Human αANP [^{125}I] RIA KIT

(REF: RK-512)

The [^{125}I]human αANP RIA system (dual range) provides direct quantitative *in vitro* determination of human αANP in urine, plasma, tissues and other biological samples. Human αANP may be measured in the range 1–32 fmol (3.1–98.6 pg) per tube using a 3 day (delayed addition) protocol. Alternatively, human αANP can be measured in the range 1–64 fmol (3.1–197 pg) per tube using an overnight assay protocol. Each pack contains sufficient material for 100 assay tubes. 42 unknowns can be measured in duplicate.

Introduction

The observation of electron-dense granules in atrial, but not ventricular cardiocytes (atrial-specific granules) has led to an enormous amount of interest in the role of the heart as an endocrine organ.

The function of atrial-specific granules was first postulated by de Bold, who later demonstrated the natriuretic, diuretic and hypotensive effects of atrial extracts. The occurrence of specific atrial factors controlling salt and water balance was suggested from these observations.

Since de Bold's pioneering experiments, research in this field has increased exponentially, initial attempts being directed towards the elucidation of the structure of the 'atrial natriuretic factor' (ANF).

Using recombinant DNA technology and cloning techniques, a number of groups identified human ANF as consisting of a family of peptides derived from a common, 151 amino acid preprohormone in atrial granules. This precursor undergoes N-terminal hydrolysis to give a 126-amino acid pre-hormone which may occur as a circulating form as well as in atrial granules. The pre-hormone undergoes further, extensive N-terminal hydrolysis and a wide range of C-terminal peptides ranging from 21–48 amino acids in length have been reported. Until recently however, little agreement had been reached on either terminology or the physiologically significant form(s) of the hormone. It is now generally accepted that the 28 amino acid peptide known as α-atrial natriuretic peptide (αANP), is the circulating form of the hormone.

Studies of the in vitro biological activity of atrial natriuretic peptides have revealed a range of functions of possible in vitro significance. These functions include natriuresis, diuresis, kaliuresis and hypotensive effects; vasodilation, inhibition of angiotensin-, norepinephrine- and potassium-induced vasoconstriction; inhibition of adenylate cyclase activity and aldosterone production and recently as a putative neurotransmitter.

Pharmacological levels of atrial natriuretic peptides in vitro have been shown to increase sodium, calcium, magnesium and phosphate excretion and urinary flow, whilst lowering arterial blood pressure, in a number of species including man and dog. Decreases in plasma renin and aldosterone levels have also been reported in some species. Plasma levels of atrial natriuretic peptides have been shown to be elevated in cases of chronic renal failure, paroxysmal atrial tachycardia, heart failure and during pregnancy.

Release of atrial natriuretic peptides has been demonstrated after volume loading and at high plasma sodium ion concentration. Specific receptors have been identified in several tissues including the heart, hypothalamus, renal and vascular tissue, the adrenal cortex and the brain. Ligand-receptor interaction has been shown to cause elevation of intracellular cGMP in some tissues.

Principle of method

This assay is based on the competition between unlabelled human αANP and a fixed quantity of 125I-labelled human-ANP for a limited number of binding sites on an αANP-specific antibody. In order to achieve greater sensitivity, a disequilibrium technique is used. Unlabelled human αANP (standard or sample) is pre-incubated with the antibody for 24 hours before addition of the 125I-tracer. This technique reduces the number of binding sites available to the tracer, being dependent on the quantity of unlabelled human αANP present.

This situation of 'disequilibrium' leads to a more sensitive dose response curve.

The antibody bound human αANP is then reacted with the separating second antibody reagent, which contains second antibody bound to magnetizable polymer particles. Separation of the antibody bound fraction is effected by either application of a magnetic field or centrifugation, followed by decantation of the supernatant.

Measurement of the radioactivity in the pellet enables the amount of labelled human αANP in the bound fraction to be calculated. The concentration of unlabelled αANP in the sample is then determined by interpolation from a standard curve.

