

ET Cell Culture

Enzyme immunoassay for the quantitative determination
of Endothelin (1-21) in cell culture supernatants

Cat. No. BI-20092

12 x 8 tests

BIOMEDICA
BIOMEDICA
GRUPPE 

Biomedica Medizinprodukte
Gesellschaft mbH & Co KG
A-1210 Wien, Divischgasse 4
Tel. +43/1/291 07 50
Fax +43/1/291 07 71
exp.biomedica@bmgrp.at

www.biomedica.co.at

Contents

- 1.) Introduction
- 2.) Principle of the assay
- 3.) Contents of the kit
- 4.) Additional material and equipment required
- 5.) Reagents and sample preparation - assay procedure
- 6.) Calculation of results
- 7.) Assay characteristics
- 8.) Technical hints
- 9.) Precautions
- 10.) References
- 11.) Incubation scheme

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 cytoplasm, 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).

There is an ever growing interest for in vitro studies of Endothelin using cell culture model systems to investigate basic properties of the endothelium (7,8,9) or interactions with biological molecules e.g. oxidized LDLs (10). A special focus of Endothelin cell culture measurements exist in monitoring and investigating the effects of various drugs (beta-blocker, ET antagonists etc.) used for the treatment of hypertension or heart failure (11,12,13).

2.) Principle of the assay

The Endothelin (1-21) test kit is an enzyme immunoassay designed to determine Endothelin directly in cell culture supernatants. In a first step, sample (cell culture supernatant) and a horse radish peroxidase labeled (HRP) 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 non-specificly bound material, Tetramethylbenzidine (TMB) is added to the wells as substrate. Endothelin is quantitated by an enzyme catalysed colour 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 strip holder, packed in alubag with desiccant. Wells are coated with a polyclonal anti-Endothelin antibody
- 10x washing buffer; concentrated
The bottle contains 100 ml of washing buffer concentrate
- Endothelin standard (amber vial with red cap) The vial contains synthetic human Endothelin-1 (1-21) in buffer, lyophilized. The concentration after reconstitution in cell culture media is stated on the label.
- Conjugate-Detection antibody (glass vial)
The vial contains HRP-labeled monoclonal mouse anti-Endothelin antibody sufficient for 96 determinations, lyophilised
- Conjugate Buffer (red solution)
The bottle contains 12 ml of conjugate buffer for reconstitution of the conjugate
- Substrate
The vial contains 22 ml TMB solution, ready for use
- Stop solution
The vial contains 7 ml of stop solution, ready for use
- 1 self-adhesive plastic film
- 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 multipipette

ELISA reader equipped with 450 nm filter

Graph paper or software for calculation of results

5.) Reagents and sample preparation - assay procedure

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.
- Dilute washing buffer concentrate to 500 ml (add the 50 ml of concentrate to 450 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.

1. Redissolve the Conjugate-Detection antibody in 11 ml of conjugate buffer (red solution). Leave for 30 min. at room temperature (18-26°C), shake well. The reconstituted conjugate is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!
2. Redissolve the Endothelin standard (amber vial with red cap) in 5 ml of cell culture medium (e.g. RPMI1640/10%FCS) used for growing the cells under investigation and leave for 30 min. at room temperature (18-26°C), shake well. The Endothelin standard then contains approximately 100 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!
3. Prepare a 1+1 serial dilution of the Endothelin standard solution with cell culture medium down to appr. 3 fmol/ml (e.g. 100 / 50 / 25 / 12.5 / 6.25/3.12 fmol/ml). Cell culture medium is used as a zero standard.
4. Dilute cell culture supernatant according to the expected concentration with the culture medium if necessary. Dilution of supernatant is dependent on amount of ET secreted by the respective cell type.
5. Mark positions for blank, standards and samples on the protocol sheet supplied.
6. 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 alu bag supplied. Strips are stable until expiry date stated on the label.
7. Pipette 100 µl of standards or samples in the respective wells.
8. Add 100 µl detection antibody-conjugate to all wells except blank, mix well.
9. Cover strips with plastic film and incubate 3 hours at room temperature (18-26°C). Make sure all wells are sealed well with the film to avoid evaporation.
10. Discard contents of the wells and wash 5x with minimum 300 µl diluted wash buffer. Remove any remaining fluid by hitting plate against paper towel after the 5th wash.
11. Add 200 µl substrate to all wells and incubate for 30 min. at room temperature (18-26°C) in the dark.
12. Add 50 µl stop solution to all wells, shake well.
13. 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.
14. If the extinction of the highest standard exceeds the measuring range of the photometer, absorption can be measured immediately at 405 nm against 690 or 620 nm as reference.

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 shape of the calibration curve may vary depending on the composition of the culture medium used. Therefore linear regression or 4PL algorithm is recommended. QC-Data for the Kit are acquired using RPMI-1640/10%FCS.

If cell culture supernatants are diluted, the dilution factor must be considered in calculating the results.

