

RayBio[®]
Human/Mouse/Rat RELM-alpha
Enzyme Immunoassay Kit

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

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

**RayBio[®] RELM-alpha Enzyme
Immunoassay Kit Protocol**

(Cat#: EIA-RELA-1)



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**Tel: (Toll Free) 1-888-494-8555 or 770-729-2992; Fax: 770-206-2393;
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RayBiotech, Inc.

**RayBio® Human/Mouse/Rat RELM-alpha Enzyme
Immunoassay Kit Protocol**

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I. INTRODUCTION

RELM alpha (also known as FIZZ1, HIMF) belongs to the family of tissue-specific cytokine-rich secretory proteins termed RELM-FIZZ (found in inflammatory zone). There is high homology among the three known members of the RELM-FIZZ family, Resistin, RELM alpha and RELM beta. They are all 85-94 amino acid secreted proteins with a highly conserved C-terminal domain characterized by 10 cysteine residues with unique spacing motif.

Studies on RELM-FIZZ Family have shown that RELM alpha may play important role in obesity. RELM alpha is exclusively produced by adipocytes, in contrast to RELM beta which is expressed in the epithelium of the colon and small bowel. RELM alpha inhibits the differentiation of 3T3-L1 preadipocytes into adipocytes but has no effect on proliferation of 3T3-L1 preadipocytes. These studies suggest that RELM alpha may be involved in the control of the adipogenesis as well as in the process of muscle differentiation.

In addition to its abundant expression in mature adipose tissues, RELM alpha can also be detected in bronchial epithelium and type II pneumocytes. In the lung, RELM-alpha is induced by hypoxia and strongly activated Akt phosphorylation in PI3K/Akt pathway. Furthermore, RELM alpha shows vasoconstrictive effect by increasing pulmonary arterial pressure and vascular resistance. These studies suggest RELM alpha regulates apoptosis and may participate in lung alveolarization and maturation.

