Endothelin-1,2 (High sensitivity)[\(^{125}\)I] RIA KIT

(REF: RK-545)

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

The [\(^{125}\)I]ET-1,2 (High sensitivity) RIA system provides direct quantitative in vitro determination of Endothelin-1 (ET-1), ET-2 and Big ET-1 in biological samples. ET-1 can be assayed in the range of 0.25-32 fmol/tube (0.625-79.74 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 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 2942 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-ET peptide that requires an unusual proteolytic processing between a Thr and Val peptide toxins. In fact, ET-91 shows a striking resemblance to a group of peptide toxins from snake venom.

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

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 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°C.

2. 1 vial 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 pH7.4 containing 0.1% (w/v) sodium azide. Store at 2-8°C.

3. 1 vial ANTISSERUM, lyophilized, reconstitution with 11 ml assay buffer. The final solution contains rabbit anti-endothelin serum in 0.02 M borate buffer pH7.4 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 0.02 M borate buffer pH7.4 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.

Pack leaflet

Materials, tools and equipment required
Pipeets or pipetting equipment with disposable tips (100µl, 200µl, 250µl, 500µl, 2.0ml and 11ml); disposable polypropylene or polysyntere 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 about 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 eluate 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 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 100ml measuring cylinder and dilute to 100ml with distilled or deionized water. Mix well. Assay buffer is used to redissolve all other components. The reagents (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 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 8 standard levels of ET-1 ranging from 0.25 fmol to 32 fmol/tube (0.623-79.74 pg/tube).

Table of divination, the preparation of ET-1 (fmol/tube) and the quality of the samples. The actual expiry date is given on the package label and on the quality certificate.

Note: The 32 fmol/tube standard is prepared by taking aliquots directly from the dilution curve of ET-1 (320 fmol/ml) vial. 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 (Mx) 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, and incubate overnight (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 overnight (16-24 hours) at 2-8°C.

Assay procedure

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 centrifugation or magnetic separation as described below.

Magnetic separation

Attach the magnet 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 magnet 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.

13. 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.

Centrifugation

Centrifuge all tubes at 4°C for 10 minutes at 1500 xg. 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. Repeat this doubling dilution successively with the remaining tubes.

Calculate percent of tracer binding Bo/TC

Calculate the percent NSB/TC using the following equation:

\[
\text{NSB/TC} \% = \left( \frac{\text{NSB (cpm)}}{\text{TC (cpm)}} \right) \times 100
\]

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:

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

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).

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

Table 1. Typical assay data

<table>
<thead>
<tr>
<th>Tube</th>
<th>Conc. (fmol/tube)</th>
<th>Mean counts (cpm)</th>
<th>B/TC (%)</th>
<th>B/Bo (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>-</td>
<td>13176</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NSB</td>
<td>-</td>
<td>202</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Bo</td>
<td>-</td>
<td>5342</td>
<td>39</td>
<td>-</td>
</tr>
<tr>
<td>S1</td>
<td>0.25</td>
<td>5260</td>
<td>-</td>
<td>98</td>
</tr>
<tr>
<td>S2</td>
<td>0.5</td>
<td>5045</td>
<td>-</td>
<td>94</td>
</tr>
<tr>
<td>S3</td>
<td>1</td>
<td>4484</td>
<td>-</td>
<td>83</td>
</tr>
<tr>
<td>S4</td>
<td>2</td>
<td>3949</td>
<td>-</td>
<td>73</td>
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<tr>
<td>S5</td>
<td>4</td>
<td>2953</td>
<td>-</td>
<td>54</td>
</tr>
<tr>
<td>S6</td>
<td>8</td>
<td>1992</td>
<td>-</td>
<td>35</td>
</tr>
<tr>
<td>S7</td>
<td>16</td>
<td>1353</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>S8</td>
<td>32</td>
<td>897</td>
<td>-</td>
<td>14</td>
</tr>
</tbody>
</table>

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Figure 1: A typical standard curve

(Do not use to calculate unknown samples!)

Calculation of results

Calculate the average count per minute (CPM) per each pair of assay tubes.

Table 1. Typical assay data
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 <2%.
The NSB was independent of tracer batch 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).

Addition 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).

Sample validation data
We have assayed normal plasma samples following Amperp 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.

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 (0.5pg/tube) (4 replicates of zero). For a 100µl sample of a 1ml extract of plasma reconstituted to 250µl this is therefore equivalent to 0.5 fmol/ml (1.246 pg/ml) of original sample.

Specificity
The cross-reactivity, as determined by the concentration giving 50% B/Bo with water (see safety data sheet for specific advice). 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.

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:

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