

TiterZyme® EIA

human Endothelin-1

Enzyme Immunometric Assay Kit

Catalog No. 900-020

96 Determination Kit

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Description

Assay Designs' human Endothelin-1 TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of human Endothelin-1 in biological fluids. In addition, this kit detects Endothelin-1 levels in bovine, canine, murine, porcine, rabbit and rat samples. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to Endothelin-1 immobilized on a microtiter plate to bind the Endothelin-1 in the standards or sample. A recombinant human Endothelin-1 Standard is provided in the kit. After a short incubation the excess sample is washed out and a polyclonal antibody to Endothelin-1 labeled with the enzyme Horseradish peroxidase is added. This labeled antibody binds to the Endothelin-1 captured on the plate. After a short incubation the excess labeled antibody is washed out and substrate is added. The substrate reacts with the labeled antibody bound to the Endothelin-1 captured on the plate. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of Endothelin-1 in either standards or samples. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

The discovery by Rubanyi's group³ in 1985 of a peptide termed endothelium-derived contracting factor (EDCF) and the isolation, sequencing, cloning and naming endothelin by Yanagisawa⁴ of the most potent vasoconstrictor has led to greater understanding of many physiological effects. Endothelin-1 (ET-1) is a 21-amino acid peptide, that with 2 other iso-peptides, have been shown to be derived from the expression of three separate genes⁵. The genes make three pre-propeptides that are cleaved into three different big endothelin molecules, each 39 amino acids long. These propeptides are cleaved by endothelin-converting enzyme into the three peptides, ET-1, ET-2 and ET-3⁶. A number of reviews of the structure, function, and molecular biology of the endothelin family of peptides and propeptides are available⁷⁻⁹. ET-1 has been shown to have potent effects on smooth muscle cells, fibroblasts and to be involved in many disease processes, particularly cardiovascular diseases. It has been shown to be important in congestive heart failure¹⁰, renal failure¹¹, and pulmonary hypertension^{12,13}. In a 1995 report it has also been shown to be elevated in the plasma of patients with metastatic prostate cancer¹⁴.

Precautions

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1. Stop Solution is a 1 normal (1N) sulfuric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles, such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The human ET-1 Standard provided, Catalog No. 80-0217, should be handled with care, because of the known and unknown effects of ET-1.

Materials Supplied

1. **human Endothelin-1 Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0215**
A strip microtiter plate coated with rabbit antibody specific to human Endothelin-1.
2. **human Endothelin-1 Labeled Antibody Concentrate, 0.4 mL, Catalog No. 80-1371**
Rabbit antibody to Endothelin-1 conjugated to Horseradish peroxidase.
3. **Assay Buffer, 30 mL, Catalog No. 80-0170**
Phosphate buffered saline containing proteins and detergents.
4. **Labeled Antibody Diluent, 12 mL, Catalog No. 80-0182**
Phosphate buffered saline containing proteins and detergents.
5. **Wash Buffer Concentrate, 50 mL, Catalog No. 80-0171**
Phosphate buffered saline containing detergents.
6. **human Endothelin-1 Standard, 2 each, Catalog No. 80-0217**
Two vials containing 100 pg each of recombinant human Endothelin-1.
7. **TMB Substrate, 15 mL, Catalog No. 80-1342**
A solution of 3,3',5,5' tetramethyl benzidine (TMB) and hydrogen peroxide. Ready to use.
8. **Stop Solution, 12 mL, Catalog No. 80-0176**
A 1N solution of sulfuric acid in water. Keep tightly capped. Caution: **Caustic**.
9. **human Endothelin-1 Assay Layout Sheet, 1 each, Catalog No. 30-0055**
10. **Plate Sealer, 2 each, Catalog No. 30-0012**

Storage

All components of this kit are stable at 4 °C until the kit's expiration date.

