Human ACE
Colorimetric ELISA Kit

Catalog #: OKAG00191

Detection and Quantification of Human ACE Concentrations in Cell Lysates, Sera and Plasma.

Please read the provided manual as suggested experimental protocols may have changed.

Research Purposes Only. Not Intended for Diagnostic or Clinical Procedures.

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INTRODUCTION

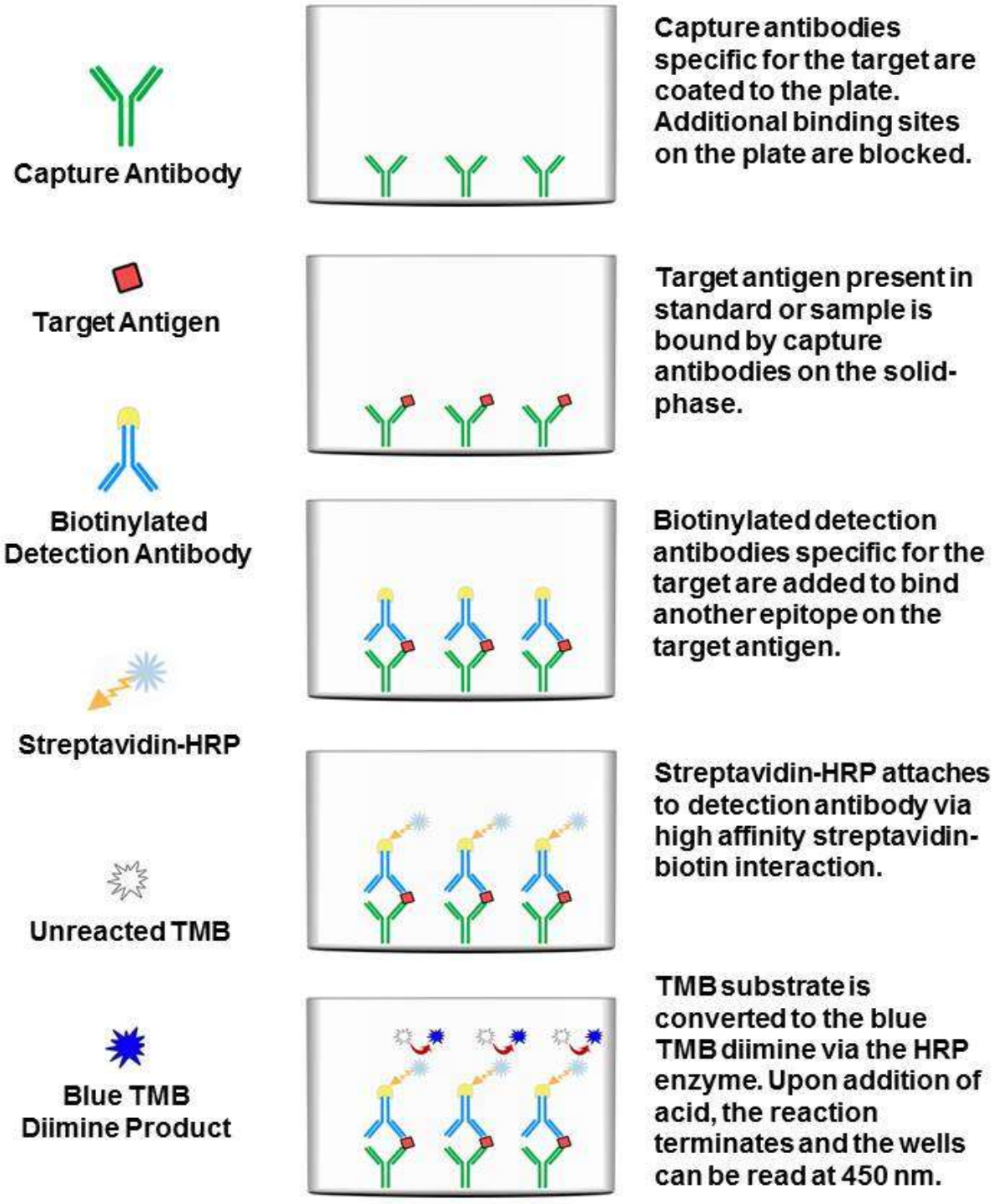
Human ACE or Angiotensin-Converting Enzyme converts angiotensin I to angiotensin II by release of the terminal His-Leu; this results in an increase of the vasoconstrictor activity of angiotensin. Moreover, it is able to inactivate bradykinin, a potent vasodilator, and has glycosidase activity that releases GPI-anchored proteins from the membrane by cleaving the mannose linkage in the GPI moiety. Release of a C-terminal dipeptide called oligopeptide-|-Xaa-Yaa when Xaa is not Pro, and Yaa is neither Asp nor Glu causes the conversion of angiotensin I to angiotensin II, with increase in vasoconstrictor activity, but no action on angiotensin II. The protein is able to bind 2 zinc ions per subunit while Isoform Testis-specific only binds 1 zinc ion per subunit. Moreover, ACE is able to bind 3 chloride ions per subunit as it is strongly activated by chloride and specifically inhibited by lisinopril, captopril and enalaprilat. ACE is ubiquitously expressed, with highest levels in lung, kidney, heart, GI system and prostate. Isoform Testis-specific is expressed in spermatocytes and adult testis. Human ACE can be phosphorylated by CK2 on Ser-1299; which allows membrane retention. Genetic variations in ACE may be a cause of susceptibility to ischemic stroke (ISCHSTR), also known as cerebrovascular accident or cerebral infarction. A stroke is an acute neurologic event leading to death of neural tissue of the brain and resulting in loss of motor, sensory and/or cognitive function. Ischemic strokes, resulting from vascular occlusion, are considered to be a highly complex disease consisting of a group of heterogeneous disorders with multiple genetic and environmental risk factors. Defects in ACE are a cause of renal tubular dysgenesis (RTD), a severe autosomal recessive disorder of renal tubular development characterized by persistent fetal anuria and perinatal death, probably due to pulmonary hypoplasia from early-onset oligohydramnios (the Potter phenotype). Genetic variations in ACE are associated with susceptibility to microvascular complications of diabetes type 3 (MVCD3). These are pathological conditions that develop in numerous tissues and organs as a consequence of diabetes mellitus. They include diabetic retinopathy, diabetic nephropathy leading to end-stage renal disease, and diabetic neuropathy. Diabetic retinopathy is a major cause of new-onset blindness among diabetic adults and is characterized by vascular permeability and increased tissue ischemia and angiogenesis.