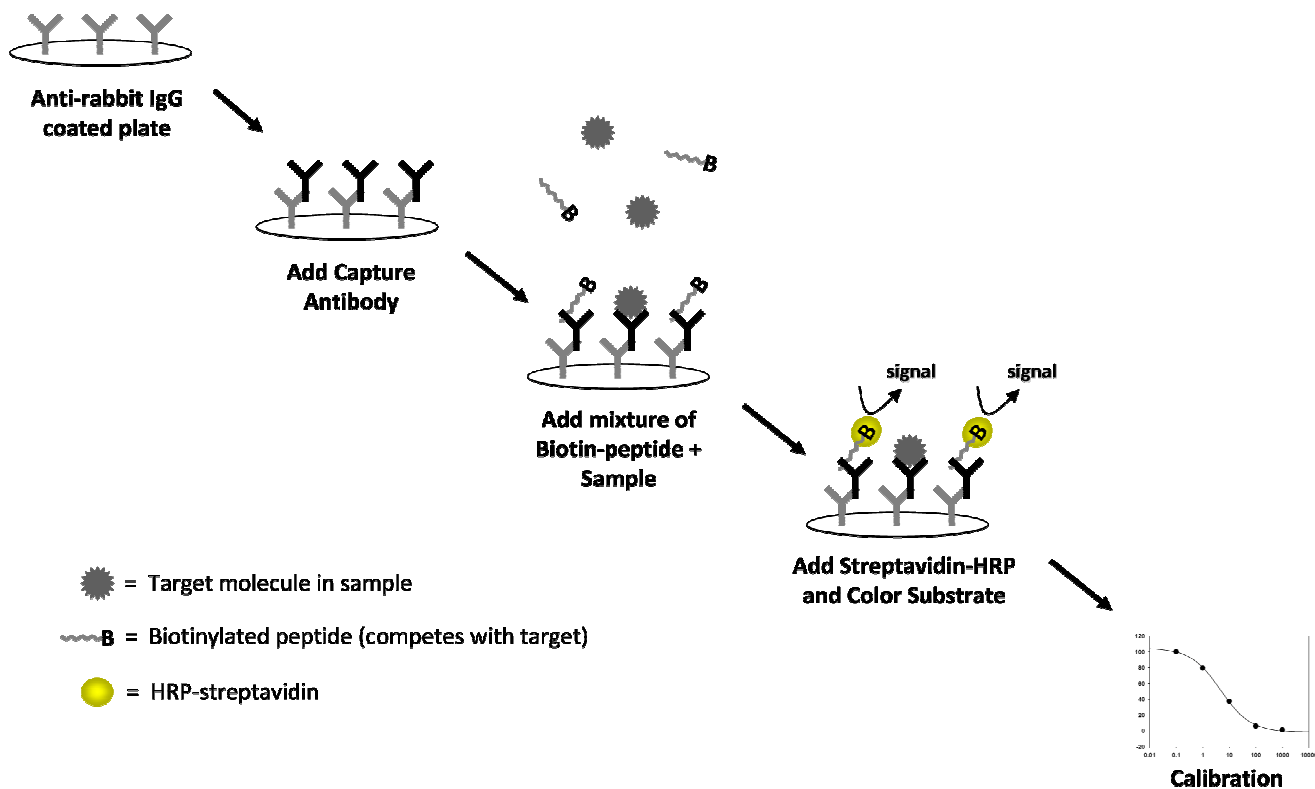
II. GENERAL DESCRIPTION

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

The microplate in the kit is pre-coated with anti-RELM-alpha antibody. After a blocking step, both biotinylated RELM-alpha peptide and peptide standard or targeted peptide in samples interacts competitively with the RELM-alpha antibody. Uncompeted (bound) biotinylated RELM-alpha 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 RELM-alpha peptide in the standard or samples. This is due to the competitive binding to RELM-alpha antibody between biotinylated RELM-alpha peptide and peptides in standard or samples. A standard curve of known concentration of RELM-alpha peptide can be established and the concentration of RELM-alpha peptide in the samples can be calculated accordingly.

EIA-RELA-1 detects RELM alpha (88aa). No other active isoforms have been reported.

Principle of Competitive EIA



III. REAGENTS

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

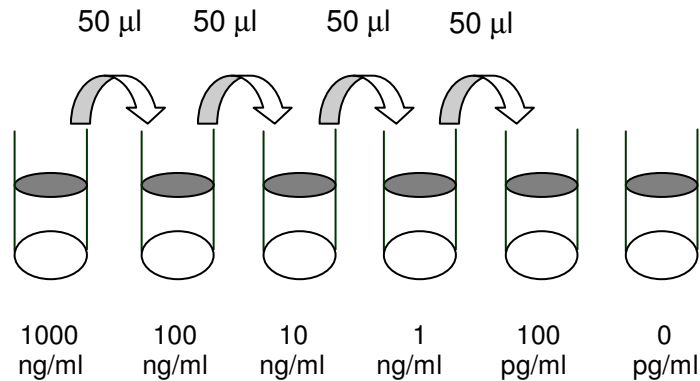
If testing plasma or serum samples, use Assay Diluent A to dilute Item F and Item C. If testing cell culture media or other sample types, use Assay Diluent B to dilute Item F and Item C. For sample and positive control dilutions, refer to steps 5, 6 and 8 of Reagent Preparation.

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 vial of Biotinylated RELM-alpha (Item F) before use. Add 10 μ l of Item F to 5 ml of the appropriate Assay Diluent. Pipette up and down to mix gently. *The final concentration of biotinylated RELM-alpha will be 20 ng/ml.*

This solution will only be used as the diluent in step 4 of Reagent Preparation.

