ARBOR ASSAYS

DetectX®

Atrial Natriuretic Peptide (ANP)

Enzyme Immunoassay Kit

1 Plate Kit	Catalog Number K026-H1
5 Plate Kit	Catalog Number K026-H5

SPECIES INDEPENDENT

Sample Types Validated:

Plasma, Urine and Tissue Culture Media

Please read this insert completely prior to using the product.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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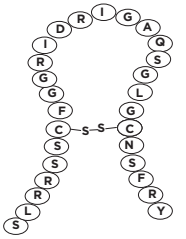
Background

Atrial Natriuretic Peptide (ANP) was discovered in the early 1980s and described in publications in 1983/4[1,2]. ANP is derived from a 151 amino acid long protein called preproANP[3]. The prepro protein is cleaved to generate proANP. This principle storage form of the peptide is the proANP form which is 126 amino acids long. ANP is derived from amino acids 99-126 to form the 28 amino acid peptide with a disulfide bond between amino acids 7 and 23. ANP is the predominant member of a family of structurally and functionally related peptide hormones that exert a wide array of effects on cardiovascular and renal function. The combined actions of ANP on vasculature, kidneys, and adrenals serve both acutely and chronically to reduce systemic blood pressure as well as intravascular volume[4,5]. ANP and the related brain natriuretic peptide bind to their common receptor, membrane-type guanylyl cyclase-A and binding leads to biological actions through a cGMP-dependent pathway.

Atrial Natriuretic Peptide



1. de Bold AJ, and Flynn TG. "Cardionatrin I-a novel heart peptide with potent diuretic and natriuretic properties.", 1983, Life. Sci., 33:297-302.

2. Kangawa K, and Matsuo H. "Purification and complete amino acid sequence of alpha-human atrial natruretic polypeptide (alphaANP)." 1984, Biochem. Biophys. Res. Commun., 118:131—139.

3. Oikawa, S, et al., "Cloning and sequence analysis of cDNA encoding a precursor of human atria1 natriuretic polypeptide.", 1984, Nature, 309: 724-726.

4. Ballerman, BJ., and Brenner. BM, "Biologically active artial peptides.", 1985. J. Clin. Invest., 76:2041-2048.

5. Ballerman, BJ., and Brenner. BM. "Role of atria1 peptides in body fluid homeostasis.", 1986, Circ. Res., 58: 619-630

Assay Principle

The DetectX® ANP Immunoassay kit is designed to quantitatively measure ANP present in plasma, urine and tissue culture media samples. Please read the complete kit insert before performing this assay. An ANP standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. Standards or diluted samples are pipetted into a clear microtiter plate coated with an antibody to capture rabbit antibodies. An ANP-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of an antibody to ANP to each well. After an hour incubation the plate is washed and substrate is added. The substrate reacts with the bound ANP-peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450nm wavelength. The concentration of the ANP in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

Related Products

KITS

Cyclic GMP EIA Kits (1 or5 Plates)	Catalog Number K020-H1/H5
Cyclic GMP CLIA Kits (1 or5 Plates)	Catalog Number K020-C1/C5
Prostaglandin E_2 (PGE_2) EIA Kits (1 or5 Plates)	Catalog Number K018-H1/H5
Prostaglandin E_2 High Sensitivity Kits (1 or5 Plates)	Catalog Number K018-HX1/HX5
Prostaglandin E_2 (PGE_2) CLIA Kits (1 or5 Plates)	Catalog Number K018-C1/C5
Serum Creatinine Detection Kits	Catalog Number KB02-H1/H2
Urea Nitrogen (BUN) Detection Kit	Catalog Number K024-H1

Supplied Components

Coated Clear 96 Well Plates
Clear plastic microtiter plate(s) coated with goat anti-rabbit IgG.

Kit K026-H1 **OR** -H5	1 **OR** 5 Each	Catalog Number X016-1EA

ANP Standard
ANP at 1,800 ng/mL in a special stabilizing solution.

Kit K026-H1 **OR** -H5	125 µL **OR** 625 µL	Catalog Number C095-125UL **OR** -625UL

DetectX® ANP Antibody
A rabbit polyclonal antibody specific for ANP.

Kit K026-H1 **OR** -H5	3 mL **OR** 13 mL	Catalog Number C093-3ML **OR** -13ML

DetectX® ANP Conjugate
An ANP-peroxidase conjugate in a special stabilizing solution.

