ab108797 – ANP (NPPA) Rat ELISA Kit

Instructions for Use

For the quantitative measurement of rat ANP in plasma, serum, tissue extract and cell culture supernatants.

This product is for research use only and is not intended for diagnostic use.

Version 8 Last Updated 4 January 2022

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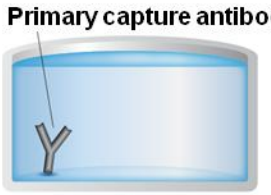
1. BACKGROUND

Abcam's ANP (NPPA) Rat *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of ANP concentrations in cell culture supernatants, tissue-extract, serum and plasma.

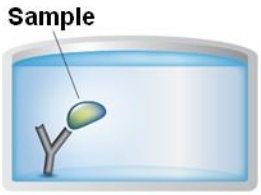
An ANP specific antibody has been precoated onto 96-well plates and blocked. Standards or test samples are added to the wells and subsequently an ANP specific biotinylated detection antibody is added and then followed by washing with wash buffer. Streptavidin-Peroxidase Conjugate is added and unbound conjugates are washed away with wash buffer. TMB is then used to visualize Streptavidin-Peroxidase enzymatic reaction. TMB is catalyzed by Streptavidin-Peroxidase to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the amount of ANP captured in plate.

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. 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. Increased ANP plasma level has been identified as predictors of cardiac dysfunction and prognosis in congestive heart failure and ischemic heart disease. Lower plasma levels of ANP will lead to sodium retention, and an increase in plasma volume, resulting in an increase blood pressure.

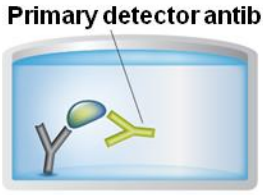
2. ASSAY SUMMARY



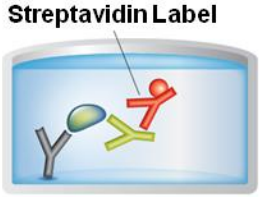
Prepare all reagents, samples and standards as instructed.



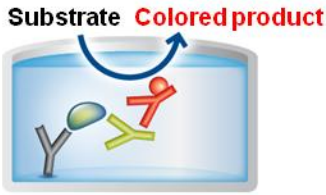
Add standard or sample to each well used. Incubate at room temperature.



Wash and add prepared biotin antibody to each well. Incubate at room temperature.



Wash and add prepared Streptavidin-Peroxidase Conjugate. Incubate at room temperature.



Add Chromogen Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 4°C immediately upon receipt, apart from the SP Conjugate & Biotinylated Antibody, which should be stored at -20°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9 & 10.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
ANP Microplate (12 x 8 well strips)	96 wells	4°C
ANP Standard	1 vial	4°C
10X Diluent N Concentrate	30 mL	4°C
40X Biotinylated Rat ANP Antibody	150 µL	-20°C
100X Streptavidin-Peroxidase Conjugate (SP Conjugate)	80 µL	-20°C
Chromogen Substrate	7 mL	4°C
Stop Solution	11 mL	4°C
20X Wash Buffer Concentrate	2 x 30 mL	4°C
Sealing Tapes	3	N/A

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 1 µL to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- 6 tubes to prepare standard or sample dilutions.

7. LIMITATIONS

- Do not mix or substitute reagents or materials from other kit lots or vendors.

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Equilibrate all reagents to room temperature (18-25°C) prior to use. Prepare fresh reagents immediately prior to use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

9.1 **1X Diluent N**

Dilute the 10X Diluent N Concentrate 1:10 with reagent grade water. Mix gently and thoroughly. *Store for up to 1 month at 4°C.*

9.2 **1X Wash Buffer**

Dilute the 20X Wash Buffer Concentrate 1:20 with reagent grade water. Mix gently and thoroughly.

9.3 **1X Biotinylated ANP Detector Antibody**

9.3.1 The stock Biotinylated ANP Antibody must be diluted with 1X Diluent N according to the label concentration to prepare 1X Biotinylated ANP Antibody for use in the assay procedure. Observe the label for the "X" concentration on the vial of Biotinylated ANP Antibody.

