BioAim Scientific Inc

Porcine ANP EasyTest™ Competitive ELISA Kit

Cat.No: 2070012

Instruction Manual (Last revised Nov 12, 2015)

For research use only

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I. INTRODUCTION

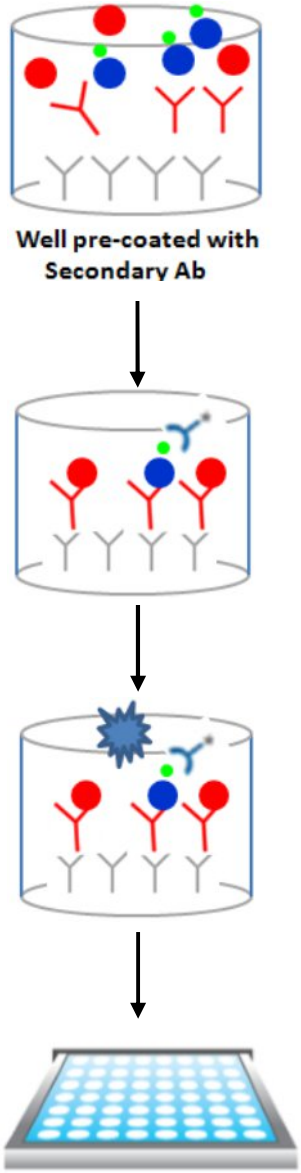
Atrial natriuretic peptide (ANP), atrial natriuretic factor (ANF), is a powerful vasodilator, and a polypeptide hormone secreted by heart muscle cells. ANP is a 28-amino acid peptide with a 17-amino acid ring in the middle of the molecule. The ring is formed by a disulfide bond between two cysteine residues at positions 7 and 23. ANP is closely related to BNP (brain natriuretic peptide) and CNP (C-type natriuretic peptide), which all share a similar amino acid ring structure.

ANP is secreted in response to stretching of the atrial wall，reduced Sympathetic stimulation of β adrenoceptors, hypernatremia, Angiotensin-II, Endothelin and exercise. It is released by muscle cells in the atria of the heart in response to high blood volume. ANP acts to reduce the water, sodium and adipose loads on the circulatory system, thereby reducing blood pressure. ANP has exactly the opposite function of the aldosterone secreted by the zona glomerulosa in regard to its effect on sodium in the kidney.

Three types of atrial natriuretic peptide receptors have been identified on which natriuretic peptides act. They are all cell surface receptors and designated as NPR1, NPR2 and NPR3.The binding of ANP to its receptor causes the conversion of GTP to cGMP and raises intracellular cGMP. As a consequence, cGMP activates a cGMP-dependent kinase (PKG or cGK) that phosphorylates proteins at specific serine and threonine residues. In the medullary collecting duct, the cGMP generated in response to ANP may act not only through PKG but also via direct modulation of ion channels.

The BioAim porcine ANP EasyTest^{TM} Competitive ELISA kit can quantitatively measure ANP in porcine serum or plasma. Other matrices, such as urine and tissue, may be suitable but have not been validated. It is a simple and rapid technology for the quantitation of antigen in a range of sample matrices. The whole process takes 2 hours with high accuracy and precision.

II. PRINCIPLE



1. Microplate was pre-coated with secondary Ab;
2. Add Anti-ANP Ab first, then sample/Standard and biotinylated peptide;
3. Incubate 1 h at room temperature.

1. Wash;
2. Add Streptavidin-HRP;
3. Incubate 45min at room temperature.

1. Wash;
2. Add TMB substrate;
3. Incubate 15 min at room temperature.

1. Add stop solution;
2. Read plate at 450nm.

= Anti-rabbit IgG
= Primary antibody
= Sample /standard peptide
= Biotinylated peptide
= Streptavidin -HRP
= TMB substrate

III. MATERIALS SUPPLIED

1. Porcine ANP Microplate: 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. Anti-ANP antibody: 6µl/vial, 2 vials.
3. Standard ANP peptide: 10µl/vial, 2 vials.
4. Biotinylated ANP peptide: 10µl/vial, 2 vials.
5. HRP-Streptavidin concentrate: 20 µl 200x concentrated HRP-conjugated Streptavin.
6. 5x Assay Diluent: 1vial, 15 ml.
7. 20x Wash Buffer Concentrate: 1 Vial, 25 ml.
8. TMB Substrate: 12 ml.
9. Stop Solution: 8 ml of 0.2 M sulfuric acid.
10. User Manual.
11. Plate sealer: 2 pieces.

