ENDOTHELIN
THE NEXT GENERATION

Enzyme immunoassay for the quantitative determination of Endothelin (1-21) in biological fluids

Kat. Nr. BI-20052
12 x 8 tests

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1. Introduction

Endothelin (ET), a potent vasoconstrictor peptide, was originally isolated from cultured porcine aortic endothelial cells by Yanagisawa et al. (1). Since their report in 1988, two additional isoforms of endothelin, endothelin-2, and endothelin-3 have been purified. All isoforms are composed of 21-amino acids with two intra-chain disulfid bridges, linking paired cysteine amino acid residues. ET-2 exhibits the closest structural similarity to ET-1, differing by only two amino acid residues, while ET-3 differs by six amino acids (2). The genes encoding these peptides have been identified in the human, porcine and rat genome (2).
All of the endothelin peptides are synthesized through proteolytic processing of a 203-residue peptide termed preproendothelin. The polypeptides are termed Big Endothelin and consist of 38-amino acid residues. Big ET (1-38) is cleaved, after synthesis in the cytoplasma, by the proteolytic action of a membrane-bound metalloproteinase, the Endothelin Converting Enzyme (ECE). The cleavage takes place in the intracellular component and on the cell surface and leads to the active ET (1-21) and to the C-terminal fragment (22-38). The physiological importance of cleavage of Big ET is indicated by the reported 140-fold increase in vasoconstrictor activity upon cleavage to ET (3). The half-life of ET in the plasma is less than one minute, whereas clearance of Big ET is much slower (4).
Endothelin has been identified in a variety of tissues, including lung, kidney, brain, pituitary and peripheral endocrine tissues and placenta (5, 6). ET-1, in contrast to ET-2 and ET-3 is also produced by endothelial cells. The vascular endothelium has proven to be the most abundant source of ET-1 in vivo (1).
The biological role of ET extends beyond regulating vascular tone also in its growth regulatory properties. The peptide interacts in an autocrine/paracrine manner with specific ET receptors found on numerous cells, including smooth muscle cells, myoctyes, and fibroblasts (7, 8).
The biology, biochemistry and the pathophysiological role of endothelins are summarized in recent reviews (2, 9, 10).

Clinical applications:
Endothelin and Big Endothelin have been shown to be of prognostic value in heart failure (11), and acute myocardial infarction (12). Moreover, ET has been suggested to be a marker for early reocclusion after PTCA (13), for coronary atherosclerosis and coronary endothelial dysfunction (14), for liver damage (15), and declining renal function (16). High plasma levels of ET have been reported in different states of ischemia (17), hemodialysis and essential hypertension (18). Elevated ET has also been demonstrated during cardiac, liver, renal, and bone marrow transplantation (19). A significant role of ET in the pathogenesis of cancer has recently been suggested (20).

2. Principle of the assay

The Endothelin (1-21) test kit is an enzyme immunoassay designed to determine Endothelin directly in biological fluids (human EDTA-plasma, urine, cell culture supernatants). To eliminate possible interfering effects due to different matrices, a precipitation step has been validated for EDTA-plasma from monkey, rat, and dog.
To provide maximum sensitivity the kit incorporates an immunoaffinity purified polyclonal capture antibody and a monoclonal detection antibody, both highly specific for Endothelin (1-21). In the first step, sample and the monoclonal detection antibody are added simultaneously to the wells. Endothelin, if present in the sample, binds to the precoated capture antibody and

forms a sandwich with the detection antibody. After a washing step, which removes all nonspecific bound material, a peroxidase conjugated antibody detects the presence of bound detection antibodies. After removal of unbound conjugate through washing, Tetramethylbenzidine (TMB) is added to the wells as substrate. Endothelin is quantitated by an enzyme catalized color change detectable on a standard ELISA reader. The amount of color developed is directly proportional to the amount of Endothelin present in the sample.