9.3.2 Calculate the necessary amount of 1X Diluent N to dilute the Biotinylated ANP Antibody to prepare a 1X Biotinylated ANP Antibody solution for use in the assay procedure according to how many wells you wish to use and the following calculation:

Number of Wells Strips	Number of Wells	(V_T) Total Volume of 1X Biotinylated Antibody (µL)
4	32	1,760
6	48	2,640
8	64	3,520
10	80	4,400
12	96	5,280

Any remaining solution should be frozen at -20°C.

Where:

C_S = Starting concentration (X) of stock Biotinylated ANP Antibody (variable)

C_F = Final concentration (always = 1X) of 1X Biotinylated ANP Antibody solution for the assay procedure

V_T = Total required volume of 1X Biotinylated ANP Antibody solution for the assay procedure

V_A = Total volume of (X) stock Biotinylated ANP Antibody

V_D = Total volume of 1X Diluent N required to dilute (X) stock Biotinylated ANP Antibody to prepare 1X Biotinylated ANP solution for assay procedures

Calculate the volume of (X) stock Biotinylated Antibody required for the given number of desired wells:

$$(C_F / C_S) \times V_T = V_A$$

Calculate the final volume of 1X Diluent N required to prepare the 1X Biotinylated ANP Antibody:

$$V_T - V_A = V_D$$

Example:

NOTE: This example is for demonstration purposes only. Please remember to check your antibody vial for the actual concentration of antibody provided.

C_S = 50X Biotinylated ANP Antibody stock

C_F = 1X Biotinylated ANP Antibody solution for use in the assay procedure

V_T = 3,520 µL (8 well strips or 64 wells)

$$(1X/50X) \times 3{,}520\ \mu L = 70.4\ \mu L$$

$$3{,}520\ \mu L - 70.4\ \mu L = 3{,}449.6\ \mu L$$

V_A = 70.4 µL total volume of (X) stock Biotinylated ANP Antibody required

V_D = 3,449.6 µL total volume of 1X Diluent N required to dilute the 50X stock Biotinylated Antibody to prepare 1X Biotinylated ANP Antibody solution for assay procedures

9.3.3 First spin the Biotinylated ANP Antibody vial to collect the contents at the bottom.

9.3.4 Add calculated amount V_A of stock Biotinylated ANP Antibody to the calculated amount V_D of 1X Assay Diluent N. Mix gently and thoroughly.

9.4 **1X SP Conjugate**

Spin down the 100X Streptavidin-Peroxidase Conjugate (SP Conjugate) briefly and dilute the desired amount of the conjugate 1:100 with 1X Diluent N.

Any remaining solution should be frozen at -20°C.

10. STANDARD PREPARATIONS

- **Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.**
- **Any remaining standard should be stored at -20°C after reconstitution and used within 30 days.**
- **This procedure prepares sufficient standard dilutions for duplicate wells.**

10.1 Reconstitution of the ANP Standard vial to prepare the 2 ng/mL ANP **Standard #1:**

10.1.1 First consult the ANP Standard vial to determine the mass of protein in the vial.

10.1.2 Calculate the appropriate volume of 1X Diluent N to add when resuspending the ANP Standard vial to produce a 2 ng/mL ANP **Standard #1** by using the following equation:

C_S = Starting mass of ANP Standard (see vial label) (ng)

C_F = 2 ng/mL ANP **Standard #1** final required concentration

V_D = Required volume of 1X Diluent N for reconstitution (µL)

Calculate total required volume 1X Diluent N for resuspension:

$$(C_S / C_F) \times 1{,}000 = V_D$$

Example:

NOTE: This example is for demonstration purposes only. Please remember to check your standard vial for the actual amount of standard provided.

C_S = 20 ng of ANP Standard in vial

C_F = 2 ng/mL ANP **Standard #1** final concentration

V_D = Required volume of 1X Diluent N for reconstitution

$$(20 \text{ ng} / 2 \text{ ng/mL}) \times 1{,}000 = 10{,}000 \text{ µL}$$

10.1.3 First briefly spin the ANP Standard Vial to collect the contents on the bottom of the tube.

10.1.4 Reconstitute the ANP Standard vial by adding the appropriate calculated amount V_D of 1X Diluent N to the vial to generate the 2 ng/mL ANP **Standard #1**. Mix gently and thoroughly.