Contents of the kit

1. 1 vial TRACER, lyophilized, reconstitution with 11 ml assay buffer, containing ~48 kBq, 1.3 µCi (3-[125I]iodotyrosy(28) αANP (human). The final solution contains (3-[125I]iodotyrosyl28) αANP in 0.025 M phosphate buffer pH7.2 containing 0.1%(w/v) sodium azide. Store at 2-8 °C.

2. 1 vial STANDARD, lyophilized, reconstitution with assay buffer, volume stated on the vial label. The final solution contains human αANP at a concentration of 640 fmol/ml in 0.025 M phosphate buffer pH 7.2 containing 0.1% (w/v) sodium azide. Store at 2-8°C.

3. 1 vial ANTISERUM, lyophilized, reconstitution with 11 ml assay buffer. The final solution contains rabbit anti-αANP serum in 0.025 M phosphate buffer pH7.2 containing 0.1% (w/v) sodium azide. Store at 2-8 °C.

4. 1 vial ASSAY BUFFER, concentrate (10 ml), dilution to 100 ml. On dilution this will give 0.025 M phosphate buffer pH7.2, containing 0.1% (w/v) sodium azide. Store at 2-8 °C.

5. 1 vial SEPARATING SECOND ANTIBODY REAGENT (30 ml), ready for use, containing donkey anti-rabbit serum coated on to magnetizable polymer particles with sodium azide, colour coded, blue-green. Store at 2-8 °C.

Quality certificate
Pack leaflet

Materials, tools and equipment required

Pipettes or pipetting equipment with disposable tips (100µl, 200µl, 400µl, 500µl, 2.0ml and 11ml); disposable polypropylene or polystyrene tubes (12 x 75 mm); refrigerator; glass measuring cylinder (100ml); test tube rack; vortex mixer; plastic foil; separators, comprising magnetic base and assay rack - these are for use in the magnetic separation protocol; adsorbent tissue; gamma counter.

Note: For the centrifugal protocol, the following additional equipment will be required:
Decantation racks; refrigerated centrifuge capable of >2000 xg.

Specimen collection, storage

1. Carry out all operations at 4°C.
2. Collect blood in plastic tubes containing EDTA such that the final concentration is 1 mg EDTA/ml of blood.
3. A protease should be included in the collection tube. A variety of inhibitors in various combinations have been reported, including: pepstatin (5 µM), phenylmethyl sulphonyl fluoride (10 mM, aprotinin (trasylol) (500-1000 KIU/ml), soya bean trypsin inhibitor (50 BAEE U/ml).
4. Centrifuge tubes immediately at 2000 xg, 4°C for 30 minutes.
5. Isolate plasma and store in single-use aliquots at -15°C to -30°C.

Sample purification

This protocol should only be used with Amprep minicolumns. The properties of other minicolumns are different and may result in different recoveries. Samples may need to be diluted prior to assay depending on the expected concentration. Assay buffer remaining after reconstitution of assay components may be used for this.

It remains the responsibility of the researcher to validate any sample processing method employed.

1. Equilibrate the column by washing with 2 ml methanol followed by 2 ml water. For this and subsequent washes maintain the flow rate of <5 ml minute.
2. Acidify 1 ml plasma with 0.25 ml 2 M HCl and centrifuge before loading on to the column. (Larger plasma volumes can be used.)

If so scale-up the volume of acid used to dilute the sample. However, the wash and elution volumes can be kept constant.

3. Wash with 5 ml water +0.1% (v/v) trifluoroacetic acid (TFA).
4. Wash with 4 ml 60% acetonitrile (or methanol) in water +0.1% (v/v) TFA, and collect eluent in a glass or polypropylene tube.
5. Dry down under nitrogen.
6. Reconstitute in 250 µl assay buffer and take 2 x 100 µl for analysis.

Preparation of reagents, storage

Storage: *see Contents of the kit*. At these temperatures each reagent is stable until expiry date. The actual expiry date is given on the package label and on the quality certificate.

Store reconstituted components at -15°C to -30°C, separating reagent and assay buffer at 2–8°C. Reconstituted components may be re-used within 28 days of dilution.