Materials Needed but Not Supplied

1. Deionized or distilled water. No difference in assay results are seen with distilled water.
2. Precision pipets for volumes between 100 μ L and 1,000 μ L.
3. Disposable test tubes for dilution of samples and standards.
4. Repeater pipet for dispensing 100 μ L.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. A 37 °C incubator.
8. Adsorbent paper for blotting.
9. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
10. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' TiterZyme® EIA is compatible with Endothelin-1 samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. **Human Endothelin-1 has identical amino acid sequence to bovine, canine, murine, porcine, rabbit and rat Endothelin-1.**

In humans, normal plasma levels of Endothelin-1 have been reported to be in the range 1-3 pg/mL. In certain disease states, levels may increase 3 fold or more. Culture fluids, serum and plasma are suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay if diluted into Assay Buffer. Users should only use standard curves generated in Assay Buffer to calculate concentrations of Endothelin-1.

Plasma samples should be drawn into chilled EDTA tubes (1 mg/mL blood) containing Aprotinin (500 KIU/mL or 10.6 TIU/mL of blood). Centrifuge the blood at 1,600 x g for 15 minutes at 0 °C. Transfer the plasma to a plastic tube and store at -70 °C or lower for long term storage.

Some samples, such as plasma and urine, normally have very low levels of Endothelin-1 present and for these samples as well as samples with high levels of protein (e.g. serum and plasma), extraction may be necessary for accurate measurement. Extraction of the sample should be carried out using a similar protocol to the one described below.¹⁵

1. Add an equal volume of 20% acetic acid (AA) to the sample. Centrifuge at 3,000 x g for 10 minutes at 4 °C to clarify and save the supernatant.
2. Equilibrate a 200 mg C₁₈ Sep-Pak column with one column reservoir volume (CV) methanol (MeOH), followed by one CV water and one CV 10% MeOH.
3. Apply the supernatant to the Sep-Pak column and wash with one CV 10% AA. Remove the excess AA by applying reduced pressure. Discard washes.
4. Wash column with two CVs ethyl acetate and remove the excess by applying reduced pressure.
5. Elute the sample slowly by applying 3 mL MeOH/0.05 M ammonium bicarbonate (80/20 v/v). Collect the eluant in a plastic tube.
6. Evaporate to dryness using a centrifugal concentrator under vacuum. Store at -20 °C.
7. Reconstitute with Assay Buffer and measure immediately.

Please note that recovery of peptides from extraction processes can be variable. It is important to optimize any process to obtain optimum recoveries. Extraction efficiencies can be determined by spiking a known amount of Endothelin-1 into paired samples and determining the recovery of this known amount of added Endothelin-1.

Procedural Notes

1. Do not mix reagents from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses plates with removable strips. Unused strips must be kept desiccated at 4 °C in the sealed bag provided. The strips should be used in the frame provided.
8. **Prior to addition of standard, antibody or substrate, ensure that there is no residual wash buffer in these wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Prepare Wash Buffer by diluting 25 mL of the supplied concentrate with 975 mL of deionized water. This can be stored at 4 °C until the kit expiration date, or for 3 months, whichever is earlier.

2. Endothelin-1 Standards

Add 500 µL of deionized water to the Endothelin-1 Standard. Let it sit at room temperature for 5 minutes. Mix it gently. This solution contains 200 pg/mL Endothelin-1.

Label eight 12 x 75 mm glass tubes #1 through 8. Pipet 220 µL of Assay Buffer into tubes #1 through #8. Add 220 µL of the 200 pg/mL standard to tube #1. Vortex. Add 220 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #8.

The concentration of Endothelin-1 in tubes #1 through #8 will be 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 pg/mL respectively. See Endothelin-1 Assay Layout Sheet for dilution details. STORE STANDARD AT -20°C, avoid repeated freeze/thaws.