Source: Entrez Gene; Swiss-Prot

ASSAY PRINCIPLES

The Human ACE ELISA Kit contains the components necessary for quantitative determination of natural or recombinant Human ACE concentrations within any experimental sample including cell lysates, serum and plasma. This particular immunoassay utilizes the quantitative technique of a “Sandwich” Enzyme-Linked Immunosorbent Assay (ELISA) where the target protein (antigen) is bound in a “sandwich” format by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies added subsequently by the investigator. The capture antibodies coated to the bottom of each well are specific for a particular epitope on Human ACE while the user-added detection antibodies bind to epitopes on the captured target protein. Amid each step of the procedure, a series of wash steps must be performed to ensure the elimination of non-specific binding between proteins to other proteins or to the solid phase. After incubation and “sandwiching” of the target antigen, a peroxidase enzyme is conjugated to the constant heavy chain of the secondary antibody (either covalently or via Avidin/Streptavidin-Biotin interactions), allowing for a colorimetric reaction to ensue upon substrate addition. When the substrate TMB (3, 3’, 5, 5’-Tetramethylbenzidine) is added, the reaction catalyzed by peroxidase yields a blue color that is representative of the antigen concentration. Upon sufficient color development, the reaction can be terminated through addition of Stop Solution (2 N Sulfuric Acid) where the color of the solution will turn yellow. The absorbance of each well can then be read by a spectrophotometer, allowing for generation of a standard curve and subsequent determination of protein concentration.

ASSAY FORMAT



ASSAY RESTRICTIONS

- This ELISA kit is intended for research purposes only, NOT diagnostic or clinical procedures of any kind.
- Materials included in this kit should NOT be used past the expiration date on the kit label.
- Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.
- Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.
- The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any biological samples. However, the possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.

