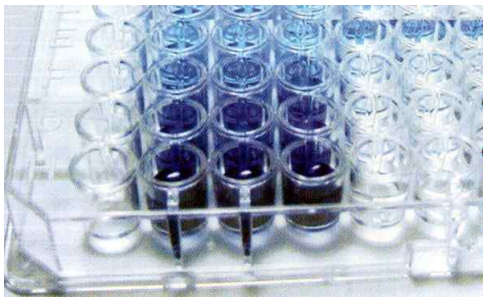
FTO (mouse) (IntraCellular) ELISA Kit

Cat. No. AG-45A-0028EK-KI01



Instruction Manual

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NOT FOR USE IN DIAGNOSTIC PROCEDURES

AdipoGen™

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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.

Assay Principles

The mouse intracellular FTO (iFTO) ELISA is to be used for quantitative determination of iFTO in mouse cell lysates.

This kit is an enzyme-linked immunosorbent assay (ELISA). A monoclonal antibody specific for mouse iFTO has been pre-coated onto 96 well microplate. Standards and samples are pipetted into the wells and any iFTO present is bound by immobilized antibody. Bound iFTO is captured by anti-mouse iFTO polyclonal antibody. HRP conjugated anti-rabbit IgG is added. After washing, a substrate solution is added. The colors develop in proportion to the bounded iFTO quantity. The color development is stopped and the intensity of color is measured.

Kit Components

1) Antibody coated 96-well plate, 12 X 8-well strips
2) 5X Wash concentrate, 100 ml
3) 5X Diluent, 50 ml
4) 10X Lysis buffer, 12 ml
5) Secondary antibody, 12 ml
6) 100X Detector, 150 µl
7) Standard, recombinant mouse FTO expressed by *E. coli* cells, 1 vial, lyophilized
8) QC sample = a positive control of recombinant mouse FTO protein, 1 vial, lyophilized (For actual concentration of QC sample, see the 'Certificate of analysis' enclosed.)
9) Substrate, 12 ml
10) Stop solution, 12 ml

Reagents Description

Antibody coated 96-well plate, 12x8-well strips, with absorbed monoclonal antibody against mouse FTO.

5X Wash concentrate, buffered detergent solution, supplied as a 5X concentrate
5X Diluent, for sample and reagent dilution
10X Lysis buffer
1X Secondary antibody, polyclonal antibody against recombinant mouse FTO
100X detector, HRP conjugated anti-rabbit IgG
Standard, 20.0 ng, recombinant mouse FTO
QC sample, recombinant mouse FTO
Substrate solution, chromogenic reagents
Stop solution, 1M H_3PO_4

Storage of Reagents

Reagents must be stored at 2-8°C when not in use. Reagents must be brought to room temperature before use. Do not expose reagents to temperatures greater than 25°C. Diluted wash solution may be stored at room temperature for up to one month.

