DRG® Human Endothelin-1 (EIA-3111)

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

Introduction

The Endothelin-1 (ET-1) Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of ET-1 in plasma, serum, and culture fluids. **ET-1 has identical amino acid sequence for human, mouse, rat, cow, dog, pig, and rabbit.** Please read the complete kit insert before performing this assay.
Endothelins (ET) were first identified as endothelin-derived relaxation factors then again as an endothelin-derived contracting factor. In 1988, the isolation and sequence of a 21 amino acid peptide was reported and identified by the more common name endothelin 1. Three distinct endothelin genes encode unique but highly related peptides, ET-1, ET-2, and ET-3[1]. The predominant physical feature of ET's is the central helical core that is stabilized and includes two intra-chain disulfide bonds. This conformation is necessary for high affinity binding by the ETA receptor, but is not for binding by ETB[2]. Translated as a pre-pro peptide and translocated into circulation as the pro form Big ET-1, bioactive ET-1 is released when Big ET-1 is cleaved by endothelin-converting enzymes at the site of action. Once released, ET-1 is able to elicit different vaso-actions depending on which receptor is bound[3]. The basal circulating level of ET-1 is reported to be < 1 to 3 pg/mL but is known to be elevated in atrial and pulmonary hypertensions, atherosclerosis, congestive heart failure, cancer[4] and variably related to lung diseases such as COPD and asthma[5].

Principle

Samples and standards are added to wells coated with a monoclonal antibody specific for ET-1. The plate is then incubated.
The plate is washed, leaving only bound ET-1 on the plate. A solution of HRP labeled monoclonal antibody to ET-1 is then added. This binds the ET-1 captured on the plate. The plate is then incubated.
The plate is washed to remove excess HRP labeled antibody. TMB Substrate solution is added. The substrate generates a blue color when catalyzed by the HRP.
Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of ET-1 in the sample.

Materials Supplied

1. **Assay Buffer**, 100 mL,
   Tris buffered saline containing detergents
2. Endothelin-1 **Standard**, 0.25 mL,
   One vial containing 1,000 pg/mL of recombinant ET-1
3. Endothelin-1 Clear **Microtiter Plate**, One Plate of 96 Wells,
   A plate of break-apart strips coated with a mouse monoclonal antibody specific to ET-1
4. **Wash Buffer** Concentrate, 100 mL,
   Tris buffered saline containing detergents
5. Endothelin-1 **Antibody** Concentrate, 100 µL,
   A solution of HRP labeled rat monoclonal antibody to ET-1
6. **Antibody Diluent**, 10 mL
   Tris buffered solution containing detergents
7. **TMB Substrate**, 10 mL,
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide

8. **Stop Solution**, 10 mL,
   A 1N solution of hydrochloric acid in water
9. Endothelin-1 **Assay Layout Sheet**, 1 each
10. **Plate Sealer**, 3 each

NOTE:
Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.
The standard should be handled with care due to the known and unknown effects of the molecule.
Protect substrate from prolonged exposure to light.
Stop solution is caustic. Keep tightly capped.

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
2. Acetic Acid
3. Methanol
4. Ethyl acetate
5. Ammonium bicarbonate
6. 200 mg C18 Sep-Pak column
7. Microcentrifuge
8. Microcentrifuge tubes
9. Centrifugal evaporator
10. Precision pipets for volumes between 50 µL and 1000 µL
11. Repeater pipet for dispensing 100 µL
12. Disposable beakers for diluting buffer concentrates
13. Graduated cylinders
14. Lint-free paper for blotting
15. Microplate reader capable of reading at 450 nm
16. Graph paper for plotting the standard curve

Reagent Preparation

- Bring all reagents to room temperature for at least 30 minutes prior to opening.
- Plastic tubes must be used for standard preparation.
- Pre-rinse each pipet tip with reagent.
- Use fresh pipet tips for each sample, standard, and reagent.

1. Wash Buffer

Prepare the wash buffer by diluting 50 mL of the supplied Wash Buffer Concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Standards

Label eight 12 x 75 mm polypropylene tubes #1 through #8.
Pipet 450 µL of the assay buffer into tube #1.
Pipet 250 µL of the assay buffer into tubes #2 through #8.
Add 50 µL of the 1,000 pg/mL standard stock into tube #1 and vortex thoroughly.
Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex thoroughly.
Continue this for tubes #4 through #8.
Diluted standards should be used within 30 minutes of preparation.

Standard #	1	2	3	4	5	6	7	8
Conc. (pg/mL)	100	50	25	12.5	6.25	3.13	1.56	0.78

3. ET-1 Antibody

Prepare the antibody by diluting 10 µL of the supplied antibody concentrate with 1 mL of antibody diluent for every mL of 1X needed.
The diluted antibody must be used within 8 hours. Only prepare what is needed each day. Discard any unused, diluted antibody.

