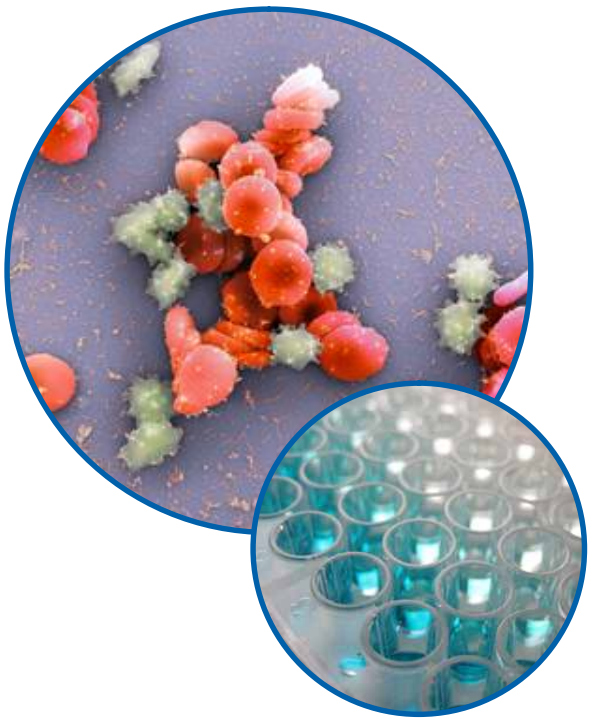


AdipoGen™

MANUAL

FTO (human) (IntraCellular) ELISA Kit

For research use only. Not for diagnostic use.

Version 2 (14-March-2011)

Cat. No. AG-45A-0025EK-KI01

Table of Contents

1. Intended Use

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

2. Introduction

FTO, Fat mass-and obesity-associated gene, was discovered as a responsible gene causing the mouse 'fused toes' mutation. The predicted 502-amino acid Fto protein has a calculated molecular mass of 58 kD and contains an N-terminal bipartite nuclear localization signal. RT-PCR detected Fto expression throughout mouse embryonic development and in all adult mouse tissues examined except heart and skin. Using RT-PCR, it was found that FTO was widely expressed in a variety of human tissues, with highest levels in brain and pancreatic islets (1). It has been shown by bioinformatics analysis that FTO shares sequence motifs with iron- and 2-oxoglutarate (2OG)-dependent oxygenases (2). They found that recombinant murine Fto catalyzes the iron- and 2OG-dependent demethylation of 3-methylthymine in single-stranded DNA, with concomitant production of succinate, formaldehyde, and carbon dioxide. Studies of wildtype mice indicated that Fto mRNA is most abundant in the brain, particularly in hypothalamic nuclei governing energy balance, and that Fto mRNA levels in the arcuate nucleus are regulated by feeding and fasting. In genomewide association studies of type 2 diabetes involving genotype data from a variety of international consortia, FTO was confirmed as a diabetes susceptibility locus (3, 4). It would be interesting to see whether metabolic dysfuntion or inflammatory stimuli alters the intracellular levels of FTO.

3. General References

(1) Cloning of Fatso (Fto), a novel gene deleted by the fused toes (Ft) mouse mutation: T. Peters, et al.; Mamm. Genome 10, 983 (1999)

(2) The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase: T. Gerken, et al.; Science 318, 1469 (2007)

(3) Variation in FTO contributes to childhood obesity and severe adult obesity: C. Dina, et al.; Nature Genet. 39, 724 (2007)

(4) A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity: T.M. Frayling, et al.; Science 316, 889 (2007)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human FTO in cells. A monoclonal antibody specific for FTO 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, FTO is recognized by the addition of a purified polyclonal antibody specific for FTO (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-rabbit 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 FTO 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 FTO 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-rabbit IgG)	(150 µl)
1 vial human FTO Standard (lyophilized)	(20 ng)
1 vial human FTO 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 1mM PMSF immediately before use.
- **Detector 100X (HRP Conjugated anti-rabbit 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 FTO Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 20 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) (**20 ng/ml**) in Diluent 1X. A seven-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
  - Suggested standard points are:

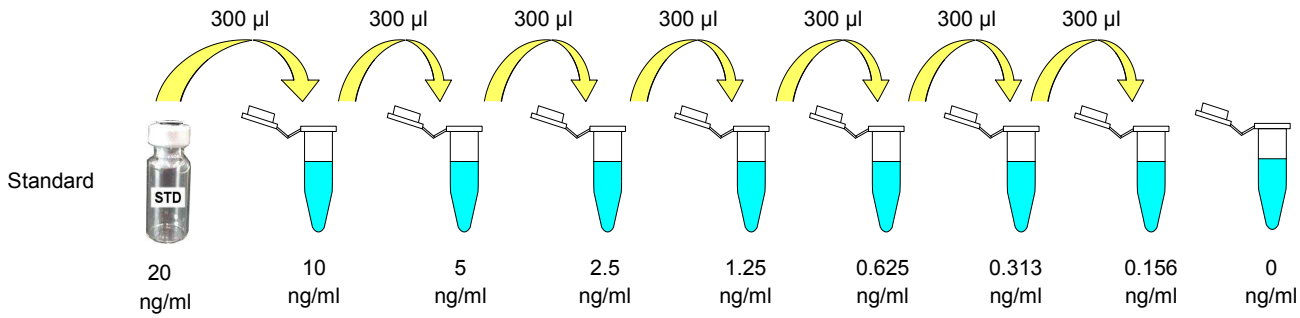
    10 , 5 , 2.5 , 1.25 , 0.625 , 0.313 , 0.156 and 0 ng/ml.

