

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

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

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

**RayBio[®] Angiotensin II Enzyme
Immunoassay Kit Protocol**

(Cat#: EIA-ANGII-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.

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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.

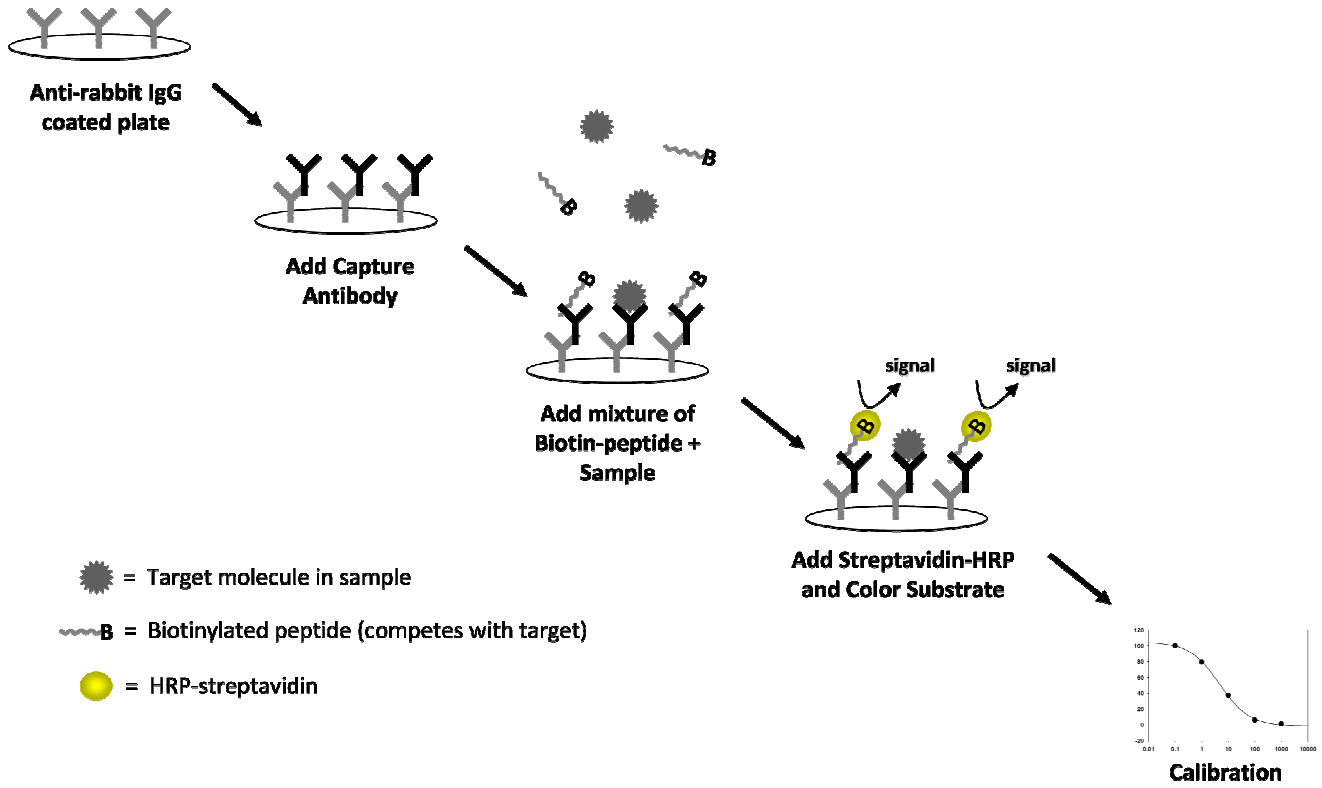
II. GENERAL DESCRIPTION

The RayBio® Angiotensin II Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Angiotensin II peptide based on the 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-Angiotensin II antibody, both biotinylated Angiotensin II peptide and peptide standard or targeted peptide in samples interacts competitively with the Angiotensin II antibody. Uncompeted (bound) biotinylated Angiotensin II 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 Angiotensin II peptide in the standard or samples. This is due to the competitive binding to Angiotensin II antibody between biotinylated Angiotensin II peptide and peptides in 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.

