

Instruction

# EURIA-ANP

ANP radioimmunoassay

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***For Research Use Only. Not for use in diagnostic procedures.***

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## **PURPOSE OF RESEARCH PRODUCT**

The Euro-Diagnostica ANP kit contains reagents and instructions for the determination of human ANP in plasma samples. After extraction with Sep-pak C18, ANP concentrations are measured by radioimmunoassay.

The result shall not be used for clinical diagnosis or patient management.

## **RESEARCH APPLICATION**

Mammalian atria contain specific granules, which resemble secretory granules in endocrine cells (1,2). DeBold et al. (3) showed that the number of these atrial granules was inversely correlated to the volume of the extracellular fluid. From atrial extracts, peptides have been isolated, sequenced, and synthesized (4-10). In human atria, three peptides seem to be important: a 28-amino acids component (alfa ANP), and a biosynthetic precursor (gamma-ANP, 126 amino acids). Biological activity was highest for alfa-ANP (11). In vitro and animal experiments have shown that alfa-ANP may possess important biological actions: it has diuretic and natriuretic activities, relaxes smooth muscle, its secretion is stimulated by volume loading, and it opposes the aldosterone secretion (reviewed in 12-17). These effects are thought to be mediated by alfa-ANP circulating in peripheral blood. Measurements of this peptide in human plasma (18-30) could be of value in determining the role of ANP in the endocrine system (31-34). This kit describes a simple, sensitive and reliable radioimmunoassay for ANP in human plasma.

## **PRINCIPLE OF THE TEST**

The method is based on a radioimmunoassay that is specific for h-ANP. Quantification of ANP is achieved using a non-equilibrium radioimmunoassay with delayed addition of the iodinated tracer. The bound and free hormone are separated by the second antibody/polyethylene glycol system. Centrifugation separates the bound and free hormone fractions. The precipitate, which contains the bound fraction, is counted. The final concentration of ANP is calculated and expressed in picograms/mL plasma (pg/mL).

## PRECAUTIONS

***For research use only. Not for use in diagnostic procedures.***

As the regulations may vary from one country to another, it is essential that the person responsible for the laboratory are familiar with current local regulations, concerning all aspects of radioactive materials of the type and quantity used in this test.

This kit contains components of human origin. They have been tested by immunoassay for hepatitis B surface antigen, antibodies to HCV and for antibodies to HIV-1 and HIV-2 and found to be negative. Nevertheless, all recommended precautions for the handling of blood derivatives, should be observed.

Steps should be taken to ensure the proper handling of the radioactive material, according to local and/or national regulations. Only authorized personnel should have access to the reagents.

The following precautions should be observed when handling radioactive materials:

- Radioactive material should be stored in specially designated areas, not normally accessible to unauthorized personnel.
- Handling of radioactive material should be conducted in authorized areas only.
- Care should be exercised to prevent ingestion and contact with the skin and clothing. Do not pipette radioactive solutions by mouth.
- Drinking, eating or smoking should be prohibited where radioactive material is being used.
- Hands should be protected by gloves and washed after using radioactive materials.
- Work should be carried out on a surface covered by disposable absorbing material.
- Spills of radioactive material should be removed immediately, and all contaminated materials disposed as radioactive waste. Contaminated surfaces should be cleaned with a detergent.

## SPECIMEN COLLECTION

EDTA-plasma is recommended for the human ANP radioimmunoassay. Collect blood by venipuncture on crushed ice. EDTA is used as anticoagulant. Centrifuge within one hour at 4° C for 15 min. at 1500 g. Add 200 KIU of aprotinin/mL (Trasylol, Bayer) plasma and store in polystyrene tubes at -20° C or lower.

**MATERIALS AND EQUIPMENT REQUIRED BUT NOT PROVIDED**

Disposable polystyrene tubes; 12 x 75 mm.

Gamma-counter.

Vortex mixer.

Pipetting devices to deliver 100, 200 and 500  $\mu$ L.

Centrifuge 1500 g

Seppak C18 cartridge

Acetic Acid 96% to be diluted to 4%

Ethanol 86%, ethanol 100%

Methanol 100%

Device for use in the extraction/purification procedure to provide vacuum.

