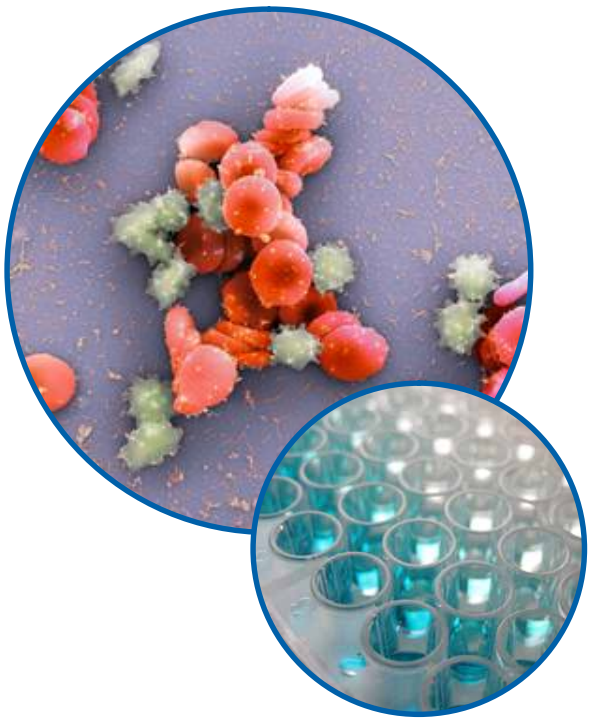


AdipoGen™

MANUAL

Resistin (human) ELISA Kit

For research use only. Not for diagnostic use.

Version 2 (14-March-2011)

Cat. No. AG-45A-0023EK-KI01

Table of Contents

1. Intended Use

The Resistin (human) ELISA Kit is to be used for the *in vitro* quantitative determination of human Resistin in serum, plasma and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

Obesity is a well-known risk factor of type 2 diabetes mellitus and is strongly associated with insulin resistance. Resistin (also called FIZZ3/ADSF) is an adipocyte-derived peptide first identified during a search for targets of thiazolidinediones. Steppan *et al*. reported that serum concentrations of resistin are markedly increased in obese mice and are decreased by treatment with thiazolidinediones (1). It was also found that administration of an anti-resistin antibody increases insulin-stimulated glucose uptake in obese mice and that treatment of normal mice with recombinant resistin impairs insulin action. Thus, resistin might link obesity with insulin resistance and diabetes in mice models. However, subsequent studies in rodent models (2-4) have produced disparate findings on the role of resistin in obesity and insulin resistance. In humans, while the expression of resistin in human adipocytes is very low compared with that seen in rodents and does not differ between normal, insulin-resistant or type 2 diabetic individuals, a more recent study using a large size of case suggests that the plasma resistin levels are increased in type 2 diabetes (5-8). Genetic case-control studies have demonstrated that genetic variations in the resistin gene are associated with insulin resistance and obesity (9-11). More recently it has been shown that resistin acts on liver and antagonizes insulin signaling, thereby increasing gluconeogenesis and hepatic glucose output (12). This is the first study showing of the role of resistin in modulating physiological glucose metabolism. Therefore determination of the plasma resistin levels may be important for understanding onsets of metabolic diseases such as type 2 diabetes or obesity.

3. General References

(1) The hormone resistin links obesity to diabetes: C.M. Steppan, et al.; Nature 409, 307 (2001)

(2) Suppressed gene expression of adipocyte resistin in an insulin-resistant rat model probably by elevated free fatty acids: C.C. Juan, et al.; Biochem. Biophys. Res. Commun. 289, 1328 (2001)

(3) Decreased resistin expression in mice with different sensitivities to a high-fat diet: S.L. Lay, et al.; Biochem. Biophys. Res. Commun. 289, 564 (2001)

(4) Adipose tissue resistin expression is severely suppressed in obesity and stimulated by peroxisome proliferator-activated receptor gamma agonists: J.M. Way, et al.; J. Biol. Chem. 276, 25651 (2001)

(5) Resistin gene expression in human adipocytes is not related to insulin resistance: J. Janke, et al.; Obes. Res. 10, 1 (2002)

(6) Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle: I. Nagaev, et al.; Biochem. Biophys. Res. Commun. 285, 561 (2001)

(7) Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans: D.B. Savage, et al.; Diabetes 50, 2199 (2001)

