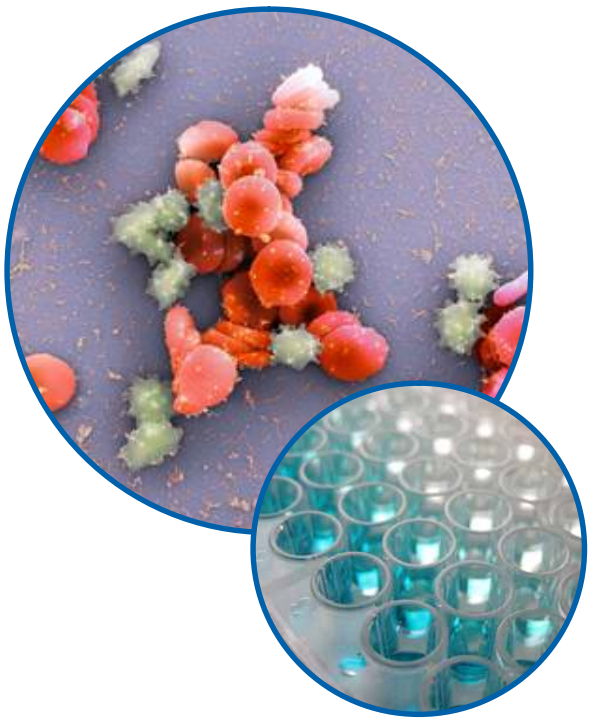


AdipoGen™

MANUAL

GPX1 (human) (IntraCellular) ELISA Kit

For research use only. Not for diagnostic use.

Version 2 (14-March-2011)

Cat. No. AG-45A-0034EK-KI01

Table of Contents

1. Intended Use

The GPX1 (human) (IntraCellular) ELISA Kit is to be used for the *in vitro* quantitative determination of human GPX1 in cell lysates or cell-based assays (screening). This ELISA Kit is for research use only.

2. Introduction

Glutathione peroxidase1 (GPX1) functions in the detoxification of hydrogen peroxide, and is one of the most important antioxidant enzymes in humans. This protein is one of only a few proteins known in higher vertebrates to contain selenocysteine, which occurs at the active site of glutathione peroxidase and is coded by UGA, that normally functions as a translation termination codon (1-2). It has been (De Haan et al. (1998) demonstrated a role for GPX1 in protection against oxidative stress by showing that Gpx1 -/- mice are highly sensitive to an oxidant compared to wildtype controls (3). The oxidant transcriptionally upregulated Gpx1 in normal cells, reinforcing a role for GPX1 in protection against its toxicity. Cortical neurons from Gpx1 -/- mice are more susceptible to peroxide whereas the wildtype controls were unaffected. This data suggest that GPX1 in protection against some oxidative stressors and in protection of neurons against peroxide. Chronic hyperglycemia causes oxidative stress, which contributes to damage in various tissues and cells, including pancreatic b-cells. The expression levels of antioxidant enzymes in the islet are low compared with other tissues, rendering the b-cell more susceptible to damage caused by hyperglycemia. A direct evidence for involvement of GPX1 in beta cell function was given by creating a transgenic mouse bearing b-cell-specific expression of GPX1 (4). The biological effectiveness of the overexpressed GPx-1 transgene was documented when b-cells of transgenic mice were protected from streptozotocin. When breeded with db/db mice hyperglycemia in db/db-GPx(+) mice was ameliorated compared with db/db-GPx(-). b-cell volume and insulin granulation and immunostaining were greater in db/db-GPx(+) animals compared with db/db-GPx(-) animals, demonstrating that GPX1 overexpression protects b-cell against deterioration during hyperglycemia.

