

RayBio[®] Human/Mouse/Rat Angiotensin II Enzyme Immunoassay Kit

Catalog #: EIA-ANGII, EIAM-ANGII, EIAR-ANGII

User Manual
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Caution:
Extraordinarily useful information enclosed



ISO 13485 Certified

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Please read the entire manual carefully before starting your experiment

I. Introduction

Angiotensin, a key player in the renin-angiotensin system, is a peptide hormone that causes vasoconstriction, increased blood pressure, and release of aldosterone from the adrenal cortex. It is derived from the precursor molecule angiotensinogen produced in the liver.

Angiotensin II is formed from Angiotensin I, which is removed of two terminal residues by the enzyme Angiotensin-converting enzyme (ACE). Angiotensin II acts as an endocrine, autocrine/ paracrine, and intracrine hormone. Angiotensin II is degraded to angiotensin III by angiotensinases that are located in red blood cells and the vascular beds of most tissues. It has a half-life in circulation of around 30 seconds, while in tissue, it may be as long as 15-30 minutes.

The effect of obesity on Angiotensin II has recently been reported. Obese patients show heightened renal vasodilation to blockade of the renin-angiotensin system, suggesting deficits in vascular responses to angiotensin II. This may be due to increased reactivity of renal vasoconstriction to ANG II.

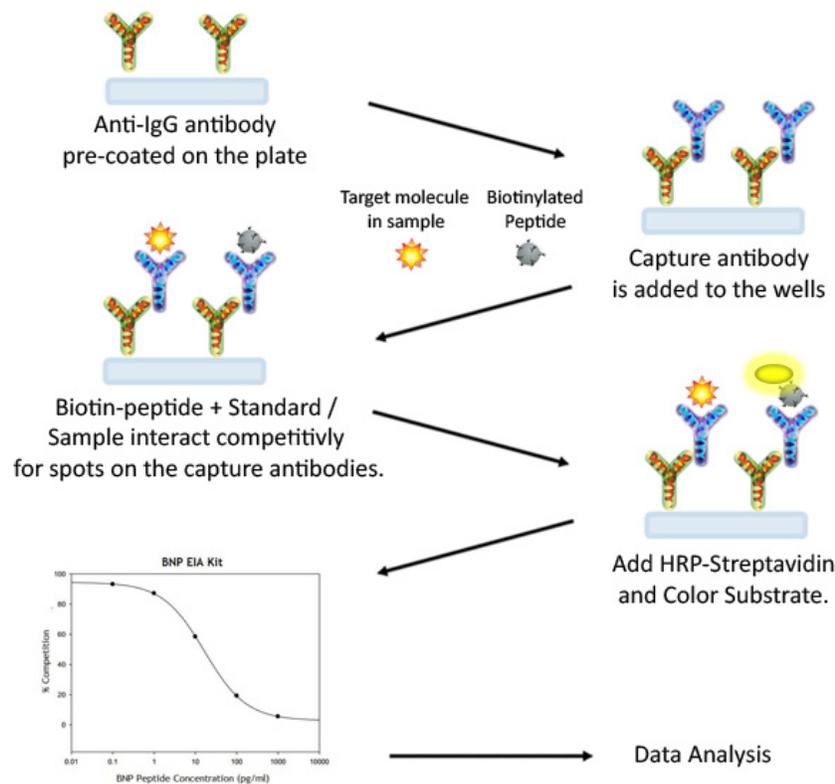
Angiotensin II has been associated with a number of important physiological processes in heart, brain, adrenal gland and kidney. For cardiovascular effect, Angiotensin II is a potent direct vasoconstrictor, constricting arteries and veins and increasing blood pressure. It is also the most important Gq stimulator of the heart during hypertrophy. For neural effects, Angiotensin II increases thirst sensation (dipsogen) through the subfornical organ (SFO) of the brain, decreases the response of the baroreceptor reflex, and increases the desire for salt. It increases secretion of ADH in the posterior pituitary and secretion of ACTH in the anterior pituitary. For adrenal effects, Angiotensin II acts on the adrenal cortex, causing it to release aldosterone. For renal effects, Angiotensin II has a direct effect on the proximal tubules to increase Na⁺ absorption.

II. General Description

The RayBio® Angiotensin II Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Angiotensin II peptide based on the competitive enzyme immunoassay principle.