10.2 Allow the reconstituted 2 ng/mL ANP **Standard #1** to sit for 10 minutes with gentle agitation prior to making subsequent dilutions

10.3 Label five tubes #2 – 6.

10.4 Add 360 µL of 1X Diluent N to tubes #2 – 6.

10.5 To prepare **Standard #2**, add 120 µL of the **Standard #1** into tube #2 and mix gently.

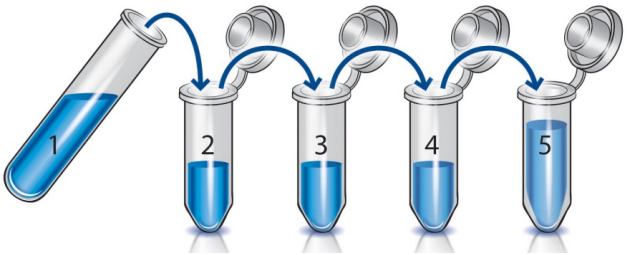
10.6 To prepare **Standard #3**, add 120 µL of the **Standard #2** into tube #3 and mix gently.

10.7 Using the table below as a guide, prepare subsequent serial dilutions.

10.8 1X Diluent N serves as the zero standard, 0 ng/mL (tube #6)

Standard Dilution Preparation Table

Standard #	Volume to Dilute (µL)	Volume Diluent N (µL)	Total Volume (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Step 10.1				2.00
2	120	360	480	2.00	0.500
3	120	360	480	0.500	0.125
4	120	360	480	0.125	0.031
5	120	360	480	0.031	0.008
6	-	360	360	-	0.00



11. SAMPLE PREPARATION

11.1 Plasma

Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3,000 x *g* for 10 minutes and assay undiluted plasma for medium and high level of ANP. Store samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

For low level ANP extraction

Buffer A: 1% trifluoroacetic acid (TFA, HPLC Grade) in H_2O.

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

11.1.1 Acidify the sample with equal amount of Buffer A (1 mL sample: 1 mL Buffer A). Mix and centrifuge samples at 6,000 x *g* for 20 minutes at 4°C.

11.1.2 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.

11.1.3 Load the acidified plasma solution onto the pre-treated C18 column.

11.1.4 Slowly wash the column with 3 mL of Buffer A twice.

11.1.5 Elute the peptide slowly with 3 mL of Buffer B once and collect the eluant.

11.1.6 Evaporate and dry the eluant in a freeze dryer or use a suitable substitute method.

11.1.7 Keep the dried extract at -20°C and perform the assay as early as possible. Reconstitute the dried extract with 200 µL of 1X Diluent N before the assay. Check sample pH with pH papers. If sample pH is below 6.5, neutralize the sample with 20 µL of 1M NaH_2PO_4. If the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the sample accordingly.

11.2 Serum

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3,000 x *g* for 10 minutes. The sample is suggested for use at 1x for medium and high level of ANP. The undiluted 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.

11.3 **Cell cultures**

Centrifuge cell culture media at 1500 rpm for 10 minutes at 4°C to remove debris. Collect supernatants and assay. If necessary, dilute samples into Diluent N; user should determine optimal dilution factor depending on application needs Store samples at -80°C. Avoid repeated freeze-thaw cycles.

11.4 **Tissue**

Extract tissue samples with 0.1 M phosphate-buffered saline (pH 7.4) containing 1% Triton X-100 and centrifuge at 14,000 x *g* for 20 min. Collect the supernatant and measure the protein concentration. Freeze remaining extract at -80°C. Avoid repeated freeze thaw cycles.

