Quantikine™ ELISA

Endothelin-1 Immunoassay

Catalog Number DET100
SET100
PDET100

For the quantitative determination of Endothelin-1 concentrations in cell culture supernates, serum, plasma, and urine.

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 (aa) residues, is a pleiotropic molecule best known for its action as a potent vasoconstrictor (1). Originally isolated from porcine aortic endothelial cells, ET-1 is one of a family of three proteins encoded by distinct genes that also includes Endothelin-2 (ET-2) and Endothelin-3 (ET-3) (2, 3). ET-2 and ET-3 differ from ET-1 by 2 and 6 amino acids, respectively (1, 2). All members of the Endothelin family contain two essential disulfide bridges and six conserved aa residues at the C-terminus. Human ET-1 is initially synthesized as a pre-pro-polypeptide of 212 amino acids (2, 4). It is proteolytically cleaved by a signal peptidase to produce pro-ET-1 and further processed by a Furin-like protease to yield a 38 aa peptide termed Big ET-1 (5, 6). Big ET-1 is then cleaved by the membrane-bound metalloprotease Endothelin-converting enzyme (ECE-1), producing the potent 21 aa mature form ET-1 (aa 1-21) (7, 8). Alternatively, ET-1 may exist in an active 31 aa form (ET-1 (aa 1-31)) following cleavage of Big ET-1 by chymase (9-12). The vascular endothelium is an abundant source of ET-1 (3, 13). It may also be expressed by leukocytes, smooth muscle cells, mesangial cells, cardiac myocytes, and astrocytes (14, 15). ET-1 can be induced in endothelial cells by many factors including mechanical stimulation, various hormones, and pro-inflammatory cytokines (16). Production is inhibited by nitric oxide (NO), Prostacyclin, and atrial natriuretic peptide (ANP) (17-19).

Two receptors for the Endothelin family have been cloned and designated ETA and ETB (20-23). ETA and ETB belong to the large family of heptahelical G protein-coupled receptors. The ETA receptor shows a higher affinity for ET-1 than for ET-2 and lowest affinity for ET-3, while the ETB receptor shows approximately equal affinity for each of the three Endothelins (21, 22, 24). ETA is primarily responsible for the vasoconstrictor effects of ET-1 and is expressed by blood vessel smooth muscle cells (25, 26). The ETB receptor is also present in smooth muscle and the endothelia of blood vessels, kidney, lung, and brain (27). ET-1 has the ability to activate an array of signaling cascades including classical phosphatidylinositol turnover pathways leading to downstream PKC activation and Ca^{2+} mobilization (28-32). Other potential signaling mediators activated or produced by ET-1 include PI 3-kinase/Akt, NO, FAK, and Rho GTPases (32-37). ET-1 signaling may also be mediated indirectly via transactivation of the EGF receptor leading to downstream signaling by Ras and MAP kinases (38, 39). Injection of a single dose of ET-1 produces an initial decrease in systemic blood pressure followed by a prolonged increase in blood pressure (16, 40, 41). Blockade of Endothelin receptors with a systemic injection of an ETA/ETB antagonist causes progressive vasodilation, and elevated levels of ET-1 are found in some forms of human hypertension (42, 43). ET-1 also stimulates cardiac contraction and the growth of cardiac myocytes, regulates the release of vasoactive substances, and stimulates smooth muscle cell mitogenesis (32, 44-46). It also acts as a pro-survival factor for endothelial cells and regulates secretion by hypothalamic and pituitary cells (47, 48). ET-1 may control inflammatory responses by promoting the adhesion and migration of neutrophils and stimulating the production of pro-inflammatory cytokines (49-53). It has also been implicated in cancer progression at several levels including regulating the proliferation and migration of tumor cells and acting as a pro-angiogenic factor (54, 55). In addition, ET-1 has putative roles in other pathologies including septic shock, atherosclerosis, heart failure, renal insufficiency, pulmonary hypertension, and cerebrovascular conditions associated with subarachnoid hemorrhage (15, 56-63).

The Quantikine™ Endothelin-1 immunoassay is a 4.5 hour solid phase ELISA designed to measure ET-1 in cell culture supernates, serum, plasma, and urine. It contains synthetic ET-1 and antibodies raised against synthetic ET-1. This immunoassay has been shown to accurately quantitate synthetic and natural ET-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Endothelin-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Endothelin-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Endothelin-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Endothelin 1 bound in the initial step. 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.
- It is important that the calibrator diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine™ Immunoassay, the possibility of interference cannot be excluded.

