DetectX® Atrial Natriuretic Peptide (ANP)

ELISA Kit

1 Plate Kit – Catalog No. K071-H1
5 Plate Kit – Catalog No. K071-H5

Sample Types Tested:
Plasma, Urine, 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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SUPPLIED COMPONENTS & STORAGE

		K071-H1	K071-H5	Description
Goat anti-Rabbit Clear Coated 96-well Plate	Quantity	1	5	Strip well plates coated with goat anti-rabbit IgG
	Catalog No.	X016-1EA	X016-1EA	
ANP Standard	Volume	125 µL	625 µL	ANP at 1,800 ng/mL in stabilizing solution
	Catalog No.	C095-125UL	C095-625UL	
DetectX® ANP Antibody	Volume	3 mL	13 mL	Rabbit polyclonal antibody specific for ANP in stabilizing solution
	Catalog No.	C262-3ML	C262-13ML	
DetectX® ANP Conjugate	Volume	3 mL	13 mL	ANP-peroxidase conjugate in stabilizing solution
	Catalog No.	C094-3ML	C094-13ML	
Assay Buffer Concentrate 5X	Volume	28 mL	55 mL	5X concentrate that must be diluted
	Catalog No.	X065-28ML	X065-55ML	
Wash Buffer Concentrate 20X	Volume	30 mL	125 mL	20X concentrate that must be diluted
	Catalog No.	X007-30ML	X007-125ML	
Extraction Solution	Volume	50 mL	250 mL	Solution for extracting ANP from plasma samples
	Catalog No.	X123-50ML	X123-250ML	
TMB Substrate	Volume	11 mL	55 mL	3,3',5,5'-Tetramethylbenzidine, a peroxidase substrate
	Catalog No.	X019-11ML	X019-55ML	
Stop Solution	Volume	5 mL	25 mL	1M solution of hydrochloric acid CAUSTIC
	Catalog No.	X020-5ML	X020-25ML	
Plate Sealer	Quantity	1	5	-
	Catalog No.	X002-1EA	X002-1EA	

Once opened, the kit can be stored at 4˚C up to the expiration date on the kit label.

OTHER MATERIALS REQUIRED

- Distilled or deionized water
- Adjustable pipettes with disposable tips capable of dispensing 25 µL, 50 µL, and 100 µL. Repeater pipettes or multichannel pipettes with corresponding tips are also recommended.
- Glass or high-quality polypropylene test tubes for standard and sample preparation
- An orbital microplate shaker
- A plate reader capable of measuring absorbance at 450 nm
- Software for converting optical density (OD) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.
- A Speedvac/centrifugal concentrator or Nitrogen (N_2) gas and gas manifold for evaporation
- Optional: automated plate washer. Refer to Plate Washing Instructions for more details.
    - https://bit.ly/3tBT7N4
- For plasma samples, add 0.5 µl/mL of protease inhibitor cocktail (such as Sigma P1860-1ML) at the time of sample collection.

PRECAUTIONS

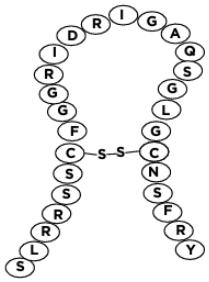
- Read this insert completely prior to using the product.
- This kit may not perform as described if any reagent or procedure is replaced or modified. Do not interchange reagents from different kit lots.
- Take appropriate safety precautions, such as: avoid breathing fumes, wear personal protective equipment (gloves, clothing, eye and face protection), and familiarize yourself with SDS documents.
    - https://www.ArborAssays.com/documentation/msds/K071-H_MSDS.pdf
- Ensure all buffers used for samples are azide free and that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer. Buffers, including other manufacturers' wash buffers, that contain sodium azide will inhibit color production from the enzyme.
- **Take appropriate precautions when handling the Stop Solution, which is a caustic acid.**

BACKGROUND

Atrial Natriuretic Peptide (ANP) was discovered in the early 1980s and first described in publications in 1983 and 1984[1,2]. ANP is derived from a 151 amino acid long protein called preproANP[3]. The prepro protein is cleaved to generate proANP.