4. Preparation of Standards: Label 6 microtubes with the following concentrations: 1000 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 μ l of biotinylated RELM-alpha solution into each tube, except for the 1000 ng/ml (leave this one empty). *It is very important to make sure the concentration of biotinylated RELM-alpha is 20 ng/ml in all standards.*
 - a. Briefly centrifuge the vial of RELM-alpha (Item C). In the tube labeled 1000 ng/ml, pipette 8 μ l of Item C and 792 μ l of 20 ng/ml biotinylated RELM-alpha solution (prepared in step 5 above). This is your RELM-alpha stock solution (1000 ng/ml RELM-alpha, 20 ng/ml biotinylated RELM-alpha). Mix thoroughly. This solution serves as the first standard.
 - b. To make the 100 ng/ml standard, pipette 50 μ l of RELM-alpha stock solution the tube labeled 100 ng/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 μ l of biotinylated RELM-alpha and 50 μ l of the prior concentration until 100 pg/ml is reached. Mix each tube thoroughly before the next transfer.

d. The final tube (0 pg/ml RELM-alpha, 20 ng/ml biotinylated RELM-alpha) serves as the zero standard (or total binding).



5. 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 6 and 8.
6. 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 4 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% of competition) if diluted as described above. It may be diluted further if desired, but be sure the final concentration of biotinylated RELM-alpha is 20 ng/ml.
7. 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.

8. Sample Preparation: Use Assay Diluent A + biotinylated RELM-alpha to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated RELM-alpha as the diluent. *It is very important to make sure the final concentration of the biotinylated RELM-alpha is 20 ng/ml in every sample.* EXAMPLE: to make a 4-fold dilution of sample, mix together 5 μ l of 10-fold diluted Item F (prepared in step 7), 182.5 μ l of appropriate Assay Diluent, and 62.5 μ l of your sample; mix gently. The total volume is 250 μ l, 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 RELM-alpha to a final concentration of 20 ng/ml.

EXAMPLE: Add 5 μ l of 10-fold diluted Item F to 245 μ l 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.

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

For example: For a 4000-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 3.9 ml 1x Assay Diluent B to prepare a final 4000-fold diluted HRP-Streptavidin solution.

Note: Do not use Assay Diluent A for HRP-Streptavidin preparation in Step 9.

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 μ l of each standard (see Reagent Preparation step 4), positive control (see Reagent Preparation step 6) and sample (see Reagent Preparation step 8) 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.
3. Discard the solution and wash 4 times as directed in Step 3.
4. Add 100 μ l of prepared HRP-Streptavidin solution (see Reagent Preparation step 11) to each well. Incubate with gentle shaking for 45 minutes at room temperature. It is recommended that incubation time should not be shorter or longer than 45 minutes.
5. Discard the solution and wash 4 times as directed in Step 3.
6. 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 with gentle shaking (1-2 cycles/sec).
7. Add 50 μ 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 μ l standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.