Kit K026-H1 **OR** -H5	3 mL **OR** 13 mL	Catalog Number C094-3ML **OR** -13ML

Acetic Acid
0.1M Acetic Acid solution for precipitating proteins in samples.

Kit K026-H1	25 mL	Catalog Number X098-25ML

Assay Buffer (or Concentrate)
One plate kit uses a ready-to-use Assay Buffer. Five plate kit uses a 5X concentrate that should be diluted with deionized or distilled water.

Kit K026-H1	28 mL	Catalog Number X059-28ML
Kit K026-H5	55 mL (Conc)	Catalog Number X065-55ML

Wash Buffer Concentrate
A 20X concentrate that should be diluted with deionized or distilled water.

Kit K026-H1 **OR** -H5	30 mL **OR** 125 mL	Catalog Number X007-30ML **OR** -125ML

TMB Substrate

Kit K026-H1 **OR** -H5	11 mL **OR** 55 mL	Catalog Number X019-11ML **OR** -55ML

Stop Solution
A 1M solution of hydrochloric acid. CAUSTIC.

Kit K026-H1 **OR** -H5	5 mL **OR** 25 mL	Catalog Number X020-5ML **OR** -25ML

Plate Sealer

Kit K026-H1 **OR** -H5	1 **OR** 5 Each	Catalog Number X002-1EA

Storage Instructions

All components of this kit should be stored at 4°C until the expiration date of the kit.

Other Materials Required

Distilled or deionized water.

Water bath capable of maintaining 85°C.

Microcentrifuge capable of producing 2,200 x g.

Protease inhibitor cocktail (PIC), such as Sigma P1860-1ML.

Repeater-type pipet with disposable tips capable of dispensing 25, 50 and 100 µL.

Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.

Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.

0.1M Acetic Acid is supplied as a reagent to precipitate plasma proteins. It is mildly acidic. Do not allow it to come in contact with skin or eyes.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on Page 8.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

Sample Types

This assay has been validated for plasma, urine and for tissue culture samples. Samples containing visible particulate should be centrifuged prior to using.

ANP is identical in a large number of species. Human, pig, sheep and dog ANP have identical sequences and this kit will measure samples from these species identically. Rat and mouse ANP molecules differ at amino acid 12 where isoleucine replaces methionine in human ANP and show 100% cross reactivity in the kit. The end user should evaluate recoveries of ANP in other sample matrices being tested.

Sample Preparation

Plasma Samples

Collected plasma samples should be treated with a protease inhibitor cocktail (see page 6) to minimize loss of ANP from the sample. Add 0.5 µL of Sigma P1860 PIC, or similar preparation, for every mL of plasma. Plasma samples should be treated to remove endogenous proteins. Two methods can be used:

1. Reverse Phase Cartridge Separation (for low concentration samples)

Samples should be acidified and applied to a washed C18 reverse phase cartridge. Please follow manufacturers recommendations for use. We have a detailed Peptide/Protein Extraction Protocol available on our website at: http://www.ArborAssays.com/resources/lit.asp.

2. Acetic Acid Treatment (for elevated concentration samples)

Add an equal volume of the 0.1M Acetic Acid provided (X098-25ML) to the plasma sample.

Heat to 85°C for 10 minutes. Centrifuge at 2,200 x g for 10 minutes.

Dilute supernatant ≥ 1:5 in Assay Buffer for a final dilution of ≥ 1:10.

Urine Samples

Urine samples should be diluted at least 1:5 with the provided Assay Buffer before running in the kit. For normalization to creatinine as a urine volume marker, please see our NIST-calibrated 2 plate and 10 plate Urinary Creatinine Detection kits, K002-H1 and K002-H5.

Tissue Culture Media

For measuring ANP in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM depending on ANP levels. We have validated the assay using RPMI-1640.

Use all diluted samples within 2 hours of preparation.

Reagent Preparation

Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine ANP concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Assay Buffer (for K026-H5)

Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable for 3 months at 4°C.

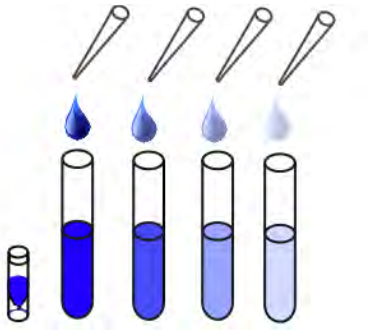
Wash Buffer

Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

Standard Preparation

Label six test tubes as #1 through #6. Pipet 450 µL of Assay Buffer into tube #1 and 200 µL into tubes #2 to #6. **The ANP stock solution contains an organic solvent. Pre-rinse the pipet tip several times to ensure accurate delivery.** Carefully add 50 µL of the ANP stock solution to tube #1 and vortex completely. Take 100 µL of the ANP solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #6. The concentration of ANP in tubes 1 through 6 will be 180, 60, 20, 6.67, 2.22, and 0.741 ng/mL.