IV. STORAGE

1. The kit can be stored for up to 6 months at 2º to 8ºC from the date of shipment.
2. Standard can be stored at -20 ºC or -80 ºC. Use freshly prepared standard within 12 hours (stored at 2~8ºC).
3. Opened Microplate Wells or reagents may be store for up to 1 month at 2 to 8ºC. Return unused strip to the pouch containing desiccant pack, reseal along entire edge and keep in 2~8ºC.
4. Avoid repeated freeze-thaw cycles.

V. ADDITIONAL MATERIALS REQUIRED

1. Distilled or deionized water.
2. Precision pipettes, with disposable plastic tips.
3. Beakers, flasks, cylinders necessary for preparation of reagents.
4. Microplate washing device (multichannel pipette or automated microplate washer).

5. Microplate shaker.
6. Microplate reader capable of reading at 450 nm.
7. Materials used for sample preparation.

VI. PRECAUTIONS

1. All reagents must be at room temperature (18ºC to 25ºC) before running assay.
2. Do not mix or substitute reagents with those from other lots or other sources.
3. Do not use kit reagents beyond expiration date on label.
4. Do not expose kit reagents to strong light during storage or incubation.
5. Use disposable pipette tips for each transfer to avoid microbial contamination or cross contamination of reagents.
6. Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results.
7. Avoid contact of stop solution with skin or eyes. If contact occurs, immediately flush area with copious amounts of water.
8. Do not use TMB substrate solution if it has begun to turn blue.
9. Do not expose bleach to work area during actual test procedure because of potential interference with enzyme activity.

VII. SAMPLE PREPARATION

In general, samples can be assayed with no prior purification. However, many plasma components can cause interference with the assay. Therefore purification is necessary for accurate measurement. The protocol shown is one such purification method. However, you may choose a different protocol based on your own requirements and expertise.

Protocol for 100 mg columns. Adjust volumes accordingly if using 200 mg columns.

Materials

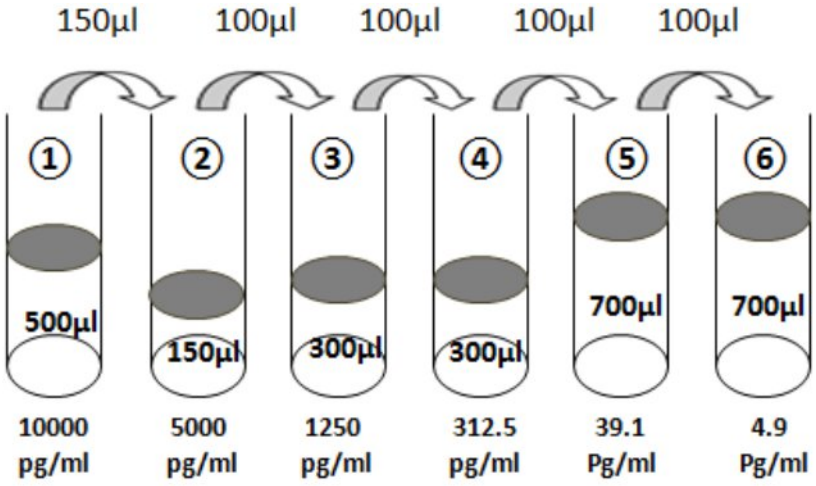
100 or 200 mg C18 Sep-Pak columns (e.g. Waters #WAT023590 or WAT054945)
Column activation: Methanol
Column washing and binding: 0.1% Trifluoroacetic acid (TFA), 99.9% Water
Elution: 60% Acetonitrile (ACN), 40% water
Vacuum centrifuge or nitrogen gas