3. Contents of the kit

- 12 x 8 well microtiter strips in stripholder, packed in alubag with dessicant. Wells are coated with a polyclonal rabbit anti-Endothelin antibody
- Plastic bag for the storage of strips
- 10x washing buffer; concentrated
  The bottle contains 100 ml of washing buffer concentrate
- Assay buffer
  The bottle contains 100 ml of assay buffer, ready to use
- Detection antibody (green cap)
  The vial contains monoclonal mouse anti-Endothelin antibody sufficient for 96 determinations, lyophilized with green dye
- 6 standards ranging from 0 to 10 fmol/ml (white caps)
  The vials contain synthetic human Endothelin-1 (1-21) in human plasma, lyophilized.
  The concentrations after reconstitution are stated on the label
- 2 controls (yellow caps)
  The vials contain synthetic human Endothelin-1 (1-21) in human plasma, lyophilized.
  The concentrations after reconstitution are stated on the label
- Conjugate
  The vial contains 22 ml anti-mouse IgG antibody conjugated to horseradish peroxidase, ready for use
- Substrate
  The vial contains 22 ml TMB solution, ready for use
- Stop solution
  The vial contains 7 ml of stop solution, ready for use
- PAA (Precipitating Agent Additive)
  The bottle contains 12 ml PAA. Add 80 ml of acetone p.a. before use
- Endothelin stock (amber vial with red cap)
  The vial contains synthetic human Endothelin-1 (1-21) in buffer, lyophilized. The concentration after reconstitution in the appropriate matrix is stated on the label
- PB-Buffer (Protocol B-Buffer)
  The bottle contains 5 ml of PB-Buffer, ready for use
- Extra high plasma standard (amber vial)
  The vial contains synthetic human Endothelin-1 (1-21) in human plasma, lyophilized. The concentration after reconstitution in assay buffer is stated on the label
- 2 self-adhesive plastic films
- Protocol sheet
- Instructions for use (package insert)

4. Additional material and equipment required

Distilled water
Variable volume pipettes in the range of 50 µl to 1000 µl
Multichannel or multipette
Manual or automatic microwell washer
ELISA reader equipped with 450 nm filter
Graph paper or software for calculation of results

Only for determination of Endothelin in precipitated plasma:
Acetone p.a.
Polypropylene tubes with capacity of at least 3.5 ml
Refrigerated centrifuge capable of 3000 x g
Speedvac concentrator or nitrogen gas

5. Reagent and sample preparation - performance of the assay

This test system is not designed for serum, heparin- or citrate-plasma

NOTICE: This test system offers five different protocols:
A) Direct measurement of Endothelin in human EDTA-plasma samples
B) Measurement of Endothelin in EDTA-plasma samples after precipitation
C) Direct measurement of Endothelin in cell culture supernatants
D) Direct measurement of Endothelin in urine
E) Direct measurement of Endothelin in human EDTA-plasma with high ET-concentrations (>10 fmol/ml)

Protocol A: Direct measurement of Endothelin in human EDTA-plasma samples
No sample preparation required

Freshly collected EDTA-plasma is put on ice immediately and centrifuged within one hour. Store samples at -20 C if not assayed on the same day. Lipemic or hemolytic samples may give erraneous results. Samples should be mixed well before assaying.

We recommend duplicates for all values.

1. Redissolve standards 0 to 5 (white caps) in 1.5 ml of assay buffer and leave at room temperature (18-26°C) for 30 min., shake well. Reconstituted standards are stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!
2. Redissolve controls (yellow caps) in 1.5 ml of assaybuffer and leave at room temperature (18-26°C) for 30 min., shake well. Reconstituted controls are stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!
3. Redissolve detection antibody (green cap) in 5.5 ml of assay buffer and leave at room temperature (18-26°C) for 30 min., shake well. Reconstituted antibody is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!

- ➢ Mark positions for blank, standards, controls and samples on the protocol sheet supplied.
- ➢ Take microtiter strips out of the alu bag and mark as appropriate. Mark 2 wells as blank. Store unused strips with the desiccant at 4°C in the plastic bag supplied. Strips are stable until expiry date stated on the label.

4. Dilute washing buffer concentrate to 1000 ml (add the 100 ml of concentrate to 900 ml of distilled water) mix well, avoid formation of foam. Crystals in the buffer concentrate will dissolve at room temperature. Buffer is stable at 4°C until expiry date stated on the label.

- ➢ All reagents and samples must have room temperature (18-26°C) before used in the assay, this usually takes about 30 min.

