

Human Endothelin-1 Immunoassay

Catalog Number BBE5

SBBE5

PBBE5

For the quantitative determination of Endothelin-1 (ET-1) in extracted EDTA plasma.

This package insert must be read in its entirety before using this product.

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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INTRODUCTION

Endothelin-1 (ET-1), a peptide of 21 amino acid residues, is the most potent vasoconstrictive substance known. Originally isolated from porcine aortic endothelial cells (1), ET-1 is now known to be one of a family of three mammalian vasoactive peptides that also includes Endothelin-2 (ET-2) and Endothelin-3 (ET-3) (2). These related peptides differ from ET-1 at two and six amino acid residue positions, respectively. A fourth peptide, vasoactive intestinal contractor (VIC), is sometimes classified as rat ET-2 (3). All members of the endothelin family contain two essential disulfide bridges and six conserved amino acid residues at the C-terminus. Additionally, all of the endothelin family members are synthesized initially as prepropeptides of approximately 200 amino acid residues encoded by separate genes (2). These are proteolytically cleaved (4) to produce biologically-inactive propeptides of approximately 40 amino acid residues termed "big endothelins". Big ET-1 is cleaved by the proteolytic action of a membrane-bound metalloprotease [endothelin-converting enzyme (ECE-1)] producing the 21 amino acid residue active peptide (5). The biochemistry and biology of the endothelins have been the subject of several reviews (2, 6, 7).

The endothelins are produced by a variety of tissues *in vivo*, including lung and kidney (7), brain, pituitary and peripheral endocrine tissues (8) and placenta (9). ET-1, in contrast to ET-2 and ET-3, is also produced by endothelial cells, the vascular endothelium being the most abundant source of ET-1 *in vivo* (1, 6). There are also reports of ET-1 production *in vitro* by pancreatic cancer cells (10), mast cells (11) and a variety of endothelial, epithelial and smooth muscle cells (12).

The best known action of ET-1 is as a vasoconstrictor. Injection of a single dose of ET-1 produces an initial decrease in systemic blood pressure followed by a prolonged increase in blood pressure, lasting for 1 to 3 hours (13, 14). From the slow onset and long-lasting effects of endothelins, it appears that these peptides are involved in long-term changes and not in acute responses to stimuli. In addition to their actions as vasoconstrictors, endothelins also produce a variety of other biological effects. These include stimulation of cardiac contraction, regulation of release of vasoactive substances, and stimulation of smooth muscle mitogenesis (7, 12). Endothelins also stimulate contraction of most smooth muscles and stimulate secretion by tissues including kidney, liver and adrenals (7). Endothelins also act in the brain, stimulating secretion by hypothalamic and pituitary cells (8).

Two receptors for endothelins have been characterized and designated ET_A and ET_B. Although these receptors are structurally and functionally distinct, they share some similarities. Each contains seven stretches of 20 - 27 hydrophobic amino acid residues, which are likely to represent transmembrane domains (13). Both have an N-terminal signal sequence and a long N-terminal extracellular domain (13). The ET_A receptor shows a higher affinity for ET-1 than for ET-2 and lowest affinity for ET-3. The ET_B receptor shows approximately equal affinity for each of the three endothelins (12, 13). Both receptors are expressed in a wide variety of tissue types, in some cases with distinct expression and in some cases showing a degree of overlapping expression (6). Studies indicate that endothelium-dependent relaxation is mediated by the ET_B receptor (6). Both the ET_A and the ET_B receptors play varying roles in mediation of vasopressor actions depending on the species and vasculature involved (6).

Elevated levels of ET-1 in blood have been reported for a variety of disease conditions, possibly as part of a response to stress (10, 12, 15 - 23).

