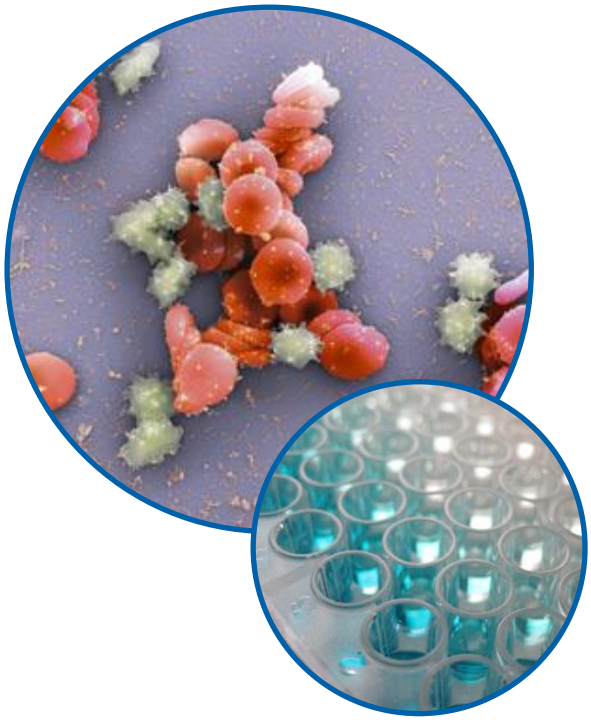


AdipoGen™

MANUAL

ACE2 (human) (IntraCellular) ELISA Kit

For research use only. Not for diagnostic use.

Version 1 (29-April-2011)

Cat. No. AG-45A-0039EK-KI01

Table of Contents

1. Intended Use

The ACE2 (human) (IntraCellular) ELISA Kit is to be used for the *in vitro* quantitative determination of human ACE2 in cell lysates or cell-based assays (screening). This ELISA Kit is for research use only.

2. Introduction

The cDNA encoding ACE2 was isolated by a homology search to the zinc metalloprotease angiotensin-I converting enzyme (ACE) (1). ACE2 is a type 1 membrane-bound protein in that it contains a N-terminal signal peptide and a putative transmemebrane domain. ACE2 was identified a receptor for SARS coronavirus that efficiently binds the S1 domain of the SARS coronavirus S protein (2). Northern blot analysis detected high expression of ACE2 in kidney, testis, and heart, and moderate expression in colon, small intestine, and ovary. It has been shown that a soluble, truncated form of ACE2 lacking transmembrane and cytosolic domains exhibited a glycosylated carboxypeptidase that was able to cleave angiotensin I and angiotensin II, but not bradykinin (1). ACE2 was not inhibited by benzylsuccinate, a carboxypeptidase A inhibitor, or by other ACE inhibitors such as lisinopril (1). ACE2 is expressed predominantly in vascular endothelial cells of the heart and kidney. Whereas ACE converts angiotensin I to angiotensin II, which has 8 amino acids, ACE2 converts angiotensin I to angiotensin 1-9, which has 9 amino acids. Whereas angiotensin II is a potent blood vessel constrictor, angiotensin 1-9 has no effect on blood vessels but can be converted by ACE to a shorter peptide, angiotensin 1-7, which is a blood vessel dilator, meaning that ACE2 is an endogenous inhibitor of ACE (3-4). ACE2-deficient mice showed that after acute angiotensin II (AT2) infusion plasma concentrations of AT2 remarkably increased in Ace2-deficient mice compared to controls, resulting in a severe hypertension the kidney. This data suggested that ACE2 is a functional component of the renin-angiotensin system, metabolizing AT2 and thereby contributing to the regulation of blood pressure (5-6). A late stage of diabetes is characterized by kidney failure called diabetic retinopathy. A recent data showed that infusion of recombinant ACE2 into the mice afflicted with diabetic retinopathy significantly improved the disease severity (7). The levels of ACE2 mRNA and protein were decreased in the kidneys of patients with type 2 diabetes and kidney dysfunction (8), suggesting that tissue ACE2 levels is a good predictor for diabetic retinopathy.