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 reservoirs for each reagent.
- 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 the 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.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # DET100	CATALOG # SET100	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Endothelin-1 Microplate	893911	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for Endothelin-1.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Endothelin-1 Conjugate	893912	1 vial	6 vials	21 mL/vial of a monoclonal antibody specific for Endothelin-1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Endothelin-1 Standard	893913	1 vial	6 vials	Synthetic Endothelin-1 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-105	895958	1 vial	6 vials	18 mL/vial of a buffered protein solution with blue dye and preservatives.	
Calibrator Diluent RD5-48	895911	1 vial	6 vials	21 mL/vial of a concentrated buffered protein solution with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DET100 contains sufficient materials to run an ELISA on one 96 well plate.
SET100 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDET100). Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak contains reagents sufficient for the assay of 50 microplates (96 wells/plate). The package inserts supplied are the same as those supplied in the single kit packs and because of this, a few minor differences related to the number of reagents and their container sizes should be noted.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.
  Note: *Additional wash buffer is available for purchase (R&D Systems®, Catalog # WA126).*

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
Endothelin-1 Microplate	893911	50 plates
Endothelin-1 Conjugate	893912	50 vials
Endothelin-1 Standard	893913	25 vials
Assay Diluent RD1-105	895958	50 vials
Calibrator Diluent RD5-48	895911	50 vials
Wash Buffer Concentrate	895126	9 bottles
Color Reagent A	895000	50 vials
Color Reagent B	895001	50 vials
Stop Solution	895032	50 vials
Plate Sealers	N/A	100 sheets
Package Insert	752141	2 booklets

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 100 mL and 500 mL graduated cylinders
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards
- Endothelin-1 Controls (optional; R&D Systems®, Catalog # QC82)

PRECAUTIONS

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

Endothelin-1 is found in saliva. A face mask and gloves must be used to protect kit reagents from contamination.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Mouse/Rat Serum - Allow blood samples to clot for 2 hours at room temperature before centrifugation for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Mouse/Rat Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Do not use grossly hemolyzed samples.

Human Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Mouse/Rat Urine - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional particulates that may appear after storage.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *Endothelin-1 is found in saliva. A face mask and gloves must be used to protect kit reagents from contamination.*

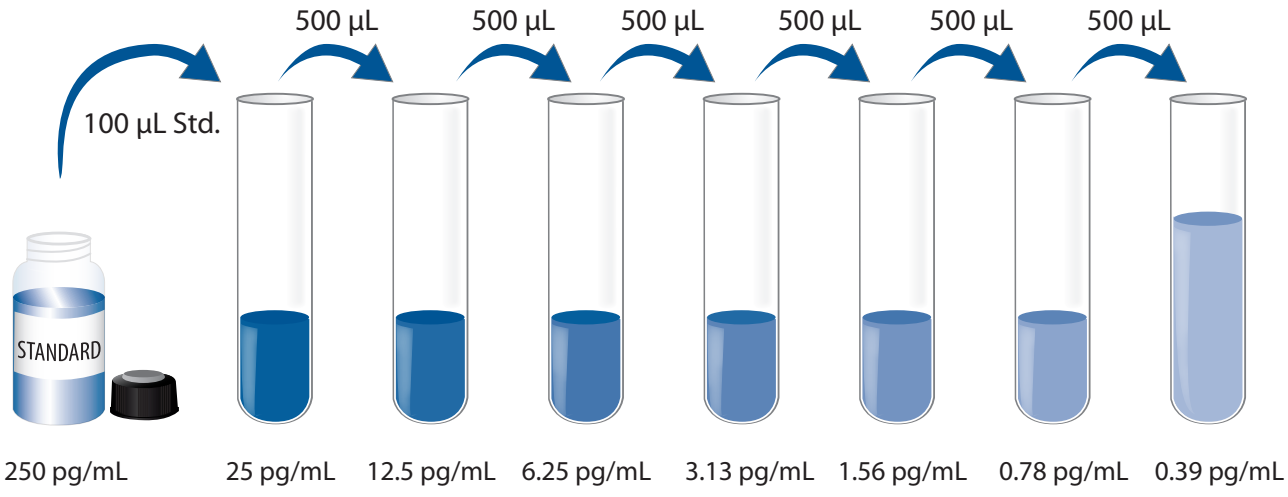
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-48 (diluted 1:5) - Add 5 mL of Calibrator Diluent RD5-48 to 20 mL of deionized or distilled water to prepare 25 mL of Calibrator Diluent RD5-48 (diluted 1:5).

Endothelin-1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Endothlin-1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 250 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-48 (diluted 1:5) into the 25 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 25 pg/mL standard serves as the high standard. Calibrator Diluent RD5-48 (diluted 1:5) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

Note: *Endothelin-1 is found in saliva. A face mask and gloves must be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 150 µL of Assay Diluent RD1-105 to each well.
4. Add 75 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Endothelin-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 3 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 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.
10. 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 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 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.

