Human EDN2 (Endothelin-2) ELISA Kit

A Complete ELISA kit for the detection of Human EDN2

(Cat. #IT3266)

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INTRODUCTION

The Human EDN2 (Endothelin-2) ELISA Kit is based on a sandwich enzyme-linked immunosorbent assay (ELISA) technology. A 96-well plate is coated with a specific antibody against the protein of interest (EDN2), which will bind the protein and this is detected by a biotin conjugated antibody against the protein of interest. The biotin moiety is subsequently detected, following washing, by the addition of streptavidin coupled horseradish peroxidase (HRP). The supplied TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is directly proportional to the concentration of protein of interest captured on the plate.

ITEMS SUPPLIED

Description	Size
Coated Microtiter Plate	1
ELISA Standard (Lyophilized)	2 vials
ELISA Standard Diluent	20ml
Wash Buffer [25X]	30ml
Biotinylated Detection Antibody	120µl
Detection Antibody Diluent	10ml
ELISA Detection Reagent	120µl
ELISA Detection Reagent Diluent	10ml
ELISA Detection Substrate (TMB)	10ml
ELISA Stop Solution	10ml
Microplate Sealing Tape	5

NOTE: *ELISA Detection Reagent is a HRP-Streptavidin Conjugate.*

STORAGE CONDITIONS

The kit is shipped on blue ice. Upon arrival, store kit at 4˚C for up to 6 months.

SPECIFICATIONS

- **Reactivity:** Human
- **Range:** 15.6-1000pg/ml
- **Sensitivity:** 9.375pg/ml

ADDITIONAL ITEMS NEEDED

- Microplate reader (wavelength: 450nm)
- 37°C incubator
- Automated plate washer (Optional)
- Precision single and multi-channel pipette and disposable tips
- Clean tubes and Eppendorf tubes
- Deionized or distilled water

PRECAUTIONS

- We recommend performing pilot experiments using standards and a small number of samples.
- After opening and before using, keep plate dry.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- ELISA Detection Substrate (TMB) must be protected from light.
- False positives may arise if washing steps are not completed.
- The use of duplicate well assays ire recommended for both standard and sample testing.
- Do not let the plate dry out during the assay as this may inactivate active components.
- Do not reuse tips and tubes to avoid cross contamination.

PREPARATION BEFORE USE

Bring all reagents to room temperature before use.

Wash Buffer

Dilute 30mL Wash Buffer [25X] into 750 mL of Wash Buffer with deionized or distilled water. If crystals have formed in the concentrate, you can warm in a 40°C water bath (Heating temperature should not exceed 50°C) and mix it gently until the crystals have completely dissolved. The solution should be cooled to room temperature before use. Store diluted wash buffer at 4°C.

ELISA Standard

1. ***50pg/ml of ELISA Standard:*** Add 1 ml of ELISA Standard Diluent into an ELISA Standard tube, keep the tube at room temperature for 10 min and mix thoroughly.
2. ***Dilute ELISA Standard:*** Label 6 Eppendorf tubes (1-6) and aliquot 0.3 ml of the ELISA Standard Diluent into each tube. Add 0.3 ml of the above 50pg/ml standard solution into 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.3 ml from 2nd tube to 3rd tube and mix thoroughly, and so on. The resulting standard will be repeating 1:1 dilutions of the starting standard
   NOTE: *The standard solutions are best used within 2 hours. The standard solution should be at 4°C for up to12 hours. Or store at -20 °C for up to 48 hours. Avoid repeated freeze-thaw cycles.*

Preparation of Biotinylated Detection Antibody

Prepare within 1 hour before starting the experiment.

1. Calculate the total volume of the working solution: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume)
2. Dilute the Biotinylated Detection Antibody with the Detection Antibody Diluent at 1:100 and mix thoroughly. (i.e. Add 1µl of Biotinylated Detection Antibody into 99µl of Diluent.)

Preparation of ELISA Detection Reagent

Prepare within 30 minutes before starting the experiment.

1. Calculate the total volume of the working solution: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume)
2. Dilute the ELISA Detection Reagent with ELISA Detection Reagent Diluent at 1:100 and mix thoroughly. (i.e. Add 1µl of ELISA Detection Reagent into 99µl of ELISA Detection Reagent Diluent.)