5. Pipette 200 µl of standards, controls and samples in the respective wells.
6. Add 50 µl detection antibody (green) to all wells except blank, mix well.
7. Cover strips with plastic film and incubate overnight (16-24 hours) at room temperature (18-26°C). Make sure all wells are sealed well with the film to avoid evaporation.
8. Discard contents of the wells and wash 5x with minimum 300 µl diluted washing buffer. Remove any remaining washing buffer by hitting plate against paper towel after the last wash.
9. Add 200 µl conjugate (red) to all wells.
10. Cover strips with plastic film and incubate for 1 hour at 37°C in an incubator/shaker.
    If no shaker is available, incubate for 3 hours at 37°C without shaking.
11. Discard contents of the wells and wash 5x with minimum 300 µl diluted washing buffer. Remove any remaining washing buffer by hitting plate against paper towel after the last wash.
12. Add 200 µl substrate to all wells and incubate for
    30 min. at room temperature (18-26°C) in the dark.
13. Add 50 µl stop solution to all wells, shake well.
14. Determine absorption immediately with an ELISA reader at 450 nm against 690 or 620 nm as reference.
    If the correction wavelength of 620 or 690 nm is not available read only at 450nm.
15. If the extinction of the highest standard exeeds the measuring range of the photometer, absorption can be measured immediately at 405 nm against 690 or 620 nm as reference.
16. Remarks to the results:

- ➢ It is recommended to reassay plasma samples with high ET-values after precipitation (protocol B)
- ➢ When concentrations of more than 10 fmol/ml are expected, samples have to be diluted and assayed according to protocol E.

Protocol B: Measurement of Endothelin after precipitation in:

- EDTA-plasma from monkey, rat, dog
- human EDTA-plasma with extremely high Endothelin levels after direct measurement

Notice: non identified interfering substances in some plasma samples (e.g. uremic patients) may cause erroneous results. It is recommended to reassay plasma samples with high ET levels after precipitation.

Freshly collected **EDTA-plasma** is put on ice immediately and centrifuged within one hour. Store samples at -20°C if not assayed on the same day. Lipemic or hemolytic samples may give erraneous results. Samples should be mixed well before assaying.

DAY 1: Precipitation of plasma samples

1. Add 80 ml of acetone p.a. to the bottle with PAA (Precipitating Agent Additive), mix well. Precipitating agent is now ready for use.
2. Transfer 1 ml samples in a PP (Polypropylene) tube (minimum capacity 3.5 ml).
3. Add 1.5 ml of precipitating agent prepared as described above to the 1 ml of sample. Mix thoroughly on a vortex mixer. If less than 1 ml of sample is available the precipitation step can be performed with respective smaller volumes.
4. Cool samples down to 4°C and centrifuge for 20 min. at 3000 rcf (=rotational centrifugal force = g) at 4°C.
5. Transfer supernatant into another PP tube.
6. Dry all samples in a speedvac concentrator or a steam of nitrogen overnight. Samples may be heated up to 37°C for drying. Make sure samples are completely dried.
   Dried samples are stable for one week in a desiccator at 4°C.

DAY 2: Performance of the assay

- ➢ We recommend duplicates for all values.

1. Redissolve Endothelin stock (amber vial with red cap) in 2 ml of PB-Buffer and leave for 30 min. at room temperature (18-26°C), shake well. The stock solution then contains approximately 10 fmol/ml Endothelin (exact value is stated on the label). Reconstituted ET stock is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles.
   Do not use the plasma-standards 0-5 (white caps) and controls (yellow caps)!
2. Redissolve dried samples in 500 µl of assay buffer, mix well and spin down unsoluble substances.
3. Prepare a serial dilution of the Endothelin stock solution with the PB-Buffer down to appr. 0.6 fmol/ml (e.g. 10 / 5 / 2.5 / 1.25 / 0.625 fmol/ml). PB-Buffer is used as a zero standard.
4. Proceed to step 3 in protocol A (page 29).

Protocol C: Direct measurement of Endothelin in cell culture supernatants

- Store samples at -20°C if not assayed on the same day.
- We recommend duplicates for all values.
- Centrifuge cell culture supernatant to remove cell particles.

