Endothelin-1,2 [^{125}I] RIA KIT

(REF: RK-535)

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

The [^{125}I]ET-1,2 RIA system provides direct quantitative *in vitro* determination of Endothelin-1 (ET-1) and Endothelin-2 (ET-2) in biological samples. ET-1 can be assayed in the range of 0.5-64 fmol/tube (1.25-160 pg/tube) using a 3-day delayed addition protocol. Each kit contains materials sufficient for 100 determinations permitting the construction of one standard curve and the assay of 40 unknowns in duplicate.

Introduction

Endothelin-1 (ET-1) is a recently described potent vasoconstrictor peptide produced by vascular endothelial cells. It is an acidic 21 amino acid peptide with a molecular weight of 2492 Da, and contains two sets of intrachain disulphide bonds, an unusual feature for a mammalian endogenous peptide, but a configuration often found in many peptide toxins. In fact, ET-1 shows a striking similarity to a group of peptide toxins from snake venom.

The C-terminal Trp21 and the intramolecular loop structure are both important for vasoconstrictor activity.

ET-1 is produced in vascular endothelial cells from a larger prepro-peptide that requires an unusual proteolytic processing between a Trp and Val residue of a 39-residue intermediate (big endothelin). Human big ET consists of 38 amino acids and is similarly processed. ET was originally purified from porcine aortic endothelial cells and was later found to be identical to human ET-1. Rat ET was sequenced and found to be homologous to porcine ET. Since then, this family has been expanded and renamed after the discovery of three ET genes in humans, of which porcine ET is ET-1, [Trp6,Leu7]ET is ET-2 and rat ET ([Thr2,Phe4,Thr5,Tyr6,Lys7,Tyr14]ET) is ET-3.

ET-1 is the most potent vasoconstrictor known to date, causing a strong and sustained vasoconstrictor response in most arteries and veins of many mammalian species and exhibiting extremely longlasting pressor activities in vitro. This activity is mediated by an increase in the intracellular concentration of Ca2+, by influx of extracellular Ca2+ through plasma membrane channels, and/or mobilization of intracellular Ca2+ by phospholipase C-stimulated inositol trisphosphate formation. However, it also has an extensive range of binding sites, not confined to vascular tissue, suggesting a wider range of activities than simply vasoconstriction. In fact, from recent in vitro experiments, ET-1 has been reported to stimulate the release of several hormones including atrial natriuretic peptide (ANP) from rat cardiac myocytes, eicosanoids and endothelium-derived relaxing factor (EDRF) from vascular beds and to modulate the release of noradrenaline from sympathetic termini. It also has effects on kidney cells, including the stimulation of mitogenesis in rat glomerular mesangial cells, the inhibition of renin release from rat glomerulus and causes acute renal failure when perfused through isolated rat kidneys. Finally, it stimulates the proliferation of vascular smooth muscle cells and contracts both airway and intestinal smooth muscle.

There is a great deal of interest in the physiological role of ET-1 in vivo, in both animal models (for example, rat) and in humans where very little is known about the levels in both normal and disease states (such as hypertension or after haemodialysis for example). Thus an immunoassay is required which is both specific and sensitive. This assay system fulfils both of these conditions and will enable researchers to study the release of ET under both normal and pathological conditions.

Principle of method

This assay is based on the competition between unlabelled ET-1 and a fixed quantity of [^{125}I] – labelled ET-1 (synthetic) for a limited number of binding sites on an ET-1 specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound will be inversely proportional to the concentration of added non-radioactive ligand.

The antibody bound ET-1 is then reacted with the separating second antibody reagent. Separation of the antibody bound fraction is effected by either magnetic separation or centrifugation of the separating reagent suspension and decantation of the supernatant.

Measurement of the radioactivity in the pellet enables the amount of labelled ET-1 in the bound fraction to be calculated. The concentration of unlabelled ET-1 in the sample is then determined by interpolation from a standard curve. The standard curve and samples should be prepared simultaneously.

Contents of the kit

1. 1 vial TRACER, lyophilized, reconstitution with 11 ml assay buffer, containing ~ 48 kBq, 1.3 µCi [^{125}I]ET-1 (synthetic). The final solution contains [^{125}I]ET-1 in 0.02 M borate buffer pH7.4 containing 0.1%(w/v) sodium azide. Store at 2-8 ^{o}C.