Sample Handling

Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Repeated freeze/thaw cycles should be avoided.
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 samples.
Plasma samples should be drawn into chilled EDTA tubes (1 mg/mL blood) containing Aprotinin (500 KIU/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. Aliquot to avoid repeated freeze/thaw cycles.
In humans, normal plasma levels of Endothelin-1 have been reported to be in the Range of 1-3 pg/mL. In certain disease states, levels may increase 3 fold or more. Samples with very low levels of ET-1 or with high levels of protein (e.g. serum and plasma), may require extraction for accurate measurement. Extraction of the sample should be carried out using a similar protocol to the one described below[6].

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; save the supernatant.
2. Equilibrate a 200 mg C18 Sep-Pak column with one column reservoir volume (CV) 100% 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 100% 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.
7. If samples cannot be assayed immediately, store at -20°C.
8. Reconstitute with at least 250 µL of the assay buffer and measure immediately.

ET-1 was spiked into 1 mL of sample which was extracted and reconstituted with .25 mL of the assay buffer or diluted in the assay buffer and measured in the assay.

NOTE: If the end user chooses to vary from the extraction procedure noted, it is up to the end user to determine the appropriate dilution of samples and assay validation.

Sample	% Recovery	Recommended Dilution
RPMI + 10% FBS	76	None
Serum (human)	110	1:4
Plasma (human)	104	1:4
Serum (human) - extracted	79	None
Plasma (human) - extracted	72	None

A minimum 1:4 dilution is required for unextracted serum and plasma samples to remove matrix interference in the assay. Culture fluids and extracted samples may be used without dilution. The optimal dilution for a specific experiment should be determined by the investigator.

Assay Procedure

- Bring all reagents to room temperature for at least 30 minutes prior to opening.
- All standards, controls, and samples should be run in duplicate.
- Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.
- Pipet the reagents to the sides of the wells to avoid possible contamination.

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 100 µL of the assay buffer into the S0 (0 pg/mL standard) wells.
2. Pipet 100 µL of Standards #1 through #8 to the bottom of the appropriate wells.
3. Pipet 100 µL of the samples to the bottom of the appropriate wells.
4. Seal the plate. Incubate for 1 hour at room temperature *.
5. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 4 more times for a total of 5 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.
   Ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.
6. Pipet 100 µL of diluted antibody into each well except the blank.
7. Seal the plate. Incubate for 30 minutes at room temperature.
8. Wash as above (Step 5).
9. Pipet 100 µL of substrate solution into each well.
10. Incubate for 30 minutes at room temperature.
11. Pipet 100 µL of stop solution into each well.

12. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

* If desired, the sealed plate may be incubated overnight at 4ºC during the first incubation step. This will result in ~67% increased optical density values.

Calculation of Results

- Make sure to adjust sample concentrations by the dilution factor or concentration factor used during sample preparation.

Several options are available for the calculation of the concentration of ET-1 in 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 concentrations can be calculated as follows:

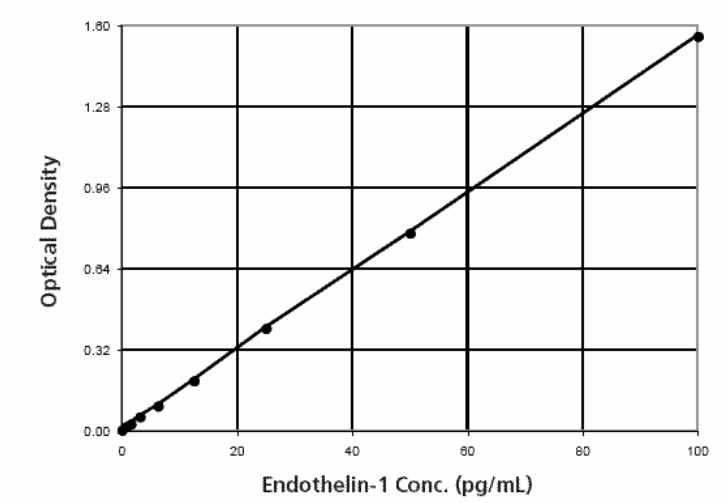
1. Calculate the average Net OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.
   Average Net OD = Average OD - Average Blank OD
2. Using linear graph paper, plot the average Net OD for each standard versus ET-1 concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