**QUALITY CONTROL**

Controls should be carried out in each assay run. Two lyophilized controls are included in the kit. For values; refer to the QC sheet and labels on vials with controls. Additional external controls may be used as recommended by the manufacturer and in accordance with reference laboratories' practice to monitor the accuracy and precision of reagents and techniques.

**SHELFLIFE AND STORAGE**

This kit is stable until the stated expiry date if stored as specified. Upon receipt of the kit, all reagents should be stored at 2-8° C.

**CONTENTS OF THE KIT**

Item	Nr. of Vials	Containing
Assay buffer	1	0.2 M Borate + 0.2% BSA, pH 8.4, lyophilized
Anti-human ANP serum (sheep)	1	lyophilized antiserum in 0.5 M Borate + 0.2% BSA
Human-ANP <sup>125</sup> I tracer	1	lyophilized <sup>125</sup> I-ANP in 0.5 M Borate + 0.2% BSA
Precipitating reagent	1	ready for use horse anti-sheep antiserum in 0.2 M Borate + 0.2% BSA
Two reference controls: Low and high	2	lyophilized ANP controls
Human ANP stock standard	1	lyophilized ANP human standard

## PREPARATION OF REAGENTS

The kit contains reagents sufficient for 100 tubes.

**NOTE: prepare reagents after extraction procedure has been completed.**

(reconstitute 30 minutes before use)

### 1. Assay buffer (zero standard) (Reagent C)

Reconstitute the buffer with 30 mL distilled or deionized water. Allow the buffer to stand for 20 min. for complete reconstitution. Mix gently, do not shake. The buffer is used for preparing the standard curve (one to one serial dilution), zero standard and non-specific binding. After extraction and evaporation the samples are dissolved in this buffer. After use, store the assay buffer at 2-8° C.

### 2. Anti-human ANP antiserum (Reagent A)

Reconstitute the content of the vial with 21 mL distilled water. Allow the antibody to stand for 20 min. for complete reconstitution. Mix gently, do not shake. After use, store at 2-8° C. The antibody is raised in sheep against human ANP.  
Colour: Yellow.

### 3. Human ANP <sup>125</sup>I tracer (Reagent B)

Reconstitute with 21 mL distilled water. Allow the tracer to stand for 20 minutes for complete reconstitution. Mix gently, do not shake. After use, store at -20° C. The tracer contains 37 KBq of radioactivity.  
Colour: Blue.

### 4. Precipitating reagent (Reagent D)

52 mL horse anti-sheep-Ig antiserum in 0.2 M Borate buffer pH 8.4 with 0.2 % BSA and 0.1% Triton. Contains 11% (w/v) Polyethylene glycol 6000.  
Mix thoroughly before use. Store at 2-8° C.  
Colour: Red

### 5. Controls (Reagent F-G)

Reconstitute with 1.0 mL distilled water. Mix gently. For exact values, refer to quality control sheet and vial labels. After use store at -20° C or lower.

### 6. Human ANP stock standard (Reagent E)

Reconstitute the standard with 1.0 mL distilled water. Mix gently. This stock standard contains 100 pg/100 µL (A). Make serial dilutions in polystyrene tubes. A scheme for preparation of standard solution is shown next. Store at -20° C.

**PERFORMANCE****Do not extract standards and controls!****A. Extraction procedure for samples**

1. Extraction on Seppak C-18 cartridge.  
Subsequently pretreat with:
  - 5 mL 4% acetic acid (96%) in 86% ethanol
  - 5 mL methanol
  - 5 mL distilled water
  - 5 mL 4% acetic acid (96%)
2. Acidify 0.5 mL plasma with 1.5 mL 4% acetic acid and apply to the cartridge.
3. Wash twice with 3 mL distilled water.
4. Elute with 3 mL acetic acid in 86% ethanol.
5. Evaporate under a stream of nitrogen during 1 hour at 37° C.  
Add 1 mL of ethanol 100%, mix and evaporate to dryness.
6. Dissolve residue in 250 µL assay buffer.
7. Introduce 100 µL samples into the radioimmunoassay procedure.

Each laboratory should establish the extraction recovery under its own experimental conditions.