2. 1 vial STANDARD, lyophilized, reconstitution with 4.0 ml assay buffer. The final solution contains ET-1 (synthetic) at a concentration of 2.56 pmol/ml in 0.02 M borate buffer pH7.4 containing 0.1%(w/v) sodium azide. Store at 2-8 ^{o}C.

3. 1 vial ANTISERUM, lyophilized, reconstitution with 11 ml assay buffer. The final solution contains rabbit anti-E-1 serum in 0.02 M borate buffer pH7.4 containing 0.1%(w/v) sodium azide. Store at 2-8 ^{o}C.

4. 1 vial ASSAY BUFFER concentrate (5 ml), dilution to 50 ml. On dilution this will give 0.02 M borate buffer pH7.4 containing 0.1%(w/v) sodium azide. Store at 2 -8 ^{o}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 ^{o}C.

Pack leaflet

Materials, tools and equipment required

Pipettes or pipetting equipment with disposable tips (100µl, 200µl, 250µl, 500µl, 4.0ml and 11ml); disposable polypropylene or polystyrene tubes (12 x 75 mm); refrigerator; glass measuring cylinder (50 ml); 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 >1500 xg.

Specimen collection and sample preparation

It is advised that if measurements are to be made in body fluids such as plasma, the sample should be collected into tubes containing either lithium/heparin or 7.5mM EDTA and aprotinin (500KIU/ml). Blood should be centrifuged immediately at 2000 xg for 10 minutes at 4°C to remove cells and the plasma stored below -15°C prior to analysis.

To extract ET from plasma, the following protocol is recommended, using Amprep™ 500mg C2 columns:

1) Equilibrate the column by washing with 2ml methanol followed by 2ml water. For this and subsequent washes maintain the flow rate of <5ml/minute).

2) Acidify 1ml plasma with 0.25ml 2M HCl and load 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 5ml water + 0.1% trifluoroacetic acid (TFA).

4) Wash with 2ml 80% acetonitrile (or methanol) in water + 0.1% TFA and collect eluent in a glass or polypropylene tube.

5) Dry down under nitrogen.

6) Reconstitute in 250µl assay buffer and take 2x100µl for analysis.

This protocol should 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.

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. Reconstituted components should be stored at 2-8°C and may be reused within 28 days of dilution.

Preparation: Equilibrate all reagents and samples to room temperature prior to use. Assay buffer: Transfer the contents of the bottle, with washings, to a 50 ml measuring cylinder and dilute to 50 ml with distilled or deionized water. Mix well. Assay buffer is used to redissolve 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.

Preparation of working standards

1. Label 9 polystyrene or polypropylene tubes 0.5, 1 ,2, 4, 8, 16, 32, 64 and 128.

2. Pipette 500 µl of assay buffer into all tubes.

3. Into the 128 tube pipette 500 µl of stock standard (2.56 pmol/ml) and vortex thoroughly.

4. Transfer 500 µl from the 128 tube to the 64 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 8 standard levels of ET-1 ranging from 0.5-64 fmol.

Note: The 128 standard is not used for the standard curve. It is diluted further to give the 64 tube and this is the top standard. Working standards should be freshly prepared before each assay, and not re-used.

Assay procedure

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

1. Equilibrate all reagents to room temperature.
2. Prepare reagents and assay standards as described in the previous section.
3. Label 12x75 mm disposable tubes in duplicate for total count (TC), non-specific binding (NSB), zero standard (Bo), standards and samples.
4. Pipette **200 µl** assay buffer into NSB tubes and **100 µl** assay buffer into Bo tubes.
5. Starting with the most dilute, pipette **100** µl of each standard (S1-8) into the appropriately labelled tubes.
6. Pipette **100 µl** unknown sample (M_x) directly into appropriately labelled tubes.
7. Pipette **100 µl** antiserum into all tubes except NSB and 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.
9. Pipette **100 µl** of tracer into **all** tubes. The TC tubes should be stoppered and put aside for counting.
10. Vortex mix all tubes thoroughly. Cover the tubes, and incubate for 16-24 hours at 2-8°C.
11. 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 all tubes thoroughly and incubate at room temperature for 10 minutes.
12. Separate the antibody bound fraction using either magnetic separation or centrifugation as described below.

Magnetic separation

Attach the rack on the magnetic 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 re-invert 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.

Table 1. Assay Protocol, Pipetting Guide (all volumes are in microlitres)

Tubes	TC	NSB	Bo	Stan - dard	Sam-ple
Buffer	-	**200**	**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**	**100**
Vortex mix, cover tubes and incubate for 16-24 hours at 2-8 °C.					
Separa-ting reagent	-	**250**	**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 xg .					
Decant tubes and blot on filter paper.					
Count radioactivity (60 sec/tube).					
Calculate the results.					