The principle 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 main member of a family of structurally and functionally related peptide hormones that exert many effects on cardiovascular and renal function. The combined actions of ANP on vasculature, kidneys, and adrenals serve acutely and chronically to reduce systemic blood pressure and intravascular volume[4,5]. ANP and the related brain natriuretic peptide bind to their common receptor, membrane-type guanylyl cyclase-A, which leads to biological actions through a cGMP-dependent pathway.

Atrial Natriuretic Peptide



ASSAY PRINCIPLE

The DetectX® ANP ELISA Kit is designed to quantitatively measure ANP present in plasma, urine, and tissue culture media samples. The ANP ELISA Kit is a competitive ELISA with a run time of 1.5 hours. Please read this 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. As the ANP concentration in the sample increases, the bound ANP-peroxidase conjugate decreases, resulting in a decrease in signal and vice versa.

After an 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 450 nm wavelength. The concentration of ANP in the sample is calculated, after making suitable correction for the dilution, using software available with most plate readers.

REAGENT PREPARATION

Except for the reagents listed below, all kit components are ready for use.

Reagent	Preparation	Stability
1X Assay Buffer	Warm 5X Assay Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 5X Assay Buffer Concentrate with 4 volumes deionized water.	1X Assay Buffer is stable for 3 months at 4°C
1X Wash Buffer	Warm 20X Wash Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 20X Wash Buffer Concentrate with 19 volumes deionized water.	1X Wash Buffer is stable for 3 months at room temperature

SAMPLE PREPARATION

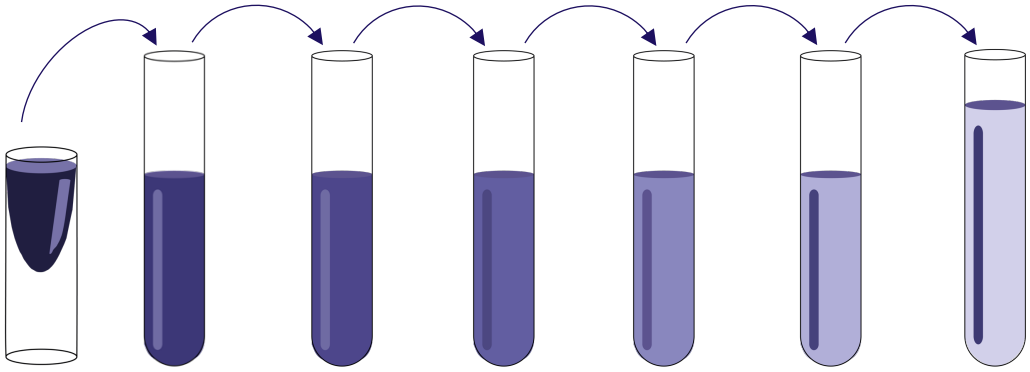
For samples containing particulates, centrifuge prior to use. Upon collection, all samples should be frozen rapidly and stored at -80˚C until testing.

Sample Type	Procedure
Plasma (Serum not recommended)	• Extract sample by mixing 1 volume sample with 1.5 volumes Extraction Solution. • Vortex briefly then nutate at room temperature for 90 minutes. • Centrifuge for 20 minutes at 4˚C at 1,660 x g. • Transfer the supernatant to a clean tube. • Evaporate the supernatant in a Speedvac at 37˚C until dry. • Reconstitute the sample with a minimum of 200 µL 1X Assay Buffer. ○ Starting sample volume and reconstitution volume will vary based on expected ANP levels in sample. • Samples may require further dilution with 1X Assay Buffer to fall within the standard curve range. • Alternatively, use a solid phase C18 column extraction protocol like the Peptide/Protein Extraction Protocol found at www.ArborAssays.com/resources/#protocols prior to using this kit.
Urine	• Prepare a minimum 5-fold dilution of sample by adding 50 µL urine to 200 µL 1X Assay Buffer. • Samples may require further dilution with 1X Assay Buffer to fall within the standard curve range. • Use our Urinary Creatinine Detection Kits (K002-H) to measure urine creatinine for normalization of ANP in urine specimens.
Tissue Culture Media (TCM)	• This assay has been validated using RPMI-1640. Other types of TCM should be validated before use. • Samples should be diluted in TCM and read off a standard curve generated in the same TCM. • Alternatively, prepare a minimum 2-fold dilution of sample by adding 100 µL TCM to 100 µL 1X Assay Buffer. • Samples may require further dilution with TCM or 1X Assay Buffer to fall within the standard curve range.

