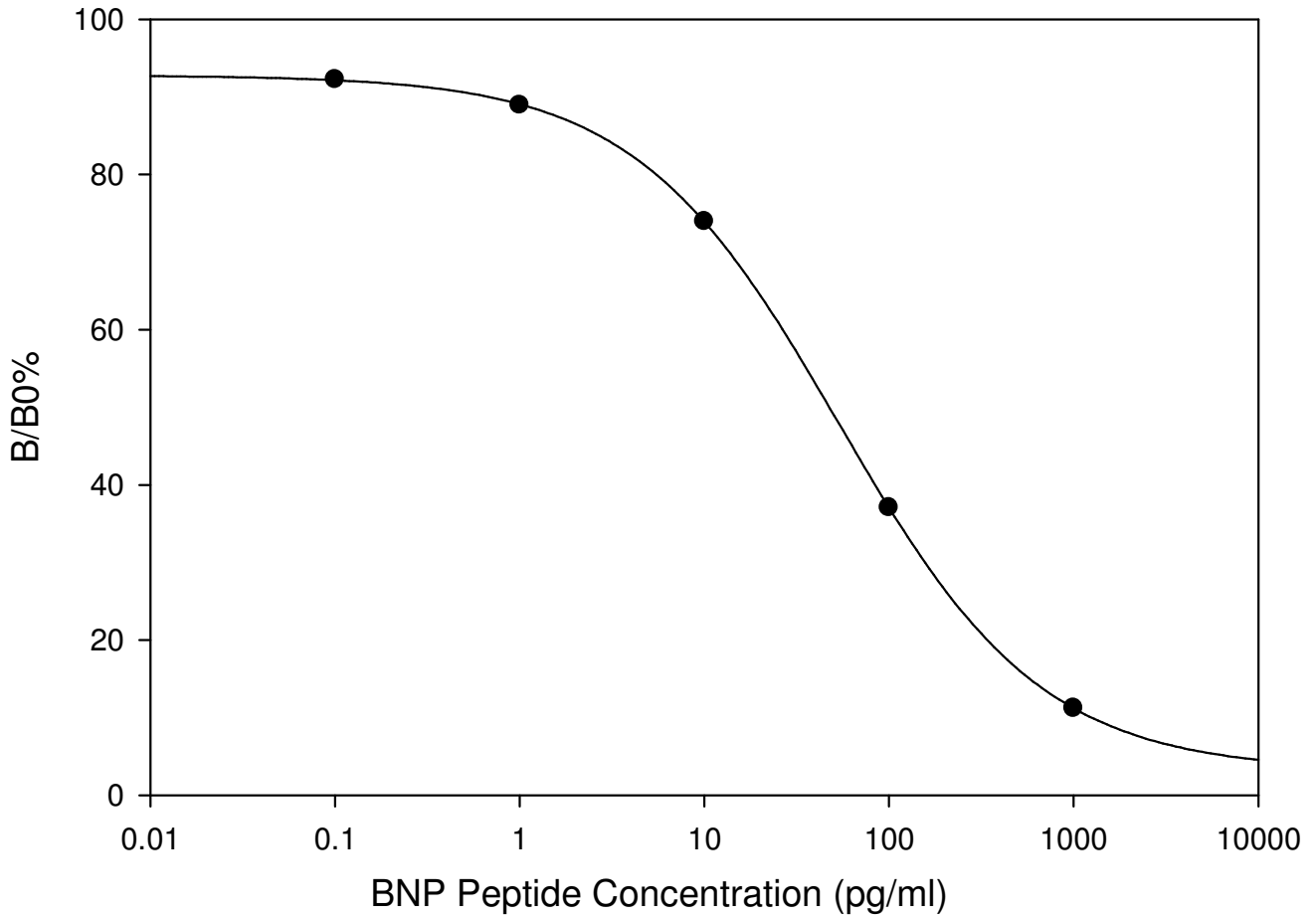
Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis 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<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.

## BNP EIA



### **B. SENSITIVITY**

The minimum detectable concentration of BNP is 1.45 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

1. Maisel A, Krishnaswamy P, Nowak R, McCord J, Hollander J, Duc P, Omland T, Storrow A, Abraham W, Wu A, Clopton P, Steg P, Westheim A, Knudsen C, Perez A, Kazanegra R, Herrmann H, McCullough P (2002). "Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure". *N Engl J Med* 347 (3): 161–7.
2. Maisel A, Hollander JE, Guss D, McCullough P, Nowak R, Green G, Saltzberg M, Ellison SR, Bhalla MA, Bhalla V, Clopton P, Jesse R (2004). "Primary results of the Rapid Emergency Department Heart Failure Outpatient Trial (REDHOT). A multicenter study of B-type natriuretic peptide levels, emergency department decision making, and outcomes in patients presenting with shortness of breath". *J. Am. Coll. Cardiol.* 44 (6): 1328–33.
3. Bibbins-Domingo K, Gupta R, Na B, Wu AH, Schiller NB, Whooley MA (2007). "N-terminal fragment of the prohormone brain-type natriuretic peptide (NT-proBNP), cardiovascular events, and mortality in patients with stable coronary heart disease". *JAMA* 297 (2): 169–76.

## XII. TROUBLESHOOTING GUIDE

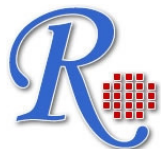
<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
1. Poor standard curve	<ol style="list-style-type: none"> <li>1. Inaccurate pipetting</li> <li>2. Improper standard dilution</li> </ol>	<ol style="list-style-type: none"> <li>1. Check pipettes</li> <li>2. Ensure briefly spin the vial of Item C and dissolve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

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.



©2008 RayBiotech, Inc.

3607 Parkway Lane, Suite 200  
Norcross, GA 30092  
Tel: 770-729-2992, 1-888-494-8555  
Fax: 770-206-2393  
Web: [www.raybiotech.com](http://www.raybiotech.com)