In this assay, a biotinylated Angiotensin II peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated Angiotensin II peptide competes with endogenous (unlabeled) Angiotensin II for binding to the anti-Angiotensin II antibody. After a wash step, any bound biotinylated Angiotensin II then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated Angiotensin II peptide and inversely proportional to the amount of endogenous Angiotensin II in the standard or samples. A standard curve of known concentration of Angiotensin II peptide can be established and the concentration of Angiotensin II peptide in the samples can be calculated accordingly.

III. How It Works



IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

Component	Size / Description	Storage / Stability After Preparation
Microplate	96 wells (12 strips x 8 wells) coated with secondary antibody.	1 month at 4°C*
Wash Buffer	25 ml of 20X concentrated solution.	1 month at 4°C
Standard Angiotensin II Peptide	2 vials of Angiotensin II Peptide. 1 vial is enough to run each standard in duplicate.	The first standard: 2-3 days at 4°C Additional dilutions: Do not store
Anti-Angiotensin II Polyclonal Antibody	2 vials of anti-Angiotensin II.	1 month at 4°C
Assay Diluent C	30 ml diluent for standards and serum or plasma.	N/A
Assay Diluent B	15 ml of 5X concentrated buffer. Diluent for standards, cell culture media or other sample types, and HRP-Streptavidin.	1 month at 4°C
Biotinylated Angiotensin II Peptide	2 vials of Biotinylated Angiotensin II Peptide, 1 vial is enough to assay the whole plate.	2-3 days at 4°C
HRP-Streptavidin	600 µl 100X concentrated HRP-conjugated streptavidin.	Do not store and reuse
Positive Control	1 vial of Positive Control.	2-3 days at 4°C
TMB One-Step Substrate Reagent	12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution.	N/A
Stop Solution	8 ml of 0.2 M sulfuric acid.	N/A

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
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. Plastic wrap

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

Note: **Assay Diluent C** should be used for dilution of samples, biotinylated peptide, and standard peptide when testing **plasma or serum samples**. **1X Assay Diluent B** should be used for dilution of samples, biotinylated peptide, and standard peptide when testing **cell culture media or other sample types**.

A. Preparation of Plate and Anti-Angiotensin II Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.
2. Label removable 8-well strips as appropriate for your experiment.
3. 5X Assay Diluent B should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti-Angiotensin II antibody vial. Then add 50 μ l of 1X Assay Diluent B to the vial to prepare the antibody concentrate. Pipette up and down to mix gently.
5. The antibody concentrate should then be diluted 100-fold with 1X Assay Diluent B. This is your anti-Angiotensin II antibody working solution, which will be used in step 2 of Assay Procedure (Section VIII).

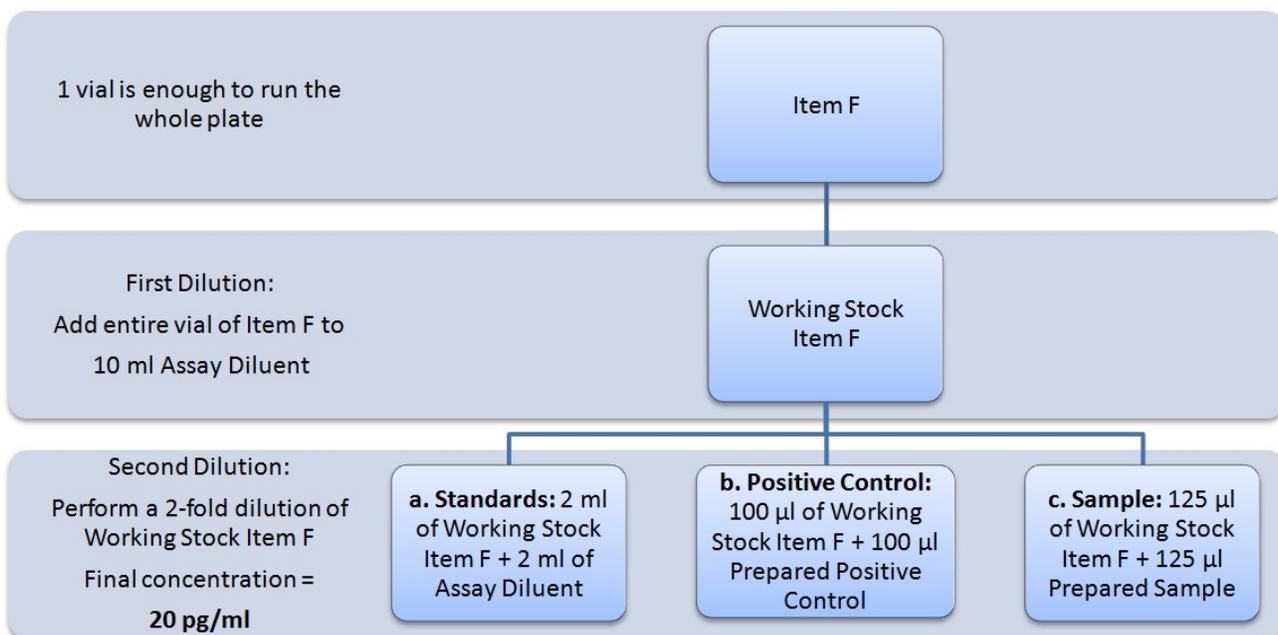
Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)