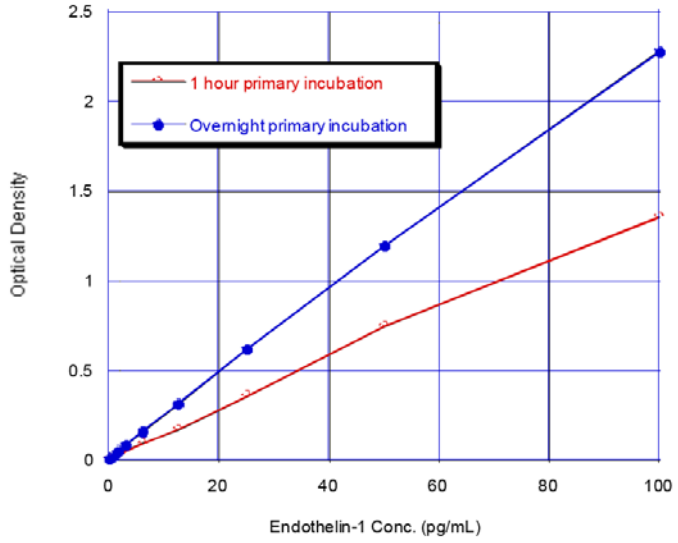
Typical Results

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

Sample	Net OD	ET-1 (pg/mL)
S1	1.563	100
S2	0.786	50
S3	0.409	25
S4	0.202	12.5
S5	0.103	6.25
S6	0.060	3.13
S7	0.032	1.56
S8	0.022	0.78
S0	0.007	0
Unknown 1	0.555	34.83
Unknown 2	0.027	1.19



Results below demonstrate expected difference in primary incubation options. There is an approximate 67% increase in optical density with the overnight primary incubation.



Performance Characteristics

Specificity

The cross reactivities for a number of related compounds were determined by diluting cross reactants in the assay buffer at a concentration of 10,000 pg/mL. These samples were then measured in the assay. Typical expression ratios of immunoreactive endothelin in normal plasma are; ET-1:Big ET (1:2), ET-1:ET-2 (5:1), and ET-1:ET-3 (2:1).[4,7].

Compound	Cross Reactivity
ET-1	100%
ET-2	21%
ET-3	3.6%
human Big ET-1	<0.1%
rat Big ET-1	<0.1%
human Big ET-2	<0.1%
human Big ET-3 Amide	<0.1%

Sample Values

The following samples were tested for the presence of ET-1.

Sample	# of Samples Tested	Range (pg/mL)	Mean (pg/mL)
Human EDTA Plasma	6	1.1 - 2.4	1.8
Human Serum	6	1.2 - 2.5	1.8

Concentrations are corrected for extraction efficiency.

Sensitivity

The sensitivity of the assay, defined as the concentration of ET-1 measured at 2 standard deviations from the mean of 24 zeros along the standard curve, was determined to be 0.41 pg/mL.

Linearity

A buffer sample containing ET-1 was serially diluted 1:2 in the assay buffer and measured in the assay. The results are shown in the table below.

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	---	80.25 pg/mL	---
1:2	40.12 pg/mL	37.62 pg/mL	93.8 %
1:4	20.06 pg/mL	19.08 pg/mL	95.1 %
1:8	10.03 pg/mL	9.97 pg/mL	99.4 %
1:16	5.02 pg/mL	5.12 pg/mL	102.0 %
1:32	2.51 pg/mL	2.56 pg/mL	102.0 %
1:64	1.25 pg/mL	1.49 pg/mL	119.2 %

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing ET-1 in a single assay.

pg/mL	%CV
35.9	6.7
2.3	8.9
1.1	8.8

Inter-assay precision was determined by measuring buffer controls of varying ET-1 concentrations in multiple assays over several days.

pg/mL	%CV
35.1	8.3
2.5	5.9
1.2	15.6

References

1. Rubanyi GM, Botelho LH. "Endothelins." FASEB J. 1991 Sep; 5(12):2713-20.
2. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T. "The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes." PMAS. 1989 Apr; 86(8):2863-7.
3. Rubanyi GM, Polokoff MA. "Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology." Pharmacol Rev. 1994 Sep;46(3):325-415.
4. Rossi GP, Seccia TM, Albertin G, Pessina AC. "Measurement of endothelin: clinical and research use." Ann Clin Biochem. 2000 Sep; 37:608-26.
5. Fagan KA, McMurtry IF, Rodman DM. "Role of endothelin-1 in lung disease." Respir Res. 2001;2(2):90-101.
6. Rolinski B, Sadri I, Bogner J, Goebel FD. "Determination of endothelin-1 immunoreactivity in plasma, cerebrospinal fluid and urine." Res Exp Med. 1994;194(1):9-24.
7. Suzuki N, Matsumoto H, Miyauchi T, Kitada C, Tsuda M, Goto K, Masaki T, Fujino M. "Sandwich-enzyme immunoassays for endothelin family peptides."J Cardiovascular Pharmacol. 1991: 17(Suppl7):S420.

Limited Warranty

DRG warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.
DRG must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if DRG is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

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Symbols used with DRG Assays

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

Symbol	Portugues	Dansk	Svenska	Ελληνικά
	Consulte as instruções de utilização	Se brugsanvisning	Se bruksanvisningen	Εγχειρίδιο χρήστη
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό
RUO				
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
		Indeholder tilsttrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevarings-temperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Distributed by				
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..