7.) Assay characteristics

Standard range: 0 to ~100 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 %

Sample volume:

100 µl cell culture supernatant

Detection Limit: (0fmol/ml +2 SD): 1 fmol/ml

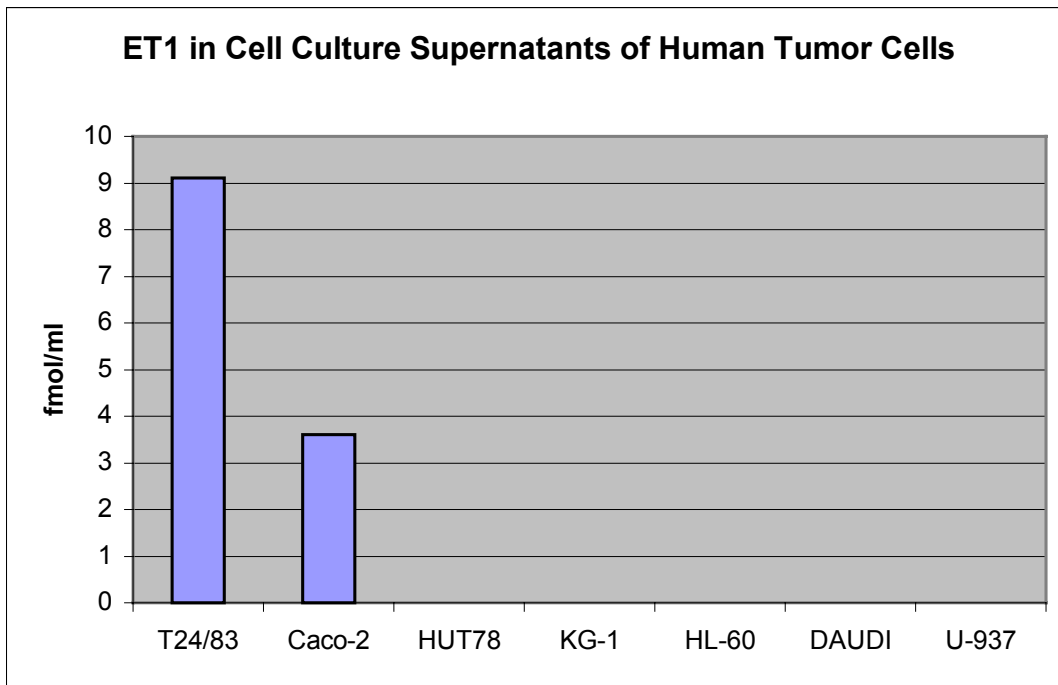
Precision:

	16 fmol/ml	5 fmol/ml
Intra-Assay (n=5)	4.0 %	6.3 %
Inter-Assay (n=6)	7.0 %	8.0 %

Experimental Data:

To prove the usefulness of the assay for cell culture measurements we investigated tumour cell lines (bladder and colon carcinoma) and compared their Endothelin secretion with those of tumour cell lines grown in homogenous suspension. A significant difference of the ET1 content of the supernatants was found (see table and chart).

	type	grown as	morphology	ET1 (fmol/ml)
T24/83	bladder carcinoma	monolayer	epithelial	9.1
Caco-2	colon carcinoma	monolayer	epithelial	3.6
HUT78	lymphoma	suspension	lymphoblast	<0.5
KG-1	leukemia	suspension	myoblast	<0.5
HL-60	lymphoma	suspension	lymphoblast	<0.5
DAUDI	Burkitt lymphoma	suspension	lymphoblast	<0.5
U-937	lymphoma	suspension	histomonocytoid	<0.5



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.
- 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 colourless until added to the plate.
- Stop solution should be added to the plate in the same order as the substrate solution.

9.) Precautions

All liquid reagents contain 0.01% thimerosal or 0.095% sodium acid as preservative.

- Thimerosal and sodium acid 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

1. Yanagisawa M. et al. (1988), Nature 332: 411-415
2. Inoue A. et al. (1989), Proc Natl Acad Sci USA 86: 2863-2867
3. Kimura S. et al. (1989), J Cardiovasc Pharmacol 13: 5-7
4. De Nucci C. et al. (1988), Proc Natl Acad Sci USA 9797-9800
5. Stojilkovic S. and K.J. Catt (1992), Trends Pharmacol Sci 13: 385-391
6. Hensen A. et al. (1991), Acta Physiol Scand. Suppl. 602: 1-61
7. Kiessling F. et al. (1999), Cell & Tissue Research, 297, 1: 131-140
8. Masayoshi S. et al. (1997), Mol. Endocrinology, 12, 2 : 172-180
9. Cervar M. et al. (1999), Cell & Tissue Research, 295, 2: 297-305
10. Unoki H. et al. (1999), Cell & Tissue Research, 295, 1: 89-99
11. Garlich C.D. et al. (1999), Eur. J. Clin. Invest., 29, 1: 12-16
12. Hasselblatt M. et al. (1998), Brain Res, 785, 2: 253-61
13. Ohlstein E.H. et al. (1998), J. Mol. Cell Cardiol, 30, 1: 167-173

11.) Incubation scheme

Sample incubation

	Blank	Standard	Sample
Standard	-	100 µl	-
Sample	-	-	100 µl
Conjugate	-	100 µl	100 µl

Cover strips with the supplied plastic foil and incubate for 3 hrs at room temperature (18-26°C)
 Discard the content of the wells and wash 5 times with 300 µl diluted wash buffer.
 Remove any remaining fluid by hitting plate against paper towel after the last wash.

Substrate incubation

Substrate	200 µl	200 µl	200 µl
-----------	--------	--------	--------

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

Stop and read

Stop solution	50 µl	50 µl	50 µl
---------------	-------	-------	-------

Mix, and read absorption with an ELISA microwell reader at 450 nm and 620 nm or 690 nm as a reference. If the readings exceed the measuring range of the photometer, absorption can be measured at 405 nm against 690 nm or 620 nm as reference immediately.