Endothelin 1-21 Specific [ ] RIA KIT  
(REF: RK-555)

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

The [125I]ET 1-21 specific RIA system provides direct quantitative in vitro determination of Endothelin 1-21 in biological samples. ET 1-21 can be assayed in the range of 0.25-16 fmol/tube (0.623-39.87 pg/tube) using an overnight delayed addition protocol. Each kit contains materials sufficient for 100 determinations permitting the construction of one standard curve and the assay of 41 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 venoms.

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 vasconstrictor 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 triphosphate 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 glomeruli 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.

ET-like immunoreactivity has been identified in the plasma of normal and hypertensive subjects and has been shown to be elevated in haemodialysis patients. It has been demonstrated that plasma ET-like immunoreactivity consists of both ET-1 and its precursor Big ET. As Big ET is thought to be inactive it is of importance to determine if the contributions of active and inactive ET forms to plasma immunoreactivity vary independently in any situations. This immunoassay has been successfully applied to the demonstration of conversion in vivo of circulating big ET to ET-1.

Another area of research where it is very important to distinguish big ET and ET-1 is in the study of the enzyme responsible for this cleavage, endothelin converting enzyme (ECE). Several studies have implicated enzymes such as pepsin and cathepsin D as being responsible for this cleavage and radioimmunoassay using specific c assays has been a very useful tool in this work.

### Principle of method

This assay is based on the competition between unlabelled ET-1 and a fixed quantity of [125I] labelled ET-3 (synthetic) for a limited number of binding sites on an ET 1-21 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 1-21 is then reacted with the separating second antibody reagent. Separation of the antibody bound fraction is effectuated 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-3 in the bound fraction to be calculated. The concentration of unlabelled ET-1 21 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 1 ml assay buffer, containing ~ 48 kBq, 1.3 µCi [125I]-ET-3 (synthetic).
2. The solution contains [125I]-ET-3 in 0.02 M borate buffer pH 7.4 containing 0.1% (w/v) sodium azide. Store at -2-8°C.
3. 2 vials STANDARD, lyophilized, reconstitution with 2.0 ml assay buffer. The final solution contains ET-1 (synthetic) at a concentration of 320 fmol/ml in 0.02 M borate buffer pH 7.4 containing 0.1% (w/v) sodium azide. Store at 2-8°C.
4. 3 vials ANTISERUM, lyophilized, reconstitution with 11 ml assay buffer. The final solution contains rabbit anti-endothelin serum in 0.02 M borate buffer pH 7.4 containing 0.1% (w/v) sodium azide. Store at 2-8°C.
5. 4 vials ASSAY BUFFER concentrate (10 ml), dilution to 100 ml. On dilution this will give 0.02 M borate buffer pH 7.4 containing 0.1% (w/v) sodium azide. Store at 2-8°C.
6. 5 vials SEPARATING ANTIBODY REAGENT (30 ml), ready for use, containing donkey anti-rabbit serum coated on to magnetizable polymer particles with sodium azide and colour-coded, blue-green. Store at 2-8°C.

### Pack leaflet

### Materials, tools and equipment required

Pipettes or pipetting equipment with disposable tips (100µl, 200µl, 250µl, 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 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. Some users may wish to add aprotinin (500KIU/ml), however we have found it makes little difference. 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. It may be stable for several months stored in this way.

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, centrifuge at 10000 xg for 5 minutes at room temperature 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 elute in a glass or polypropylene tube.
5. Dry down under nitrogen or in a centrifugal evaporator.
6. Reconstitute in 250µl assay buffer and take 2x100µl for analysis. Larger reconstitution volumes may be used depending on the expected concentration. Assay buffer remaining after component reconstitution is provided for this.

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 elution 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 of working standards
1. Label 7 polystyrene or polypropylene tubes 0.25, 0.5, 1, 2, 4, 8 and 16.
2. Pipette 500 µl of assay buffer into all tubes.
3. Into the 16 tube pipette 500 µl of stock standard (320 fmol/ml) and vortex thoroughly.
4. Transfer 500 µl from the 16 tube to the 8 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 7 standard levels of ET-1 ranging from 0.25 fmol to 16 fmol/tube (0.623-39.87 pg/tube).

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

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

1. Place the tubes in a rack and centrifugation at 1500 x g for 5 minutes.
2. Centrifuge at 4°C for 10 minutes at 1500 x g 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.
3. 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.
4. Determine the radioactivity present in each tube by counting for at least 60 seconds in a gamma scintillation counter.

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.

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:

\[ \text{Bo/TC} = \frac{\text{Bo (cpm)} - \text{NSB (cpm)}}{\text{TC (cpm)}} \times 100 \]

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

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.

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Figure 1: A typical standard curve

(Do not use to calculate unknown samples!)
Non-specific binding

The non-specific binding (NSB) defined as the proportion of tracer bound in the absence of antibody was determined to be <2.3%, (n=36). 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 0.2 fmol/tube (0.5 pg/tube) (4 replicates of zero). For a 100 µl sample of a 1 ml extract of plasma reconstituted to 250 µl this is therefore equivalent to 0.5 fmol/ml (1.2 pg/ml) of original sample.

Specificity

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>(%) Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelin-1 (synthetic)</td>
<td>100</td>
</tr>
<tr>
<td>Endothelin-2 (synthetic)</td>
<td>144</td>
</tr>
<tr>
<td>Endothelin-3 (synthetic)</td>
<td>52</td>
</tr>
<tr>
<td>Big endothelin-1 (human)</td>
<td>0.4</td>
</tr>
<tr>
<td>Big endothelin-22-38 (human)</td>
<td>&lt;0.003318</td>
</tr>
<tr>
<td>Big endothelin-1 (porcine)</td>
<td>0.26</td>
</tr>
<tr>
<td>Big endothelin-22-39 (porcine)</td>
<td>&lt;0.00312</td>
</tr>
<tr>
<td>ANP (human, synthetic)</td>
<td>&lt;0.00625</td>
</tr>
<tr>
<td>BNP (porcine, synthetic)</td>
<td>&lt;0.00625</td>
</tr>
<tr>
<td>Sarafotoxin S6b</td>
<td>12</td>
</tr>
<tr>
<td>Vasoactive intestinal contractor (mouse)</td>
<td>100</td>
</tr>
</tbody>
</table>

Precision

The intra-assay precision for duplicate determinations was calculated by repeatedly measuring buffer controls in the assay. The results are shown below (mean values as fmol/tube):

<table>
<thead>
<tr>
<th>Control</th>
<th>Number of replicates</th>
<th>Mean ± 2SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>8.28±0.396</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>3.04±0.09</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>0.98±0.06</td>
<td>6.2</td>
</tr>
</tbody>
</table>

The between assay precision was assessed by repeated measurement of the same samples in successive assays. The results are shown below (mean values as fmol/tube):

<table>
<thead>
<tr>
<th>Control</th>
<th>Number of replicates</th>
<th>Mean ± 2SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33</td>
<td>6.87±0.95</td>
<td>13.8</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>3.95±0.38</td>
<td>9.5</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>1.38±0.24</td>
<td>17.3</td>
</tr>
</tbody>
</table>

Sample validation data

We have assayed normal plasma samples following Amprep extraction as described. Mean plasma values of 6fmol/ml (15pg/ml) were obtained for 1ml samples after extraction. The recovery of the extraction procedure as determined by spiking experiments using labelled ET-1 was 79%. Extracts were shown to dilute in parallel to the standard curve following reconstitution.

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 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. 262628-22-8

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:


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.