⚠ **Use all samples within 2 hours of dilution.**

STANDARD PREPARATION

1. Label tubes Standard 1 through Standard 6.
2. Add 450 µL 1X Assay Buffer to Standard 1 tube.
3. Add 200 µL 1X Assay Buffer to Standard 2 – 6 tubes.
4. Add 50 µL of the ANP stock solution to Standard 1 tube. Vortex thoroughly.
   ⚠ **The ANP stock solution contains an organic solvent. Pipet the stock solution up and down several times prior to dispensing to ensure accurate delivery.**
5. Transfer 100 µL of Standard 1 into Standard 2 tube to make a 3-fold dilution. Vortex thoroughly.
6. Transfer 100 µL of the mixed solution from Standard 2 into Standard 3 tube to make a 3-fold dilution. Vortex thoroughly.
7. Continue serially diluting into the remaining tubes. This process and the final concentrations are summarized in the table below.



	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
1X Assay Buffer (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 Concentration (ng/mL)	180	60	20	6.7	2.2	0.7

⚠ **Use all Standards within 2 hours of dilution.**

ASSAY PROTOCOL

Before You Begin:

- **Ensure all reagents have been warmed to room temperature.**
- **Dilute samples as described in Sample Preparation.**
- **Run all standards and samples in duplicate.**
- Use the blank plate template on the back page of this booklet to design your plate layout and aid in proper sample and standard identification.
- Be sure to shake the plate as directed. Failing to shake the plate or altering the shaking speed during incubations will result in decreased signal.
- Set plate parameters on the plate reader for a 96-well Corning CoStar 2592 plate. See ArborAssays.com for plate dimension data.
- Determine the number of strip wells to be used and return unused strip wells to the foil pouch with desiccant. Seal the foil pouch and store at 4ºC. Desiccant color will change from blue to pink if the foil pouch is not properly sealed.
- If you are only using part of a strip well plate, at the end of the assay discard the used wells and retain the plate frame for use with the remaining unused wells.

1. Add 50 µL of Samples or Standards into duplicate wells.
2. Add 75 µL 1X Assay Buffer into duplicate NSB (non-specific binding) wells.
3. Add 50 µL 1X Assay Buffer into duplicate Zero Standard (maximum binding or B0) wells.
4. Add 25 µL of the DetectX® ANP Conjugate to each well.
5. Add 25 µL of the DetectX® ANP Antibody to each well, **except the NSB wells**.
6. Cover the plate with the plate sealer and shake at room temperature at 700-900 rpm for **1 hour**.
7. Remove the plate sealer, aspirate the plate, and wash each well 4 times with 300 µL 1X Wash Buffer. Tap the plate dry on clean absorbent towels.
8. Add 100 µL TMB Substrate to each well.
    - The substrate solution will begin to turn blue.
9. Incubate at room temperature for **30 minutes** without shaking.
10. Add 50 µL Stop Solution to each well.
    - The substrate solution will begin to turn yellow.
11. Read the optical density at 450 nm within 10 minutes.

CALCULATION OF RESULTS

Follow the instructions below or use this online tool: https://www.myassays.com/assay.aspx?id=1315

1. Use four-parameter logistic curve (4PLC) software to calculate the ANP concentration for each sample. Gather all raw data OD readings from each Sample and Standard, including the Zero Standard (B0), and NSB.
2. Average the duplicate OD readings for each Sample, Standard, B0, and NSB (Mean OD).