B. Preparation of Biotinylated Angiotensin II Peptide

6. Briefly centrifuge the vial of Biotinylated Angiotensin II Peptide before use.

7. See the image below for proper preparation of the biotinylated peptide. Transfer the entire contents of the Biotinylated Angiotensin II Peptide vial into a tube containing 10 ml of the appropriate Assay Diluent. This is your Working Stock of Biotinylated Angiotensin II Peptide. Pipette up and down to mix gently. *The final concentration of biotinylated Angiotensin II will be **40pg/ml** .*

- a. Second Dilution of Biotinylated Angiotensin II Peptide for Standards: Add 2 ml of Working Stock Biotinylated Angiotensin II Peptide to 2 ml of the appropriate Assay Diluent. The final concentration of biotinylated Angiotensin II will be **20 pg/ml**.
- b. Second Dilution of Biotinylated Angiotensin II Peptide for Positive Control: Add 100 μ l of Working Stock Biotinylated Angiotensin II Peptide to 100 μ l of the prepared Positive Control. (See section D for Positive Control preparation) The final concentration of biotinylated Angiotensin II will be **20 pg/ml**.
- c. Second Dilution of Biotinylated Angiotensin II Peptide for samples: Add 125 μ l of Working Stock Biotinylated Angiotensin II Peptide to 125 μ l of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated Angiotensin II will be **20 pg/ml**.

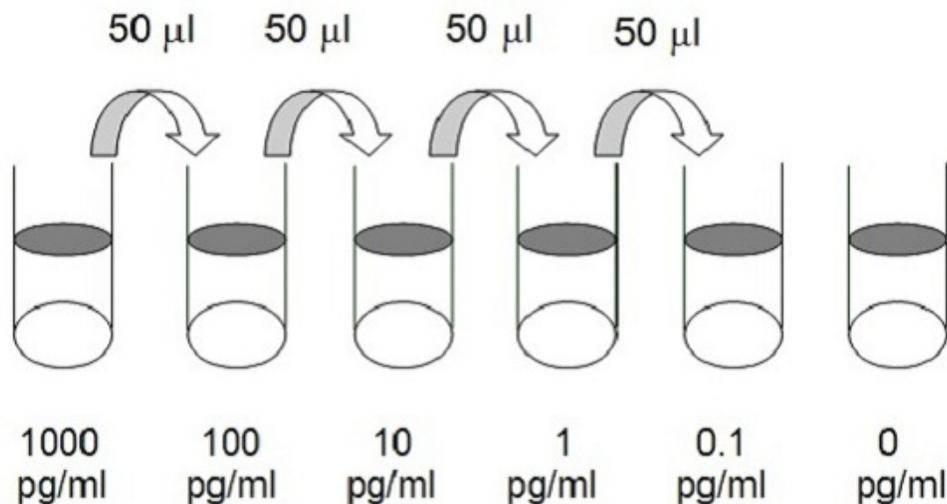


C. Preparation of Standards

- Label 6 microtubes with the following concentrations: 1,000 pg/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml, 0.1 pg/ml and 0 pg/ml. Pipette 450 μ l of biotinylated Angiotensin II Biotinylated Peptide working solution (prepared in step 7a) into each tube, except the 1,000 pg/ml (leave this one empty).

It is very important to make sure the concentration of biotinylated Angiotensin II is 20 pg/ml in all standards.

- Briefly centrifuge the vial of Angiotensin II Standard. Pipette 8 μ l of Standard Peptide and 792 μ l of 20 pg/ml biotinylated Angiotensin II working solution (prepared in step 7a) into the tube labeled 1000 pg/ml. Mix thoroughly. This solution serves as the first standard (1000 pg/ml Angiotensin II standard, 20 pg/ml biotinylated Angiotensin II).
- To make the 100 pg/ml standard, pipette 50 μ l of the 1000 pg/ml Angiotensin II standard into the tube labeled 100 pg/ml. Mix thoroughly.
- Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 μ l of biotinylated Angiotensin II and 50 μ l of the prior concentration until the 0.1 pg/ml is reached. Mix each tube thoroughly before the the next transfer.