The Human ET-1 Immunoassay is a 90 minute (total incubation time) solid phase ELISA that is designed to measure ET-1 in extracted EDTA plasma. It contains synthetic human ET-1 and antibodies raised against synthetic human ET-1. This immunoassay has been shown to quantitate synthetic and natural human ET-1 accurately.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative enzyme immunoassay technique. An antibody specific for ET-1 has been pre-coated onto a microplate. Standards, samples, Control and Conjugate are pipetted into the wells and any ET-1 present is sandwiched by the immobilized antibody and the enzyme-linked antibody specific for ET-1. Following a wash to remove any unbound substances and/or antibody-enzyme reagent, substrate is added to the wells and color develops in proportion to the amount of ET-1 bound. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- If samples generate values higher than the highest standard, further dilute with Sample Diluent and repeat the assay.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Description	Part #	Cat. # BBE5	Cat. # SBBE5
ET-1 Microplate - 96 well microplate (12 strips of 8 wells) coated with rat antibody to human ET-1.	890397	1 plate	6 plates
ET-1 Standards - Lyophilized synthetic human ET-1 containing blue dye. The concentrations of ET-1 after reconstitution are shown on the vial labels.	890249 - 890254	1 vial of each level	6 vials of each level
Sample Diluent - 20 mL/vial of buffered protein base with blue dye and preservatives.	895155	1 vial	6 vials
ET-1 Conjugate Concentrate - 0.3 mL/vial of antibody to ET-1 conjugated to horseradish peroxidase in buffer with preservatives.	890248	1 vial	6 vials
Conjugate Diluent - 11 mL/vial of diluent for HRP-Conjugate Concentrate, with red dye and preservatives.	895156	1 vial	6 vials
ET-1 Control - Lyophilized synthetic human ET-1. The concentration of the control should fall within the range specified on the vial label if the assay is valid.	890255	1 vial	6 vials
Wash Buffer Concentrate - 20 mL/vial of a 25-fold concentrated buffered surfactant with preservatives.	895154	1 vial	6 vials
Substrate - 11 mL/vial of stabilized substrate solution (tetramethylbenzidine).	895002	1 vial	6 vials
Stop Solution - 11 mL/vial of acid solution.	895004	1 vial	6 vials
Plate Covers - Adhesive strips.	—	8 strips	48 strips

BBE5 contains sufficient materials to run an ELISA on one 96 well plate.

SBBE5 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PBBE5). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use kit past expiration date.	
Opened/ Reconstituted Reagents	Diluted ET-1 Conjugate	May be stored for up to 1 month at 2 - 8° C.*
	Diluted Wash Buffer	
	Substrate	
	Stop Solution	
	Sample Diluent	
	Standards	Aliquot and store for up to 1 month at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
	Endothelin-1 Control	
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack and reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 620 nm or 650 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Refrigerated spark-proof centrifuge.
- Centrifugal evaporator.
- 500 mL graduated cylinder.
- **Polypropylene** test tubes for dilution.

REAGENTS REQUIRED BUT NOT SUPPLIED

- 1 N HCl
- Acetone

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Avoid microbial contamination when removing aliquots from reagent vials.

Use of a fume hood is recommended when using solvents for sample extraction.

Endothelin-1 is a bioactive peptide toxin and should be handled as a biological hazard.

SAMPLE COLLECTION AND STORAGE

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Extract plasma and assay immediately or, prior to extraction, aliquot and store at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Note: *Heparin and citrate plasma have not been validated for use in this assay. Do not use lipemic, grossly hemolyzed or turbid samples.*

SAMPLE PREPARATION (5 hour extraction procedure)

This assay is not suitable for the direct assay of plasma samples. An extraction procedure is required.

The following example of an extraction procedure has been used to generate the sample value data in this booklet. It remains the responsibility of the individual laboratory to validate any sample preparation procedure followed.

1. Add 1 mL of plasma and 1.5 mL of extraction solvent (acetone:1 N HCl:Water (40:1:5)) to a **polypropylene tube**.
2. Mix by inversion.
3. Centrifuge for 20 minutes at 2000 x g in a refrigerated centrifuge at 2 - 8° C.
4. Decant supernatant into a polypropylene tube.
5. Dry down supernatant in a centrifugal evaporator (minimum drying time 4 hours at 37° C).
6. Reconstitute pellet in 0.25 mL sample diluent and vortex for 30 seconds.
7. Assay immediately for best results. If necessary, reconstituted samples can be stored overnight at 2 - 8° C.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer Concentrate - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into distilled or deionized water to prepare 500 mL of Wash Buffer.