(8) 5' flanking variants of resistin are associated with obesity: J.C. Engert, et al.; Diabetes 51, 1629 (2002)

(9) Plasma resistin levels are elevated in the subjects with type 2 diabetes mellitus: B.S. Youn, et al.; J. Clin. Endocrinol. Metab. 89, 150 (2004)

(10) Common genetic polymorphisms in the promoter of resistin gene are major determinants of plasma resistin concentrations in humans: Y.M. Cho, et al.; Diabetologia 47, 559 (2004)

(11) Human resistin gene: molecular scanning and evaluation of association with insulin sensitivity and type 2 diabetes in Caucasians: H. Wang, et al.; J. Clin. Endocrinol. Metab. 87, 2520 (2002)

(12) Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production: M.W. Rajala, et al.; J. Clin.Invest. 111, 225 (2003)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human resistin in biological fluids. A monoclonal antibody specific for resistin 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, resistin is recognized by the addition of a biotinylated polyclonal antibody specific for resistin (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 resistin 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 resistin Antibody	(12 x 8-well strips)
1 bottle Wash Buffer 10X	(50 ml)
1 bottle Diluent 5X	(50 ml)
1 bottle Detection Antibody	(12 ml)
1 vial Detector 100X (HRP Labeled Streptavidin)	(150 µl)
1 vial human resistin Standard (lyophilized)	(16 ng)
1 vial human resistin QC sample (lyophilized)	
1 bottle Substrate Solution I (TMB)	(6 ml)
1 bottle Substrate Solution II (Peroxidase)	(6 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

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.
- **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.*

- **Substrate Solution I and II** have to be mixed together in equal volumes within 15 minutes of use.

  NOTE: *Freshly prepare just before use the Substrate Solution and protect from light!*

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

  - This reconstitution produces a stock solution of 16 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*

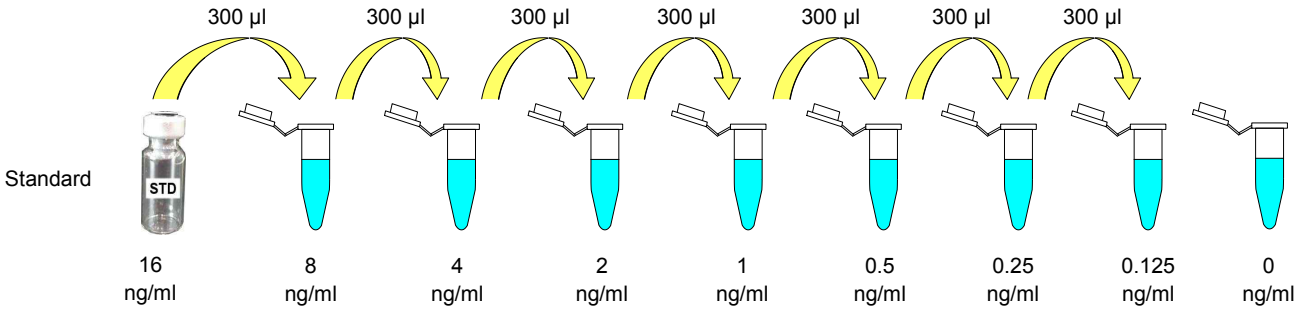
  - Dilute the standard protein concentrate (STD) (**16 ng/ml**) in Diluent 1X. A seven-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
  - Suggested standard points are:
    8 , 4 , 2 , 1 , 0.5 , 0.25 , 0.125 and 0 ng/ml.

- **Human resistin 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
8 ng/ml	300 µl of resistin (16 ng/ml)	300 µl of Diluent 1X
4 ng/ml	300 µl of resistin (8 ng/ml)	300 µl of Diluent 1X
2 ng/ml	300 µl of resistin (4 ng/ml)	300 µl of Diluent 1X
1 ng/ml	300 µl of resistin (2 ng/ml)	300 µl of Diluent 1X
0.5 ng/ml	300 µl of resistin (1 ng/ml)	300 µl of Diluent 1X
0.25 ng/ml	300 µl of resistin (0.5 ng/ml)	300 µl of Diluent 1X
0.125 ng/ml	300 µl of resistin (0.25 ng/ml)	300 µl of Diluent 1X
0 ng/ml	300 µl of Diluent 1X	Empty tube



8.2. Sample Collection, Storage and Dilution

Serum : Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Plasma : Collect plasma using heparin, EDTA, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -20°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma or **Cell Culture Supernatant** have to be diluted in Diluent 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/20 dilution of serum or plasma is 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 resistin 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 serum, plasma or cell culture supernatant 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 mixed substrate solution.
☐	12. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 20 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 resistin concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the resistin 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 resistin in the samples.