MATERIALS INCLUDED

Component	Quantity Per Plate	Container
Microstrips Coated w/ Capture Antibody	12 x 8-Well Microstrips	-
Protein Standard	Lyophilized	Red
Biotinylated Detection Antibody	Lyophilized	Yellow
400x Streptavidin-HRP	30 µl	Blue
Wash Buffer (15x)	50 ml	Clear
Assay Diluent	50 ml	Clear
Ready-to-Use Substrate	12 ml	Brown
Stop Solution	12 ml	Clear
Adhesive Plate Sealers	2 Sheets	-
Technical Manual	1 Manual	-

ADDITIONAL MATERIALS REQUIRED

The following materials and/or equipment are NOT provided in this kit but are necessary to successfully conduct the experiment:

- Microplate reader able to measure absorbance at 450 nm (with correction wavelength set to 540 nm or 570 nm)
- Micropipettes with capability of measuring volumes ranging from 1 µl to 1 ml
- Deionized or sterile water
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent paper or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 ml
- Bench-top centrifuge (optional)
- Bench-top vortex (optional)
- Orbital shaker (optional)

HEALTH AND SAFETY PRECAUTIONS

- Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.

- Stop Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent. Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.

STORAGE INFORMATION

Note: If used frequently, reagents may be stored at 4°C.

Unopened Kits: Store at 4°C for 6 months.

Component	Storage Time	Storage Information
Microstrips Coated w/ Capture Antibody	6 Months	4°C
400x Streptavidin-HRP		
Wash Buffer (15x)		
Assay Diluent		
Ready-to-Use Substrate		
Stop Solution		
Protein Standard	Lyophilized: 6 Months Reconstituted: 1 Month	4°C
Biotinylated Detection Antibody		
Adhesive Plate Sealers	-	-
Technical Manual	-	-

SAMPLE PREPARATION AND STORAGE

If samples are to be used within 24 hours, aliquot and store at 4°C. If samples are to be used over a long period of time, aliquot and store between -20°C and -80°C, depending on the duration of storage.

Note: Samples containing a visible precipitate or pellet must be clarified prior to use in the assay.

Caution: Avoid repeated freeze/thaw cycles to prevent loss of biological activity of proteins in experimental samples.

Cell Lysate and Supernatants

Remove large cell components via centrifugation and perform the assay. Cell lysates and supernatants require a dilution using Assay Diluent. A serial dilution may be performed to determine a suitable dilution factor for the sample.

Serum Preparation

Allow samples to clot in a serum separator tube (SST) for 30 minutes. After sufficient clotting, centrifuge at 1000 x g for 15 minutes and remove serum from SST in preparation for the assay. A serial dilution may be performed to determine a suitable dilution factor for the sample.

Plasma Preparation

Use heparin, citrate or EDTA as an anticoagulant to gather plasma from original biological sample. After collection of the plasma, centrifuge for 15 minutes at 1000 x g. This step must be performed within 30 minutes of plasma collection.

Serum and Plasma Sample Dilution Recommendation

Dilute the plasma or serum samples with 10-50% animal serum in PBS. Do not reconstitute or dilute the detection antibody or Streptavidin-HRP in the buffer with animal serum. However, it is important to use the same diluent for the samples and the standard so it reflects the same environment of the samples being measured.

SAMPLE EXPERIMENT LAYOUT

	1	2	3	4	5	6
A	Standard (High Point)	Standard (High Point)	Standard (High Point)	Sample	Sample	Sample
B	Standard (1:2)	Standard (1:2)	Standard (1:2)	Sample	Sample	Sample
C	Standard (1:4)	Standard (1:4)	Standard (1:4)	Sample	Sample	Sample
D	Standard (1:8)	Standard (1:8)	Standard (1:8)	Sample	Sample	Sample
E	Standard (1:16)	Standard (1:16)	Standard (1:16)	Sample	Sample	Sample
F	Standard (1:32)	Standard (1:32)	Standard (1:32)	Sample	Sample	Sample
G	Standard (1:64)	Standard (1:64)	Standard (1:64)	Sample	Sample	Sample
H	Negative Control	Negative Control	Negative Control	Sample	Sample	Sample

IMMUNOASSAY PROTOCOL

Note: If possible, all incubation steps should be performed on an orbital shaker to equilibrate solutions when added to the microplate wells. Also, all provided solutions should be at ambient temperature prior to use.