Use all Standards within 2 hour of preparation.



	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Assay Buffer Volume (µL)	**450**	200	200	200	200	200
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5
Volume of Addition (µL)	**50**	100	100	100	100	100
Final Conc (ng/mL)	180	60	20	6.67	2.22	0.741

Assay Protocol

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to foil pouch with desiccant. Seal the ziploc plate bag and store at 4˚C.

2. Pipet 50 µL of samples or standards into wells in the plate.

3. Pipet 75 µL of Assay Buffer into the non-specific binding (NSB) wells.

4. Pipet 50 µL of Assay Buffer into wells to act as maximum binding wells (Bo or 0 pg/mL).

5. Add 25 µL of the DetectX® ANP Conjugate to each well using a repeater pipet.

6. Add 25 µL of the DetectX® ANP Antibody to each well, **except the NSB wells**, using a repeater pipet.

7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour.

8. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.

9. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.

10. Incubate the plate at room temperature for 30 minutes without shaking.

11. Add 50 µL of the Stop Solution to each well, using a repeater pipet.

12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

13. Use the plate reader's built-in 4PLC software capabilities to calculate ANP concentration for each sample.

Calculation of Results

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

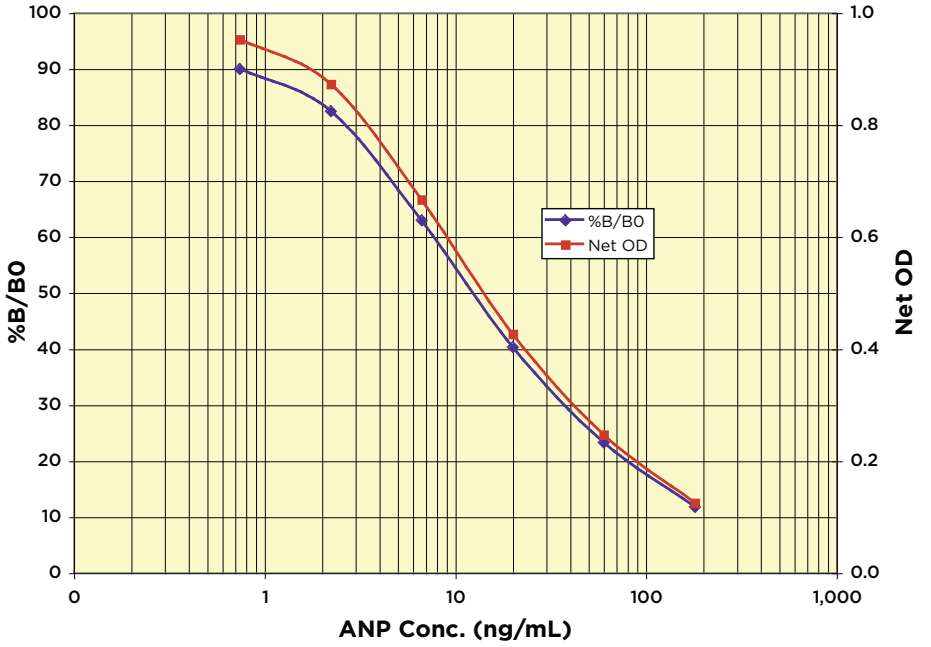
Typical Data

Sample	Mean OD	Net OD	% B/B0	ANP Conc. (ng/mL)
NSB	0.046	0	-	-
Standard 1	0.171	0.125	11.80	180
Standard 2	0.293	0.247	23.32	60
Standard 3	0.473	0.427	40.32	20
Standard 4	0.713	0.667	62.98	6.67
Standard 5	0.919	0.873	82.44	2.22
Standard 6	0.999	0.953	89.99	0.741
B0	1.105	1.059	100	0
Sample 1	0.417	0.371	34.99	26.85
Sample 2	0.625	0.579	54.67	10.28

Always run your own standard curve for calculation of results.
Do not use this data.

Conversion Factor: 100 ng/mL of human ANP is equivalent to 32.5 nM.

Typical Standard Curves



Always run your own standard curves for calculation of results.
Do not use this data.