3. General References

(1) Selenocysteine: the 21st amino acid: A. Bock, et al.; Molec. Microbiol. 5, 515 (1991)

(2) Selenium repletion and glutathione peroxidase-differential effects on plasma and red cell enzyme activity: H.J. Cohen, et al.; Am. J. Clin. Nutr. 41, 735 (1985)

(3) Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide: J.B. de Haan, et al.; J. Biol. Chem. 273, 22528 (1998)

(4) B-cell-specific overexpression of glutathione peroxidase preserves intranuclear MafA and reverses diabetes in db/db mice: J.S. Harmon, et al.; Endocrinology 150, 4855 (2009)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human GPX1 in cells. A polyclonal antibody specific for GPX1 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, GPX1 is recognized by the addition of a purified polyclonal antibody specific for GPX1 (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-IgG (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 GPX1 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 GPX1 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 Conjugated anti-IgG)	(150 µl)
1 vial human GPX1 Standard (lyophilized)	(8 ng)
1 vial human GPX1 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 Conjugated anti-IgG)** 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 GPX1 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 8 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*

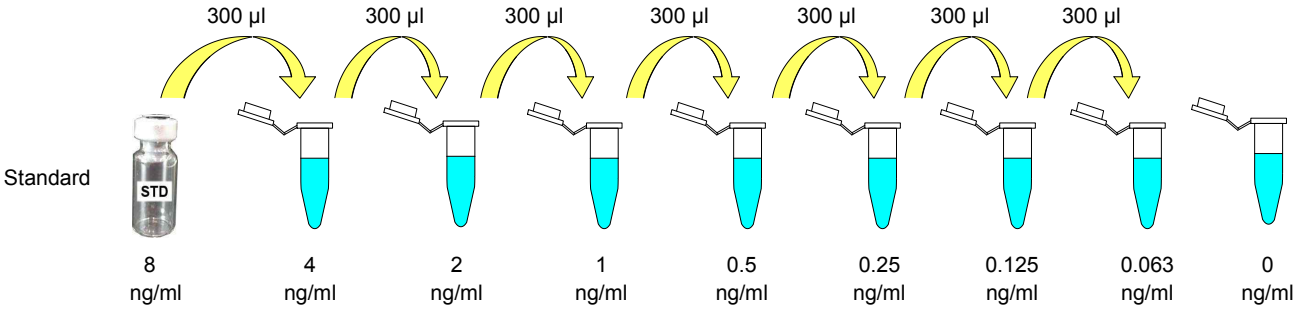
  - Dilute the standard protein concentrate (STD) (**8 ng/ml**) in Diluent 1X. A seven-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
  - Suggested standard points are:
    4 , 2 , 1 , 0.5 , 0.25 , 0.125 , 0.063 and 0 ng/ml.

- **Human GPX1 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
4 ng/ml	300 µl of GPX1 (8 ng/ml)	300 µl of Diluent 1X
2 ng/ml	300 µl of GPX1 (4 ng/ml)	300 µl of Diluent 1X
1 ng/ml	300 µl of GPX1 (2 ng/ml)	300 µl of Diluent 1X
0.5 ng/ml	300 µl of GPX1 (1 ng/ml)	300 µl of Diluent 1X
0.25 ng/ml	300 µl of GPX1 (0.5 ng/ml)	300 µl of Diluent 1X
0.125 ng/ml	300 µl of GPX1 (0.25 ng/ml)	300 µl of Diluent 1X
0.063 ng/ml	300 µl of GPX1 (0.125 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, 1/10 to 1/1,000 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 GPX1 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 **overnight at 4°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 10 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 GPX1 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the GPX1 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 GPX1 in the samples.

