Enzyme immunoassay for the quantitative
determination of Big Endothelin-1 (1-38) biological
fluids

Cat. No. BI-20072
12 x 8 tests

BIOMEDICA

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1. Introduction

Big Endothelin-1 (Big ET), a small 38-amino-acid peptide, is the biological precursor of Endothelin (1-21), the most potent vasoconstrictor known today. Endothelin was originally isolated from cultured porcine aortic endothelial cells by Yanagisawa et al. (1). Endothelins are produced by various cell types including vascular endothelial cells and non vascular cells, such as Mesanglial, kidney and epithelial cells (2, 3, 4). The biological role of ET extends beyond regulating vascular tone also in its growth regulatory properties (5, 6). The peptide interacts in an autocrine/paracrine manner with ET receptors found on numerous cells, including smooth muscle cells, myocytes and fibroblasts (7, 8). Evidence now suggests that ET influences DNA synthesis, the expression of protooncogenes, cell proliferation and hypertrophy. Moreover, ET also acts in synergism with various factors such as EGF, PDGF, bFGF, TGF's, insulin, etc., to potentiate cellular transformation or replication (5, 9).
After synthesis in the cytoplasma, Big ET is cleaved between the aminoacids Trp21 and Val22 by 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); (1, 10). The physiological importance of cleavage of Big ET is indicated by the reported 140-fold increase in vasoconstrictor activity upon cleavage to ET-1 (11), although both peptides can be determined in about equimolar concentrations in the plasma. It was demonstrated that the half-life of ET (1-21) in the plasma is less than one minute, whereas clearance of Big ET is much slower (12). In addition, plasma levels of Big ET are significantly elevated even one hour after systemic applications (13).
The biological, biochemical and pathophysiological role of Endothelins is summarized in recent reviews (14,15).
<u>Clinical applications:</u>
Elevated levels of Big ET have been detected in situations of cardiovascular stress such as acute myocardial infarction, renal insufficiency or during and after graft rejection. Increased plasma levels of ET have also been reported in conditions such as hypercholesterolemia, atherosclerosis, pulmonary hypertension, and scleroderma. The proportion of active ET to Big ET and to the C-terminal fragment in the circulation of healthy human subjects has been reported to be 1:1:3 (16). Until now, no specific test system has been available to measure Big ET alone. The BIOMEDICA Big ET-1 (1-38) ELISA is the first enzyme-linked immunosorbent assay for the direct measurement of Big Endothelin-1 in biological fluids.

2. Principle of the assay

The Big Endothelin-1 (1-38) test kit is an enzyme immunoassay designed to determine Big Endothelin-1 (1-38) 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 non human EDTA-plasma.
To provide maximum sensitivity the kit incorporates an immunoaffinity purified polyclonal capture antibody and a monoclonal detection antibody, both highly specific for Big Endothelin-1 (1-38). In the first step, sample and the monoclonal detection antibody are added simultaneously to the wells. Big 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 a substrate. Big Endothelin-1 is quantitated by an enzyme catalyzed color change detectable on a standard ELISA reader. The amount of color developed is directly proportional to the amount of Big Endothelin-1 present in the sample.

3. Contents of the kit

12 x 8 well microtiter strips in stripholder, packed in zipped alubag with desiccant. Wells are coated with a polyclonal rabbit anti-Big Endothelin-1 antibody

- 10x washing buffer
  The vial 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 anti-Big Endothelin antibody sufficient for 96 determinations, lyophilized with a green dye
- 6 standards (white caps)
  The vials contain synthetic human Big Endothelin-1 (1-38), lyophilized in human plasma. The concentrations after reconstitution are stated on the label
- 1 control (yellow caps)
  The vials contain synthetic human Big Endothelin-1 (1-38), lyophilized in human plasma. 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 of 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 6 ml precipitating agent additive PAA. Add 40 ml of acetone p.a. before use
- Big Endothelin-1 stock (amber vial with red cap)
  The vial contains synthetic human Big Endothelin-1 (1-38) in buffer, lyophilized. The concentration after reconstitution 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 Pipettes in the range of 50 µl to 1000 µl with disposable tips
Multichannel or multipette
Incubator/shaker for 37°C
Manual or automatic microwell washer
ELISA reader equipped with 450 nm filter
Graph paper or software for calculation of results
Only for determination of Big Endothelin-1 in precipitated plasma:
Acetone p.a.
Polypropylene tubes with capacity of at least 2 ml
Refrigerated centrifuge capable of 3000 x g
Speedvac concentrator or nitrogen gas

5. Reagent and Sample preparation - Performance of the test

Freshly collected EDTA-plasma is put on ice immediately. Store samples at -20°C if not assayed on the same day. Do not use lipemic or hemolytic samples.
<u>This test system is not designed for serum, heparin- or citrate plasma.</u>
NOTICE: This test system offers four different protocols:

A) Direct measurement of Big Endothelin-1 in human EDTA-plasma samples
   CAUTION: The measuring range of this assay can be varied! Protocol Aa offers a measuring range from 0.05 to 15.6 fmol/ml.
   For even better sensitivity follow the assay procedure of protocol Ab with a measuring range of 0.025 to 6.25 fmol/ml. Standard 5 is omitted in this protocol.
B) Measurement of Big Endothelin-1 in non human EDTA-plasma samples after precipitation
C) Direct measurement of Big Endothelin-1 in cell culture supernatants

D) Direct measurement of Big Endothelin-1 in urine

Protocol A)
Direct determination of Big Endothelin-1 in human EDTA-Plasma:

We recommend duplicates for all values.
Allow all reagents to reach room temperature (18 - 26°C) before use. This usually takes 30 min.