Reconstitution of Provided Materials

1. Reconstitute the Protein Standard in 83µl of ddH_2O for a concentration of 220ng/ml.

2. Reconstitute the Biotin-Conjugated Detection Antibody in 55µl of ddH_2O for a concentration of 36ug/ml.

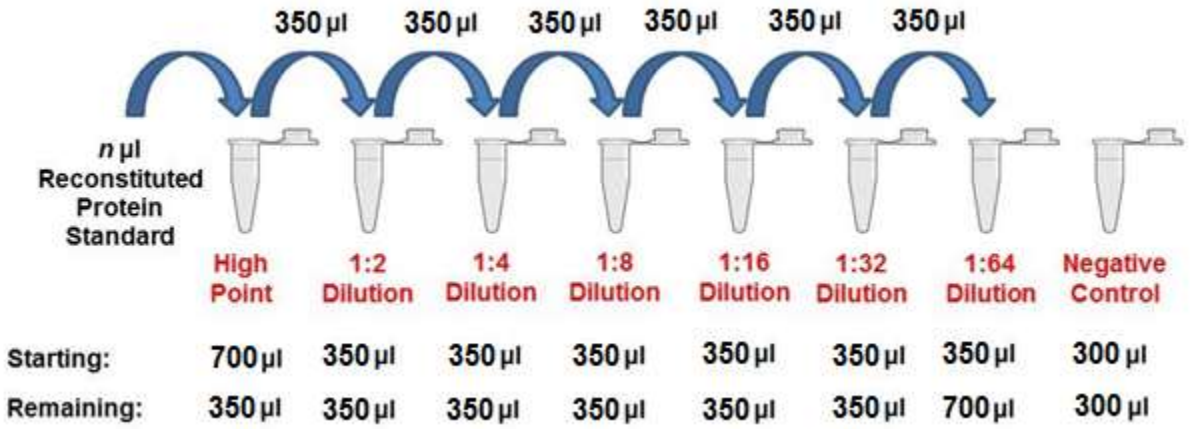
3. Dilute the 15x Wash Buffer to 1x Wash Buffer using 14 volumes of ddH_2O and 1 volume of 15x Wash Buffer. Use as necessary.

Addition of Known Standard and Unknown Sample to Immunoassay

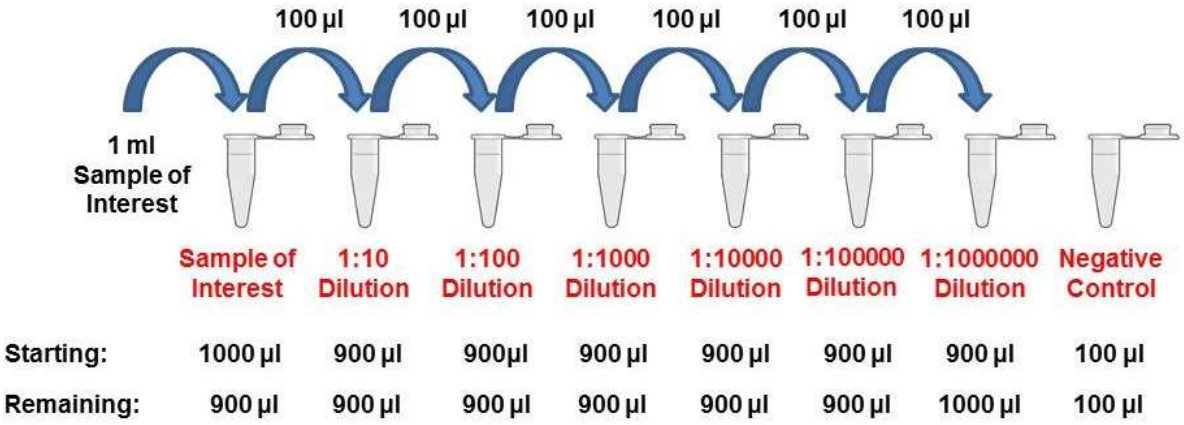
The Human ACE ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human ACE proteins within the range of 125-8000 pg/ml.