10. Typical Data

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

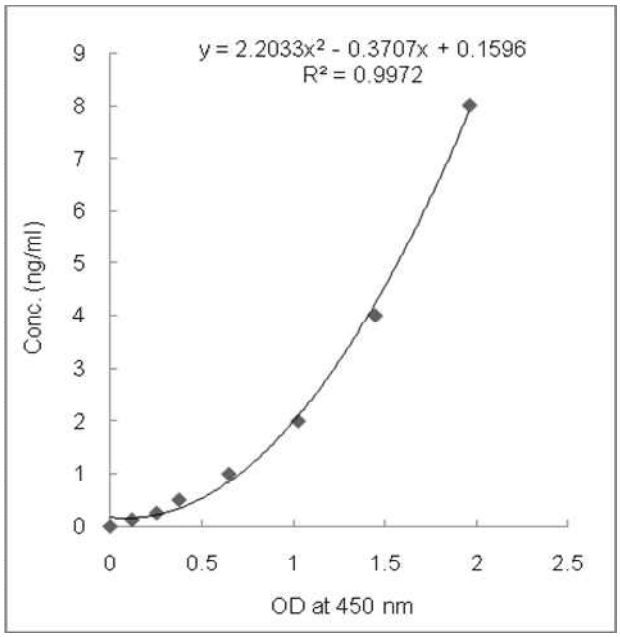


Figure: Standard curve

Standard hResistin (ng/ml)	Optical Density (mean)
8	1.958
4	1.444
2	1.024
1	0.647
0.5	0.375
0.25	0.253
0.125	0.119
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of resistin that can be detected by this assay is 100 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: 0.125 ng/ml – 8 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human resistin. It does not cross-react with mouse resistin, rat resistin, human RELM-β, mouse RELM-α, mouse RELM-β, rat RELM-α, human leptin, human adiponectin.

D. Intra-assay precision:

Five samples of known concentrations of human resistin were assayed in replicates 10 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	10.78	0.31	2.86	10
2	19.23	0.99	5.17	10
3	21.49	0.67	3.12	10
4	5.19	0.20	3.77	10
5	12.57	0.47	3.73	10

E. Inter-assay precision:

Five samples of known concentrations of human resistin were assayed in 10 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	6.80	0.49	7.20	10
2	22.96	1.24	5.40	10
3	6.49	0.27	4.20	10
4	15.32	1.07	6.97	10
5	25.66	1.11	4.35	10

F. Recovery:

When samples (serum or plasma) are spiked with known concentrations of human resistin, the recovery averages 96% (range from 93% to 108%).

Samples	Average recovery (%)	Range (%)
1	94.4	93-96
2	96.6	95-97
3	98.9	92-108

G. Linearity:

Different human serum samples containing resistin were diluted several fold (1/10 to 1/40) and the measured recoveries ranged from 88% to 122%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1 : 10	5.592	5.592	100
	1 : 20	2.796	2.632	94.15
	1 : 40	1.398	1.234	88.29
2	1 : 10	6.129	6.129	100
	1 : 20	3.064	3.712	121.11
	1 : 40	1.532	1.795	117.13
3	1 : 10	6.325	6.325	100
	1 : 20	3.162	3.657	115.66
	1 : 40	1.581	1.627	102.90

H. Expected values:

Resistin levels range in plasma and serum from **1 to > 20 ng/ml** (from healthy donors).