1. Dilute wash buffer concentrate 1:10 (1 part of buffer + 9 parts distilled water) to a final volume of 1000 ml with distilled water, mix well. Crystals in the buffer concentrate will dissolve at room temperature. Diluted buffer is stable at 4°C until expiry date stated on the label.
2. Redissolve standards 0 to 5 (white caps) in 1ml 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!
3. Redissolve control (yellow cap) in 1ml of assaybuffer and leave at room temperature (18-26°C) for 30 min., shake well. Reconstituted control is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!
4. Redissolve detection antibody (green cap) in 5,5 ml of diluted washing 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, control and samples on the protocol sheet supplied.
- Take microtiter strips out of the zipped alu bag and mark as appropriate. Mark 2 wells as blank. Store unused strips with the desiccant at 4°C in the zipped alu bag. Strips are stable until expiry date stated on the label

Protocol Aa (0.05 to 15.6 fmol/ml):

1. Pipette 100 µl of standard, control or sample in respective wells.
2. Add 50 µl detection antibody (green) to all wells except blank.
3. Cover strips with plastic film and incubate for 3 hours at 37°C in an incubator/shaker.
4. Discard contents of the wells and wash 5 x with at least 300 µl diluted washing buffer. Remove any remaining washing buffer by hitting plate against paper towel after the last wash
5. Add 100 µl conjugate (red) to all wells.
6. Cover strips with plastic film and incubate for 1 hour at 37°C in an incubator/shaker.
7. Discard contents of the wells and wash 5 x with at least 300 µl diluted washing buffer. Remove any remaining washing buffer by hitting plate against paper towel after the last wash
8. Add 100 µl substrate to all wells and incubate for 30 min. at room temperature (18 - 26°C) in the dark.
9. Add 50 µl stop solution to all wells, shake well
10. Determine absorption with an ELISA reader at 450 nm against 620 or 690 nm as reference immediately. If the correction wavelength of 620 or 690 nm is not available read only at 450 nm.

If the extinction of the highest standard exceeds the measuring range of the photometer, absorption can be measured immediately at 405 nm against 620 or 690 nm as reference.

Protocol Ab (0.025 to 6.25 fmol/ml):

1. Pipette 200 µl of each standard, control or sample in respective wells. Do not use standard 5 !
2. Add 50 µl detection antibody to all wells except blank.
3. Cover strips with plastic film and incubate for 3 hours at 37°C in an incubator/shaker.
4. Discard contents of the wells and wash 5 x with at least 300 µl diluted washing buffer. Remove any remaining washing buffer by hitting plate against paper towel after the last wash.
5. Add 200 µl conjugate (red) to all wells.
6. Cover strips with plastic film and incubate for 1 hour at 37°C in an incubator/shaker.
7. Discard contents of the wells and wash 5 x with at least 300 µl diluted washing buffer. Remove any remaining washing buffer by hitting plate against paper towel after the last wash.
8. Add 200 µl substrate to all wells and incubate for 30 min. at room temperature (18 - 26°C) in the dark.
9. Add 50 µl stop solution to all wells, shake well
10. Determine absorption with an ELISA reader at 450 nm against 620 or 690 nm as reference immediately. If the correction wavelength of 620 or 690 nm is not available read only at 450 nm.

If the extinction of the highest standard exceeds the measuring range of the photometer, absorption can be measured immediately at 405 nm against 620 or 690 nm as reference.

Protocol B:
Determination of Big Endothelin-1 in non-human EDTA-Plasma after precipitation

DAY 1: Precipitation of plasma samples

1. Add 40 ml of acetone p.a. to the bottle with PAA (Precipitating Agent Additive), mix well. Precipitating agent is now ready for use.
2. Redissolve standards 0 to 5 (white caps) in 1 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!
3. Redissolve control (yellow cap) in 1 ml of assaybuffer and leave at room temperature (18-26°C) for 30 min., shake well. Reconstituted control are stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!
4. Transfer 0.5 ml of standards, control and samples in a PP (Polypropylene) tube (minimum capacity 2 ml).
5. Add 0.75 ml of precipitating agent prepared as described above to the standards, control and samples. Mix thoroughly on a vortex mixer.
6. Cool standards, control and samples down to 4°C and centrifuge for 20 min. at 3000 x g at 4°C.
7. Transfer supernatant into another PP tube.
8. Dry all standards, control and samples in a speedvac concentrator or a steam of nitrogen overnight. Samples may be heated up to 37°C for drying. Make sure contents of all tubes 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.
Redissolve dried standards, control and samples in 500 µl of assay buffer, mix well and spin down insoluble substances.
Redissolved standards, control and samples are assayed as described in protocol Aa (see page 28).