10. Typical Data

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

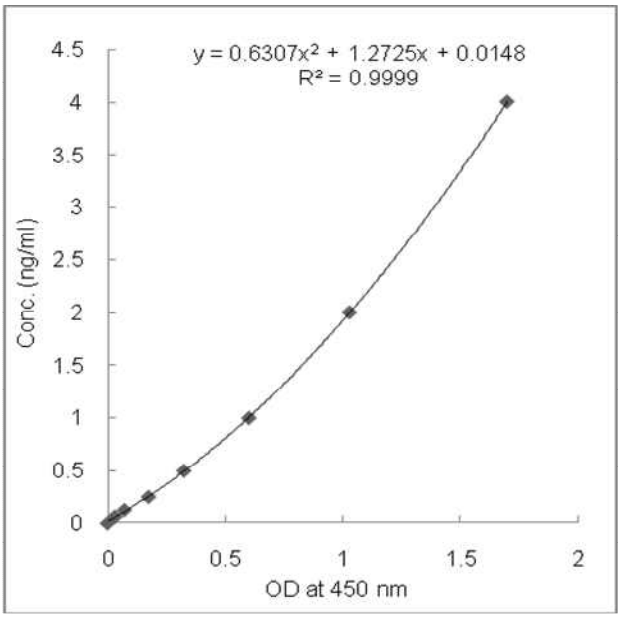


Figure: Standard curve

Standard hGPX1 (ng/ml)	Optical Density (mean)
4	1.7
2	1.03
1	0.602
0.5	0.325
0.25	0.175
0.125	0.072
0.063	0.032
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of GPX1 that can be detected by this assay is 45 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: 0.063 ng/ml – 4 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human GPX1. It does not cross-react with human GPX2, human GPX3, human GPX4, human IL-33, human ST2, human adiponectin, human RBP4, human Nampt, human vaspin, human progranulin, human resistin, human clusterin, human ANGPTL3, human CTRP5, human ACE2, human leptin, mouse GPX3, mouse Nampt, rat Nampt.

D. Intra-assay precision:

Six samples of known concentrations of human GPX1 were assayed in replicates 5 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A549 cells	26.872	0.868	3.229	5
HT-29 cells	13.368	0.326	2.437	5
HepG2 cells	2.674	0.096	3.608	5
HeLa cells	15.074	0.410	2.722	5
293 cells	69.178	3.057	4.419	5
THP-1 cells	127.700	10.853	8.499	5

E. Inter-assay precision:

Six samples of known concentrations of human GPX1 were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
A549 cells	26.516	1.296	4.887	5
HT-29 cells	13.803	1.015	7.357	5
HepG2 cells	2.577	0.152	5.889	5
HeLa cells	16.435	1.128	6.861	5
293 cells	68.704	4.397	6.400	5
THP-1 cells	123.496	8.054	6.522	5

F. Recovery:

When samples (cell lysates) are spiked with known concentrations of human GPX1, the recovery averages 99% (range from 95% to 105%).

Samples	Average recovery (%)	Range (%)
A549 cells	98.063	95-105
HT-29 cells	101.512	95-105
HepG2 cells	100.800	95-105
HeLa cells	98.987	95-105
293 cells	96.559	95-105
THP-1 cells	100.387	95-105

G. Linearity:

Different human cell lysates samples containing GPX1 were diluted several fold (1/20 to 1/80) and the measured recoveries ranged from 91% to 110%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
A549 cells	1 : 20	27.183	27.183	100
	1 : 40	13.592	13.264	97.589
	1 : 80	6.796	6.194	91.142
THP-1 cells	1 : 20	233.388	233.388	100
	1 : 40	116.694	128.013	109.700
	1 : 80	58.347	61.347	105.142
293 cells	1 : 20	64.141	64.141	100
	1 : 40	32.071	35.205	109.775
	1 : 80	16.035	17.474	108.969