Suggested Protocol

1. In a 1.5 mL centrifuge tube, add 500µl 0.1% TFA to 500µl plasma sample. Mix and centrifuge at maximum speed, cold (4°C), for 20 minutes.
2. Carefully remove the supernatant and store on ice until Sep-Pak column is ready.
3. Equilibrate a Sep-Pak column by washing with 0.5 ml Methanol once followed by 1 ml 0.1 % TFA 3 times. Don't allow the SPE cartridge to dry.
4. Load the acidified plasma (1ml) and let it through the column by gravity flow.
5. Slowly wash column 2 times with 1 ml 0.1 % TFA and discard the wash.
6. Elute peptide slowly using two applications of 1ml 60% ACN.
7. Dry peptide by vacuum centrifugation or by a suitable substitute method. It is imperative that all the solvent be removed as even trace quantities can affect the assay.
8. Add 500 µl the assay buffer to re-suspend the pellet at 1 X concentration and assay immediately. If necessary, sample can be concentrated in this step by resuspending in 250 µl assay buffer, which will result in two-fold concentration.

VIII.REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18~25°C) before use.
2. **Assay diluent**
   Dilute the concentrated assay diluent 1:5 with distilled water (e.g. 10ml plus 40ml).
3. **Wash buffer**
   Dilute the concentrated wash buffer 1:20 with distilled water (e.g. 20ml plus 380ml).
4. **Anti-ANP antibody**
   Briefly centrifuge the vial before use. Add 1494 µl 1x assay diluent to the vial. Pipette up and down to mix thoroughly. A vial can be used for around 60 wells.
5. **Standard ANP peptide**
   a. Briefly spin the standard vial before use. Add 490 µl 1x Assay Diluent to prepare a 10000pg/ml standard solution. Gently vortex to mix. This is standard #1.
   b. Label 5 tubes #2 through #6. Pipette 150 µl 1x assay diluent into tube #2, pipette 300 µl 1x assay buffer into tube #3 and #4. Pipette 700 µl 1x assay diluent into tubes #5 and #6.
   c. Take 150 µl from standard #1 and add to tube #2. Mix thoroughly. Add 100 µl from tube #2 to tube #3. Mix thoroughly. Continue this for tubes #4 through #6.



The concentrations of ANP in the tubes are labeled above. Diluted standards should be used within 60 minutes of preparation.

6. **Biotinylated ANP peptide**
   Briefly centrifuge the vial before use. Add 1490 µl of 1x assay diluent to make the final concentration enough for 60 wells.
7. **Streptavidin-HRP**
   The HRP-Streptavidin concentrate should be diluted 200- fold with 1x assay diluent. Do not store diluted Streptavidin-HRP.

IX. ASSAY PROCEDURE

1. All reagents must be brought to room temperature (18-25°C) prior to use. Place the required number of microwells in the holder. It is recommended that all samples, standards, and blanks be run in duplicate.
2. Pipette 25 µl of anti-ANP antibody into all wells.
3. Pipette 75 ul of 1x assay diluent into the blank wells.
4. Pipette 50 µl of 1x assay diluent into the Bo (0 ng/ml standard) wells.
5. Pipette 50 µl of Standards #1 through #6 or from #6 to #1 (in reverse order of serial dilution) to the appropriate wells.
6. Pipette 50 µl of samples to the appropriate wells.
7. Pipette 25 µl of biotinylated peptide into each well except the Blank wells.
8. Seal the plate. Incubate for 1 hour at room temperature with gentle shaking.
9. Empty the contents of the wells and wash by adding 300 µl of wash buffer to every well. Repeat 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
10. Pipette 100 µl of diluted streptavidin-HRP solution to each well.