**B.**

<b>Preparation of standard solutions, example</b>		
Dilution		Conc. ANP
Example of dilution	Stock Standard A	100 pg/100 µL
250 µL stock solution A + 250 µL assay buffer	Standard B	50 pg/100 µL
250 µL stock solution B + 250 µL assay buffer	Standard C	25 pg/100 µL
250 µL stock solution C + 250 µL assay buffer	Standard D	12.5 pg/100 µL
250 µL stock solution D + 250 µL assay buffer	Standard E	6.25 pg/100 µL
250 µL stock solution E + 250 µL assay buffer	Standard F	3.12 pg/100 µL
250 µL stock solution F + 250 µL assay buffer	Standard G	1.56 pg/100 µL
250 µL stock solution G + 250 µL assay buffer	Standard H	0.78 pg/100 µL
After use, store the stock standard A at -20° C		

**C. ASSAY PROCEDURE**

1. Pipette 100 µL of ANP standard solutions, reconstituted controls and plasma extracts into labelled test tubes.
2. Add 200 µL sheep anti ANP antibody (except NSB - and TC-tubes).  
Correct NSB volume with 200 µL assay buffer.
3. Vortex and incubate all tubes at 4° C for 20-22 hours.
4. Add 200 µL of ANP [<sup>125</sup>I] Tracer to all tubes.
5. Vortex all tubes and incubate for 20-22 hours at 4° C.
6. Add 500 µL precipitating reagent to all tubes, except TC tubes.
7. Vortex, and incubate for 30-60 min. at 2-8° C.
8. Centrifuge 15 min at 1700 g,. 2-8° C.
9. Decant (or aspirate) supernatant immediately.
10. Count residue for 1 min.

Flow Chart									
Concentration in pg/100 µL	Assay buffer	Standard or Sample	Anti-serum		Tracer		Prec. reagent		
Total counts	-	-	-	Vortex and incubate 20-22 hrs at 4° C	200 µL	Vortex and incubate 20-22 hrs at 4° C	-	Incubate 30-60 min. at 2-8° C. Centrifuge 15 min. at 2-8° C at 1700 g.	Aspi- rate or decant super- natant
NSB	300 µL	-	-		200 µL		500 µL		
Standard 0	100 µL	-	200 µL		200 µL		500 µL		
Standard 0.78		100 µL	200 µL		200 µL		500 µL		
Standard 1.56		100 µL	200 µL		200 µL		500 µL		
Standard 3.12		100 µL	200 µL		200 µL		500 µL		
Standard 6.25		100 µL	200 µL		200 µL		500 µL		
Standard 12.5		100 µL	200 µL		200 µL		500 µL		
Standard 25		100 µL	200 µL		200 µL		500 µL		
Standard 50		100 µL	200 µL		200 µL		500 µL		
Standard 100		100 µL	200 µL		200 µL		500 µL		
Controls		100 µL	200 µL	200 µL	500 µL				
Unknown		100 µL	200 µL		200 µL		500 µL		

**CALCULATION OF RESULTS**

- Subtract the average count rate (cpm) of the NSB from the average count rate (cpm) of the replicates of standards, controls and samples.
- A standard curve can be generated by plotting cpm or % B/Bo of precipitated bound fraction against the concentration of the ANP calibrators.
- To obtain the ANP concentration in samples and controls, their cpm or % B/Bo of precipitated bound fractions are interpolated now from the generated standard curve.
- The calibration curve can also be constructed by computer methods. For automated data reduction, both logit/log or Spline methods can be used.

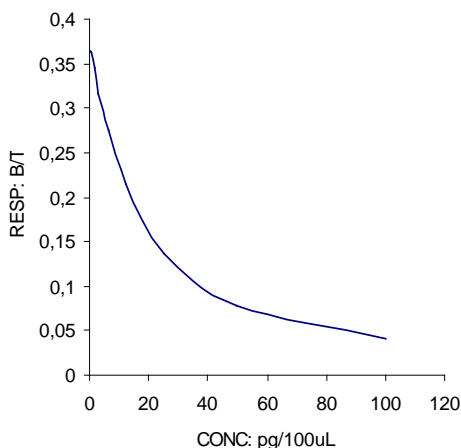
Note: ANP concentration in samples read from standard curve have to be multiplied by 5 due to the dilution factor with the extraction procedure. Correct all subject sample results by % recovery:

$$\frac{\text{ANP pg/100 } \mu\text{l} \times 5}{\% \text{ recovery}} \times 100 = \text{_____ pg/mL}$$