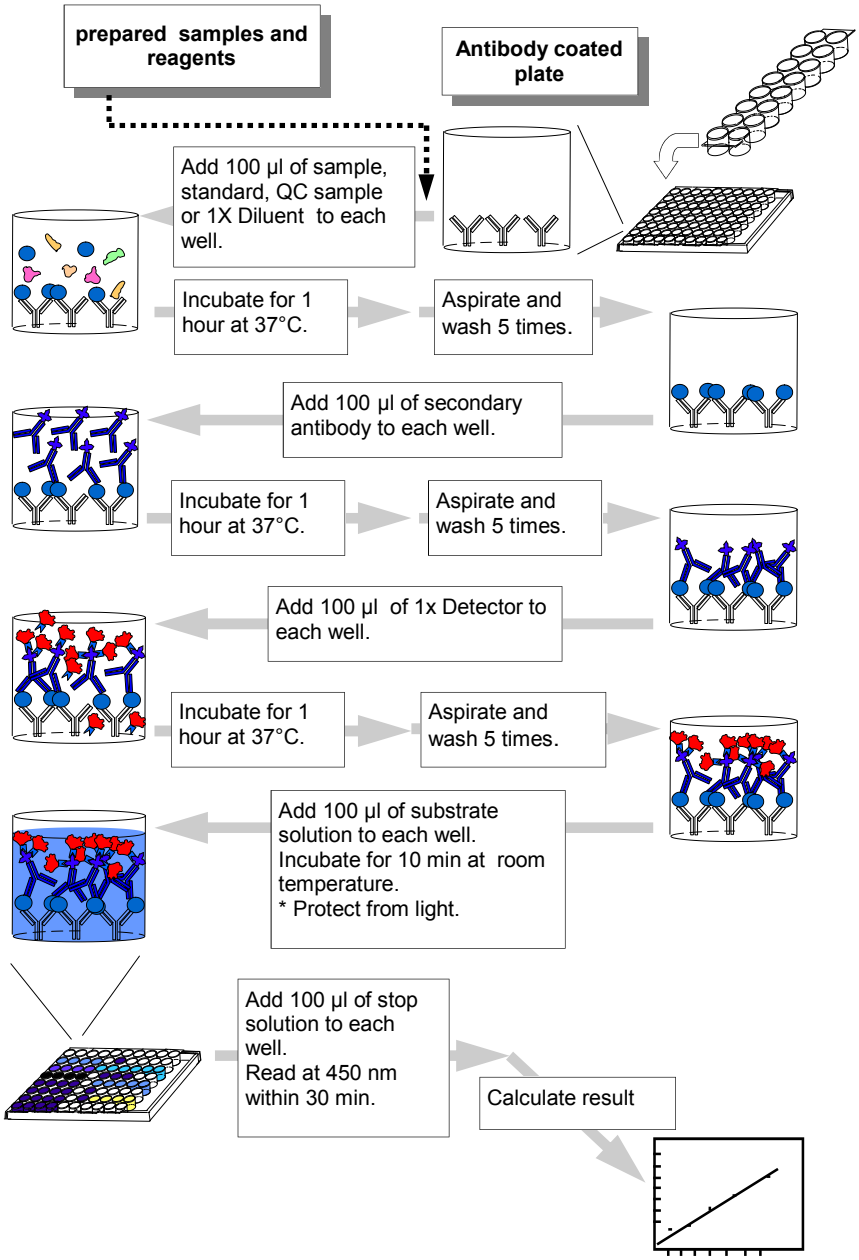
Materials Required but not Supplied

Precision single and multi-channel pipettes.
Disposable pipette tips.
Microtubes or equivalent for preparing dilutions.
Disposable plastic containers for preparing working reagents.
Reagent reservoirs.
Microwell or microstrip plate reader 450 nm
Deionized water
Phenyl methylsulfonyl fluoride (PMSF)

Sample Collection and Storage

Lysate 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 ℃. 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 ℃ and transfer the supernatant to a new tube. The supernatant is the cell lysate. Use freshly prepared cell lysate samples.

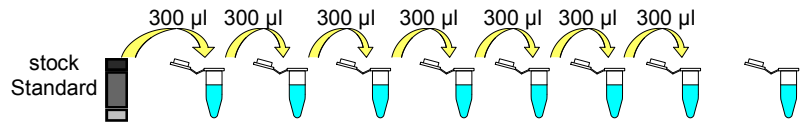
Flow Chart of Assay Procedure



Assay Procedure

1. Preparation of Reagents

1) Allow all samples and kit components to equilibrate to room temperature (20-25°C).
2) Plan the plate configuration and create a plate map. Calculate the amount of working reagents to use. It is recommended that standards and samples be run in duplicate.
3) Prepare **1X lysis buffer**. Dilute 10X Diluent 1:10 with deionized water (1 part 10X Diluent with 9 parts deionized water). Add 1 mM phenyl methylsulfonyl fluoride (PMSF) immediately before use.
4) Prepare **1X Wash Solution**. Dilute 5X Wash Concentrate 1:5 with deionized water (1 part 5X Wash Concentrate with 4 parts deionized water). The diluted 1X Wash Solution is stable for one month at room temperature.
5) Prepare **1X Diluent**. Dilute 5X Diluent 1:5 with deionized water (1 part 5X Diluent with 4 parts deionized water).
6) Prepare **1X Detector**. Dilute 100X Detector 1:100 with 1X Diluent (1 part 100X Detector with 99 parts 1X Diluent). Use the 1X Detector within one hour of preparation.
7) Warm **Substrate Solution** to room temperature before use.
8) Prepare working aliquots of the Standard as follows :
When opening the lyophilized Standard, remove cap gently as the lyophilizate may have become dislodged during shipping.



Tube #	stock	7	6	5	4	3	2	1	0
Conc.(ng/ml)	20	10	5	2.5	1.25	0.625	0.313	0.156	0
Initial vol.(µl)	-	300	300	300	300	300	300	300	300

Add 1 ml of deionized water to the Standard vial to make a stock concentration of **20 ng/ml**. Mix well.

A recommended dilution scheme is as follows :
a. Label 8 microcentrifuge tubes #0-7. Add 300 µl of the 1X Diluent to the microcentrifuge tubes #0-7, respectively.
b. Add 300 µl of the stock Standard solution to tube #7 and vortex. This is Standard tube #7 with a concentration of10 ng/ml.
c. Standards #6 to #1 are then prepared by performing a 1:2 dilution of the preceding standard. Do not add any standard to the tube #0.

9) Reconstitute QC sample in 1 ml of deionized water. Mix well.

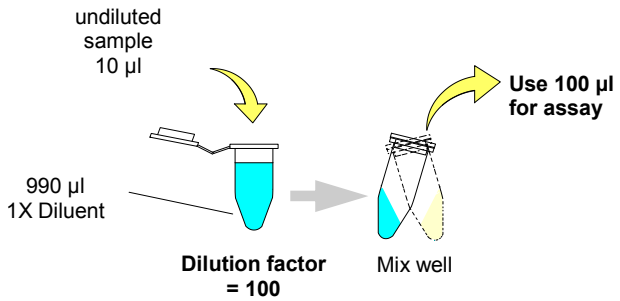
2. Sample Preparation

1) Dilute samples between 1:10 and 1:1000 and mix well.

* If samples fall the outside range of assay, a lower or higher dilution may be required.