- **Human FTO 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
10 ng/ml	300 µl of FTO (20 ng/ml)	300 µl of Diluent 1X
5 ng/ml	300 µl of FTO (10 ng/ml)	300 µl of Diluent 1X
2.5 ng/ml	300 µl of FTO (5 ng/ml)	300 µl of Diluent 1X
1.25 ng/ml	300 µl of FTO (2.5 ng/ml)	300 µl of Diluent 1X
0.625 ng/ml	300 µl of FTO (1.25 ng/ml)	300 µl of Diluent 1X
0.313 ng/ml	300 µl of FTO (0.625 ng/ml)	300 µl of Diluent 1X
0.156 ng/ml	300 µl of FTO (0.313 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 FTO 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 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 FTO concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the FTO 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 FTO in the samples.

10. Typical Data

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

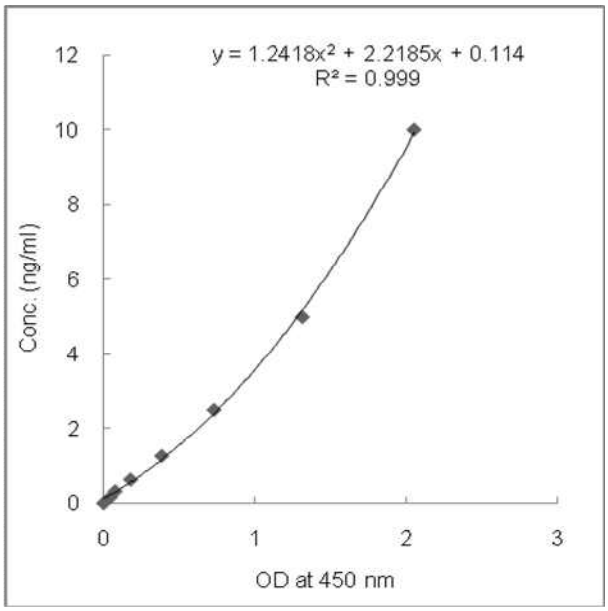


Figure: Standard curve

Standard hFTO (ng/ml)	Optical Density (mean)
10	2.058
5	1.317
2.5	0.731
1.25	0.385
0.625	0.178
0.313	0.076
0.156	0.043
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of FTO that can be detected by this assay is 50 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: 0.156 ng/ml – 10 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human FTO**.** It does cross-react with human adiponectin, human RBP4, human Nampt, human vaspin, human progranulin, human resistin, human clusterin, human GPX3, human sirtuin 1, human IDO, human IL-33, human ANGPTL3, human ANGPTL4, human FGF21, mouse progranulin, mouse ANGPTL3, mouse leptin, rat Nampt.

Mouse FTO shows weakly 5% cross-reactivity in this assay.

D. Intra-assay precision:

Four samples of known concentrations of human FTO were assayed in replicates 6 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
THP-1 cells	31.976	1.789	5.585	6
Molt4 cells	262.277	6.146	2.343	6
A549 cells	96.285	1.569	1.629	6
HepG2 cells	130.940	2.823	2.156	6

E. Inter-assay precision:

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

Samples	Means (ng/ml)	SD	CV (%)	n
Molt4 cells	226.599	20.899	9.223	4
A549 cells	92.102	8.076	8.768	4
HepG2 cells	99.771	4.978	4.989	4

F. Recovery:

When samples (cell lysates) are spiked with known concentrations of human FTO the recovery averages 100% (range from 95% to 105%).