EIA-ANGII-1 can theoretically detect all active angiotensins, including ANGI, ANGII, ANGIII and ANGIV. However, it does not detect inactive angiotensinogen.

Principle of Competitive EIA



III. REAGENTS

1. Angiotensin II 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 Angiotensin II Peptide (Item C): 2 vials, 10 μ l/vial
4. Anti-Angiotensin II polyclonal antibody (Item N): 2 vials, 5 μ l/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 Angiotensin II peptide, (Item F): 2 vials, 20 μ l/vial
8. HRP-Streptavidin concentrate (Item G): 8 μ l 5,000x concentrated HRP-conjugated Streptavidin.
9. Positive control (Item M): 1 vial, 100 μ l
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 Angiotensin II 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 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 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-Angiotensin II Antibody vial (Item N) before use. Add 50 μ l of 1x Assay Diluent B into the vial to

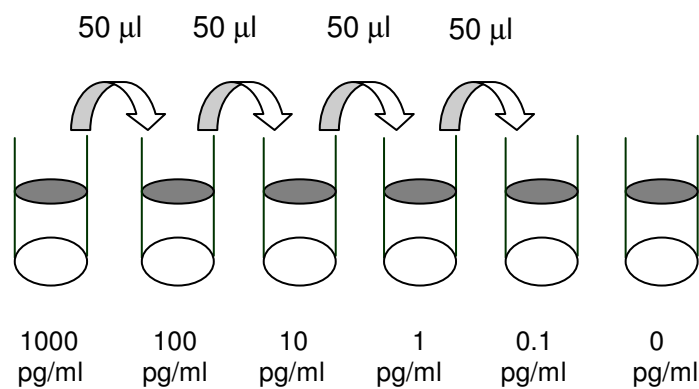
prepare a detection antibody concentrate. Pipette up and down to mix gently.

4. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent B. This is your anti-Angiotensin II 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 Angiotensin II (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 Angiotensin II will be 20 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 μ l of biotinylated Angiotensin II 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 Angiotensin II is 20 pg/ml in all standards.*
 - a. Briefly centrifuge the vial of Angiotensin II (Item C). In the tube labeled 1000 pg/ml, pipette 8 μ l of Item C and 792 μ l of 20 pg/ml biotinylated Angiotensin II solution (prepared in step 5 above). This is your Angiotensin II stock solution (1000 pg/ml Angiotensin II, 20 pg/ml biotinylated Angiotensin II). Mix thoroughly. This solution serves as the first standard.
 - b. To make the 100 pg/ml standard, pipette 50 μ l of Angiotensin II 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 Angiotensin II and 50 μl of the prior concentration until 0.1 pg/ml is reached. Mix each tube thoroughly before the the next transfer.
- d. The final tube (0 pg/ml Angiotensin II, 20 pg/ml biotinylated Angiotensin II) 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 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% competition) if diluted as described above. It may be diluted further if desired, but be sure the final concentration of biotinylated Angiotensin II is 20 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 Angiotensin II to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated Angiotensin II as the diluent. *It is very important to make sure the final concentration of the biotinylated Angiotensin II is 20 pg/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 Angiotensin II to a final concentration of 20 pg/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.
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 a 5,000-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 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 μ l anti-Angiotensin II 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 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 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 μl 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 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-Angiotensin II 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 μ l Stop Solution to each well. Read at 450 nm immediately