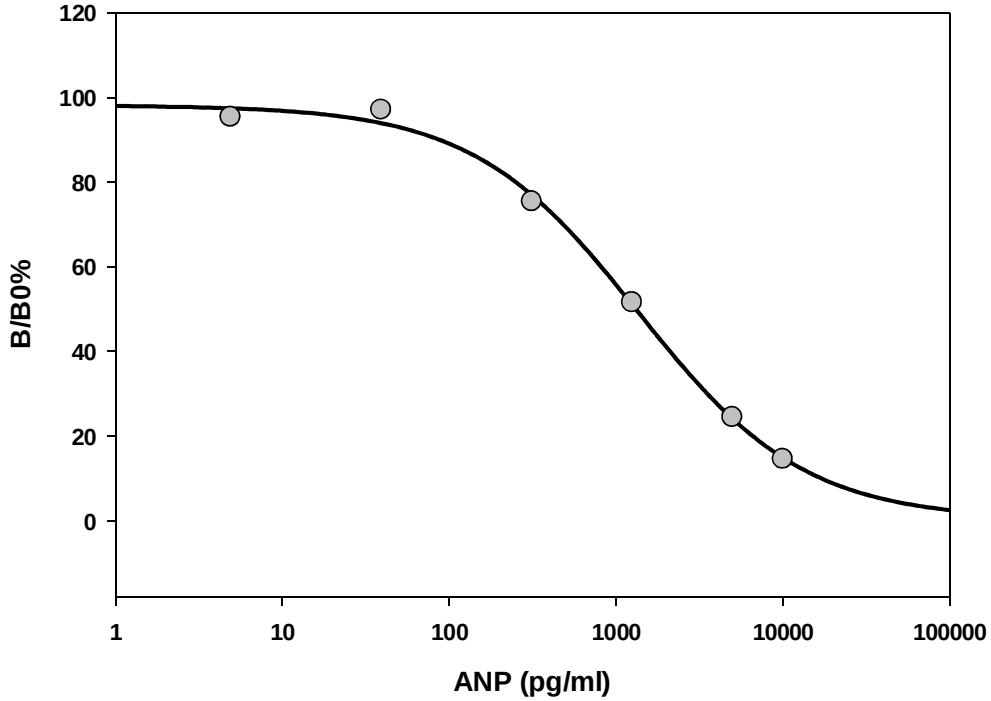
11. Seal the plate. Incubate for 45 min at room temperature with gentle shaking.
12. Wash plate as above (Step 9).
13. Pipette 100 µl of TMB Substrate Solution into each well. Incubate plate for 15 minutes at room temperature in the dark with gentle shaking.
14. Add 50 µl of stop solution to each well.
15. Read absorbance at 450nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract the optical density readings at 570nm from readings at 450nm.

X. CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of duplicate standards, samples and controls. Subtract the average blank well optical density.
2. 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 – blank OD)/ (Bo – blank OD) *100

   Where B = OD of sample or standard and Bo = OD of zero Standard, total binding.
3. A representative standard curve is shown below. This standard curve is for demonstration only. A standard curve must be run with each assay by operator.



XI. PERFORMANCE

A. Sensitivity

The minimum detectable dose of ANP was determined to be 15pg/ml. This is defined as two standard deviations above the mean optical density of 20 replicates of the zero standards.

B. Specificity

No cross-reactivity was identified with the following peptides: Vasopressin, ANGII, BNP, and CNP.