Validation Data

Sensitivity and Limit of Detection

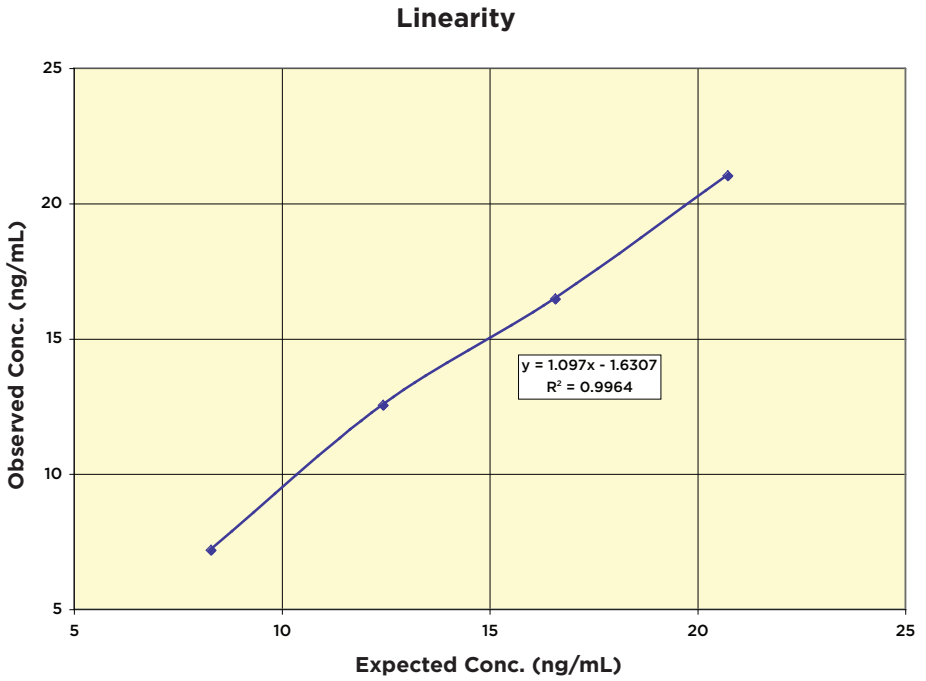
Sensitivity was calculated by comparing the OD's for twenty wells run for each of the B0 and standard #6. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve.
Sensitivity was determined as 0.25 ng/mL.

The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty runs for each of the zero standard and a low concentration human sample.
Limit of Detection was determined as 0.26 ng/mL

Linearity

Linearity was determined by taking two urine samples diluted with Assay Buffer, one with a low diluted ANP level of 4.2 ng/mL and one with a higher diluted level of 24.9 ng/mL, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

Low Urine	High Urine	Observed Conc. (ng/mL)	Expected Conc. (ng/mL)	% Recovery
80%	20%	7.2	8.3	86.4
60%	40%	12.5	12.4	100.7
40%	60%	16.5	16.6	99.2
20%	80%	21.0	20.7	101.4
			Mean Recovery	**96.9%**



Intra Assay Precision

Three human samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated ANP concentrations were:

Sample	ANP Conc. (ng/mL)	%CV
1	26.4	9.0
2	10.3	4.9
3	4.17	6.1

Inter Assay Precision

Three human samples were diluted with Assay Buffer and run in duplicates in fifteen assays run over multiple days by three operators. The mean and precision of the calculated ANP concentrations were:

Sample	ANP Conc. (ng/mL)	%CV
1	26.1	4.8
2	10.3	7.8
3	4.36	10.2

Interferents

A variety of detergents and solvents were tested as possible interfering substances in the assay.

Addition	% Added	% Change in Measured ANP
Triton-X100	0.10%	9.2%
Chaps	0.01%	-9.1%
SDS	0.05%	-10.0%
CTAC	0.0004%	-8.6%
Ethanol	1%	-1.4%
Methanol	1%	-9.3%
DMSO	1%	3.4%
DMF	1%	5.3%
Acetonitrile	1%	9.2%

Cross Reactivity

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Cross Reactant	Cross Reactivity (%)
Human ANP (1-28)	100%
Rat ANP (1-28)	99.4%
Rat ANF (8-33)	100%
Urodilantin	161.4%
Human ß-ANP (1-28)	50%
Human γ-ANP	40%
Rat ANF (18-28)	60%
Atriopeptin II	5%
BNP	< 0.001%

Limited Warranty

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

Contact Information

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