PROTOCOL

For Manual Washing

1. Discard the solution in the plate without touching the side walls.
2. Clap the plate on absorbent filter papers or other absorbent material.
3. Fill each well completely with 350µl wash buffer and soak for 1 to 2 minutes
4. Aspirate contents from the plate, and clap the plate on absorbent filter papers or other absorbent material.
5. Repeat this procedure two more times for a total of THREE washes.

For Automated Washing

Aspirate all wells, and then wash plate THREE times with 350µl wash buffer. After the final wash, invert plate, and clap the plate on absorbent filter papers or other absorbent material. It is recommended that the washer be set for a soaking time of 1 minute.

Sample Dilution Guideline

End user should estimate the concentration of the target protein in the test sample first, and select a proper dilution factor to ensure the diluted target protein concentration falls in the optimal detection range of the kit. Dilute the sample with the ELISA Standard Diluent. The test sample must be well mixed with the ELISA Standard Diluent.

- High target protein concentration (500-5000pg/ml): Dilution: 1:100. (i.e. Add 1µl of sample into 99µl of ELISA Standard Diluent)
- Medium target protein concentration (50-500pg/ml): Dilution: 1:10.(i.e. Add 10µl of sample into 90µl of ELISA Standard Diluent)
- Low target protein concentration (15.6-1000pg/ml): Dilution: 1:2.(i.e. Add 50µl of sample into 50µl of ELISA Standard Diluent)
- Very low target protein concentration (outside the lower range of the assay), it is unnecessary to dilute, or dilute at 1:2.

Assay Procedure

Before adding to wells, equilibrate the ELISA Detection Reagent working solution and TMB substrate for at least 30 min at room temperature. When diluting samples and reagents, they must be mixed completely and evenly. It is recommended to plot a standard curve for each test.

1. Wash plate 2 times before adding standard, sample and control (zero) well as directed above.
2. Aliquot 0.1ml standard solutions into the standard wells. Perform in duplicate
3. Add 0.1 ml of ELISA Standard Diluent into the control (zero) wells.
4. Add 0.1 ml of properly diluted sample into test sample wells.
   NOTE: *See Sample Dilution Guideline above*
5. Seal the plate with a cover and incubate at 37°C for 90 min.
6. Remove the cover and discard the plate content, clap the plate on the absorbent filter papers or other absorbent material. Do NOT let the wells completely dry at any time.
   NOTE**:** *Do not wash the plate at this time***.**
7. Add 0.1 ml of Biotinylated Detection Antibody working solution into the above wells (standard, test sample & zero wells). Add the solution at the bottom of each well without touching the side wall.
8. Seal the plate with a cover and incubate at 37°C for 60 min.
9. Remove the cover, and wash plate 3 times with Wash buffer.
10. Add 0.1 ml of ELISA Detection Reagent working solution into each well, cover the plate and incubate at 37°C for 30 min.

11. Remove the cover and wash plate 5 times with Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min.
12. Add 90µl of ELISA Detection Substrate (TMB) into each well, cover the plate and incubate at 37°C in the dark for 15-30 min.
    ***NOTE**: This incubation time is for reference use only; the optimal time should be determined by end user. A blue color should be seen in the first 3-4 wells (with most concentrated standard solutions); the other wells show no obvious color change.*
13. Add 50µl of Stop solution into each well and mix thoroughly. The color changes from blue to yellow immediately.
14. Read the O.D. absorbance at 450 nm in a microplate reader immediately after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The concentration of the samples can be interpolated from the standard curve.