CALCULATION OF RESULTS

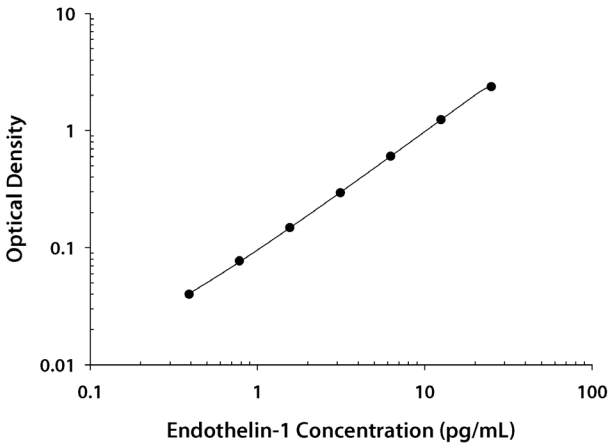
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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. The data may be linearized by plotting the log of the Endothelin-1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

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



(pg/mL)	O.D.	Average	Corrected
0	0.007 0.009	0.008	—
0.39	0.046 0.049	0.048	0.040
0.78	0.083 0.087	0.085	0.077
1.56	0.152 0.160	0.156	0.148
3.13	0.299 0.307	0.303	0.295
6.25	0.608 0.613	0.611	0.603
12.5	1.231 1.256	1.244	1.236
25	2.354 2.397	2.376	2.368

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (pg/mL)	3.00	7.34	14.7	3.05	7.43	14.4
Standard deviation	0.120	0.170	0.280	0.231	0.438	0.759
CV (%)	4.0	2.3	1.9	7.6	5.9	5.3

RECOVERY

The recovery of Endothelin-1 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	99	88 - 108%
Human serum (n=4)	98	86 - 107%
Human EDTA plasma (n=4)	93	87 - 102%
Human heparin plasma (n=4)	93	85 - 106%
Human urine (n=4)	91	85 - 109%
Mouse serum (n=4)	94	86-104%
Mouse EDTA plasma (n=4)	99	92-107%
Rat serum (n=4)	98	88-113%
Rat EDTA plasma (n=4)	99	86-113%

SENSITIVITY

Thirty-four assays were evaluated and the minimum detectable dose (MDD) of Endothelin-1 ranged from 0.031-0.207 pg/mL. The mean MDD was 0.087 pg/mL.

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

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of Endothelin-1 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Human serum (n=4)	Human EDTA plasma (n=4)	Human heparin plasma (n=4)	Human urine (n=4)
1:2	Average % of Expected	96	95	97	103	104
	Range (%)	90-109	92-99	93-106	98-107	95-108
1:4	Average % of Expected	102	102	104	107	106
	Range (%)	100-106	97-109	100-115	99-110	101-113
1:8	Average % of Expected	101	98	103	106	105
	Range (%)	98-104	91-105	92-112	96-111	100-111
1:16	Average % of Expected	101	99	101	103	103
	Range (%)	92-109	90-107	95-111	101-106	97-113

		Mouse serum (n=4)	Mouse EDTA plasma (n=4)	Rat serum (n=4)	Rat EDTA plasma (n=4)
1:2	Average % of Expected	97	99	93	96
	Range (%)	95-101	95-103	89-96	94-99
1:4	Average % of Expected	99	101	99	95
	Range (%)	95-102	98-107	94-104	92-98
1:8	Average % of Expected	95	101	104	96
	Range (%)	94-97	94-107	96-108	93-101
1:16	Average % of Expected	100	99	96	93
	Range (%)	96-106	89-112	93-99	87-97

CALIBRATION

This immunoassay is standardized against synthetic Endothelin-1.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of Endothelin-1 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Human serum (n=35)	1.24	0.47-2.00	0.35
Human heparin plasma (n=35)	1.17	0.58-1.96	0.32
Mouse serum (n=14)	3.10	1.95-4.03	0.58
Mouse EDTA plasma (n=15)	2.82	1.96-3.68	0.45
Rat serum (n=15)	1.63	1.32-2.07	0.20
Rat EDTA plasma (n=15)	1.59	0.53-2.36	0.44

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Human EDTA plasma (n=35)	1.17	97	ND-1.92
Human urine (n=25)	0.724	48	ND-1.14

ND=Non-detectable

Cell Culture Supernates:
Human peripheral blood leukocytes were cultured in DMEM supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 6 days. Aliquots of the cell culture supernates were removed and assayed for levels of Endothelin-1.

Condition	Day 1 (pg/mL)	Day 6 (pg/mL)
Unstimulated	ND	1.29
Stimulated	3.46	7.33

ND=Non-detectable

Tissue from mice or rats were homogenized and seeded into 100 mL of RPMI containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for the indicated times. Aliquots of the cell culture supernates were removed and assayed for levels of Endothelin-1.

Sample Type	Observed Levels (pg/mL)
Mouse liver (3 days)	1.05
Mouse lung (1 day)	5.15
Rat lung (1 day)	20.8

SPECIFICITY

This assay recognizes natural and synthetic Endothelin-1.

The factors listed below were prepared at 250 pg/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 250 pg/mL in a low level control were assayed for interference. No significant cross-reactivity or interference was observed.

Human peptides:
Big Endothelin-1 (aa 16-38)
Big Endothelin-2
Sarafotoxin S6b
Sarafotoxin S6c

Rat peptides:
Big Endothelin-39

Bovine peptides:
Big Endothelin-39

Human Endothelin-2 does not interfere but does cross-react approximately 23.4% in this assay.

Human/Rat Endothelin-3 does not interfere but does cross-react approximately 0.5% in this assay.

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