Instruction

EURIA-Endothelin

Endothelin radioimmunoassay

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

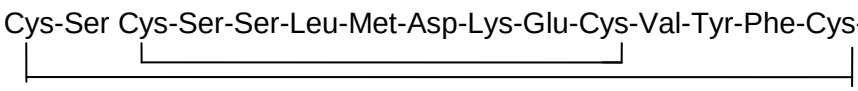
REF **RB 304 RUO**

Σ 100

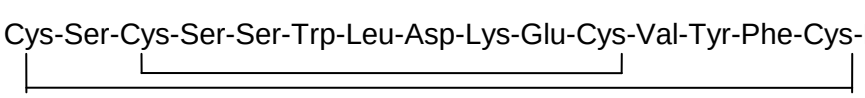
INTRODUCTION

Endothelin is synthesized in and released from endothelial cells (1). Endothelin occurs in three different molecular forms: Endothelin 1, 2 and 3. All three are 21 aminoacid residues peptides containing two disulfide bridges (2). The aminoacid sequences are as follows:

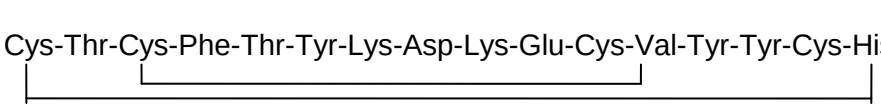
Endothelin-1 (human, porcine, canine, rat, mouse, bovine):



Endothelin-2 (human, canine):



Endothelin-3 (human, porcine, rat, rabbit):



Endothelin 1 is a potent vasoconstrictor peptide. Endothelin occurs in the circulation in very low concentrations (~5 pmol/L) and may be an important factor in the regulation of local blood circulation, of blood pressure and contractility of the heart (3, 4, 5, 6). Sensitive radioimmunoassays have been developed for assay of endothelin in human plasma (7, 8).

PRINCIPLE OF THE METHOD

The intended use of these reagents is for assay of endothelin in human plasma. Endothelin is extracted from plasma by means of Sep-pak C-18 columns or similar. Endothelin in the extracts is assayed by a competitive radioimmunoassay using a rabbit antiserum raised against an endothelin-1 albumin conjugate. Endothelin in standards and samples compete with ^{125}I-labelled endothelin-1 in binding to the antibodies. ^{125}I-endothelin binds in a reverse proportion to the concentration of endothelin in standards and samples. Antibody-bound ^{125}I-endothelin is separated from the unbound fraction using double antibody solid phase. The radioactivity of the bound fraction is measured in a gamma counter. The antiserum used in this assay cross reacts with endothelin-1 (100%), endothelin-2 (50%) and endothelin-3 (100%).

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

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.

The reagents in this kit contain sodium azide. Contact with copper or lead drain pipes may result in the cumulative formation of highly explosive azide deposits. On disposal of the reagents in the sewerage, always flush with copious amounts of water, which prevents metallic azide formation. Plumbing suspected of being contaminated with these explosive deposits should be rinsed thoroughly with 10% sodium hydroxide solution.

COMPOSITION OF THE REAGENT KIT

The reagents provided in this kit are sufficient for 100 tubes.

1. Anti-endothelin (Reagent A)
Rabbit antiserum raised against synthetic endothelin-1 conjugated to bovine albumin. 50 mL antiserum in working dilution. Dilution buffer: 0.05 M phosphate buffer, pH 7.4, 0.2% bovine serum albumin, 0.1% Triton X-100, 0.015% EDTA and 0.05% sodium azide. Contains 0.05 g/L of Brilliant Yellow. For 100 tubes.

2. ^{125}I-endothelin (Reagent B)
Contains an activity of 54 KBq or 1.45 μCi at reference date. Synthetic endothelin-1 is iodinated (^{125}I). The monoiodinated form is purified by HPLC.
Specific activity: 62-77 MBq/nmol or 1700-2100 μCi/nmol. Lyophilized in 5.0 mL 0.5M phosphate buffer, pH 7.4, with 2.0% bovine albumin, 1.0% Triton X-100, 0.15% EDTA and 0.5% sodium azide. Contains 0.15 g/L Patent blue.
Reconstitution in 52 mL distilled water. For 100 tubes.