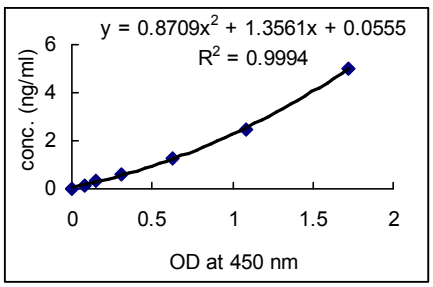
2) Use 100 µl of the final diluted sample for ELISA.

<Example - 1:100 dilution>



3. Experiment procedure

1) Remove the appropriate number of microwell strips from the sealed foil pouch.
2) Pipette 100 µl of standards #0 to #7, the reconstituted QC sample and diluted sample into the antibody-coated plate according to the plate configuration. Use a new pipette tip for each standard or sample.
3) Incubate at 37°C for 1 hour.
4) Remove the solution and wash 5 times with 300 µl of 1X Wash Solution to each well.
5) Add 100 µl Secondary Antibody to each well.
6) Incubate at 37°C for 1 hour.
7) Remove the solution and wash 5 times with 300 µl of 1X Wash Solution to each well.
8) Add 100µl 1X Detector to each well.
9) Incubate at 37°C for 1 hour.
10) Remove the solution and wash 5 times with 300 µl of 1X Wash Solution to each well.
11) Add 100 µl of the Substrate Solution to each well.
12) Incubate at room temperature for 10 min.
    * Protect from light.



13) Using the multi-channel pipette, add 100 µl Stop Solution to each well.
14) Read at 450 nm.
15) Subtract the absorbance of the blank from the readings for each standard and sample.
16) Construct a standard curve by plotting the known concentrations (Y) of standard versus the absorbance (X) of standard. A measurable range is typically shown between 0.156 ng/ml and 10 ng/ml.
17) Calculate iFTO concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation.
18) The iFTO concentrations calculated must be multiplied by dilution factor [see **2. Sample Preparation**] to obtain the concentrations of the undiluted samples.

Performance Characteristics

1) Sensitivity : 20 pg/ml

2) Precision

a. Intra-Assay (precision within an assay)
4 samples were tested 6 times to assess intra-assay precision.

Sample	Mean (ng/ml)	SD (ng/ml)	CV (%)
1	79.109	2.901	3.667
2	56.022	1.767	3.154
3	13.506	0.302	2.236

b. Inter-Assay (precision between assays)
3 samples were tested 7 times to assess inter-assay precision.

Sample	Mean (ng/ml)	SD (ng/ml)	CV (%)
1	120.499	4.719	3.916
2	49.286	2.575	5.225
3	58.256	3.669	6.299

3) Recovery
The recovery of FTO spiked to three different levels in three different samples throughout the range of assay was evaluated.

Sample No.	Average recovery (%)	Range (%)
1	96.865	95-105
2	99.043	95-105
3	99.636	95-105

4) Specificity

a. Cross Reactivity

Analyte	Max. Conc. (ng/ml)	Cross Reactivity (%)
Mouse FTO	5	100
Human FTO	50	6 <
Mouse adiponectin	50	N. R.
Mouse RBP4	50	N. R.
Mouse visfatin	50	N. R.
Mouse vaspin	50	N. R.
Mouse progranulin	50	N. R.
Mouse resistin	50	N. R.
Mouse clusterin	50	N. R.
Mouse GPX3	50	N. R.
Mouse CD137	50	N. R.
Rat adiponectin	50	N. R.
Rat visfatin	50	N. R.
Human adiponectin	50	N. R.
Human visfatin	50	N. R.
Human progranulin	50	N. R.
Mouse progranulin	50	N. R.

N. R. : No Cross-reactivity

5) Linearity - Effect of Lysate Dilution

To assess the linearity of the assay, three lysate samples were first diluted as indicated below prior to sample preparation as described in the protocol.

Sample No.	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% Of Expected
Pre B cells	1:4	20.585	20.585	100
	1:8	10.293	9.856	95.759
	1:16	5.146	5.208	101.194
RAW cells	1:4	26.360	26.360	100
	1:8	13.180	14.468	109.772
	1:16	6.590	6.825	103.566
3T3L1 cells	1:4	14.545	14.545	100
	1:8	7.273	7.293	100.274
	1:16	3.636	3.744	102.950

% of expected = observed / expected x 100%

References

1. Peters T, Ausmeier K, Rüther U. 1999 Cloning of Fatso (Fto), a novel gene deleted by the Fused toes (Ft) mouse mutation. *Mammalian Genome* 10: 983-986.

2. Gerken T, Girard CA, Tung YC et al. 2007 The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. *Science* 318: 1469-1472.

3. Dina C, Meyre D, Gallina S et al. 2007 Variation in FTO contributes to childhood obesity and severe adult obesity. *Nature Genet.* 39: 724-726.

4. Frayling TM, Timpson NJ, Weedon MN et al. 2007 A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* 316: 889-894.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Troubleshooting Guide

Problem	Possible Cause	Solution
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 appropriate for the system.
	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.
	Technique problem	Proper mixing of reagents and wash steps are critical.

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