***NOTE:** If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.*

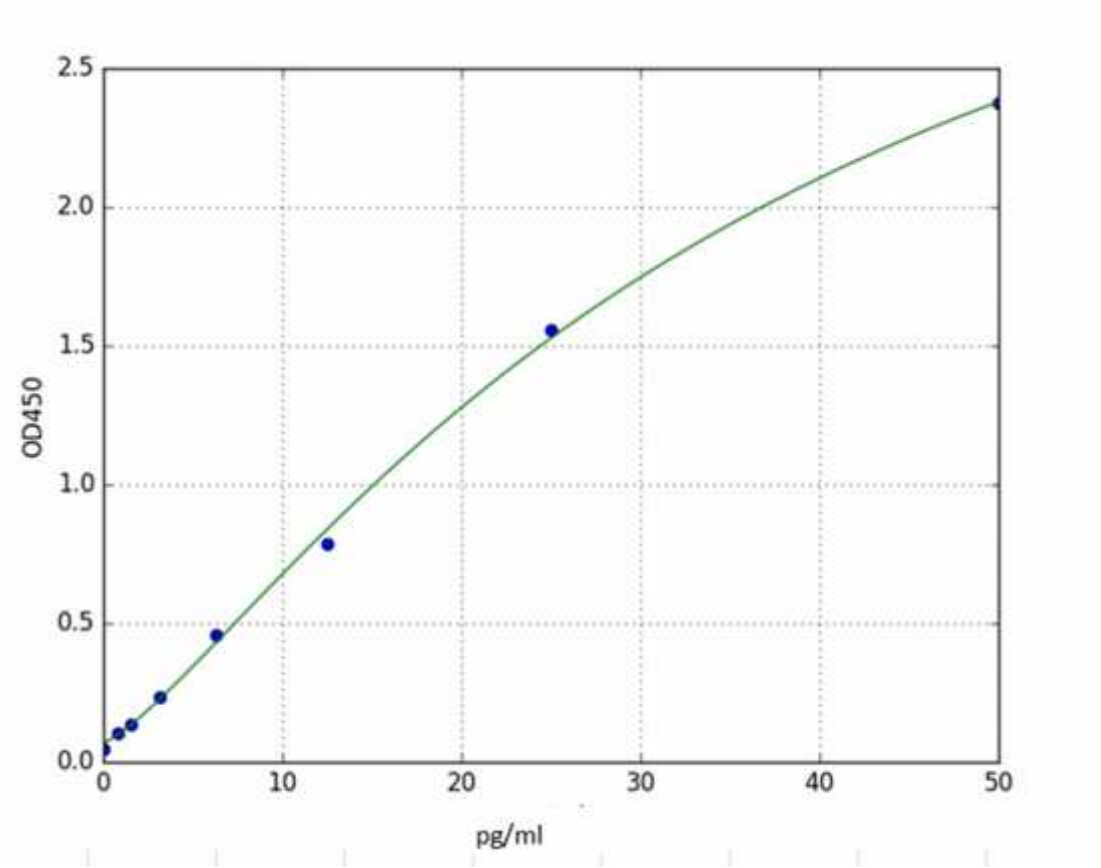
PROTOCOL SUMMARY

1. Wash plate 2 times before adding standard, sample and control (zero) wells
2. Add 100µL standard or sample to each well for 90 minutes at 37°C
3. add 100µL Biotinylated Detection Antibody working solution to each well for 60 minutes at 37°C
4. Aspirate and wash 3 times
5. Add 100µL ELISA Detection Reagent working solution to each well. Incubate for 30 minutes at 37°C
6. Aspirate and wash 5 times
7. Add 90µL ELISA Detection Substrate (TMB). Incubate 15 -30 minutes at 37°C
8. Add 50µL Stop Solution. Read at 450nm immediately
9. Calculation of results

TYPICAL DATA & STANDARD CURVE

Results of a typical standard operation of an EDN2 ELISA Kit are listed below. This standard curve was generated at our lab for demonstration purpose only. Users shall obtain standard curve as per experiment by themselves. (N/A=not applicable)

X	pg/ml	0	0.781	1.562	3.125	6.25	12.5	25	50
Y	OD450	0.049	0.105	0.134	0.236	0.457	0.788	1.556	2.373



SPECIFICITY

This assay has high sensitivity and excellent specificity for detection of EDN2. No significant cross-reactivity or interference between EDN2 and analogues was observed.

Note: Limited by current skills and knowledge, it is difficult for us to complete the cross- reactivity detection between EDN2 and all the analogues, therefore, cross reaction may still exist.

RECOVERY

Matrices listed below were spiked with certain level of EDN2 and the recovery rates were calculated by comparing the measured value to the expected amount of EDN2 in samples.