D. Positive Control Preparation

12. Briefly centrifuge the Positive Control vial.
13. Refer to step 7b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated Angiotensin II should still be 20 pg/ml.

The Positive Control is a mouse serum sample that serves as a system control to verify that the kit components are working. The resulting OD will not be used in any calculations; if no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired, but be sure the final concentration of biotinylated Angiotensin II is 20 pg/ml.

E. Sample Preparation

14. If you wish to perform a 2-fold dilution of your sample, proceed to step 7c. If you wish to perform a higher dilution of your sample, dilute your sample with the appropriate Assay Diluent before performing step 7c.

EXAMPLE (to make a 4-fold dilution of sample):

- a. Dilute sample 2-fold (62.5 μ l of sample + 62.5 μ l of the appropriate Assay Diluent.).
- b. Perform step 7c (125 μ l of working solution Biotinylated Angiotensin II Peptide + 125 μ l of sample prepared above).

The total volume is 250 μ l, enough for duplicate wells on the microplate. It is very important to make sure the final concentration of the biotinylated Angiotensin II is **20 pg/ml**.

Note: Optimal sample dilution factors should be determined empirically, however you may reference below for recommended dilution factors for serum:

Human=4X Mouse=2X Rat=2X .

If you have any questions regarding the recommended dilutions you may contact technical support at 888-494-8555 or techsupport@raybiotech.com.

F. Preparation of Wash Buffer and HRP

15. If the Wash Buffer contains visible crystals, warm to room temperature and mix gently until dissolved.
16. Dilute 20 ml of Wash Buffer concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
17. Briefly centrifuge the HRP-Streptavidin vial before use.
18. Dilute the HRP-Streptavidin concentrate 100-fold with 1X Assay Diluent B.
*Note: do **not** use Assay Diluent C for HRP-Streptavidin preparation in step 18*

VIII. 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 μ l of Anti-Angiotensin II Antibody (See Reagent Preparation step 5) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycle/sec). You may also incubate overnight at 4°C.
3. Discard the solution and wash wells 4 times with 1X Wash Solution Buffer (200-300 μ 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 μ l of each standard (see Reagent Preparation Section C), Positive Control (see Reagent Preparation Section D) and sample (see Reagent Preparation Section E) to 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) overnight or at 4°C.
5. Discard the solution and wash 4 times as directed in Step 3.
6. Add 100 μ l of prepared HRP-Streptavidin solution (see Reagent Preparation step 18) to each well. Incubate for 45 minutes at room temperature with

gentle shaking. 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 μ l of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).
9. Add 50 μ l of Stop Solution to each well. Read at 450 nm immediately.

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 μ l anti-Angiotensin II to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.
3. Add 100 μ l standard or sample 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.

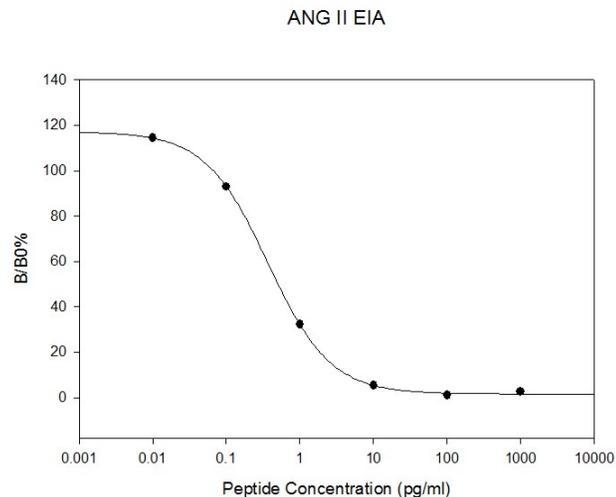
X. Calculation of Results

Calculate the mean absorbance for each set of duplicate stands, 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_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 concentrations of Angiotensin II is 0.3 pg/ml.