1. Dilute Protein Standard with Assay Diluent within the range of 8000 pg/ml to 125 pg/ml in a series of microfuge tubes. Mix each tube thoroughly by inverting several times or by vortexing lightly to ensure proper equilibration. Add 100 µl of each serial dilution step into the wells of a specified row or column of the 96-well microtiter plate in duplicate or triplicate and incubate at room temperature for 2 hours. Unknown Samples of Interest can be serial diluted with Assay Diluent to concentrations within the detection range of this assay kit and added to the plate at 100 µl per well. Blank Control is defined as 100 ul of Assay Diluent per well. Seal the microplate air-tight using parafilm if readily available. See Appendix for serial dilution diagram.

To obtain serial dilution high point, dilute reconstituted Protein Standard to the maximum concentration for serial dilution by adding *n* µl reconstituted Protein Standard to serial dilution high point tube and then raising the volume to 700 µl. Shown below is a diagram illustrating an example 2-fold serial dilution on a given reconstituted Protein Standard.



For samples of unknown protein concentrations, serial dilute the experimental sample using Assay Diluent to determine range of detection and acceptable dilutions. Shown below is a diagram illustrating a 10-fold serial dilution on a given Sample of Interest.



Addition of Detection Antibody to Capture Antibody-Bound Samples

2. Aspirate the protein standard solution out of the microplate wells. If your lab does not have a vacuum-based aspirator, you may dump the solutions from the microplate into a waste container and blot 3-4 times on a stack of paper towels until most or all of the liquid is removed from the wells. Dilute the 15x Wash Buffer to 1x using pure H_2O. Add 300-400 µl of 1x Wash Buffer to each well being used and gently shake for 5-7 minutes on an orbital shaker. Perform this wash step 4 times consecutively.

3. After the 4^{th} wash step, dilute the detection antibody solution 1:180 in Assay Diluent to 0.21ug/ml. Mix the test tube either by inverting several times or vortexing to ensure proper equilibration. Ensure that there is enough detection antibody solution for all wells being used. Add 100 µl of the diluted detection antibody solution into each well, seal the plate and incubate at room temperature for 2 hours.

Conjugation of Streptavidin-HRP to Biotinylated Detection Antibody

4. Remove the detection antibody solution out of the microplate wells by either vacuum-based aspirator or paper towel blotting. Perform 4 consecutive wash steps with gentle shaking between each wash.

5. Dilute the 400x Streptavidin-HRP by 1:400 using Assay Diluent to a 1x Streptavidin-HRP solution.

6. After the 4^{th} wash step, add 100 µl of 1x Streptavidin-HRP solution into each well and incubate at room temperature for 30 minutes.

Application of Liquid Substrate for Colorimetric Reaction

7. Remove the 1x Streptavidin-HRP solution out of the microplate wells by either vacuum-based aspirator or paper towel blotting. Prepare the Ready-to-Use Substrate by bringing it to room temperature without exposure to fluorescent or UV light as these may degrade the substrate. Perform 4 consecutive wash steps with gentle shaking between each wash.

8. After the 4th wash step, add 100 µl of Ready-to-Use Substrate solution into each well and incubate at room temperature for approximately 15-20 mins. The microplate should be kept out of direct light by either covering with an opaque object or putting it into a dark room. Closely monitor the color development as some wells may turn blue very quickly depending on analyte and/or detection antibody-HRP concentrations. Once the blue color has ceased to develop further, immediately add 100 µl of Stop Solution to each well being used. The color in the wells should immediately change from blue to yellow.

9. The microplate is now ready to be read by a microplate reader. Within 30 minutes of adding the Stop Solution, determine the optical density (absorbance) of each well by reading the plate with the 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.

   Caution: Readings made directly at 450 nm without correction may be higher and less accurate.

Generation of Standard Curve and Interpretation of Data

10. Average the duplicate or triplicate readings for each standard, control and sample and subtract the average zero standard optical density.