3. General References

(1) A human homolog of angiotensin-converting enzyme: cloning and functional expression as a captopril-insensitive carboxypeptidase: S.R. Tipnis, et al.; J. Biol. Chem. 275, 33238 (2000)

(2) Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus: W. Li, et al.; Nature 426, 450 (2003)

(3) Angiotensin-converting enzyme 2--a new cardiac regulator: M. Boehm, et al.; New. Eng. J. Med. 347, 1795 (2002)

(4) Angiotensin-converting enzyme 2 is an essential regulator of heart function: M.A. Crackower, et al.; Nature 417, 822 (2002)

(5) Altered blood pressure responses and normal cardiac phenotype in ACE2-null mice: S.B. Gurley, et al.; J. Clin. Invest. 116, 2218 (2006)

(6) Angiotensin-converting enzyme 2 protects from severe acute lung failure: Y. Imai, et al.; Nature 436, 112 (2005)

(7) Human recombinant ACE2 reduces the progression of diabetic nephropathy: G.Y. Oudit, et al.; Diabetes 59, 529 (2010)

(8) Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease: H.N. Reich, et al; Kidney Int. 74, 1610 (2008)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human ACE2 in cells. A polyclonal antibody specific for ACE2 has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, ACE2 is recognized by the addition of a biotinylated polyclonal antibody specific for ACE2 (Detection Antibody). After removal of excess biotinylated antibody, HRP labeled streptavidin (Detector) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of ACE2 in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

1 plate coated with human ACE2 Antibody	(12 x 8-well strips)
1 bottle Wash Buffer 10X	(50 ml)
1 bottle Diluent 5X	(50 ml)
1 bottle Lysis Buffer 10X	(12 ml)
1 bottle Detection Antibody	(12 ml)
1 vial Detector 100X (HRP Labeled Streptavidin)	(150 µl)
1 vial human ACE2 Standard (lyophilized)	(50 ng)
1 vial human ACE2 QC sample (lyophilized)	
1 bottle TMB Substrate Solution	(12 ml)
1 bottle Stop Solution	(12 ml)
3 plate sealers (plastic film)	

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Calibrated precision single and multi-channel pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard
- Phenyl methylsulfonyl fluoride (PMSF)

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 50 ml Wash Buffer 10X + 450 ml water) to obtain Wash Buffer 1X.
- **Diluent 5X** has to be diluted with deionized water 1:5 before use (e.g. 50 ml Diluent 5X + 200 ml water) to obtain Diluent 1X.
- **Lysis Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 12 ml Lysis Buffer 10X + 108 ml water) to obtain Lysis Buffer 1X. Add 1 mM PMSF immediately before use.
- **Detector 100X (HRP Labeled Streptavidin)** has to be diluted to the working concentration by adding 120 µl in 12 ml of Diluent 1X (1:100).

  NOTE: *The diluted Detector is used within one hour of preparation.*

- **Human ACE2 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 50 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C*

  - Dilute the standard protein concentrate (STD) (**50 ng/ml**) in Diluent 1X. A seven-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
  - Suggested standard points are:

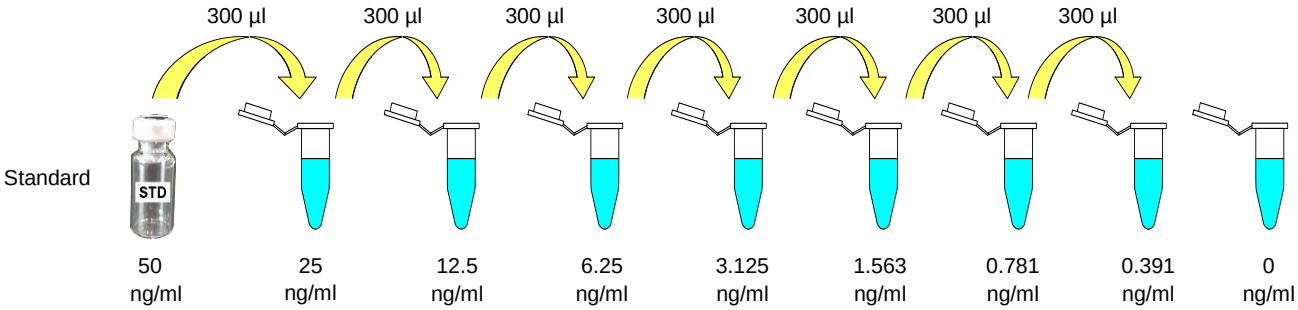
    25 , 12.5 , 6.25 , 3.125 , 1.563 , 0.781 , 0.391 and 0 ng/ml.

- **Human ACE2 QC sample** has to be reconstituted with 1 ml of deionized water.

  - Refer to the Certificate of Analysis for current QC sample concentration. Mix the QC sample to ensure complete reconstitution and allow the QC sample to sit for a minimum of 15 minutes. The reconstituted QC sample is ready to use, do not dilute it.

Dilute further for the standard curve:

To obtain	Add	Into
25 ng/ml	300 µl of ACE2 (50 ng/ml)	300 µl of Diluent 1X
12.5 ng/ml	300 µl of ACE2 (25 ng/ml)	300 µl of Diluent 1X
6.25 ng/ml	300 µl of ACE2 (12.5 ng/ml)	300 µl of Diluent 1X
3.125 ng/ml	300 µl of ACE2 (6.25 ng/ml)	300 µl of Diluent 1X
1.563 ng/ml	300 µl of ACE2 (3.125 ng/ml)	300 µl of Diluent 1X
0.781 ng/ml	300 µl of ACE2 (1.563 ng/ml)	300 µl of Diluent 1X
0.391 ng/ml	300 µl of ACE2 (0.781 ng/ml)	300 µl of Diluent 1X
0 ng/ml	300 µl of Diluent 1X	Empty tube



8.2. Sample Collection, Storage and Dilution

Cell Lysates : Grow cell until 90% confluency. Scrap cells off the plate and transfer to an appropriate tube. Keep on ice and microcentrifuge at 1,200 rpm for 5 minutes at 4°C. Remove supernatant, rinse cells once with ice-cold PBS. Remove PBS and add 200 µl ice-cold 1x lysis buffer supplemented with 1 mM phenyl methylsulfonyl fluoride (PMSF) to ten million cells of interest and incubate on ice for 30 minutes. Microcentrifuge at 12,000 rpm for 5 minutes at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Use freshly prepared cell lysate samples.

Cell Lysates have to be diluted in Diluent 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1 to 1/10 dilutions of cell lysates are recommended! If samples fall the outside range of assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 8-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 8-well strips coated with ACE2 antibody when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted lysates samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples).**
☐	3. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the Detection Antibody.
☐	6. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted Detector **(see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB Substrate Solution.
☐	12. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 30 minutes**.
☐	13. Stop the reaction by adding 100 µl of Stop Solution. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
	! CAUTION: CORROSIVE SOLUTION!
☐	14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

9. Calculation of Results

- Average the duplicate readings for each standard, QC and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding ACE2 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the ACE2 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of human ACE2 in the samples.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