Matrix	Recovery Range (%)	Average (%)
Serum (n=5)	86-104	95
EDTA Plasma (n=5)	89-104	95
Heparin Plasma (n=5)	88-104	98

LINEARITY

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of EDN2 and their serial dilutions. The results were demonstrated by percentage of calculated concentration to the expectation.

Sample	1:2	1:4	1:8	1:16
Serum (n=5)	91-100%	85-103%	89-105%	89-102%
EDTA Plasma (n=5)	82-101%	86-100%	82-98%	82-98%

Heparin Plasma (n=5)	80-100%	88-99%	80-92%	86-95%

PRECISION

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level EDN2 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level EDN2 were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/meanX100

Intra-Assay: CV<8%

Inter-Assay: CV<10%

STABILITY

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage condition.

Standard (n=5)	37°C for 1 month	4°C for 6 months
Average (%)	80	95-100

To minimize extra influence on performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is strongly suggested that the same operator performs the whole assay from the beginning to the end.

ELISA TROUBLESHOOTING GUIDE

Problem	Possible Source	Solution
Poor Standard Curve	Improper standard solution	Confirm dilutions are made correctly
	Standard improperly reconstituted	Briefly spin vial before opening, inspect for undissolved material after reconstituting
	Standard degraded	Store and handle standard as recommended
	Curve does not fit scale	Try plotting using different scales e.g. log-log, 5 parameter logistic curve fit
	Pipetting error	Use calibrated pipettes and proper pipetting technique
	Standard was incompletely reconstituted or was inappropriately stored	Reconstitute standard according to protocol. Store reconstituted standard in appropriate vials. Store reconstituted standard at -70 °C
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and correct reagent volume.
	Incubations done at inappropriate temperature, timing or agitation	Assay conditions need to be checked
No signal	Incubation time too short	Incubate samples overnight at 4°C or follow the manufacturer guidelines.
	Target present below detection limit of assay	Decrease dilution factor or concentrate samples.
	Incompatible sample type	Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect a positive

Problem	Possible Source	Solution
		control.
	Recognition of epitope impeded by absorption to plate	To enhance detection of a peptide by direct or indirect ELISA, conjugate peptide to a large carrier protein before coating onto the microtiter plate.
	Assay buffer compatibility	Ensure assay buffer is compatible with target of interest (e.g. enzymatic activity retained, protein interactions retained)
	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Avidin-HRP was not added	Add Avidin-HRP according to protocol and continue.
	Substrate solution was not added	Add substrate solution and continue.
	Wash buffer contains sodium azide	Avoid sodium azide in the wash buffer.
	Multichannel pipette errors	Calibrate the pipettes.
	Plate washing was not adequate or uniform	Make sure pipette tips are tightly secured. Confirm all reagents are removed completely in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before pipetting
	Samples may have high particular matter	Remove the particular matter by centrifugation.
	Cross-well contamination	When reusing plate sealers check that no reagent has touched the sealer. Care should be taken when using the same pipette tips used for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.
	Not enough detection reagent	Increase concentration or amount of detection reagent following manufacturer guidelines.
	Sample prepared incorrectly	Ensure proper sample preparation/dilution. Samples may be incompatible with microtiter plate assay format.
	Insufficient antibody	Try different concentrations/dilutions of antibody
	Incubation temperature too low	Ensure the incubations are carried out at the correct temperature. All reagents including plate should be at room temperature or as recommended by the manufacturer before proceeding.
	Incorrect wavelength	Verify the wavelength and read plate again
	Plate washings too vigorous	Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually.
	Wells dried out	Do not allow wells to become dry once the assay has started. Cover the plate using sealing film or tape for all incubations
	Slow color developments of enzymatic reaction	Prepare substrate solution immediately before use. Ensure the stock solution has not expired and is not contaminated. Allow longer incubation.
High variation in samples and/or standards	Bubbles in wells	Ensure no bubbles are present prior to reading plate
	Wells not washed equally/thoroughly	Check that all ports of the plate washer are not obstructed. Wash wells as recommended.
	Incomplete reagent mixing	Ensure all reagents are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes and proper technique to ensure accurate pipetting
	Edge effects	Ensure the plate and all reagents are at room temperature.
	Inconsistent sample preparation	Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaw cycles).
High background	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Prepare fresh water buffer
	Too much detection reagent	Ensure the reagent has been diluted properly or decrease the