EXAMPLE:

Sample	Replicate 1 OD	Replicate 2 OD	Mean OD
NSB	0.102	0.106	0.104
B0	1.485	1.495	1.490
Sample 1	0.480	0.460	0.470

3. Subtract the NSB from the Mean OD for each Sample, Standard, and the B0 (Net OD).

EXAMPLE:

Sample	Mean OD	NSB Mean OD	Net OD
B0	1.490	0.104	1.386
Sample 1	0.470	0.104	0.366

4. Divide the Net OD for each Sample and Standard by the Net OD for B0 and multiply by 100% (%B/B0).

EXAMPLE:

Sample	Net OD	B0 Net OD	%B/B0
Sample 1	0.366	1.386	26.4

5. Plot the standard curve with %B/B0 for the Standards on the y-axis and ANP concentration (ng/mL) on the x-axis. Perform a 4PLC fit.

   Use the sample %B/B0 readings and the 4PLC fit to calculate ANP concentrations in diluted samples. If diluted sample ANP concentrations are outside of the range of the standards, the sample should be prepared again at a more appropriate dilution.

EXAMPLE:

Sample	Net OD	%B/B0	Sample ANP Concentration (ng/mL)
Sample 1	0.366	26.4	33.8

6. If the original sample was diluted, multiply the ANP concentration by the sample dilution factor to determine the concentration of ANP in the original sample (Sample 1). If the original sample was concentrated during extraction, divide the ANP concentration by the sample concentration factor to determine the concentration of ANP in the original sample (Sample 2).

EXAMPLE:

Sample	Sample ANP Concentration (ng/mL)	Sample Modification Factor	Original Sample ANP Concentration (ng/mL)
Sample 1	33.8	10x dilution	338
Sample 2	8.1	2x concentration	4.1

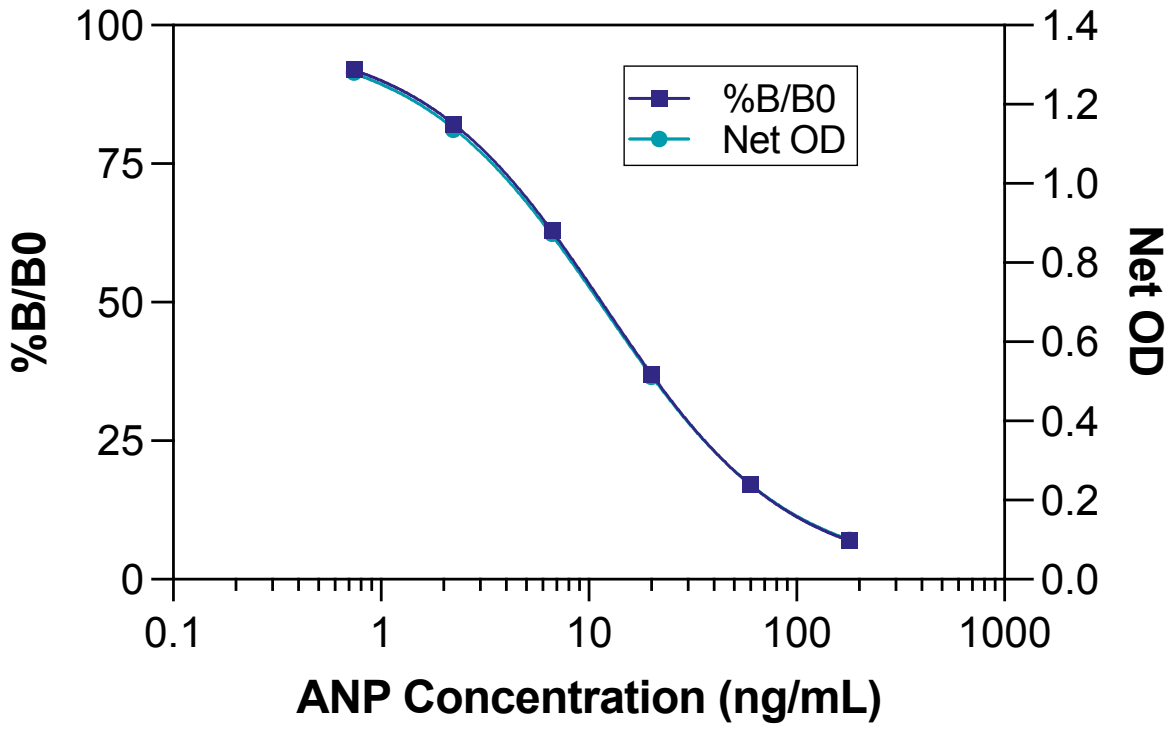
TYPICAL DATA

⚠ **Always run your own standard curve. This data should NOT be used to interpret results.**