Preparation: Equilibrate all reagents and samples to room temperature prior to use. Assay buffer: Warm the bottle containing assay buffer concentrate to 40°C or until the gel-like material melts. Temperatures above 60°C should be avoided. Transfer the contents of the bottle, with washings, to a 100 ml measuring cylinder and dilute to 100 ml with

distilled or deionised water. Mix well. Assay buffer is used to reconstitute all other components.
The other components (except Separating reagent): Carefully add the required volume of assay buffer and replace stopper. Mix the contents of the bottles by inversion and swirling, taking care to avoid foaming.
Note: The tracer is needed on the second day of the assay.

Preparation of working standards

1. Label 6 polypropylene or polystyrene tubes 1, 2, 4, 8, 16 and 32.
2. Pipette 500 µl of assay buffer into all tubes.
3. Into the 32 tube pipette 500 µl of assay standard (640fmol/ml) and vortex thoroughly.
4. Transfer 500 µl from the 32 tube to the 16 tube and vortex thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes.
6. 100 µl aliquots from each serial dilution give rise to 6 standard levels of αANP ranging from 1–32 fmol.

Note: Working standards should be freshly prepared before each assay, and not re-used.

Assay procedure

(For a quick guide , refer to Table 1.)

Day 1

1. Equilibrate all reagents to room temperature.
2. Prepare reagents and assay standards as described in the previous section.
3. Label polypropylene or polystyrene tubes in duplicate for total counts (TC), zero standard (Bo), standards and samples.
4. Pipette **100 µl** assay buffer into the zero standard (Bo) tubes.
5. Starting with the most dilute, pipette **100 µl** of each standard (S1-6) into the appropriately labelled tubes.
6. Pipette **100 µ**l unknown sample (Mx) into appropriately labelled tubes. Samples should be diluted in assay buffer.
7. Pipette **100 µl** antiserum into all tubes except the TC.
8. Vortex mix all tubes thoroughly. Cover the tubes, for example with plastic film, and incubate for 16–24 hours at 2–8°C.

Day 2

9. Remove tubes from 2–8°C.
10. Pipette **100 µl** of [125I]αANP into all tubes. The TC tubes should be stoppered and put aside for counting.
11. Vortex mix all tubes thoroughly. Cover the tubes and incubate for 24 hours at 2-8°C.

Day 3

12. Gently shake and swirl the bottle containing separating second antibody reagent (blue-green) to ensure a homogeneous suspension. Add **250 µl** into each tube except the TC, vortex mix and incubate at room temperature for 10 minutes.
13. Separate the antibody bound fraction using either magnetic separation or centrifugation as described below.

Magnetic separation

Attach the rack on to the separating reagent separator base and ensure that all tubes are in contact with the base plate. Leave for 15 minutes. After separation, do not remove the rack from the separator base. Pour off and discard the supernatant. Keeping the separator inverted, place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes.

Centrifugation

Centrifuge all tubes at 4°C for 10 minutes at 1500 xg or greater. After centrifugation, place the tubes carefully into suitable decantation racks, then pour off and discard the supernatant. Keeping the tubes inverted, place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes.
14. On completion of either magnetic or centrifugal separation, firmly blot the rims of the inverted tubes on the tissue pad to remove any adhering liquid. Do not reinvert the tubes once they have been turned upright.
15. Determine the radioactivity present in each tube by counting for at least 60 seconds in a gamma scintillation counter.

Note 1: An alternative protocol involving a single, 16–24 hour incubation may be used. Standards are prepared in the range 1–64 fmol αANP/tube (the bottle containing reconstituted assay standard is at a concentration equivalent to 64 fmol/tube). To 0.1 ml standard/sample is added 0.1 ml antiserum and 0.1 ml standard/sample is added 0.1 ml antiserum and 0.1ml tracer. Tubes are vortex-mixed and incubated for 16–24 hours at 2–8°C. Separation is achieved by addition of separating reagent as described above. This protocol has been found to give standard curves with reduced sensitivity (25 fmol/tube) at 50% displacement of tracer) and is not routinely tested by our QC department.

Note 2: Non-specific binding (NSB) is not normally determined as it has been shown to be low. If NSB is measured, tubes should be prepared as normal but 0.1ml human αANP at 32 pmol/ml should be used in place of assay standard. Antiserumblank NSB's are artificially high due to the presence of components in the resuspended antiserum which prevent non-specific binding of αANP to separating reagent.

