Endothelin-1 ELISA kit
Catalog #ADI-900-020A

96 well enzyme-linked immunosorbent assay
For use with serum, plasma, and culture fluids
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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.
INTRODUCTION

The Endothelin-1 (ET-1) Enzyme-linked Immunosorbent Assay (ELISA) 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

1. Samples and standards are added to wells coated with a monoclonal antibody specific for ET-1. The plate is then incubated.

2. 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.

3. 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.

4. 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 17**
   100 mL, Catalog No. 80-1573
   Tris buffered saline containing detergents

2. **Endothelin-1 Standard**
   0.25 mL, Catalog No. 80-1567
   One vial containing 1,000 pg/mL of recombinant ET-1

3. **Endothelin-1 Clear Microtiter Plate**
   One Plate of 96 Wells, Catalog No. 80-1572
   A plate of break-apart strips coated with a mouse monoclonal antibody specific to ET-1

4. **Wash Buffer Concentrate**
   100 mL, Catalog No. 80-1287
   Tris buffered saline containing detergents

5. **Endothelin-1 Antibody Concentrate**
   100 µL, Catalog No. 80-1565
   A solution of HRP labeled rat monoclonal antibody to ET-1

6. **Antibody Diluent**
   10 mL Catalog No. 80-1755
   Tris buffered solution containing detergents

7. **TMB Substrate**
   10 mL, Catalog No. 80-0350
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide

8. **Stop Solution 2**
   10 mL, Catalog No. 80-0377
   A 1N solution of hydrochloric acid in water

9. **Endothelin-1 Assay Layout Sheet**
   1 each, Catalog No. 30-0234

10. **Plate Sealer**
    3 each, Catalog No. 30-0012

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- 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 antigen.
- Protect substrate from prolonged exposure to light.
- Stop solution is caustic. Keep tightly capped.

Stop solution is caustic. Keep tightly capped.

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Stop solution is caustic. Keep tightly capped.

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Stop solution is caustic. Keep tightly capped.

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Stop solution is caustic. Keep tightly capped.
**STORAGE**

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

**OTHER MATERIALS NEEDED**

1. Deionized or distilled water
2. Acetic Acid
3. Methanol
4. Ethyl acetate
5. Ammonium bicarbonate
6. 200 mg C\textsubscript{18} Sep-Pak column
7. Microcentrifuge
8. Microcentrifuge tubes
9. Centrifugal evaporator
10. Precision pipets for volumes between 50 µL and 1,000 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
REAGENT PREPARATION

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. ET-1 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. The concentration of ET-1 in tubes is labeled above.

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

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 (1mg/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 1-3pg/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.

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.
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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Recovery</th>
<th>Recommended Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI + 10% FBS</td>
<td>76</td>
<td>None</td>
</tr>
<tr>
<td>Serum (human)</td>
<td>110</td>
<td>1:4</td>
</tr>
<tr>
<td>Plasma (human)</td>
<td>104</td>
<td>1:4</td>
</tr>
<tr>
<td>Serum (human) - extracted</td>
<td>79</td>
<td>None</td>
</tr>
<tr>
<td>Plasma (human) - extracted</td>
<td>72</td>
<td>None</td>
</tr>
</tbody>
</table>

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

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.¹
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.
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.

¹ If desired, the sealed plate may be incubated overnight at 4°C during the first incubation step. This will result in ~67% increased optical density values.
CALCULATION OF RESULTS

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.

\[ \text{Average net OD} = \text{Average OD} - \text{Average Blank OD} \]

2. 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.

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

Make sure to adjust sample concentrations by the dilution factor or concentration factor used during sample preparation.
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 reactant in the assay buffer at a concentration of 10,000 pg/mL. 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).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>100%</td>
</tr>
<tr>
<td>ET-2</td>
<td>21%</td>
</tr>
<tr>
<td>ET-3</td>
<td>3.6%</td>
</tr>
<tr>
<td>human Big ET-1</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>rat Big ET-1</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>human Big ET-2</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>human Big ET-3 Amide</td>
<td>&lt;0.1%</td>
</tr>
</tbody>
</table>

Sample Values

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

<table>
<thead>
<tr>
<th>Sample</th>
<th># of Samples Tested</th>
<th>Range (pg/mL)</th>
<th>Mean (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human EDTA Plasma</td>
<td>6</td>
<td>1.1 - 2.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Human Serum</td>
<td>6</td>
<td>1.2 - 2.5</td>
<td>1.8</td>
</tr>
</tbody>
</table>

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.

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

Precision

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

<table>
<thead>
<tr>
<th>pg/mL</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.9</td>
<td>6.7</td>
</tr>
<tr>
<td>2.3</td>
<td>8.9</td>
</tr>
<tr>
<td>1.1</td>
<td>8.8</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>pg/mL</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.1</td>
<td>8.3</td>
</tr>
<tr>
<td>2.5</td>
<td>5.9</td>
</tr>
<tr>
<td>1.2</td>
<td>15.6</td>
</tr>
</tbody>
</table>
REFERENCES