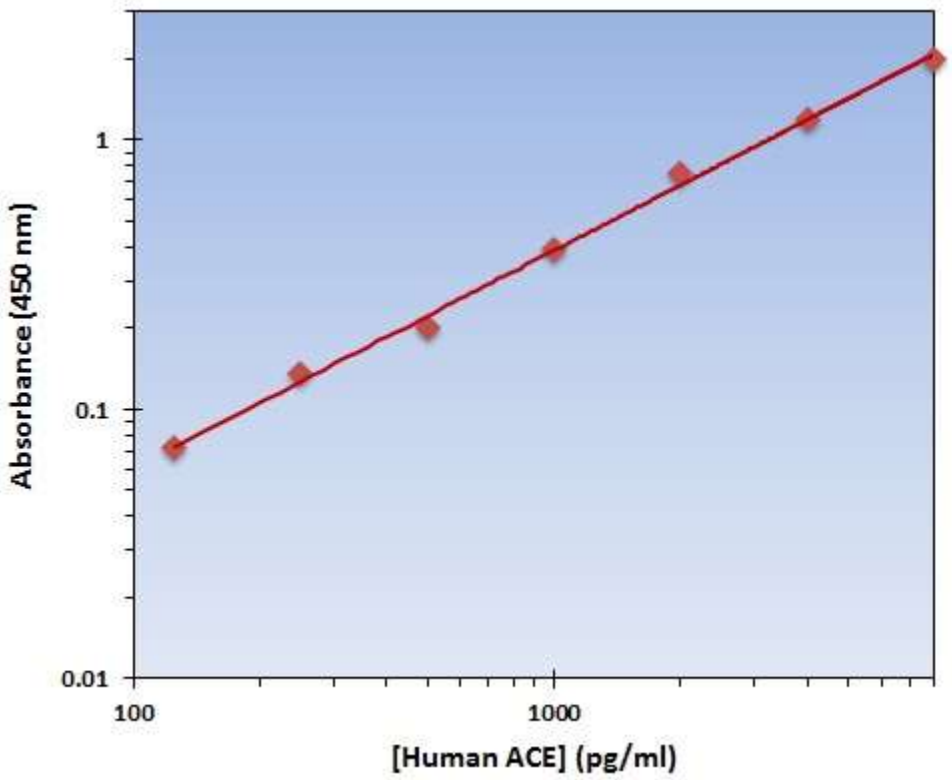
11. Generate a standard curve by using Microsoft Excel or other computer software capable of establishing a 4-Parameter Logistic (4-PL) curve fit. If using Excel or an alternative graphing tool, plot the average optical density values in absorbance units (y-axis) against the known standard concentrations in pg/ml (x-axis).

    Note: Only use the values in which a noticeable gradient can be established. Afterwards, generate a best fit curve or “trend-line” through the plotted points via regression analysis.

    Note: Shown on the next page is an example of typical data produced by analysis of the standard sample.

The data and subsequent graph was obtained after performing a cytokine ELISA for Human ACE. Each known sample concentration was assayed in triplicate.

hACE	
Conc. (pg/ml)	Avg OD 450nm
8000	2
4000	1.2
2000	0.75
1000	0.39
500	0.2
250	0.135
125	0.072



SUMMARIZED PROTOCOL

Reconstitute Biotinylated Detection Antibody and Protein Standard and dilute the 15x Wash Buffer as specified.

Perform serial dilution of Protein Standard and prepare samples as desired. See sample preparation section for instructions to dilute serum and plasma samples.

Add 100ul of Protein Standard, sample or control to each well and incubate for 2 hours at room temperature.

Aspirate Protein Standards, samples or controls out and wash plate 4 times.

Dilute Biotinylated Detection Antibody as specified. Add 100ul to each well and incubate for 2 hours at room temperature.

Aspirate Biotinylated Detection Antibody out and wash plate 4 times.

Dilute 400x Streptavidin-HRP as specified. Add 100ul of 1x Streptavidin-HRP to each well and incubate at room temperature for 30 minutes.

Aspirate 1x Streptavidin-HRP out and wash plate 4 times.

Add 100ul of Ready-to-Use Substrate to each well and incubate at room temperature for color development.

Add 100ul of Stop Solution and read plate at 450nm.

SENSITIVITY

The Human ACE ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human ACE proteins within the range of 125-8000 pg/ml.

CROSS REACTIVITY AND SPECIFICITY

The Human ACE ELISA is capable of recognizing both recombinant and naturally produced Human ACE proteins. The antigens listed below were tested at 50 ng/ml and did not exhibit significant cross reactivity or interference.
• Human: ACE-2

TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to www.avivasysbio.com or contact us at:

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ELISA PLATE TEMPLATE