Table 1. Radioimmunoassay protocol, Pipetting Guide (all volumes are in microlitres)

Tubes	TC	Bo	Stan - dard	Sam- ple
Buffer	**-**	**100**	**-**	**-**
Stan- dard	-	**-**	**100**	**-**
Sample	-	**-**		**100**
Anti- serum	-	**100**	**100**	**100**
Vortex mix, cover tubes and incubate for 16-24 hours at 2-8°C				
Tracer	**100**	**100**	**100**	**100**
Vortex mix and incubate for 24 hours at 2-8°C				
Separa ting reagent	-	**250**	**250**	**250**
Vortex mix, incubate for 10 minutes at room temperature. Separate either using magnetic separator for 15 minutes or by centrifugation for 10 minutes at >1500 g. Decant supernatants, drain for 5 minutes and count.				

Calculation of results

The calculation is illustrated using representative data. The assay data collected should be similar to the data shown in table 2.

Calculate the average counts per minute (cpm) for each set of replicate tubes.

Calculate the percent Bo/TC using the following equation:

$$\text{Bo/TC(\%)} = \frac{\text{Bo (cpm)}}{\text{TC (cpm)}} \times 100$$

If the counter background is high, it should be subtracted from all the counts.

Calculate the percent bound for each standard and sample using the following equation:

$$\text{B/Bo(\%)} = \frac{\text{Standard or sample (cpm)}}{\text{Bo (cpm)}} \times 100$$

A standard curve may be generated by plotting the percent B/Bo as a function of the log human αANP concentration.

Plot B/Bo(%) (y axis) against the fmol standard per tube (x axis). The fmol per tube value of the samples can be read directly from the graph. NSB is not normally determined, and is given for information only.

Table 2. Typical assay data

Tube	Conc. (fmol /tube)	Avarage counts/ minute (cpm)	B/TC (%)	B/Bo (%)
TC	-	23325	-	-
NSB	-	568	2.4	-
Bo	-	10846	46.5	100
S1	1	9814	-	90.5
S2	2	8665	-	79.9
S3	4	6900	-	63.6
S4	8	4369	-	40.3
S5	16	2160	-	19.9
S6	32	1364	-	12.6

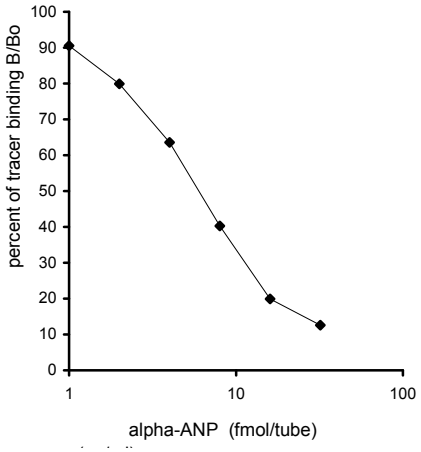

Figure 1: A typical standard curve
(*Do not use to calculate unknown samples!)*

Characterization of assay

Stability

The components of this radioimmunoassay system will have a shelf life of at least 4 weeks from the date of despatch.
Upon arrival, all components should be stored at 2–8°C where they are stable until the expiry date printed on the end pack label.
Store reconstituted reagents at -15°C to -30°C, separating reagent and assay buffer at 2–8°C. Reconstituted components may be re-used within 28 days of dilution. Working dilutions of assay standard should be freshly prepared.

Non-specific binding

The non-specific binding (NSB), defined as the proportion of tracer bound in the absence of antibody (or in the presence of a 100-fold excess of αANP), was determined to be 3.1% (n=54). The NSB was independent of tracer batch and did not change over a 14 week storage period.

Sensitivity

The sensitivity, defined as the amount of human αANP needed to reduce zero dose binding by two standard deviations, was less than 1.0 fmol per tube (<3 pg/tube) and calculated as 0.4 fmol/tube by plotting B/Bo(%) against concentration on a linear scale. This is equivalent to a sample concentration of 4 fmol (12 pg)/ml.