**EXAMPLE OF SAMPLE DATA**

	cpm	corrected cpm	% B/Bo	results pg/100 $\mu$ L
Total counts	10140			
NSB	614			
Standard 0	4320	3706	100	
Standard 0.78 pg/100 $\mu$ L	4289	3675	99,2	
Standard 1.56 pg/100 $\mu$ L	4123	3509	94,7	
Standard 3.12 pg/100 $\mu$ L	3823	3209	86,6	
Standard 6.25 pg/100 $\mu$ L	3410	2796	75,4	
Standard 12.5 pg/100 $\mu$ L	2792	2178	58,8	
Standard 25 pg/100 $\mu$ L	2006	1392	37,6	
Standard 50 pg/100 $\mu$ L	1413	799	21,6	
Standard 100 pg/100 $\mu$ L	1032	418	11,3	
Low control	3636	3022	81,6	4,9
High control	2218	1604	43,3	20,6

**EXAMPLE OF ANP STANDARD CURVE**





**ASSAY CHARACTERISTICS**

<b>Specificity</b>	
The following fragments were checked for cross-reactivity:	
Fragment	% cross reactivity
human $\alpha$ -ANP [1-28]	100
human $\alpha$ -ANP [5-28]	109
human $\alpha$ -ANP [7-28]	127
human $\alpha$ -ANP [13-28]	<0.2
human $\alpha$ -ANP [18-28]	<0.2
human $\alpha$ -ANP [1-11]	<0.2
rat $\alpha$ -ANP [1-28]	2.2
$\alpha$ -MSH	<0.2
Arg-Vasopressin	<0.2
ACTH [1-39]	<0.2

<b>Recovery</b>
Mean recovery after addition of different amounts of ANP to 1 mL of plasma is 90%.

<b>Linearity</b>			
Different samples were diluted with zero calibrator			
Sample no.	Undiluted (pg/mL)	1/2 (pg/mL)	1/4 (pg/mL)
1	21	27	23
2	16	17	18
3	15	14	14
4	145	-	164
5	62	-	68
6	173	-	170
7	185	-	187
8	53	-	57
9	133	-	149

Precision									
Within-run variation The within-run variation was calculated from 7 replicate determinations.					Between-run variation The between-run variation was calculated from 6 independent assay runs.				
	n	mean pg/mL	SD	% c.v.		n	mean pg/mL	SD	% c.v.
Sample	7	12.6	1.1	8.6	Sample	6	21.7	2.5	11.6

**Sensitivity**  
The sensitivity of the assay, judged as 3 Sd change from zero calibrator is 3.5 pg/mL.

**Expected values**  
Each laboratory should establish its own normal range.  
The normal range as determined at the department of Experimental and Chemical Endocrinology of the University of Nijmegen, The Netherlands, is 9-68 pg/mL, with a mean value of 26.0 " 15.5 pg/mL (n = 25).











**Interference**

Samples displaying cloudiness, hemolysis, hyperlipemia or containing fibrin may give inaccurate results.

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**Explanation of symbols.**

	Use-by date
	Biological risks
	Temperature limit
	Manufacturer.
	Date of manufacture.
	Batch code.
	Catalogue number.
	Consult instructions for use.
	Contains sufficient for 100 tests.
	Contains radioactive substances.

<b>REAG</b>   <b>A</b>   <b>Ab</b>	Anti-human ANP antiserum.
<b>REAG</b>   <b>B</b>   <b>Ag</b>   <sup>125</sup> I	Human ANP <sup>125</sup> I tracer.
<b>REAG</b>   <b>C</b>   <b>DAB</b>	Assay buffer
<b>REAG</b>   <b>D</b>   <b>DIL</b>	Precipitating reagent.
<b>REAG</b>   <b>E</b>   <b>CAL</b>   <b>300</b>	Human ANP stock standard.
<b>REAG</b>   <b>F</b>   <b>CONTROL</b>	Control.
<b>REAG</b>   <b>G</b>   <b>CONTROL</b>	Control.

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