Sample	Mean OD	Net OD	%B/B0	ANP Concentration (ng/mL)
NSB	0.104	0.000	-	-
Standard 1	0.205	0.101	7.2	180
Standard 2	0.344	0.240	17.3	60
Standard 3	0.616	0.512	36.9	20
Standard 4	0.977	0.873	62.9	6.7
Standard 5	1.241	1.137	82.0	2.2
Standard 6	1.384	1.280	92.3	0.7
B0	1.490	1.386	100.0	0
Sample 1	0.470	0.366	26.4	33.8
Sample 2	0.914	0.810	58.4	8.1

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

Typical Standard Curve



VALIDATION DATA

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the B0 and Standard 6. The detection limit was determined at two standard deviations from the B0 along the standard curve.

Sensitivity was determined as 0.44 ng/mL

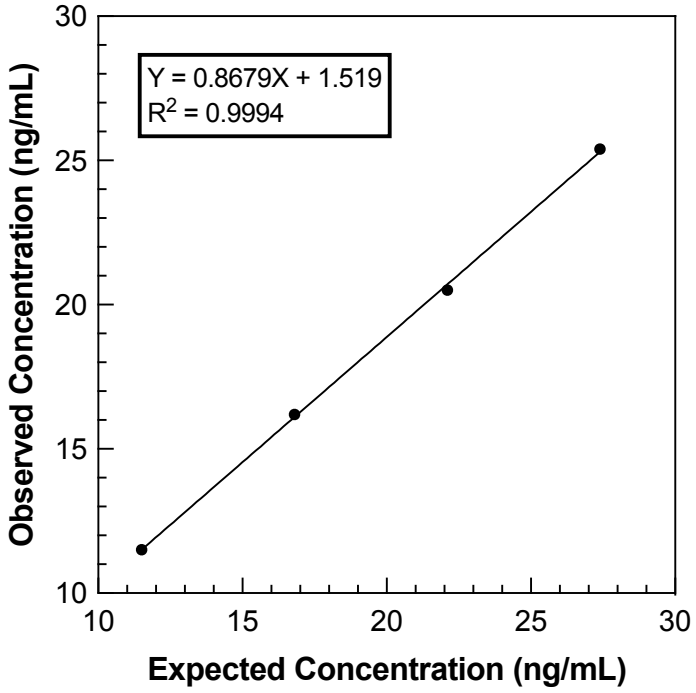
The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the B0 and a low concentration sample.

The Limit of Detection was determined as 0.49 ng/mL

Linearity

Linearity was determined in human urine by diluting two samples with known ANP concentration with 1X Assay Buffer. One sample had an ANP concentration of 6.2 ng/mL (Low Sample); a second sample had an ANP concentration of 32.7 ng/mL (High Sample). The two samples were mixed in the ratios given below and the measured concentrations were compared to the expected values for each given ratio.

Low Sample	High Sample	Expected Concentration (ng/mL)	Observed Concentration (ng/mL)	% Recovery
80%	20%	11.5	11.5	100.0
60%	40%	16.8	16.2	96.4
40%	60%	22.1	20.5	92.8
20%	80%	27.4	25.4	92.7
			Mean Recovery	**95.5%**



Intra Assay and Inter Assay Precision

For intra assay precision, three human urine samples were diluted in 1X Assay Buffer and 22 replicates were run in one assay. For inter assay precision, three human urine samples were diluted in 1X Assay Buffer and duplicates of each sample were run in 22 assays over multiple days by five operators. %CV represents the variation in concentration (not optical density) as determined using a standard curve.

	Intra Assay Precision		Inter Assay Precision	
Sample	ANP Concentration (ng/mL)	% CV	ANP Concentration (ng/mL)	% CV
1	35.5	4.2	33.8	7.6
2	16.9	2.2	15.1	10.2
3	9.3	2.2	8.1	14.9

SAMPLE VALUES

7 human urine samples were diluted in 1X Assay Buffer and tested in the assay. 18 human plasma samples were extracted, reconstituted in 1X Assay Buffer, and tested in the assay. The adjusted average concentration and sample range are shown below.