Problem	Possible Source	Solution
		recommended concentration of detection reagent.
	Blocking buffer ineffective (e.g. detection reagent binds blocker; wells not completely blocked)	Try different blocking reagent and/or blocking reagent to wash buffer.
	Salt concentration of incubation/wash buffers	Increasing salt concentrations may reduce non-specific and/or weak off-target interactions.
	Waiting too long to read plate after adding stop solution.	Read plate immediately after adding stop solution.
	Non-specific binding of antibody	Use suitable blocking buffers e.g. BSA or 5010% normal serum-species same as primary antibody if using a directly conjugated detection antibody or same as secondary if using conjugated.
	Background wells were contaminated	Avoid cross-well contamination by using the sealer appropriately. Use multichannel pipettes without touching the reagents on the plate.
	Matrix used has endogenous analyte or interference	Check the matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
	High antibody concentration	Try different dilutions for optimal results
	Substrate incubation carried out in light	Substrate incubations should be carried out in the dark or as recommended by manufacturer.
	Precipitate formed in wells upon substrate addition.	Increase dilution factor of sample or decrease concentration of substrate
	Dirty plate	Clean the plate bottom.
Low Sensitivity	Improper storage of ELISA kit	Store all reagents as recommended. Please note that all reagents may not have identical storage requirements.
	Not enough target	Concentrate sample or reduce sample dilution
	Inactive detection reagent	Ensure reporter enzyme has the expected activity.
	Plate reader settings incorrect	Ensure plate reader is set to read the correct absorbance wavelength or excitation/emission wavelengths for fluorescent detection.
	Assay format not sensitive enough	Switch to a more sensitive detection system (e.g. colorimetric to chemiluminescence / fluorescence) Switch to a more sensitive assay type (e.g. direct ELISA to sandwich ELISA). Lengthen incubation times or increase temperature.
	Target poorly absorbs to microtiter plate	Covalently link target to microtiter plate.
	Not enough substrate	Add more substrate
	Incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect as a positive control.
	Interfering buffers or sample ingredients	Check reagents for any interfering chemicals. For example, sodium azide in antibodies inhibit HRP enzyme and EDTA used as anticoagulant for plasma collection inhibits enzymatic reactions.
	Mixing or substituting reagents from different kits	Avoid mixing components from different kits.

APPENDIX: SAMPLE COLLECTION AND STORAGE

Isolate the test samples soon after collecting, then, analyze immediately (within 2 hours). Or aliquot and store at -20° C for long term. Avoid multiple freeze-thaw cycles.

- Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.
- Plasma: Collect plasma using EDTA-Na2 as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2 - 8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.
- Tissue homogenates: For general information, hemolysis blood may affect the result, so you should rinse the tissues with ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces which will be homogenized in PBS (the volume depends on the weight of the tissue. 9mL PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitor is recommended to add into the PBS.) With a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifugated for 5minutes at 5000×g to get the supernatant. We recommend including ProteaseARREST™ (Cat. # 786-108), a protease inhibitor cocktail, during the homogenization.
- Cell culture supernatant: Centrifuge supernatant for 20 minutes to remove insoluble impurity and cell debris at 1000×g at 2 - 8°C. Collect the clear supernatant and carry out the assay immediately. We recommend including TCM-ProteaseARREST™ (Cat. # 786-238), a tissue culture media protease inhibitor cocktail, during the homogenization.
- Other biological fluids: Centrifuge samples for 20 minutes at 1000×g at 2 - 8°C. Collect the supernatant and carry out the assay immediately.
- Sample preparation: Samples should be clear and transparent and be centrifuged to remove suspended solids.

NOTE: *Samples to be used within 5 days may be stored at 4°C,otherwise samples must be stored at -20°C (≤1 month) or -80°C(≤2 months) to avoid loss of bioactivity and contamination. Hemolyzed samples are not suitable for use in this assay.*

RELATED PRODUCTS

Download our Protein Assay Development or Bioassay Handbook

https://info2.gbiosciences.com/complete-assay-development-handbooke

https://info2.gbiosciences.com/complete-bioassay-handbook

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