Samples	Average recovery (%)	Range (%)
THP-1 cells	95.416	95-105
A549 cells	102.444	95-105
HepG2 cells	103.941	95-105

G. Linearity:

Different human cell lysates samples containing FTO were diluted several fold (1/50 to 1/200) and the measured recoveries ranged from 78% to 105%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
Molt4 cells	1 : 50	468.488	468.488	100
	1 : 100	234.244	211.056	90.100
	1 : 200	117.122	92.472	78.854
A549 cells	1 : 50	73.705	73.705	100
	1 : 100	36.853	30.217	81.994
	1 : 200	18.426	14.960	91.186
HepG2 cells	1 : 50	102.071	102.071	100
	1 : 100	51.036	41.192	92.473
	1 : 200	25.518	18.345	103.765

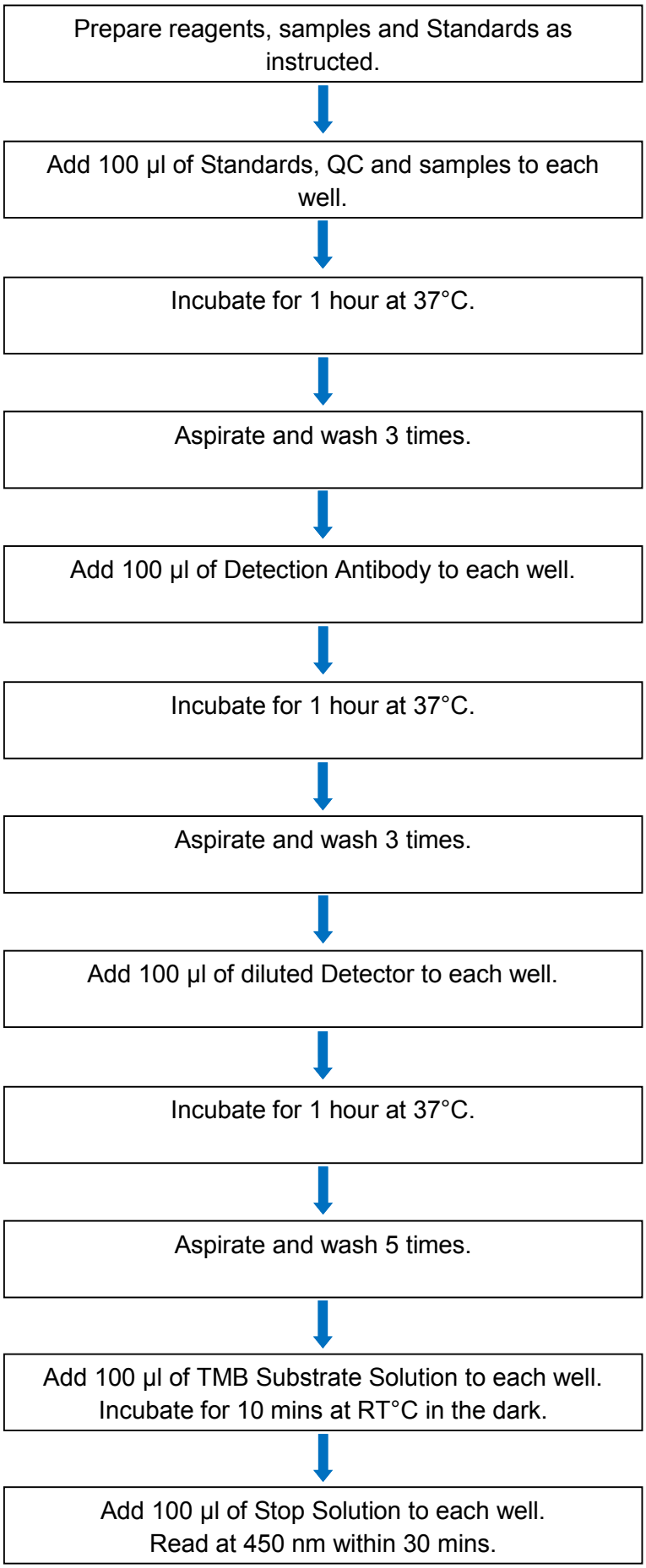
12. Technical Hints and Limitations

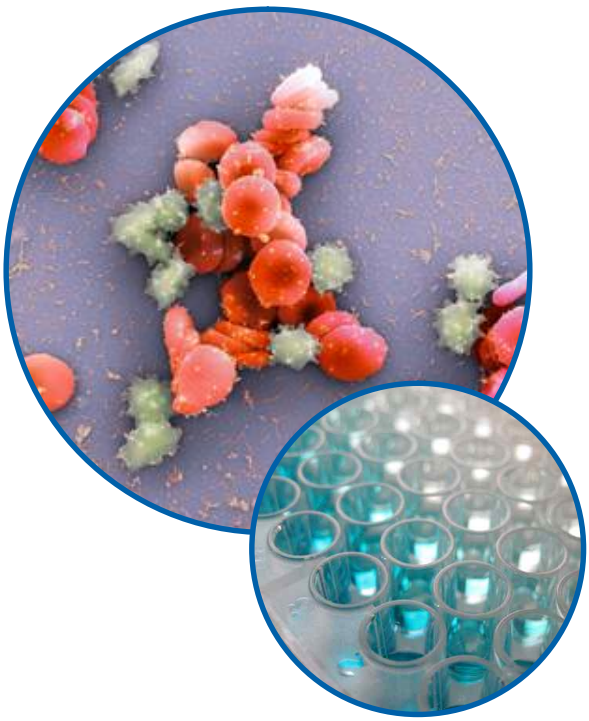
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last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