C. Reproducibility

Intra-Assay CV%: <10%

Inter-Assay CV%: <15%

XII. REFERENCES

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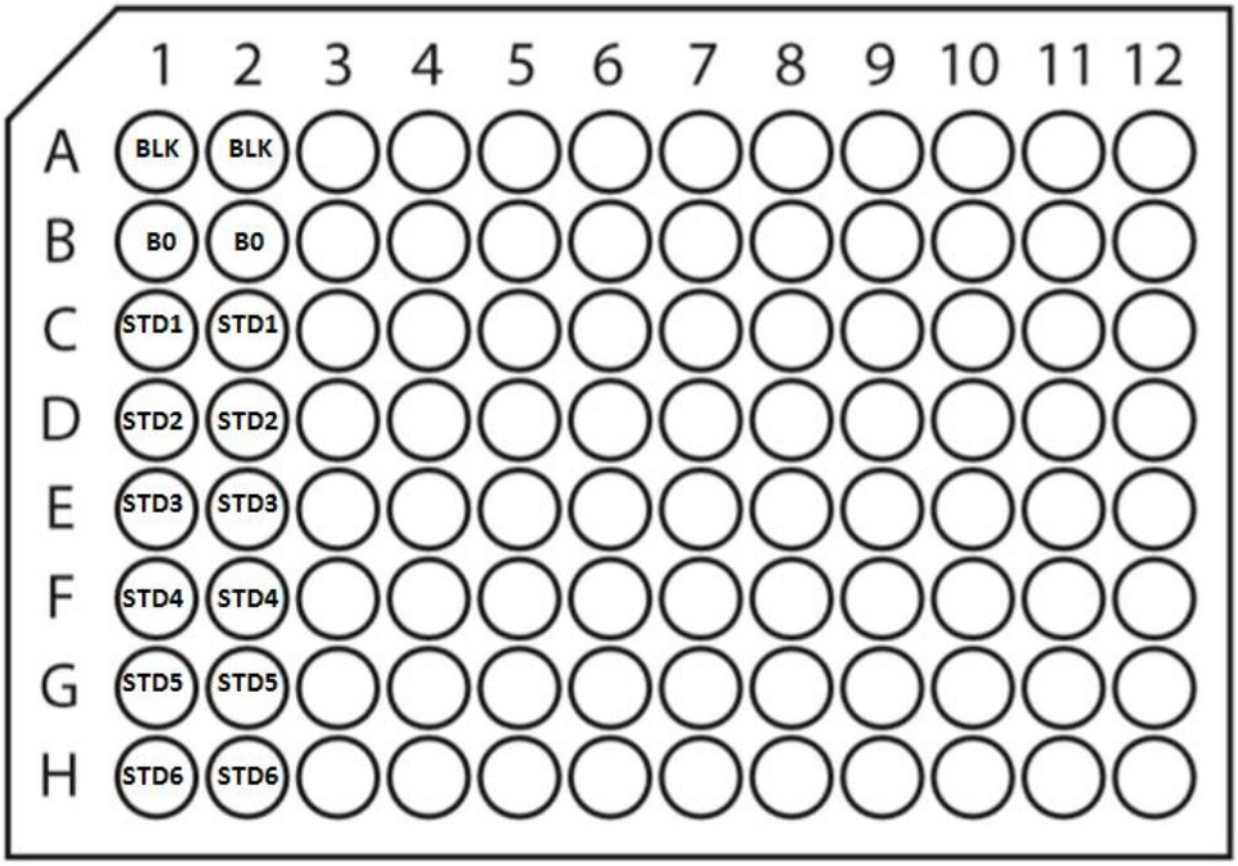
3. de Bold AJ (Nov 1985). Science 230 (4727): 767–70.
4. Yan W, Wu F, etal. (Jul 2000). Proceedings of the National Academy of Sciences of the United States of America 97 (15): 8525–9.
5. Kokkonen UM, Pösö AR, etal. (Apr 2002). Journal of Veterinary Medicine. 49 (3): 144–50.

XIII. TROUBLESHOOTING

Problem	Cause	Solution
1.Poor standard curve	1. Inaccurate pipetting 2. Improper standard dilution	1. check pipettes; 2. Ensure briefly spin the vial of standard, take the right amount to dilution.
2. Low signal	1. Too brief incubation time 2. Inadequate reagent volumes or improper dilution	1. ensure adequate incubation time; 2. Check pipettes and ensure corrected preparation.
3. Large CV	Inaccurate pipetting	1. Check pipettes; 2. Accurately perform each step.
4.High background	1. Plate is insufficiently washed; 2. Wash buffer contamination	1.Follow the manual correctly; if using a plate washer, check that all ports are working functionally; 2. Prepare fresh buffer.
5. Low sensitivity	1.EIA kit improper storage 2. Stop solution	1. Follow the manual to store each component correctly; 2. Add enough stop solution to each well.

XIV. ASSAY DIAGRAM



Bioaim Scientific Inc

Unit 6, 27 Casebridge Court
Scarborough, ON, M1B 4Y4
Canada
Tel: 416-286-6868
www.bioaimscientific.com