11.5 **Cell Lysate**

Rinse cell with cold PBS and then scrape the cell into a tube with 5 ml of cold PBS and 0.5 M EDTA. Centrifuge suspension at 1500 rpm for 10 minutes at 4°C and aspirate supernatant. Resuspend pellet in ice-cold Lysis Buffer (PBS, 1% Triton X-100, protease inhibitor cocktail). For every 1 x 106 cells, add approximately 100 µl of ice-cold Lysis Buffer. Incubate on ice for 60 minutes. Centrifuge at 13000 rpm for 30 minutes at 4°C and collect supernatant. If necessary, dilute samples into Diluent N; user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well plate strips should be returned to the plate packet and stored at 4°C.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section.

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature (18 - 25°C) prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

13.1 Prepare all reagents, working standards and samples as instructed. Equilibrate reagents to room temperature before use. The assay is performed at room temperature (18-25°C).

13.2 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.

13.3 Add 50 µL of ANP Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.

13.4 Wash five times with 200 µL of 1X Wash Buffer manually. Invert the plate each time and decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µL of 1X Wash Buffer and then invert the plate, decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid.

13.5 Add 50 µL of 1X Biotinylated ANP Antibody to each well and incubate for two hours.

13.6 Wash microplate as described above.

13.7 Add 50 µL of 1X SP Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

13.8 Wash microplate as described above.

13.9 Add 50 µL of Chromogen Substrate per well and incubate for 30 minutes or till the optimal blue colour density

develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

13.10 Add 50 µL of Stop Solution to each well. The color will change from blue to yellow.

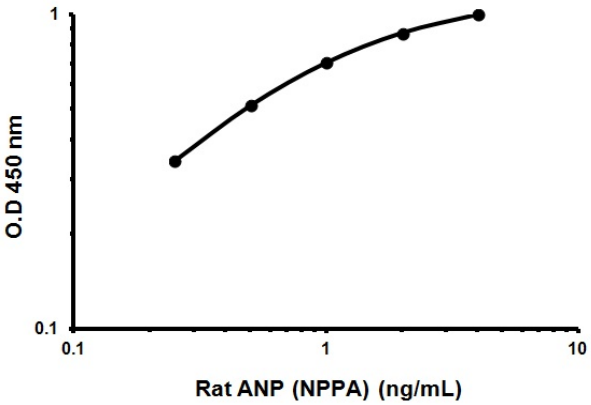
13.11 Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

14. CALCULATIONS

Calculate the mean value of the 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.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.



16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The minimum detectable dose of ANP is typically ~ 6.7 pg/mL.

RECOVERY –

Standard Added Value: 0.031 – 0.5 ng/mL

Recovery %: 86 – 109.

Average Recovery %: 102

PRECISION –

	Intra-Assay	Inter-Assay
% CV	5.6	10.1

17. ASSAY SPECIFICITY

Species	% Cross Reactivity
Canine	<40
Bovine	None
Monkey	<40
Mouse	<40
Rat	100
Swine	100
Human	<40

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Improper standard dilution	Confirm dilutions made correctly
	Standard improperly reconstituted (if applicable)	Briefly spin vial before opening; thoroughly resuspend powder (if applicable)
	Standard degraded	Store sample as recommended
	Curve doesn't fit scale	Try plotting using different scale
Low signal	Incubation time too short	Try overnight incubation at 4ºC
	Target present below detection limits of assay	Decrease dilution factor; concentrate samples
	Precipitate can form in wells upon substrate addition when concentration of target is too high	Increase dilution factor of sample
	Using incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types
	Sample prepared incorrectly	Ensure proper sample preparation/dilution
Large CV	Bubbles in wells	Ensure no bubbles present prior to reading plate
	All wells not washed equally/thoroughly	Check that all ports of plate washer are unobstructed wash wells as recommended
	Incomplete reagent mixing	Ensure all reagents/master mixes are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes and ensure accurate pipetting
	Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (eg. minimize freeze/thaws cycles)

Problem	Cause	Solution
High background/ Low sensitivity	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Make fresh wash buffer
	Waiting too long to read plate after adding STOP solution	Read plate immediately after adding STOP solution
	Improper storage of ELISA kit	Store all reagents as recommended. Please note all reagents may not have identical storage requirements.
	Using incompatible sample type (e.g. Serum vs. cell extract)	Detection may be reduced or absent in untested sample types

19. NOTES

For all technical and commercial enquires please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)