

Rat ANP ELISA Kit

Introduction

Atrial natriuretic peptide (ANP), a 28 amino acids polypeptide, is mainly secreted from the atrium of the heart where it is stored in secretory granules as a 136 amino acids pro-hormone (1). Upon its secretion, induced by increases in atrial pressure and stretch, the pro-hormone is processed by a serine protease to the active 28 amino acids peptide. The peptide binds with high affinity to the membrane receptor guanylate cyclase GC-A, leading to increased intracellular cGMP levels (2). Increased ANP plasma level has been identified as predictors of cardiac dysfunction and prognosis in congestive heart failure and ischemic heart disease (3-5). Lower plasma levels of ANP will lead to sodium retention, and an increase in plasma volume, resulting in an increase blood pressure (6).

Principal of the Assay

The Rat ANP ELISA kit is designed for detection of rat ANP in plasma, serum, tissue extract, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures ANP in less than 5 hours. A polyclonal antibody specific for rat ANP has been pre-coated onto a microplate. The ANP in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for ANP, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution.

Reagents

- **Rat ANP Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against rat ANP.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.

- **Rat ANP Standard:** Rat ANP in a buffered protein base (20 ng, lyophilized).
- **Biotinylated ANP Antibody:** A 100-fold biotinylated polyclonal antibody against rat ANP (80 µl).
- **MIX Diluent Concentrate (10x):** A 10-fold buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate:** A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution:** A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8°C or -20°C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20°C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8°C.
- Store Standard at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl and multiple channel).
- Deionized or distilled reagent grade water.

Sample Collection and Storage

- **Plasma:** Collect plasma using a final concentration of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000x g for 10 minutes and assay undiluted plasma for medium and high level of ANP. Samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles. For low level of ANP, please use ANP extraction protocol below.

Low Level ANP Extraction Protocol

Buffer A: 1% trifluoroacetic acid (TFA, HPLC Grade) in H₂O

Buffer B: 60% acetonitrile (HPLC Grade) in 1% TFA

1. Acidify the sample with equal amount of Buffer A (1 ml sample: 1 ml Buffer A). Mix and centrifuge samples at 6000 x g for 20 minutes at 4°C.
2. Pack an extraction column using 200 mg of C18 resin. Pre-equilibrate the column with 1 ml of Buffer B once and then with 3 ml of Buffer A three times.
3. Load the acidified plasma solution onto the pre-treated C18 column.
4. Slowly wash the column with 3 ml of Buffer A twice.
5. Elute the peptide slowly with 3 ml of Buffer B once and collect the eluant.
6. Evaporate and dry the eluant in a freeze dryer or use a suitable substitute method.
7. Keep the dried extract at -20°C and perform the assay as early as possible. Reconstitute the dried extract with 200 µl of MIX Diluent before the assay. Check sample pH with pH papers. If sample pH is below 6.5, neutralize the sample with 20 µl of 1M NaH₂PO₄. If the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the sample accordingly.

- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and perform the assay for medium and high level of ANP. Samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles. For low level of ANP, please use the extraction protocol as above.
- **Tissue:** Extract tissue samples with 0.1 M phosphate-buffered saline (pH7.4) containing 1% Triton X-100 and centrifuge at 14000 x g for 20 min. Collect the supernatant and measure the protein concentration. Freeze remaining extract at -20°C or below.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent Concentrate 1:10 with reagent grade water. Store for up to 1 month at 2-8°C.
- **Rat ANP Standard:** Reconstitute the 20 ng of rat ANP Standard with 5 ml of MIX Diluent to generate a standard solution of 4 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the Standard (4 ng/ml) twofold with MIX Diluent to generate 2, 1, 0.5 and 0.25 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C.

Standard Point	Dilution	[Rat ANP] (ng/ml)
P1	1 part Standard (4 ng/ml)	4.000
P2	1 part P1 + 1 part MIX Diluent	2.000
P3	1 part P2 + 1 part MIX Diluent	1.000
P4	1 part P3 + 1 part MIX Diluent	0.500
P5	1 part P4 + 1 part MIX Diluent	0.250
P6	MIX Diluent	0.000

- **Biotinylated ANP Antibody (100x):** Dilute the antibody 1:100 with MIX Diluent. Spin down the Biotinylated Antibody briefly and only dilute the desired amount of the antibody. Any remaining solution should be frozen at -20°C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20°C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.

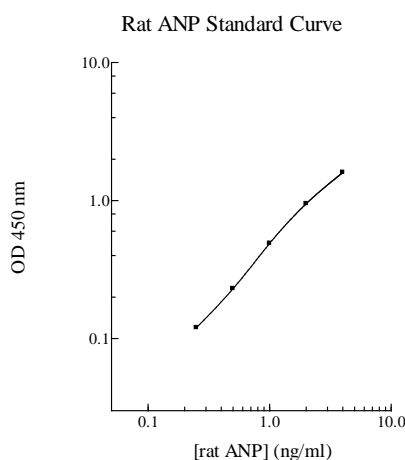
- Wash five times with 200 μ l of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 μ l of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 μ l of Biotinylated rat ANP Antibody to each well and incubate for two hours.
- Wash the microplate as described above.
- Add 50 μ l of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 μ l of Chromogen Substrate per well and incubate for approximately 10 minutes or till the optimal color density develops. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 μ l of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- The minimum detectable dose of rat ANP is typically ~ 0.2 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.9 % and 7.0% respectively.

Cross-Reactivity

Species	% Cross Reactivity
Canine	100%
Bovine	10%
Monkey	20%
Mouse	100%
Rat	100%
Swine	100%
Rabbit	100%
Human	100%

References

- (1) Atlas SA *et al.* (1992) p.1577-673
- (2) Leitman, DC *et al.* (1988) *J. Biol. Chem.* 263:3720-8
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