C. Standard Curve Range

0.1-1,000 pg/ml

D. Reproducibility

Intra-Assay: CV<10%

Inter-Assay: CV<15%

E. Assay Diagram

Recommended Plate Layout:

Blank	Blank	SA1	SA1	SA9	SA9	SA17	SA17	SA25	SA25	SA33	SA33
Total Binding	Total Binding	SA2	SA2	SA10	SA10	SA18	SA18	SA26	SA26	SA34	SA34
Standard1	Standard1	SA3	SA3	SA11	SA11	SA19	SA19	SA27	SA27	SA35	SA35
Standard2	Standard2	SA4	SA4	SA12	SA12	SA20	SA20	SA28	SA28	SA36	SA36
Standard3	Standard3	SA5	SA5	SA13	SA13	SA21	SA21	SA29	SA29	SA37	SA37
Standard4	Standard4	SA6	SA6	SA14	SA14	SA22	SA22	SA30	SA30	SA38	SA38
Standard5	Standard5	SA7	SA7	SA15	SA15	SA23	SA23	SA31	SA31	SA39	SA39
Pos Control	Pos Control	SA8	SA8	SA16	SA16	SA24	SA24	SA32	SA32	SA40	SA40

Key:

Blank = Buffer Only

Total Binding = Biotin-Angiotensin II only

Standard 1 = 1000 pg/ml

Standard 2 = 100 pg/ml

Standard 3 = 10 pg/ml

Standard 4 = 1 pg/ml

Standard 5 = 0.1 pg/ml

Pos Control = Biotin with Positive Control

XI. Specificity

This EIA kit is designed to detect human, mouse, and rat ANGII

XIV. Publications Citing This Product

1. Dhar, Priyanka, Shashi B. Singh, and Vijay K. Sharma. "Autonomic Cardiovascular Responses in Acclimatized Lowlanders on Prolonged Stay at High Altitude: A Longitudinal Follow Up Study." PLoS One. PLoS One, 03 Jan. 2014. Web.
Species: Human
Sample Type: Plasma
2. Hao L., Wang X., Cheng J., You S., Ma S., Zhong X., Quan L., Luo B. The upregulation of endothelin-1 and downregulation of miRNA-125a-5p, -155 and -199a/b-3p in human atherosclerotic coronary artery. Cardiovascular Pathology - 07 April 2014 [Epub Ahead of Print]
Species: Human
Sample Type: Other Fluid
3. Emdin M., et al. Biomarkers of activation of renin-angiotensin-aldosterone system heart failure: how useful, how feasible? Clinica Chimica Acta Dec 2014, epub ahead of print. DOI: 10.1016/j.cca.2014.10.031
Species: Human
Sample Type: N/A
4. Mascareno E, Galatioto J, Rozenberg I, et al. Cardiac Lineage Protein-1 (CLP-1) Regulates Cardiac Remodeling via Transcriptional Modulation of Diverse Hypertrophic and Fibrotic Responses and Angiotensin II-transforming Growth Factor ? (TGF-?1) Signaling Axis. The Journal of Biological Chemistry 2012;287(16):13084-13093. doi:10.1074/jbc.M111.288944.
Species: Mouse
Sample Type: Tissue Lysate

For additional publications citing this product, please contact technical support at 888-494-8555 or techsupport@raybiotech.com.

XIII. Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	<ul style="list-style-type: none"> ○ Inaccurate pipetting ○ Improper standard dilution 	<ul style="list-style-type: none"> ○ Check pipettes ○ Briefly centrifuge Standard Angiotensin II Peptide and dissolve the powder thoroughly by gently mixing
Low signal	<ul style="list-style-type: none"> ○ Improper preparation of standard and/or biotinylated antibody ○ Too brief incubation times ○ Inadequate reagent volumes or improper dilution 	<ul style="list-style-type: none"> ○ Briefly spin down vials before opening. Dissolve the powder thoroughly. ○ Ensure sufficient incubation time; assay procedure step 2 may be done overnight ○ Check pipettes and ensure correct preparation
Large CV	<ul style="list-style-type: none"> ○ Inaccurate pipetting ○ Air bubbles in wells 	<ul style="list-style-type: none"> ○ Check pipettes ○ Remove bubbles in wells
High background	<ul style="list-style-type: none"> ○ Plate is insufficiently washed ○ Contaminated wash buffer 	<ul style="list-style-type: none"> ○ Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. ○ Make fresh wash buffer
Low sensitivity	<ul style="list-style-type: none"> ○ Improper storage of the ELISA kit ○ Stop solution 	<ul style="list-style-type: none"> ○ Follow storage recommendations in sections IV and V. Keep substrate solution protected from light. ○ Add stop solution to each well before reading plate

RayBio[®] ELISA Kits

Over 3,000 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits.html for details.

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