3. Add 100 μ l prepared streptavidin solution. Incubate 45 minutes at room temperature.



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



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

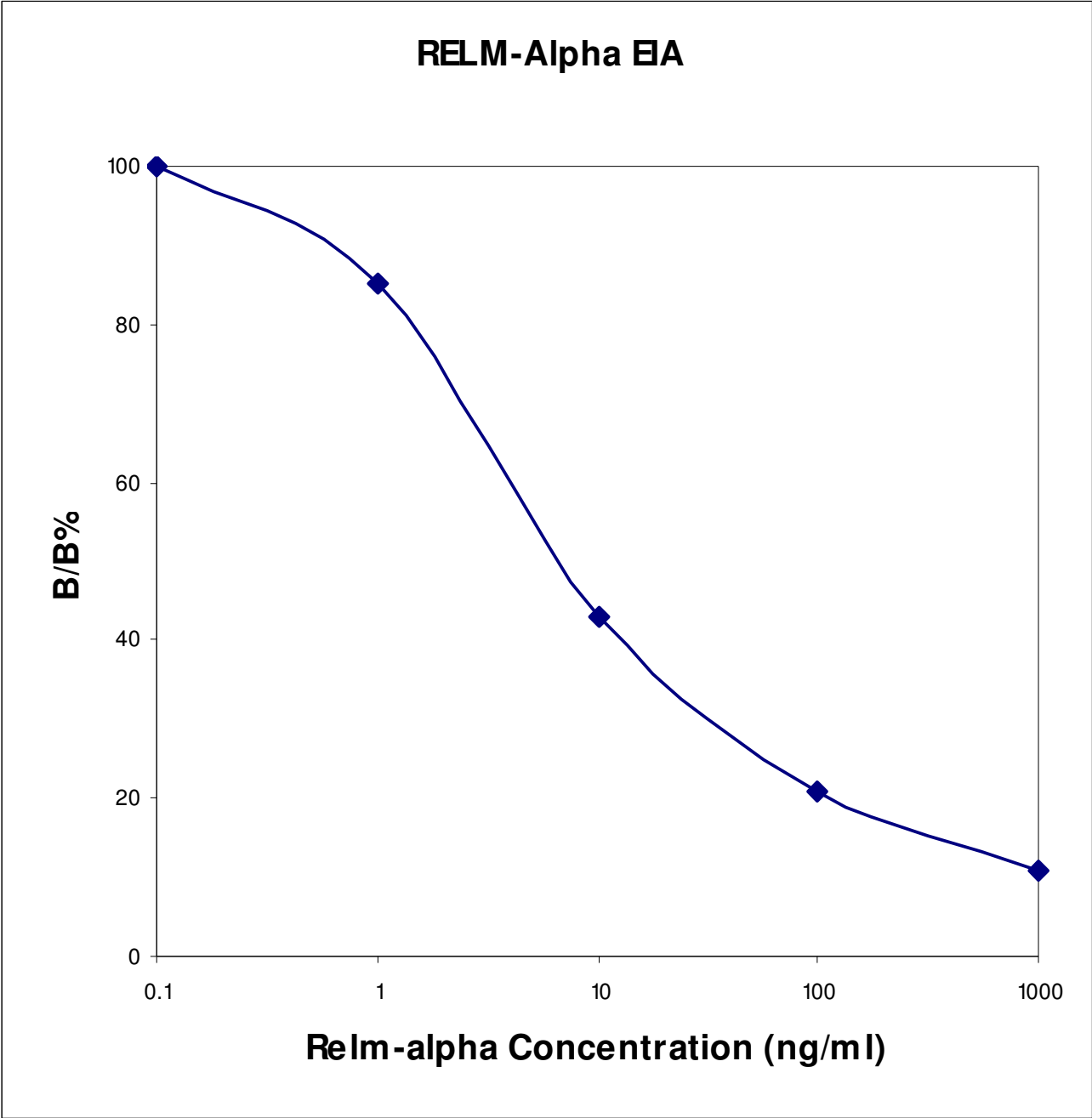
IX. CALCULATION OF RESULTS

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
B₀ = 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 concentration of RELM-alpha is 4.7ng/ml.

C. DETECTION RANGE

0.1-1000 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: Ghrelin, Nesfatin, RELM-alpha, NPY and APC.

XI. REFERENCES

1. Asano T, Sakosda H, Fujishiro M, Anai M, Kushiyaama A, Horike N, Kamata H, Ogihara T, Kurihara H, Uchijima Y (2006). Physiological significance of resistin and resistin-like molecules in the inflammatory process and insulin resistance. *Curr Diabetes Rev.* 2(4):449-54.
2. Miner JL (2004). The adipocyte as an endocrine cell. *J Anim Sci.* 82(3):935-41.

XII. TROUBLESHOOTING GUIDE

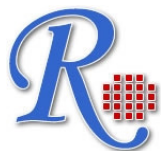
Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol 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 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 for details.

Notes:

This product is for research use only.



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