Standards - Reconstitute the ET-1 Standards immediately before use with 1.0 mL of distilled or deionized water. Allow the standards to sit at room temperature for at least 10 minutes. The contents of the vials should be further mixed by gentle agitation until all the contents are completely dissolved. **Vigorous agitation and foaming should be avoided.** The Standards are now ready for use in the assay and require no further dilution. The concentrations of the Standards are stated on the labels.

Endothelin-1 Control - Reconstitute the ET-1 Control immediately before use with 1.0 mL of distilled or deionized water. Allow the Control to sit at room temperature for at least 10 minutes and mix by gentle agitation before use.

ET-1 Conjugate Concentrate - Tap the vial of Conjugate Concentrate to dislodge any liquid from the cap. Ensure contents are mixed with a suitable pipette. Transfer 250 μ L of the Conjugate Concentrate into the bottle of Conjugate Diluent. Mix contents by gentle inversion and swirling. **Vigorous agitation and foaming should be avoided.** The Conjugate is now ready for use in the assay and requires no further dilution.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, Standards, and the Endothelin-1 Control be assayed in duplicate.

1. Prepare all reagents, extracted samples, working Standards, and Control as directed in the previous sections.
2. Remove excess microplate strips from the frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 μL diluted Conjugate to each well.
4. Add 100 μL Standard, Control, or reconstituted sample extract to each well with sufficient force to ensure mixing. **Ensure reagent addition is uninterrupted and completed within 10 minutes.**
5. Cover the plate with a plate sealer provided and incubate at room temperature for 1 hour.
6. Aspirate or decant contents from each well and wash by adding 300 μL of Wash Buffer per well. Repeat the process five times for a total of six washes. After the last wash, aspirate or decant the contents and remove any remaining Wash Buffer by tapping the inverted plate firmly on clean paper towels.
7. Add 100 μL Substrate to each well. Cover the plate with a new plate sealer and incubate at room temperature for 30 minutes.
8. Add 100 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 620 nm or 650 nm. If wavelength correction is not available, subtract readings at 620 nm or 650 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, extracted samples, Standards, and Control as instructed.



2. Add 100 μL of diluted ET-1 Conjugate to each well.



3. Add 100 μL Standard, Control, or reconstituted sample extract to each well.
Incubate 1 hour at RT.



4. Aspirate and wash 6 times.



5. Add 100 μL Substrate Solution to each well.
Incubate 30 minutes at RT.



6. Add 100 μL Stop Solution to each well.
Read at 450 nm within 30 minutes.
 λ correction 620 nm or 650 nm.

CALCULATION OF RESULTS

Calculate the mean absorbance values for each set of duplicate Standards.

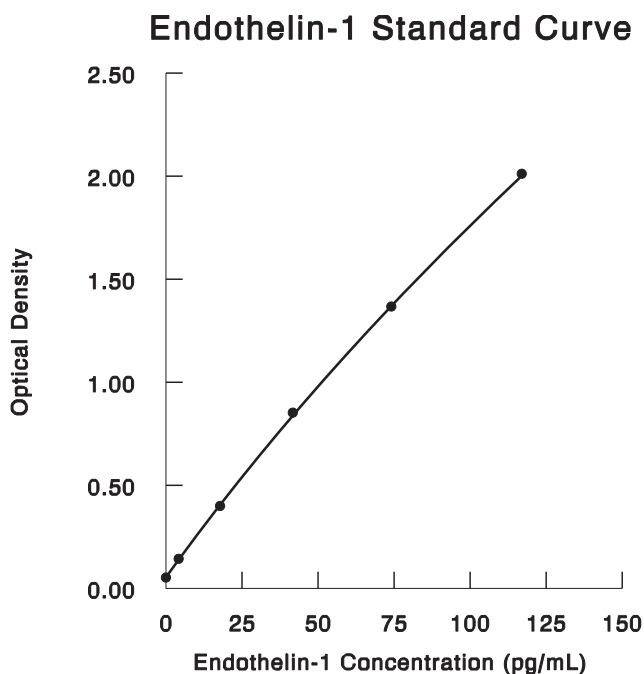
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each Standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.