Calculation of results

Calculate the average count per minute (cpm) for each pair of assay tubes.
Calculate the percent NSB/TC using the following equation:

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

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

Users may wish to subtract the average NSB cpm from all tubes except TC. If so the appropriate correction should be made. Calculate the percent Bo/TC using the following equation:

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

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

$$B/Bo(\%) = \frac{S1\text{-}8/M_x\ (cpm) - NSB\ (cpm)}{Bo\ (cpm) - NSB\ (cpm)} \times 100$$

A standard curve can be generated by plotting the percent B/Bo as a function of the log ET-1 concentration.

Plot B/Bo(%) (y-axis) against concentration fmol standard per tube (x-axis). The concentration (fmol per tube) value of the samples can be read directly from the graph (see Figure 1).

Table 2. Typical assay data

Tube	Conc. (fmol /tube)	Mean counts (cpm)	B/TC (%)	B/Bo (%)
TC	-	12524	-	-
NSB	-	178	1.7	-
Bo	-	7401	57.4	-
S1	0.5	7286	-	98
S2	1	7205	-	97
S3	2	6741	-	90
S4	4	6152	-	82
S5	8	4666	-	62
S6	16	3089	-	40
S7	32	2104	-	26
S8	64	1500	-	18

Note: The counts were obtained using new tracer. The counts will decline in line with the age of the tracer.

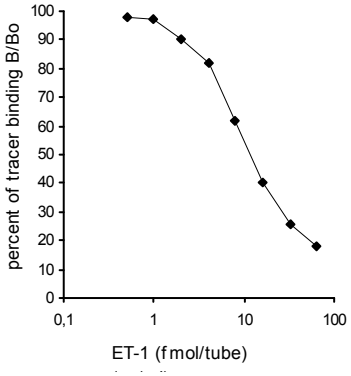


Figure 1: A typical standard curve
(*Do not use to calculate unknown samples!)*

Characterization of assay

Stability

The components of this assay 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 pack label.
Once reconstituted, all reagents should be stored at 2-8°C where they are stable for at least 28 days.

Non-specific binding

The non-specific binding (NSB) defined as the proportion of tracer bound in the absence of antibody was determined to be <1.52%.
The NSB was independent of tracer batch and did not change over 14-week storage period.

Sensitivity

The sensitivity, defined as the amount of ET-1 needed to reduce zero doze binding by two standard deviations was 1.03 fmol/tube (2.6 pg/tube).

Specificity

The cross-reactivity, as determined by the concentration giving 50% B/Bo with a number of related compounds is shown below.

Compound	(%) Cross-reactivity
Endothelin-1 (synthetic)	100
Endothelin-2 (synthetic)	204
Endothelin-3 (synthetic)	0.0024
Big endothelin-1 (human)	37.9
Big endothelin-1 (porcine)	32.9
ANP (human, synthetic)	<0.024
BNP (porcine, synthetic)	<0.024

Precision

The intra-assay reproducibility for duplicate determinations was calculated by repeatedly measuring an unknown in the assay. The result obtained was 32.66 ± 1.33 fmol, CV = 4.1%. The inter-assay reproducibility was assessed by repeated measurement of the same samples in successive assays using different batches of reagents and different operators. These results are shown below:

Control	Number of replicates	Mean ± SD	CV (%)
1	18	2.66±0.45	17.4
2	18	6.30±0.66	10.4
3	18	22.50±2.71	12.1

Additional information

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

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 satisfied.

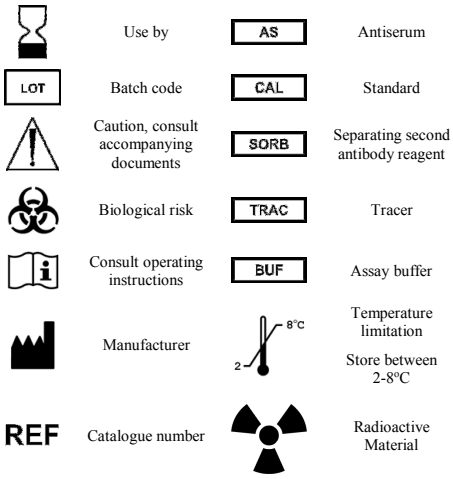
Chemical hazard

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 80 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.



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