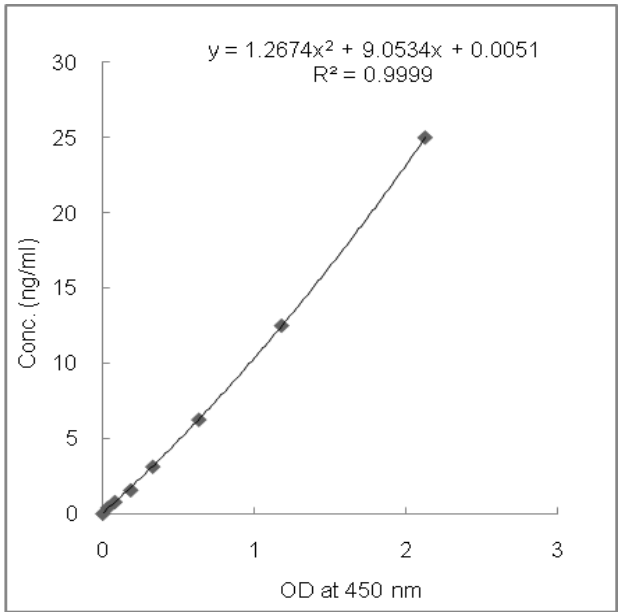


Figure: Standard curve

Standard hACE2 (ng/ml)	Optical Density (mean)
25	2.128
12.5	1.180
6.25	0.635
3.125	0.330
1.563	0.185
0.781	0.080
0.391	0.032
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of ACE2 that can be detected by this assay is 293 pg/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.391 ng/ml – 25 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human ACE2. It does not cross-react with human ACE, human adiponectin, human leptin, human resistin, human Nampt, human clusterin, human RBP4, human RELM-β, human IL-23, human ANG1, human ANG2, human FABP4, human ANGPTL6, human PAI-1, human vaspin, mouse RELM-α.

D. Intra-assay precision:

Two samples of known concentrations of human ACE2 were assayed in replicates 4 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
HEK293E cells	8.70	0.58	6.6	4
THP-1 cells	35.73	0.25	0.7	4

E. Inter-assay precision:

Two samples of known concentrations of human ACE2 were assayed in 4 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
HEK293E cells	7.97	0.63	8.0	4
cells	37.81	0.64	1.7	4

F. Recovery:

When samples (cell lysates) are spiked with known concentrations of human ACE2, the recovery averages 95% (range from 90% to 100%).

Samples	Average recovery (%)	Range (%)
HEK293E cells	94.80	90-100
THP-1 cells	94.92	90-100

G. Linearity:

Different human cell lysates samples containing ACE2 were diluted several fold (1/2 to 1/16) and the measured recoveries ranged from 96% to 103%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
HEK293E cells	1 : 2	7.75	7.75	100
	1 : 4	3.88	3.79	97.7
	1 : 8	1.94	1.99	102.9
THP-1 cells	1 : 4	22.71	22.71	100
	1 : 8	11.35	10.96	96.5
	1 : 16	5.68	5.31	93.5