3. Labeled Antibody Conjugate

Just before use, the human ET-1 Labeled Antibody Concentrate must be diluted 1:30 into the Labeled Antibody Diluent in a clean test tube and vortexed thoroughly. For example, if using one 8 well strip, dilute 30 µL of the Labeled Antibody Concentrate into 870 µL of the Labeled Antibody Diluent.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 100 µL of Assay Buffer into the S0 (0 pg/mL Standard) wells.
3. Pipet 100 µL of Standards #1 through #8 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at 4°C for overnight.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 6 more times for a total of **7 washes**. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of the Labeled Antibody into each well, except the Blank.
9. Seal the plate and incubate at 37 °C for 30 minutes.
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 8 more times for a total of **9 washes**. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µL of the Substrate Solution to each well.
12. Incubate for 30 minutes at room temperature in the dark.
13. Add 100 µL of Stop Solution to each well.
14. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of Endothelin-1 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of Endothelin-1 can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average Blank OD}$$

2. Plot the Average Net OD for each standard versus Endothelin-1 concentration in each standard.
3. Using linear graph paper, plot the Average OD for each standard versus Endothelin-1 concentration in each standard. Approximate a straight line through the points. The concentration of Endothelin-1 in the unknowns can be determined by interpolation.

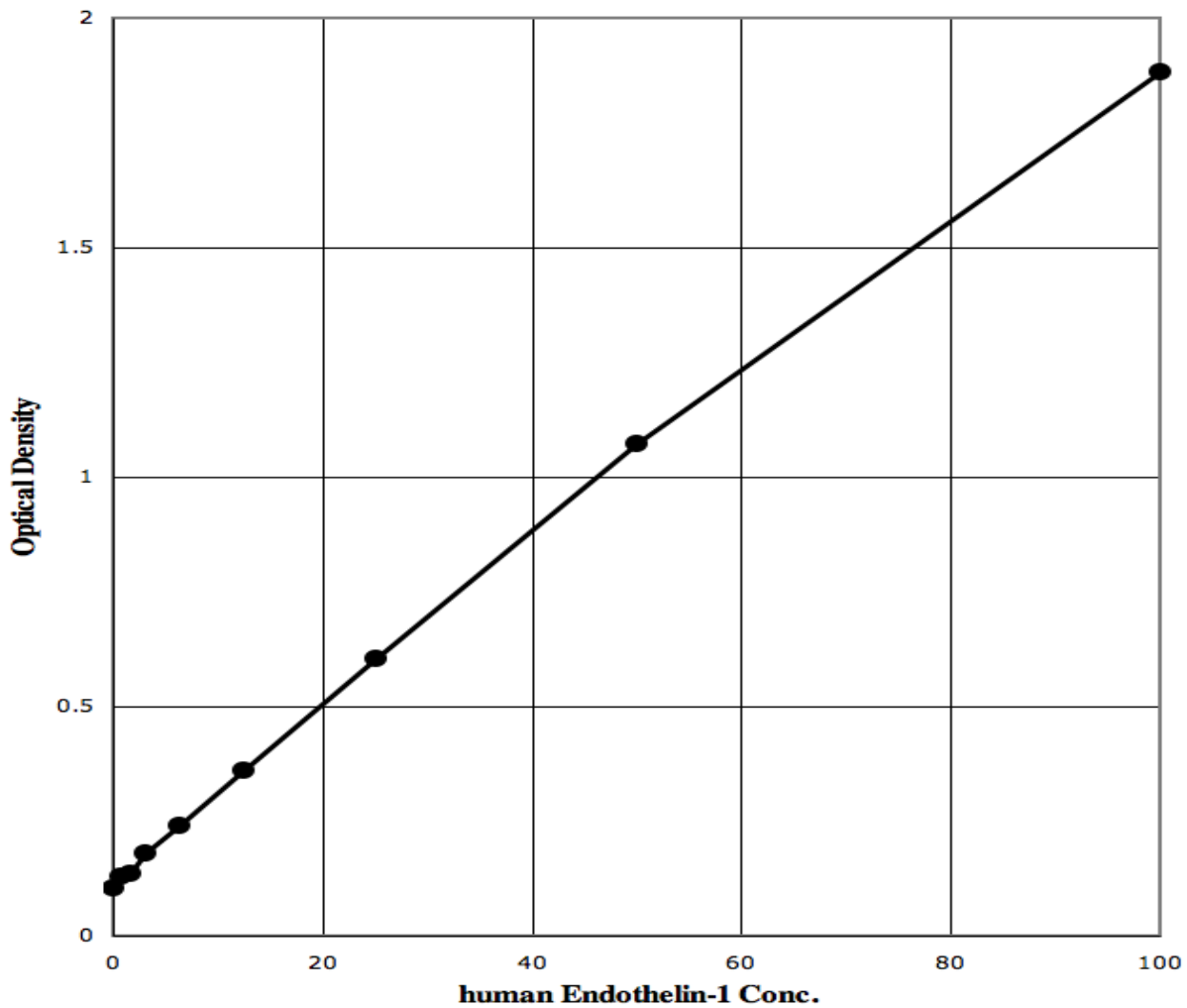
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