Protocol C:
Direct measurement of Big Endothelin-1 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 Big Endothelin-1 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 Big Endothelin-1 stock then contains approximately 10 fmol/ml Big Endothelin-1 (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 control (yellow cap)!
2. Prepare a serial dilution of the Big Endothelin-1 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 Big ET secreted by the respective cell type.
4. These standards and samples are assayed as described in protocol Aa (see page 28)

Protocol D:
Direct measurement of Big Endothelin-1 in urine

Store samples at -20°C if not assayed on the same day.
We recommend duplicates for all values.
Dilute urine samples 1 + 4 in assay buffer (i.e. 1 part of urine +4 part of assay buffer).

1. Redissolve the Big Endothelin-1 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 Big Endothelin-1 stock then contains approximately 10 fmol/ml Big Endothelin-1 (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 Big Endothelin-1 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. These standards and samples are assayed as described in protocol Aa (see page 28)

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 C and D the dilution factor has to be considered.

7. Assay characteristics

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

Measuring range: 0.05 to 15.6 fmol/ml* (protocol Aa)
0.025 to 6.25 fmol/ml* (protocol Ab)
0.05 to 10 fmol/ml* (protocols B,C,D)

Cross reactivity:

ET-1/2/3 (1-21)	< 1 %
Big ET-2 (1-37)	< 1 %
Big ET-1/2 (1-38)	< 1 %
porcine Big ET-1 (1-39)	< 1 %
human/rat Big ET-3 (1-41)	< 1 %
Big ET-1/2 (22-38)	< 1 %
Big ET-2 (22-37)	< 1 %
Big ET (22-41)	< 1 %
Sarafotoxin	< 1 %
rat BigET-1 (1-39)	6 %

Sample: 100 µl human EDTA-Plasma (protocol Aa)
200 µl human EDTA-Plasma (protocol Ab)
500 µl non-human EDTA-Plasma after precipitation (protocol B)
≥ 100 µl cell culture supernatant (protocol C)
≥ 100 µl urine (protocol D)

* User limit of measuring range is dependent on the concentration of the respective standards.

Precision:

	Mean	CV	Number
Intraassay	6.7 ± 0.26	3.9 %	n = 11
	1.2 ± 0,06	4.9 %	n = 11
Interassay	6.5 ± 0,39	6.1 %	n = 12
	1.6 ± 0.11	6.9%	n = 12

Recovery:
5 plasma have been spiked with 3 levels of synthetic Big Endothelin-1:

Spike: 1 fmol/ml	Value: 0.96 fmol/ml	96%
Spike: 3 fmol/ml	Value: 3.1 fmol/ml	103%
Spike: 7 fmol/ml	Value: 6.6 fmol/ml	94%

Detection limit:
The detection limit is the concentration of Big Endothelin-1 being 3 SD above zero. For this assay detection limit was determined to be 0.05 fmol/ml (protocol A) or 0.025 fmol/ml (protocol B).

Assay time: 5 hours

Storage: 4°C

8. Technical hints

- To avoid cross-contamination, change pipette tips between addition of standards, controls, samples, antibody, conjugate substrate and stop solution. Also use separate reservoirs for each reagent.
- Washing is a crucial step in this very sensitive assay system. Washing should be done thoroughly, 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 last wash.
- Do not mix stoppers and caps of different reagents to avoid cross-contamination.
- Do not use reagents beyond expiration date.
- Do not mix or substitute reagents with those from other lots or sources.

- Protect reagents from direct sunlight.
- 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 and speed as the substrate solution.

For recovery experiments use the Big Endothelin-1 stock (amber vial with red cap) 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.
All liquid reagents contain 0,01% Thimerosal or 0,095% sodium azide as preservative.

- Thimerosal and sodium azide are toxic! Avoid contact with skin and mucous membrane.
- 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 for Protocol Aa (0.05 to 15.6 fmol/ml)

NOTICE: Protocols A to D only differ in preparation of standards and samples. All consecutive steps are identical.

	Blank	Std./Contr.	Sample
Standard/Control	-	100 µl	-
Sample	-	-	100 µl
Detection antibody	-	50 µl	50 µl

Incubate the microwell strips covered with plastic film for 3 hours at 37°C in an incubator/shaker.
Discard liquid and wash the wells 5 times with at least 300 µl diluted washing buffer.

	Blank	Std./Contr.	Sample
Conjugate	100 µl	100 µl	100 µl

Incubate the microwell strips covered with plastic film for 1 hour at 37°C in an incubator/shaker.
Discard liquid and wash the wells 5 times with at least 300 µl diluted washing buffer.

Substrate	100 µl	100 µl	100 µl

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

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.

Incubation scheme
for Protocol Ab (0.025 to 6.25 fmol/ml)

	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 for 3 hours at 37°C in an incubator/shaker.
Discard liquid and wash the wells 5 times with at least 300 µl diluted washing buffer.

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.
Discard liquid and wash the wells 5 times with at least 300 µl diluted washing buffer.

Substrate	200 µl	200 µl	200 µl

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

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.

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