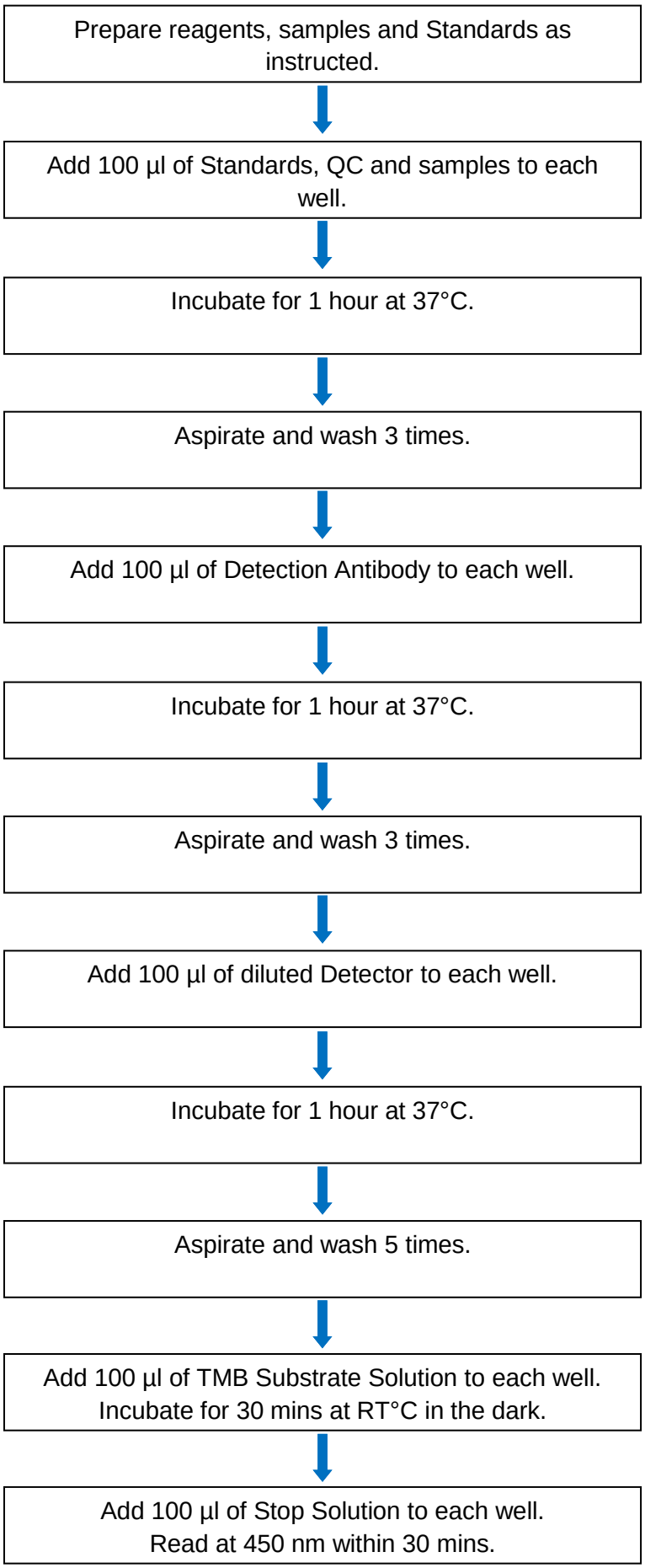
12. Technical Hints and Limitations

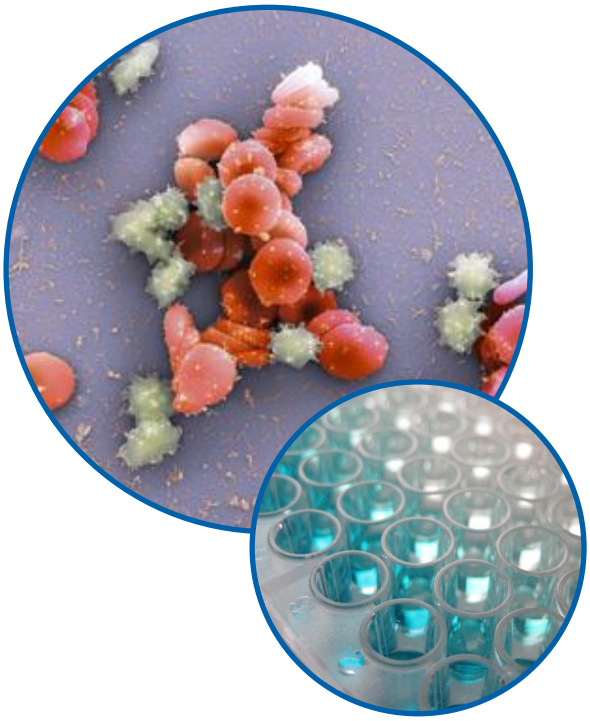
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 8-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution protected from light.
- The Stop Solution consists of phosphoric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of detector too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Assay Flow Chart





Product Specific References:

For more References please visit www.adipogen.com!

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