3. Double antibody solid phase (Reagent C)
Anti-rabbit-Ig coupled to cellulose particles in 0.01 M phosphate buffer pH 6.8 with 0.25% Human serum albumin, 0.045% NaCl, 0.05% NaN_3, 0.185% EDTA and 0.05% Tween 80.
11 mL suspension.

4. Assay buffer (Reagent D)
50 mL 0.05 M phosphate buffer, pH 7.4, with 0.2% bovine albumin, 0.1% Triton X-100, 0.015% EDTA and 0.05% sodium azide.

5. Endothelin standard (Reagent E)
Concentration: 125 pmol/L of synthetic human endothelin-1. 5.00 mL standard after reconstitution. Lyophilized in 0.05 M phosphate buffer, pH 7.4 with 0.2% bovine albumin, 0.1% Triton X-100, 0.015% EDTA and 0.05% sodium azide.

6. Controls (Reagent F-G)
Lyophilized controls with two different levels of endothelin-1. 1.00 mL of each control after reconstitution. The endothelin concentrations are given on the labels of the vials. The controls should be assayed directly without extraction. Contains 0.05% sodium azide.

EQUIPMENT AND REAGENTS REQUIRED BUT NOT PROVIDED

Disposable test tubes 11-13 x 70 mm, polystyrene.

Disposable test tubes 11-13 x 55 mm, polystyrene.

Pipettes with disposable tips, 100 and 500 µL.

Sep-pak C-18 cartridges (Waters).

Trifluoro-acetic acid (TFA).

Methanol, pro analysi.

Distilled water.

Vortex mixer.

Centrifuge, refrigerated, capable for min 1700 x g.

Glass tubes 15 mL.

Glass tubes, e.g. 11-13 x 55 mm, for standard preparation.

Heating bath.

Equipment for evaporation with a stream of N_2.

5 mL disposable plastic syringes.

Well-type gammacounter.

REAGENT PREPARATION AND STORAGE

Store all reagents at 2-8° C before reconstitution and use. The stability of the reagents is indicated on the labels of the vials. For lyophilized reagents the expiry date is valid for the unreconstituted reagents. The reconstituted reagents are stable for 8 weeks if stored properly.

The water used for reconstitution of lyophilized reagents should be distilled in an all-glass apparatus or be of corresponding purity. Dissolve the content in a vial by gentle inversion and avoid foaming.

Reagent A: Anti-endothelin
Ready for use.
Store at 2-8° C.

Reagent B: 125I-endothelin
Reconstitute with 52 mL distilled water.
Store at -18° C or lower if reused.

Reagent C: Double antibody solid phase
Ready for use. Stir continuously during pipetting this reagent.
Store at 2-8° C.

Reagent D: Assay buffer
Ready for use. Store at 2-8° C.

Reagent E: Endothelin standard
Reconstitute with 5.00 mL distilled water.
Store at -18° C or lower if reused.

Reagent F-G: Controls
Reconstitute with 1.00 mL distilled water. Store at -18° C or lower if reused.

SPECIMEN COLLECTION

Subjects should be fasting at least ten hours prior to sample collection. Veinous blood is collected in tubes containing Sodium Heparin (144 U.S.P. Units Sodium Heparin in a 10 mL tube). The samples are immediately cooled in an ice-bath. The plasma is separated by centrifugation at
2-4° C and stored at -20° C or lower. The plasma should be frozen within 1 hour from sample collection. Repeated thawing and freezing must be avoided!!!

ASSAY PROCEDURE

The assay is performed in two steps:

I. Extraction of plasma samples
II. Radioimmunoassay of extracts

For an overview see page 13.

A complete assay includes:

Standards (St-tubes): 7 different concentrations, 0, 3.9, 7.8, 15.6, 31.2, 62.5 and 125 pmol/L.