IX. CALCULATION OF RESULTS

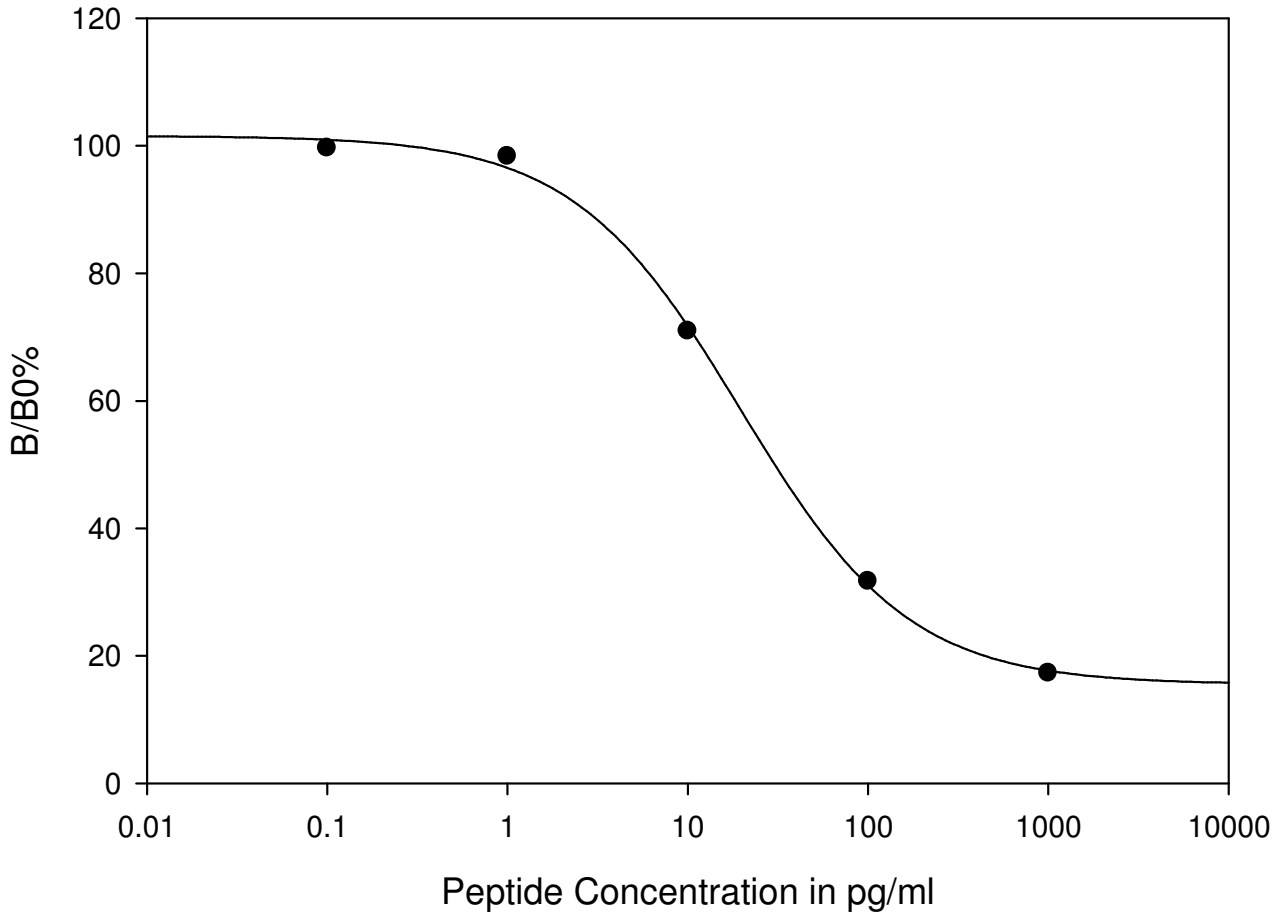
Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis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

ANGII EIA



B. SENSITIVITY

The minimum detectable concentration of Angiotensin II is 2.62 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

Skurk T, Lee YM, Hauner H (May 2001). "Angiotensin II and its metabolites stimulate PAI-1 protein release from human adipocytes in primary culture". *Hypertension* 37 (5): 1336–40.

XII. TROUBLESHOOTING GUIDE

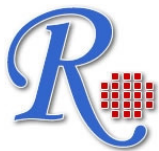
Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol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