Determine the concentration of each unknown sample by calculating the concentration of ET-1 corresponding to the mean absorbance from the standard curve. For samples, the concentration determined from the standard curve should then be corrected for reconstitution in the extraction procedure. Since samples have been extracted, the concentration read from the standard curve must be divided by the correction factor of 4.

It is recommended that the user run the Control in each assay. If the values obtained are not within the expected range, as stated on the Control vial label, the assay results may be invalid.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



Standard	(pg/mL)	O.D.	Mean
0	0	0.054 0.052	0.053
1	4.2	0.138 0.149	0.144
2	17.8	0.386 0.865	0.400
3	41.7	0.841 1.364	0.853
4	74.1	1.373 2.027	1.368
5	117	1.998	2.012

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate containers for each reagent.
- Additions at each step of the protocol should be uninterrupted.
- Sodium azide will inactivate the ET-1 Conjugate.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Avoid contact of Substrate with oxidizing agents or metal.
- Stop Solution should be added to the plate in the same order as the Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested ten times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in fifty-seven separate assays to assess inter-assay precision.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	10	10	10	57	57	57
Mean (pg/mL)	14.4	32.8	68.7	16.2	33.9	70.0
Standard deviation	0.66	1.48	2.89	1.05	1.86	3.57
CV (%)	4.6	4.5	4.2	6.5	5.5	5.1

SENSITIVITY

The minimum detectable dose (MDD) of ET-1 is typically less than 1.0 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

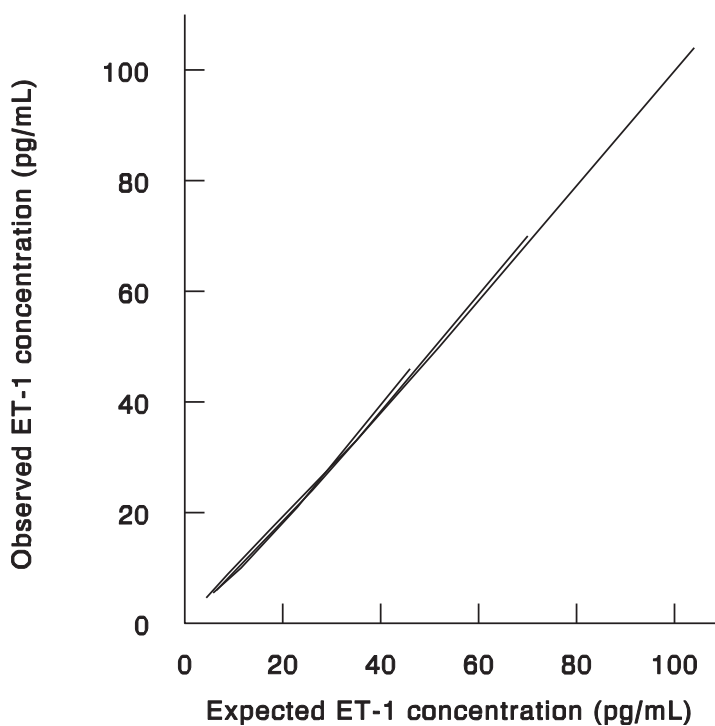
Twenty-five EDTA plasma samples were tested for ET-1 concentrations using the procedure outlined under Sample Preparation.

The mean value was 0.6 pg/mL with a range of 0.3 - 0.9 pg/mL.

Other sample types such as serum, heparin, and citrate plasma may be used, but could give different ranges. Results should only be compared with one sample type and/or within one collection method.

LINEARITY

To assess linearity of the assay, three control samples were assayed at serial 2-fold dilutions.



SPECIFICITY

This assay recognizes both synthetic and natural human ET-1. Limited levels of cross reactivity were observed with ET-1 related proteins, as indicated.

ET-1 related proteins:

Big Endothelin	< 1% cross-reactivity
Sarafotoxin	< 2% cross-reactivity
ET-2	45% cross-reactivity
ET-3	14% cross-reactivity

In normal 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. Big Endothelin circulates at twice the level of ET-1 (24).

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