1. Redissolve the Endothelin stock (amber vial with red cap) in 2 ml of cell culture medium and leave for 30 min. at room temperature (18-26°C), shake well. The Endothelin stock then contains approximately 10 fmol/Endothelin (exact value is stated on the label). Reconstituted standard is stable at -20°C until expiry date stated on the label.
   Avoid repeated freeze-thaw cycles!
   Do not use the plasma-standards 0-5 (white caps) and controls (yellow caps)!
2. Prepare a serial dilution of the Endothelin stock solution with cell culture medium down to appr. 0.6 fmol/ml (e.g. 10 / 5 / 2.5 / 1.25 / 0.625 fmol/ml).Cell culture medium is used as a zero standard.
3. Dilute cell culture supernatant according to the expected concentration with the culture medium. Dilution of supernatant is dependent on amount of ET secreted by the respective cell type. Some cell-lines are reported to secrete up to 1000 fmol/ml Endothelin
4. Proceed to step 3 of protocol A (page 29).

Protocol D: Direct measurement of Endothelin in urine

- Store samples at -20°C if not assayed on the same day. We recommend duplicates for all values.

1. Redissolve Endothelin stock (amber vial with red cap) in 2 ml of assay buffer and leave for 30 min. at room temperature (18-26°C), shake well. The standard then contains approximately 10 fmol/ml Endothelin (exact value is stated on the label). Reconstituted standard is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!
   Do not use the plasma-standards 0 -5 (white caps) and controls (yellow caps)!
2. Prepare a serial dilution of the Endothelin stock solution with assay buffer down to appr. 0.6 fmol/ml (e.g. 10 / 5 / 2.5 / 1.25 / 0.625 fmol/ml).
   Assay buffer is used as a zero standard.
3. Dilute urine samples 1 + 4 with assay buffer (1 part of urine + 4 parts of assay buffer).
4. Proceed to step 3 of protocol A (page 29).

Protocol E: Direct measurement of human EDTA-plasma samples containing high ET-concentrations of more than 10 fmol/ml (e.g. ET inhibitor studies)

Freshly collected **EDTA-plasma** is put on ice immediately and centrifuged within one hour. Store samples at -20°C if not assayed on the same day. Lipemic or hemolytic samples may give erraneous results. Samples should be mixed well before assaying.
We recommend duplicates for all values.

1. Redissolve extra high plasma standard (amber vial) in 5 ml of assay buffer and leave for 30 min. at room temperature (18-26°C), shake well. The standard then contains appr. 10 fmol/ml Endothelin (exact value is stated on the label). Reconstituted standard is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!
   Do not use the plasma-standards 0-5 (white caps) and controls (yellow caps)!
2. Prepare a serial dilution of this plasma standard with assay buffer down to appr. 0.6 fmol/ml. (e.g. 10 / 5 / 2.5 / 1.25 / 0.625 fmol/ml). Assay buffer is used as a zero standard.
3. Dilute EDTA-plasma samples with high ET-concentrations 1 + 9 with assay buffer (1 part of plasma + 9 parts of assay buffer), mix well.
4. Proceed to step 3 of protocol A (page 29).

6. Calculation of results

The extinction of the blank is subtracted from all other values. A calibration curve is constructed from the standards. Commercially available software can be used as well as graph paper. Results of the samples are read from this calibration curve.
THE CALIBRATION CURVE IS NOT LINEAR, therefore a spline or 4PL algorithm is recommended.
In protocol B the concentration factor of 2 has to be considered.
In protocols C, D, E the respective dilution factor has to be considered.

7. Assay characteristics

Normal range: 0.2 to 0.7 fmol/ml in human EDTA-plasma

Standard range: 0 to 10 fmol/ml

Cross reactivity:

ET 1 (1-21):	100 %
ET 2 (1-21):	100 %
ET 3 (1-21):	< 5 %
Big Endothelin (1-38):	< 1 %
Big Endothelin (22-38):	< 1 %

In normal human plasma samples ET-2 is estimated to be present at less than 20% of the ET-1 level. ET-3 is estimated to be present at 50% of the ET-1 level.