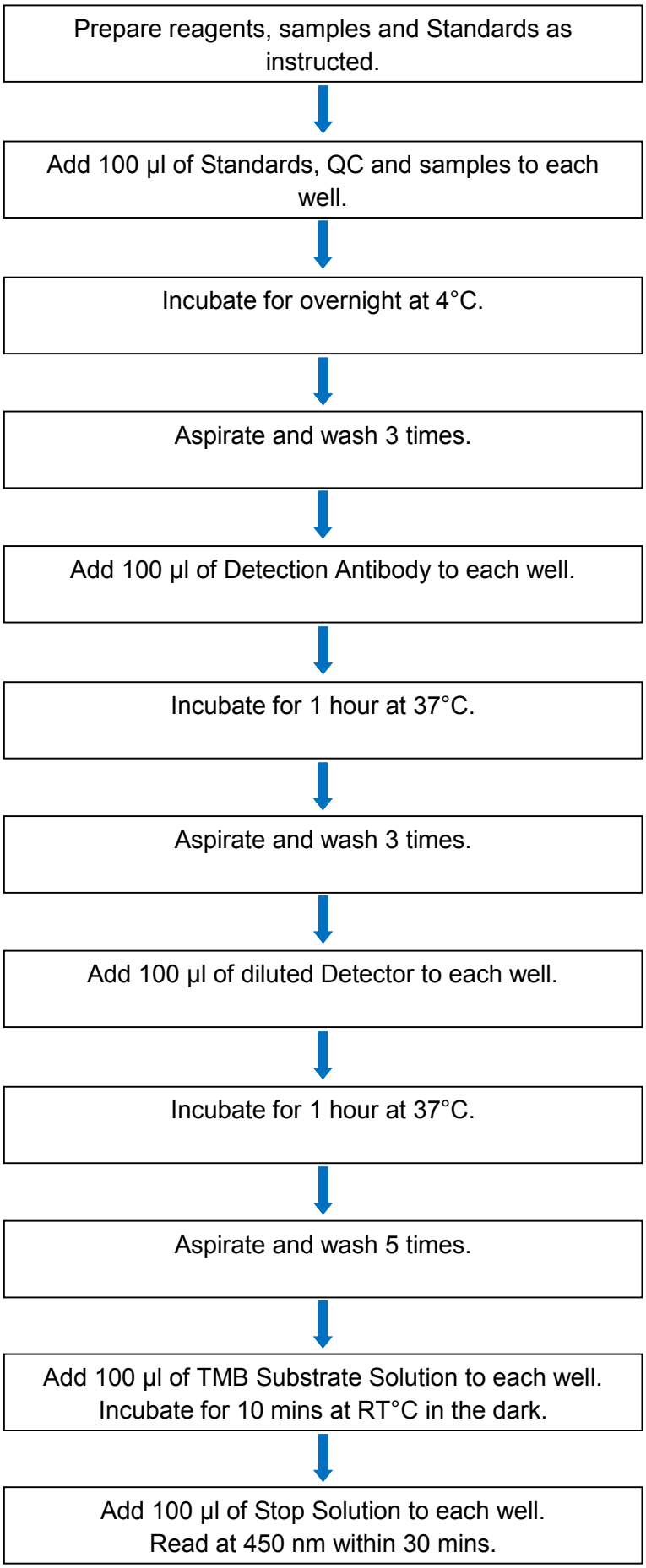
12. Technical Hints and Limitations

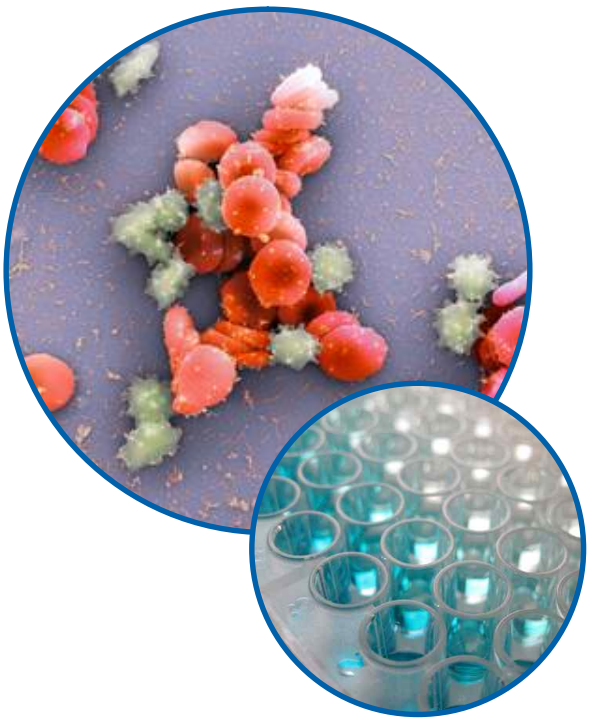
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last 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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