<u>Sample</u>	<u>Average OD</u>	<u>Net OD</u>	human Endothelin-1 (pg/mL)
Blank	(0.044)		
S0	0.148	0.104	0
S1	1.926	1.882	100
S2	1.117	1.073	50
S3	0.649	0.605	25
S4	0.404	0.360	12.5
S5	0.285	0.241	6.25
S6	0.222	0.178	3.1
S7	0.179	0.135	1.56
S8	0.173	0.129	0.78

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate Endothelin-1 concentrations; each user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols¹⁶.

Sensitivity

Sensitivity was calculated by determining the average optical density bound for eight (8) wells run with 0 pg/mL Standard, and comparing to the average optical density for eight (8) wells run with Standard #8. The detection limit was determined as the concentration of Endothelin-1 measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Average Optical Density for the S0 = 0.188 ± 0.005 (2.4%)

Average Optical Density for Standard #8 = 0.244

Delta Optical Density (0.78-0 pg/mL) = 0.056

2 SD's of the 0 pg/mL Standard = 2 x 0.005 = 0.01

Sensitivity = $\frac{0.01}{0.056} \times 0.78 \text{ pg/mL} =$ **0.14 pg/mL**

Linearity

A sample containing 200 pg/mL Endothelin-1 was diluted 7 times 1:2 into RPMI with 10% fetal bovine serum added and measured in the assay. The data was plotted graphically as actual Endothelin-1 concentration versus measured Endothelin-1 concentration.

The line obtained had a slope of 0.846 and a correlation coefficient of 0.994.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Endothelin-1 and running these samples multiple times (n=39) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Endothelin-1 in multiple assays (n=22).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Endothelin-1 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Endothelin-1 (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	4.76	8.5	
Medium	12.39	5.8	
High	50.02	4.6	
Low	4.96		8.1
Medium	12.74		4.7
High	50.57		3.1

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant in Assay Buffer. These samples were then measured in the Endothelin-1 assay, and the measured Endothelin-1 concentration calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

<u>Compound</u>	<u>Cross Reactivity</u>
Endothelin-1 (1-21)	100%
Endothelin-2 (1-21)	<0.1%
Endothelin-1 (1-31)	<0.1%
Rat Big ET-1	<0.1%
Endothelin-3 (1-21)	<0.1%
Endothelin-2 (1-31)	<0.1%
Endothelin-3 (1-31)	<0.1%
Big Endothelin-1	<0.1%
Big Endothelin-2	<0.1%
Big Endothelin-3	<0.1%
VIC (Mouse ET-2)	<0.1%

Human Endothelin-1 has identical amino acid sequence to bovine, canine, murine, porcine, rabbit and rat Endothelin-1.

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Endothelin-1 concentrations were measured in a variety of different samples including tissue culture media, human serum and human EDTA plasma. Endothelin-1 was spiked into the undiluted samples of these media which were then diluted with the kit Assay Buffer and assayed in the kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery*</u>	<u>Recommended Dilution*</u>
Tissue Culture Media	98.4	≥1:2
human Serum	75.7	≥1:32
human EDTA Plasma	72.7	≥1:8

* See Sample Handling instructions on page 4 for details.

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LIMITED WARRANTY

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For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

Material Safety Data Sheet (MSDS) available on our website or by fax.

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