Controls (C-tubes): For recovery control (C_{REC}) and radioimmunoassay control (C_{RIA})

Samples (P-tubes)

Tubes for determining the ***non-specific binding (NSB-tubes)***
Tubes for determining the ***total radioactivity (TOT-tubes).***

PERFORMANCE

I . Extraction of plasma samples

The described extraction procedure is based on the use of sep-pak C-18 cartridges available from Millipore Intertech, P.O. Box 255, Bedford M.M. 01730, USA. Millipore Intertech has local officies in most European countries.
The same number of sep-pak C-18 cartridges as the number of samples are necessary for the extraction procedure. A sep-pak cartridge rack (Millipore Intertech) simplifies the sample clean-up.

This procedure involves concentration of endothelin with a factor of 10.

1. Load the sep-pak cartridge rack with sep-pak C-18 cartridges. Connect a solvent reservoir to each cartridge. Connect the rack to a vacuum source.

2. Thaw the samples immediately before starting the extraction. Store the samples in an ice-bath (± 0) before adding TFA 1:10 according to 4.

3. Pipette 2.50 mL plasma sample in an 11-13 x 70 mm polystyrene tube.

4. Add 25 µL TFA 1:10 (v/v in water) to the plasma and vortex mix carefully.

5. Centrifuge for 10 minutes at +8° C with a minimum of 1500 x g.

6. Add 10 mL of TFA, H_2O, Methanol (0.50, 99.5, 400) to each cartridge (flow rate 10-20 mL/minute).
   Note! Allow the sep-pak cartridge to get dry in steps 6, 7, 8 and 9.

7. Add 10 mL of TFA: H_2O (0.50, 500) to each cartridge (flow rate 10-20 mL/min).

8. The centrifuged plasma samples are transferred quantitatively to respectively cartridge (4, 5) and allowed to pass through.

9. Add 4 mL of TFA: H_2O (0.50, 500) to each cartridge and allow it to pass through.

10. Elute endothelin from the sep-pak cartridge by adding 4 mL TFA: H_2O: Methanol (0.50, 99.5, 400). Elute slowly with a flowrate of 4-8 mL/minute. Collect the eluate in a glass tube.

11. Evaporate to dryness with a stream of N_2 at +45° C (heating bath). It is important not to heat when the tubes are dry.

12. Dissolve the dry extracts in 0.25 mL assay buffer (reagent D). Shake continuously in a shake apparatus for 10 minutes.

13. The reconstituted extracts can be analysed immediately by the radioimmunoassay procedure or stored frozen (-20° C) until assayed.

RECOVERY CONTROLS

It is important to determine the recovery in the extraction procedure under your own experimental conditions. To estimate the extraction recovery add 50 µL of standard 125 pmol/L to 950 µL blood donor plasma. The concentration will be 6.25 pmol/L. Handle the recovery sample by the same extraction procedure (control a). Perform in duplicate. Extract also the same blood donor plasma after adding 50 µL assay buffer to 950 µL plasma (control b). In the extraction procedure the Endothelin is concentrated by a factor of 10. Analyse the extracts as samples.

% recovery =

$$\frac{\text{(pmol/L found with addition - pmol/L found without addition) x 100}}{6.25 \times 10}$$

II. Radioimmunoassay of extracts
Reagents should be brought to room temperature prior to use. Accuracy in all pipetting steps is essential. All tests (standards, controls and samples) should be performed in duplicate.

1. Reconstitute the reagents according to the instructions on page 6.

2. Prepare the endothelin working standards by dilution of the endothelin standard 125 pmol/L (Reagent E) with the assay buffer (Reagent D) according to the following (use glass tubes for standard preparation):
   a/ 1.00 mL standard 125 pmol/L + 1.00 mL assay buffer = 62.5 pmol/L
   b/ 1.00 mL standard 62.5 pmol/L + 1.00 mL assay buffer = 31.2 pmol/L
   c/ 1.00 mL standard 31.2 pmol/L + 1.00 mL assay buffer = 15.6 pmol/L
   d/ 1.00 mL standard 15.6 pmol/L + 1.00 mL assay buffer = 7.8 pmol/L
   e/ 1.00 mL standard 7.8 pmol/L + 1.00 mL assay buffer = 3.9 pmol/L
   f/ Assay buffer = 0 pmol/L.

   Store the standards at -18° C or lower if reused.