Note: The sensitivity of the 24 hour assay was determined as 0.6 fmol (1.8 pg) per tube. This is equivalent to a sample concentration of 6 fmol (18 pg)/ml.

Specificity

Cross-reactivity (at 50% displacement).
The antiserum cross-reactivity with related and other important compounds are shown below:

Compound	Cross-reactivity (%)
Human αANP	100
Rat αANP	70
Rat ANF8-33	95
Atriopeptin 1	0.002
Atriopeptin 2	<0.1
Atriopeptin 3	<0.31
αANP (7-28)	<0.01
αANP (13-28)	<0.01
αANP (18-28)	<0.01

Note: The effect of dithiothreitol (1 mM) was investigated. It was found to interfere in the assay and its use should be avoided.

Reproducibility

The assay reproducibility has been expressed graphically in the form of a precision profile. This was determined by measuring the standard deviation between assay duplicates over 20 assays.
Between batch reproducibility was assessed by repeated analysis of the same sample in successive assays. These data are typical of the reproducibility between batches and operators.

Control	Mean (fmol /tube)	SD	CV (%)	n
1	2.30	0.38	16.5	50
2	6.70	0.99	14.8	52
3	11.47	1.27	11.1	53

Additional information

Components from various lots or from kits of different manufacturers should not be mixed or interchanged.

Warning

For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Precautions

Radioactivity

This product contains radioactive material. It is the responsibility of the user to ensure that local regulations or code of practice related to the handling of radioactive materials are

Chemical hazard

Components contain sodium azide as an antimicrobial agent. Dispose of waste by flushing with copious amount of water to avoid build-up of explosive metallic azides in copper and lead plumbing. The total azide present in each pack is 130 mg.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water (see safety data sheet for specific advice).

Safety data sheet

Product name:

Sodium azide
CAS No. 26628-22-8
R: 22-32 Toxic if swallowed. Contact with acids liberates very toxic gas.
S: (1/2)-28-45 (Keep locked up and out of the reach of children). After contact with skin, wash immediately with plenty of water. In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).

Composition:

Sodium azide solution.

Hazards identification:

Toxic if swallowed, inhaled, or absorbed through skin. May cause eye and skin irritation.

First aid measures:

In case of contact, immediately flush eyes or skin with copious amounts of water. If inhaled remove to fresh air. In severe cases seek medical attention.

Fire fighting measures:

Dry chemical powder. Do not use water.

Accidental release:

Wear suitable protective clothing including laboratory overalls, safety glasses and gloves. Mop up spill area, place waste in a bag and hold for waste disposal. Wash spill site area after material pick-up is complete.

Handling and storage:

Wear suitable protective clothing including overalls, safety glasses and gloves. Do not get in eyes, on skin, or on clothing. Wash thoroughly after handling.

Personal protection:

See above instructions for handling and storage.

Physical and chemical properties:

Formula weight: 65.01. Density: 1.850.

Stability and reactivity:

Avoid contact with metals and acid chlorides. This yields a very toxic gas.

Toxicological information:

LD50: 27 mg/kg oral, rat
LD50: 20 mg/kg skin, rabbit

Ecological information:

Not applicable

Disposal consideration:

Up to 5 vials worth of material may be disposed of directly down the sink with water. If 6 or more vials are to be disposed of they should pass through a chemical waste route.
Note: Inorganic azides will react with lead and copper plumbing fixtures to give explosive residues. Disposal of significant quantities of azides via such plumbing is not recommended.

Transport information :

No special considerations applicable.

Regulatory information:

The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.

	Use by	CONTROL	Control
LOT	Batch code	CAL	Standard
	Caution, consult accompanying documents	CT	Coated tube
	Biological risk	TRAC	Tracer
	Consult operating instructions	WASHB	Wash buffer
	Manufacturer		Temperature limitation Store between 2-8°C
REF	Catalogue number		Radioactive Material

Website: http://www.izotop.hu
Technical e-mail: immuno@izotop.hu
Commercial e-mail: commerce@izotop.hu

INSTITUTE OF ISOTOPES Ltd.
1535 Budapest. Pf.: 851.
Tel.: (36-1)392-2577, Fax: (36-1)395-9247