Sample volume:
200 µl human EDTA-plasma (direct measurement)
1 ml EDTA-plasma (measurement after precipitation)
200 µl cell culture supernatant (diluted)
200 µl urine (diluted)

Precision:

Intraassay

Mean	CV	Number
2,77 ± 0,13	4,5%	n = 18
5,42 ± 0,24	4,4%	n = 18

Interassay

Mean	CV	Number
2,85 ± 0,20	6,9%	n = 13
5,82 ± 0,45	7,6%	n = 13

Recovery:
5 different plasma have been spiked with 2 levels of synthetic Endothelin-1 and were assayed directly (protocol A).
Spike: 0.5 fmol/ml value: 0.48 fmol/ml 95%
Spike: 2 fmol/ml value: 2.08 fmol/ml 104%

5 different plasma have been spiked with synthetic Endothelin-1 and were assayed after precipitation (protocol B).
Spike: 2 fmol/ml value: 1.92 fmol/ml 96%

Detection limit:
The detection limit is the concentration of Endothelin being 3 SD above zero. For this assay the detection limit was determined to be 0.05 fmol/ml.

Incubation time: overnight / 60 min. / 30 min.

Storage: 4°C

8. Technical hints

- To avoid cross-contamination, change pipette tips between addition of standards, controls, samples, antibody, conjugate and substrate. Also use separate reservoirs for each reagent.
- Washing is a crucial step in this very sensitive assay system. Washing should be done thouroghly, if a washer is used adjust washer to a moderate washing speed and valve pressure. Do not cause foam. Make sure all wells are full. Remove any remaining washing buffer by hitting plate against paper towel after the 4th wash.
- When mixing reagents, always avoid foaming.
- Do not mix stoppers and caps of different reagents.
- Do not use reagents beyond expiration date.
- Do not mix or substitute reagents with those from other lots or sources.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate solution should remain colorless until added to the plate.
- Stop solution should be added to the plate in the same order as the substrate solution.
- For recovery experiments use Endothelin stock (amber vial) reconstituted in 2 ml of assaybuffer.
  Do not use lyophilized plasma standards for recovery experiments!

9. Precautions

All test components of human source were tested with 3rd generation tests against HIV-Ab and HBsAG; all components were found to be negative. However, standards as well as controls should be handled and disposed as if they were infectious, since no test method can offer complete assurance.

- Thimerosal and sodium acide are toxic! Avoid contact with skin and mucous membrane.
- Do not interchange kit components from different lots.

- Do not use kit components beyond the expiry date.
- Protect reagents from direct sunlight.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply cosmetics where reagents are used.
  Avoid all contact with the reagents by using gloves.
  Stop solution contains diluted sulfuric acid. Irritation to eyes and skin is possible. Flush with water after contact.

10. References

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Incubation scheme

Notice: protocols A to E differ only in the preparation of standards and samples. Subsequent incubation steps are identical for all protocols!

	Blank	Std./Contr.	Sample
Standard/Control	-	200 µl	-
Sample	-	-	200 µl
Detection antibody	-	50 µl	50 µl

Incubate the microwell strips covered with plastic film overnight (16 - 24 hours) at room temperature (18 - 26°C).
Discard liquid and wash the wells 4 times with minimum 300 µl diluted washing buffer.
Remove any remaining washing buffer by hitting plate against paper towel after the 4th wash.

	Blank	Std./Contr.	Sample
Conjugate	200 µl	200 µl	200 µl

Incubate the microwell strips covered with plastic film for 1 hour at 37°C in an incubator/shaker.
Alternative: Incubate the microwell strips covered with plastic film for 3 hours at 37°C without shaking
Discard liquid and wash the wells 4 times with minimum 300 µl diluted washing buffer.
Remove any remaining washing buffer by hitting plate against paper towel after the 4th wash.

	Blank	Std./Contr.	Sample
Substrate	200 µl	200 µl	200 µl

Incubate microwell strips 30 min. at room temperature (18-26°C) in the dark.

	Blank	Std./Contr.	Sample
Stop solution	50 µl	50 µl	50 µl

Read absorption with an ELISA microwell reader at 450 nm and 620 or 690 nm as a reference.
If the extinction of the highest standard exeeds the measuring range of the photometer, absorption can also be measured at 405 nm against 690 or 620 nm as reference. However, measurement should be done immediately.

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