3. Pipette 100 µL of standards, controls and samples in their respective tubes.

4. Pipette 100 µL of the zerostandard in the NSB-tubes.

5. Pipette 500 µL anti-endothelin (Reagent A) in all tubes except the NSB-tubes and the TOT-tubes. Vortex mix.

6. Pipette 500 µLassay buffer (Reagent D) in the NSB-tubes.

7. Incubate for 20-24 hours at 2-8° C.

8. Pipette 500 µL ^{125}I-Endothelin (Reagent B) in all tubes. Vortex mix. The TOT-tubes are sealed and kept aside.

9. Incubate for 20-24 hours at 2-8° C.

10. Pipette 100 µL double antibody solid phase (Reagent C) in all tubes except the TOT-tubes. This reagent should be stirred continuously with a magnetic stirrer during pipetting. Vortex mix.

11. Incubate for 30-60 minutes at 2-8° C.

12. Centrifuge for 15 minutes at +4° C (minimum 1700 x g).

13. Decant the supernatants immediately.

14. Count the radioactivity in all tubes in a gamma counter. Counting time: 2 minutes.

CALCULATIONS

1. Subtract the average count rate (CPM) of the NSB-standard from the count rate (CPM) of the standards, controls and samples.

2. A standard curve is generated by plotting the bound fraction CPM or B/TOT against the concentrations of the endothelin standards. An example of a standard curve is given on page 14.

3. Interpolate the endothelin concentrations of the controls and samples from the generated standard curve. Correct the observed concentrations for the samples with the concentration factor (10).
   Correct the found concentrations in the samples with respect to the recovery in the extraction procedure. It is important that each laboratory estimates the recovery under its own experimental conditions.

4. The standard curve and the calculation of the endothelin concentrations in the samples can also be done by a computer method. A spline method may be used.

ASSAY CHARACTERISTICS

Sensitivity
The lowest detectable concentration is 4 pmol/L. The figure corresponds to a decrease in binding of three x SD of the bound radioactivity in the zero-concentration standard.
Using a concentration factor of 10 in the extraction procedure this corresponds to a sensitivity of 0.4 pmol/L in the sample.

Accuracy
A mean recovery of 80% (± 5%) when known amounts of endothelin-1 in the range 20-100 pmol/L were added to plasma samples.

Precision
Intra assay variation

Level	Coefficient of variation (%CV)	N
12.7 pmol/L	9.5%	25
66.8 pmol/L	2.3%	25

Inter assay variation (total variation)

Level	Coefficient of variation (%CV)	N
12.7 pmol/L	12.0%	25
66.8 pmol/L	4.0%	25

Specificity
The following cross reactions have been found:

Compound	Cross reaction
Endothelin-1, human	100.0%
Endothelin-2, human	48 %
Endothelin-3, human	109 %
Big endothelin, human	6 %

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

QUALITY CONTROL

In order for the laboratory to completely monitor the consistent performance of the radioimmunoassay there are some important factors which must be checked.

1. Controls
The found concentrations of the controls (Reagent F and G) should be within the limits given on the labels of the vials.

2. Recovery control
The recovery should be at least 70% for a valid assay. It is important that the recovery is controlled under the user's own experimental conditions. The recovery obtained at the product development laboratory was 80 ± 5%.

3. Total counts
Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of ^{125}I-endothelin in this kit will give 25 000 CPM (-5, + 20%) at the reference date (counter efficiency = 80%).

4. Maximum binding (Bo/TOT)
Calculate for each assay the % bound radioactivity in the zero-standard: $\frac{Bo}{TOT} \times 100$.

$\frac{Bo}{TOT} \times 100$ is generally 33-50% at the reference date and may have decreased a few % at the expiry date of the kit.

5. Non-specific binding (NSB/TOT)
Calculate for each assay the % non-specific binding $\frac{NSB}{TOT} \times 100$.

$\frac{NSB}{TOT} \times 100$ is less than 5% if decanting is made properly.

6. Shape of standard curve
For example, monitor the 80, 50 and 20% points of the standard line for run to run reproducibility.