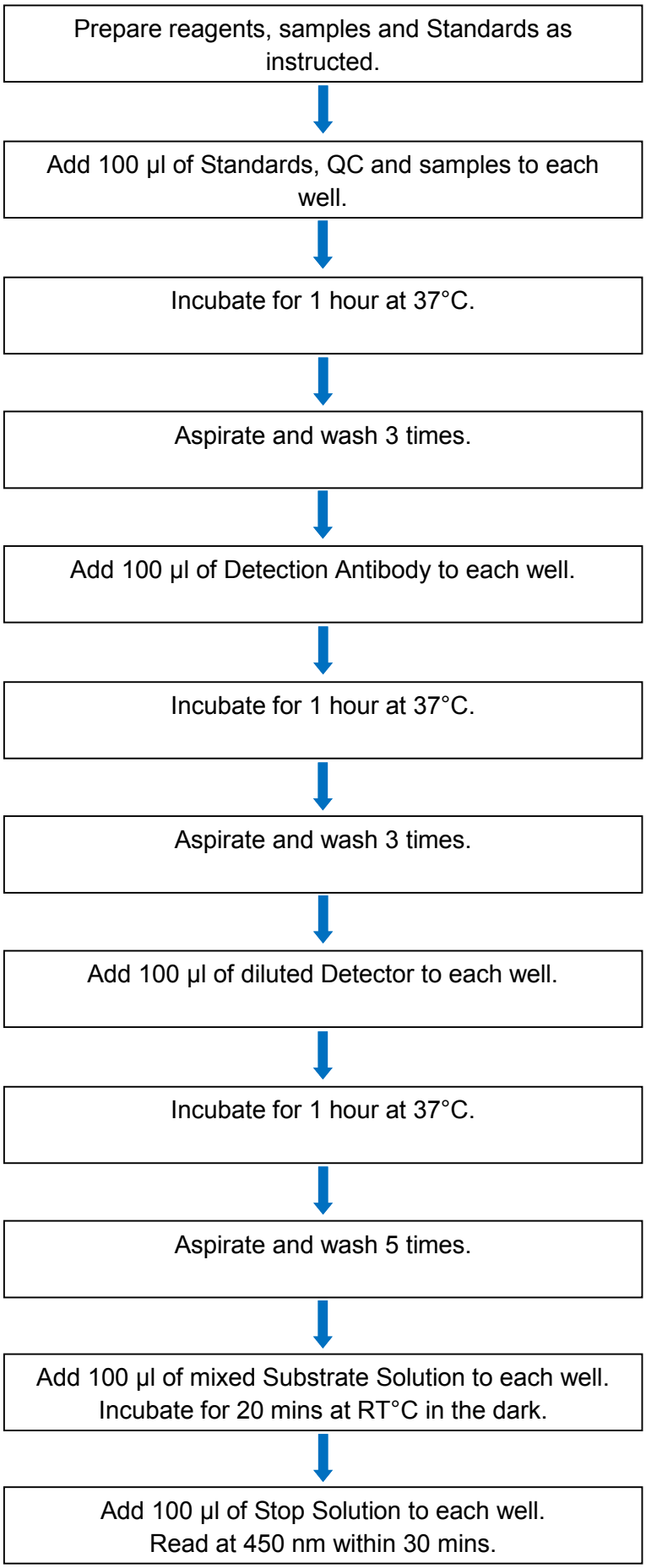
12. Technical Hints and Limitations

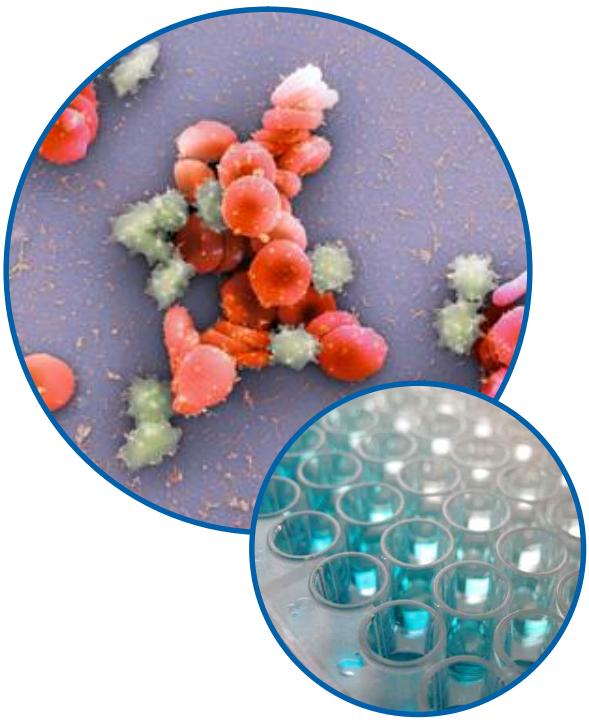
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last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

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For more References please visit www.adipogen.com!

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