Sample Type	Recommended Minimum Dilution	Adjusted Average Concentration (ng/mL)	Adjusted Average Concentration (ng/mL) Range
Urine	1:5	8.2	5.9 – 10.3
Plasma	Extracted	1.4	0.84 – 3.0

INTERFERENCE

Potentially interfering substances were evaluated in the assay and the change in concentration was calculated.

Interferent	% Added	Effect
CHAPS	0.1%	4.9% decrease
NP-40	0.1%	4.1% decrease
SDS	0.004%	7.6% decrease
Triton-X100	1.0%	4.5% increase
Tween20	0.1%	5.9% increase
Acetone	5.0%	7.0% decrease
Acetonitrile	2.5%	5.3% decrease
DMF	0.5%	1.5% increase
DMSO	1.0%	9.0% increase
Ethanol	5.0%	2.7% decrease
Methanol	2.5%	8.9% decrease

CROSS REACTIVITY

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

Cross Reactant	Cross Reactivity (%)
Atrial Natriuretic Factor (ANF) (1-28) (human, bovine, porcine)	100
ANF (1-28) (mouse, rabbit, rat)	100
β-ANP (1-28) (human)	100
ANF (8-33) (rat)	100
Atriopeptin III (rat)	100
Urodilantin	100
Auriculin A (rat)	100
ANP-21 (frog)	0.4
ANP (1-11) (human)	< 0.01
γ-ANP (1-25) (human)	< 0.01
BNP (porcine)	< 0.01

TROUBLESHOOTING

Issue	Possible Cause & Solution
Reagent Shortage	• Check under the cap for additional reagent. Pulse spin reagent containers to collect contents prior to opening when possible. • When using a multichannel pipette, return unused reagent to container for later use.
Erratic Values	• Ensure the assay plate has been properly blotted after assay washes to remove residual wash buffer. • Prerinse pipet tips with desired reagent prior to dispensing the required volume. • Deliver volume with care to prevent splashing into adjacent wells.
High Background	• Ensure assay plate has been properly washed with the number of washes indicated in the protocol. • Reagent contamination during assay setup. • Verify antibody was not added to the NSB wells.
Low Signal	• Confirm tools, equipment, reagents, and containers used do not contain any trace of sodium azide. • Altering the shaking speeds or excluding shaking during incubation steps. • Verify the plate reader wavelength is 450 nm. • Ensure reagents are at room temperature prior to use.

CITATIONS

1. de Bold, A. J., & Flynn, T. G. (1983). Cardionatrin I - a novel heart peptide with potent diuretic and natriuretic properties. *Life sciences*, *33*(3), 297–302.

2. Kangawa, K. & Matsuo, H. (1984). Purification and complete amino acid sequence of alpha-human atrial natriuretic polypeptide (alphaANP). *Biochemical and Biophysical Research Communications*, 118(1), 131–139.

3. Oikawa, S., Imai, M., Ueno, A., Tanaka, S., Noguchi, T., Nakazato, H., Kangawa, K., Fukuda, A., & Matsuo, H. (1984). Cloning and sequence analysis of cDNA encoding a precursor for human atrial natriuretic polypeptide. *Nature*, *309*(5970), 724–726.

4. Ballerman BJ, Brenner BM. Biologically active atrial peptides. *J Clin Invest*. 1985 Dec; 76(6):2041-8.

5. Ballerman, B. J. & Brenner, B. M. (1986). Role of atria1 peptides in body fluid homeostasis. *Circ Res*, 58, 619–630.

RELATED PRODUCTS

Kits	Catalog No.
C-Reactive Protein (CRP) Human ELISA Kits	K069-H1/H5
Creatinine Serum Detection Kits	KB02-H1/H2
Cyclic GMP Direct ELISA Kits	K065-H1/H5
Hemoglobin Colorimetric Detection Kits	K013-H1/H5
Hemoglobin High Sensitivity Detection Kits	K013-HX1/HX5
Myeloperoxidase (MPO) Human ELISA Kit	K060-H1
Prostaglandin E_2 (PGE_2) ELISA Kits	K051-H1/H5
Urea Nitrogen (BUN) Detection Kits	K024-H1/H5

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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.