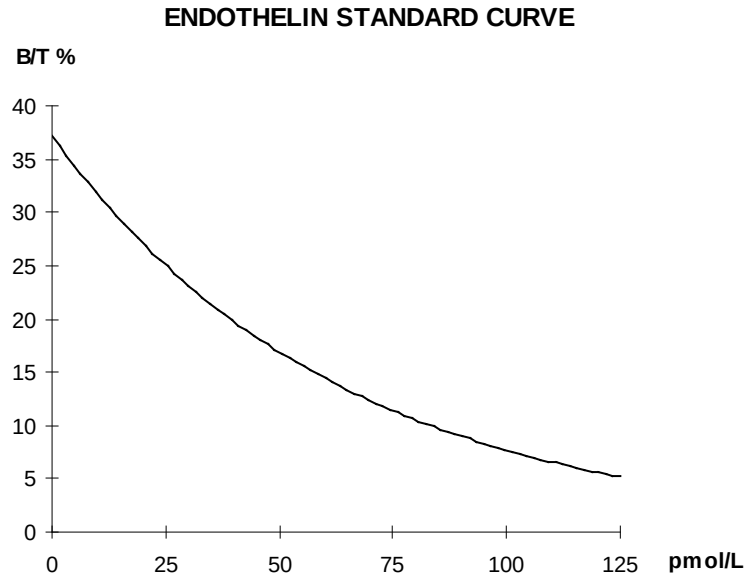
OUTLINE OF THE RIA PROCEDURE

Type of tubes	Tube no	Standard sample or control	Anti-Endothelin (A)	Assay buffer (D)		^{125}I-Endothelin (B)		Double antibody Solid phase (C)	
TOT	1-2	-	-	-	Vortex-mix and incubate for 20-24 hours at 2-8° C.	500 µL	Vortex-mix and incubate for 20-24 hours at 2-8° C.	-	Vortex-mix and incubate for 30-60 min. at 2-8° C. Centrifuge 15 min. at 1700 x g at +4° C. Decant and count the radio-activity of the precipitates
NSB_{st}	3-4	100 µL	-	500		500 µL		100 µL	
Stand 0	5-6	100 µL	500 µL	-		500 µL		100 µL	
Stand 3.9	7-8	100 µL	500 µL	-		500 µL		100 µL	
Stand 7.8	9-10	100 µL	500 µL	-		500 µL		100 µL	
Stand 15.6	11-12	100 µL	500 µL	-		500 µL		100 µL	
Stand 31.3	13-14	100 µL	500 µL	-		500 µL		100 µL	
Stand 62.5	15-16	100 µL	500 µL	-		500 µL		100 µL	
Stand 125	17-18	100 µL	500 µL	-		500 µL		100 µL	
$Control_{RIA}$ (F)	19-20	100 µL	500 µL	-		500 µL		100 µL	
$Control_{RIA}$ (G)	21-22	100 µL	500 µL	-		500 µL		100 µL	
$Control_{REC}$ (a)	23-24	100 µL	500 µL	-		500 µL		100 µL	
$Control_{REC}$ (b)	25-26	100 µL	500 µL	-		500 µL		100 µL	
P, Sample 1	27-28	100 µL	500 µL	-		500 µL		100 µL	
P, Sample 2 etc.	29-30	100 µL	500 µL	-		500 µL		100 µL	

Standard Curve Data

	Average cpm	Corrected cpm	% B/T	Results (pmol/L)
Total counts	31077			
NSB	891			
Standard 0 pmol/L	12281	11390	36.7	
Standard f 3.9 pmol/L	11790	10899	35.1	
Standard e 7.8 pmol/L	11394	10503	33.8	
Standard d 15.6 pmol/L	10295	9404	30.3	
Standard c 31.3 pmol/L	8077	7186	23.1	
Standard b 62.5 pmol/L	4625	3734	12.0	
Standard a 125 pmol/L	2595	1704	5.5	
Low control	9035	8144	26.2	24.5
High control	5514	4623	14.9	53.2

Example of standard curve



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Explanation of symbols

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

REAG A Ab	Endothelin.
REAG B Ag ^{125}I	^{125}I- endothelin.
REAG C DASP	Double antibody solid phase.
REAG D BUF AS	Assay buffer.
REAG E CAL 125	Endothelin standard, 125 pmol/L.
REAG F CONTROL	Control.
REAG G CONTROL	Control.

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