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CONTACT INFORMATION

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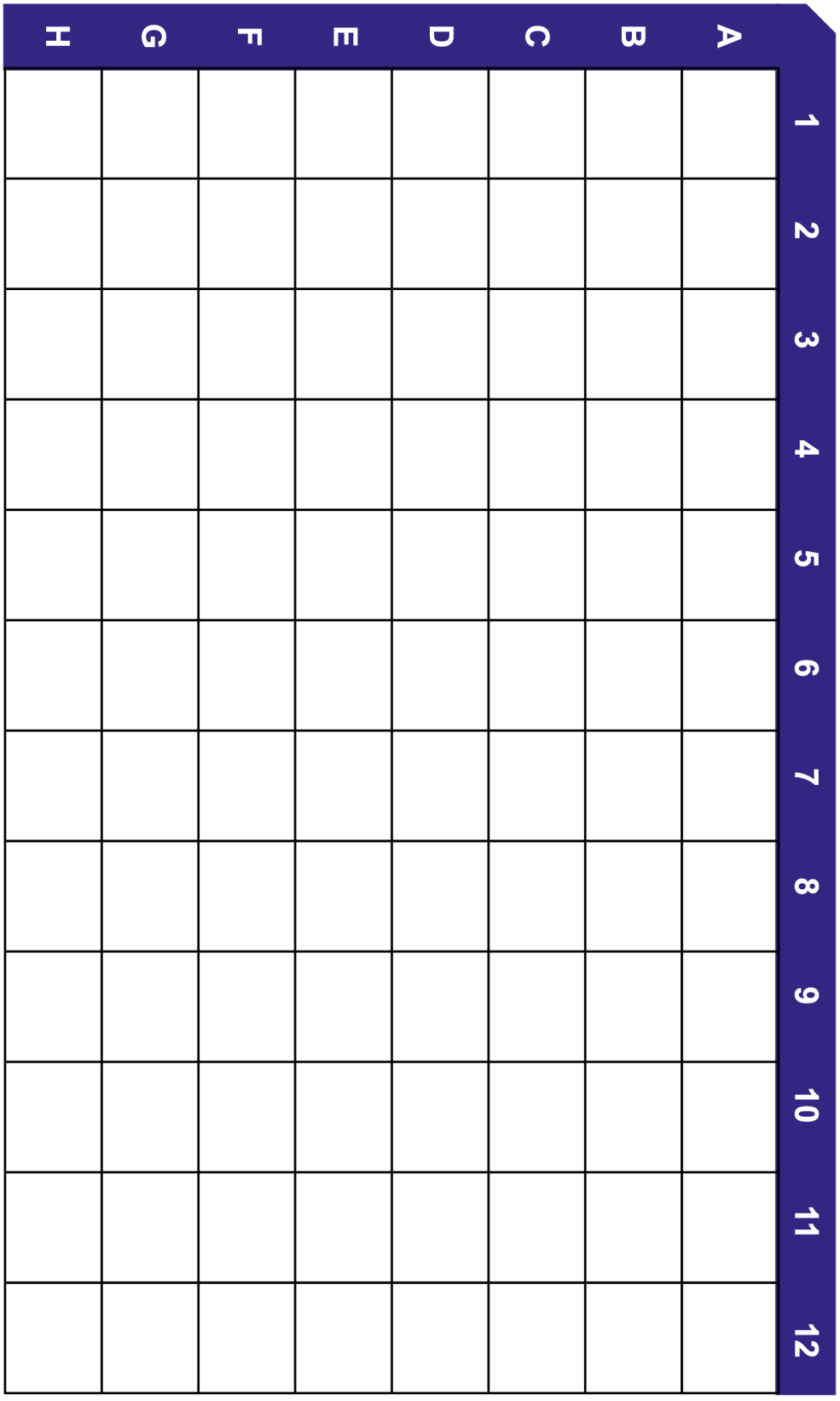
OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with assay kits and reagents for wildlife conservation research.

PLATE LAYOUT



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