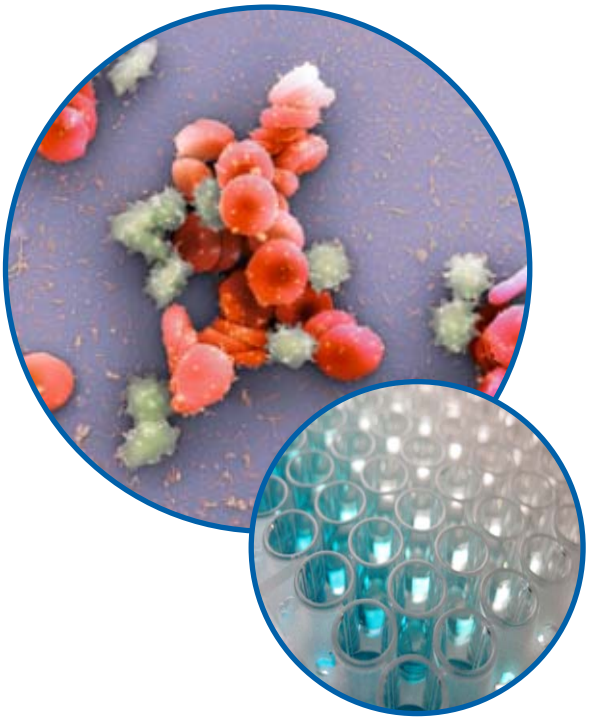


AdipoGen® LIFE SCIENCES

MANUAL

Irisin Competitive ELISA Kit

For research use only. Not for diagnostic use.

Version 4 (04-May-2015)

Cat. No. AG-45A-0046YEK-KI01

Table of Contents

1. Intended Use

The Irisin Competitive ELISA Kit is to be used for the in vitro quantitative determination of irisin in serum, plasma and cell culture supernatant of human origin. It should also work for the in vitro quantitative determination of irisin in mouse, rat and monkey biological samples. This ELISA Kit is for research use only.

2. Introduction

Fibronectin type III domain containing (FNDC) family consists of the multiple protein species but has a typified fibronectin type III domain (1-2). Among them FNDC5 also called irisin, hereinafter named irisin, has been identified a myokine which is capable of inducing browning of white adipose tissue leading to brown adipocytes and beige fat cells by stimulating UCP1 expression, via the PGC-1α transcriptional co-activator (3). Irisin is a single-pass type I membrane protein whose secretion could be by a putative signal peptide. Since irisin has been discovered as a soluble protein from the culture supernatant of the myocytes from the muscle-specific PGC-1α transgenic mice the predicted extracellular domain should be cleaved followed by being liberated from the cell surface membrane. Indeed, this was the case by having shown that the presence of the irisin exists in the culture supernatants and human/mouse sera as a 22 kDa protein (3). Irisin is able to induce UCP1 as well as a number of genes manifested in brown adipose tissues. In particular, the level of irisin was increased by muscular exercise in mouse and human, suggesting that irisin plays an important role in obesity and glucose homeostasis.

3. General References

(1) Frcp1 and Frcp2, two novel fibronectin type III repeat containing genes: A. Teufelet, et al.; Gene **297,** 79 (2002)

(2) Complete sequencing and characterization of 21,243 full-length human cDNAs: T. Ota, et al.; Nat. Genet. **36,** 40 (2004)

(3) A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis: P. Boström, et al.; Nature **481,** 463 (2012)

4. Assay Principle

This assay is a competitive Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of irisin in human biological fluids (plasma, serum, cell culture supernatant). A polyclonal antibody recognizing native irisin reacts with a series of predetermined recombinant irisin standard proteins or samples under competition in the irisin-coated plate. Their relative reactivity is plotted with that of the standard proteins.

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 irisin Recombinant protein	(6 x 16-well strips)	
2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
1 vial Detection Antibody	(30 µl)	(DET)
1 vial HRP 100X (HRP Conjugated anti-rabbit IgG)	(150 µl)	(HRP 100X)
1 vial irisin Standard (lyophilized)	(5 µg)	(STD)
1 bottle TMB Substrate Solution	(12 ml)	(TMB)
1 bottle Stop Solution	(12 ml)	(STOP)
2 plate sealers (plastic film)		
2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450 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.

- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 20 ml ELISA Buffer 10X + 180 ml water) to obtain ELISA Buffer 1X.

- **Detection Antibody (DET)** has to be diluted to 1:625 in ELISA Buffer 1X
  (16 µl DET + 10 ml ELISA Buffer 1X).
  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 100 µl in 10 ml of ELISA Buffer 1X (1:100).
  NOTE: *The diluted HRP is used within one hour of preparation.*

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

  - This reconstitution produces a stock solution of 5 µg/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) (**5 µg/ml**) in ELISA Buffer 1X. A seven-point standard curve in ELISA Buffer 1X is recommended.

  - Suggested standard points are:

    5, 2.5 , 1 , 0.5 , 0.25 , 0.1 , 0.01 and 0.001 µg/ml.

Dilute further for the standard curve:

To obtain	Add	Into
5 µg/ml	-	-
2.5 µg/ml	300 µl of Irisin (5 µg/ml)	300 µl of ELISA Buffer 1X
1 µg/ml	200 µl of Irisin (2.5 µg/ml)	300 µl of ELISA Buffer 1X
0.5 µg/ml	300 µl of Irisin (1 µg/ml)	300 µl of ELISA Buffer 1X
0.25 µg/ml	300 µl of Irisin (0.5 µg/ml)	300 µl of ELISA Buffer 1X
0.1 µg/ml	200 µl of Irisin (0.25 µg/ml)	300 µl of ELISA Buffer 1X
0.01 µg/ml	50 µl of Irisin (0.1 µg/ml)	450 µl of ELISA Buffer 1X
0.001 µg/ml	50 µl of Irisin (0.01 µg/ml)	450 µl of ELISA Buffer 1X

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 or EDTA 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 ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/4 dilution of serum or plasma is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

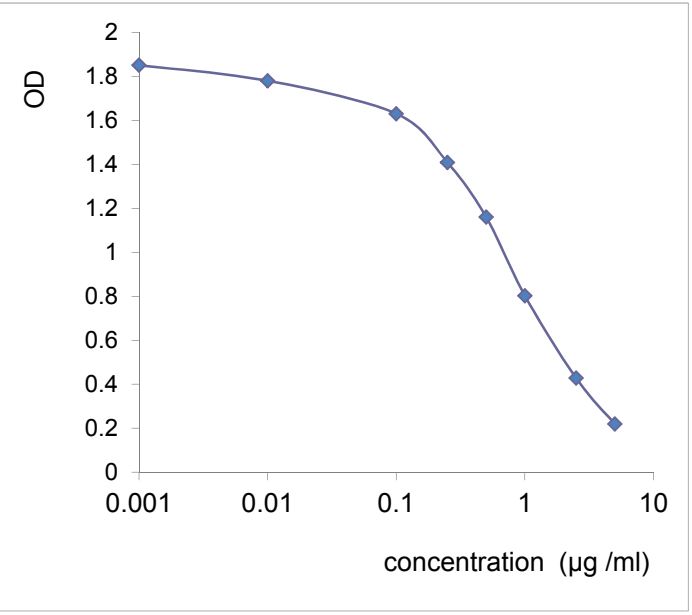
☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 16-well strips coated with irisin protein when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 50 µl of the different standards into the appropriate wells in duplicate! At the same time, add 50 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. Add 50 µl to each well of the Detection Antibody **(DET) (see 8.1. Preparation and Storage of Reagents)** and tap gently on the side of the plate to mix.
☐	4. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	5. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multi-channel 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.
☐	6. Add 100 µl to each well of the diluted HRP Conjugated anti-rabbit IgG (**HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	7. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	8. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multi-channel 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.
☐	9. Add 100 µl to each well of TMB Substrate Solution **(TMB)**.
☐	10. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 20 minutes**.
☐	11. 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!
☐	12. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

9. Calculation of Results

- Average the duplicate readings for each standard, control and sample.
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs the corresponding irisin concentration (µg/ml) on the horizontal (X) axis (see **10.** TYPICAL DATA).
- Calculate the irisin concentrations of samples by interpolation of the regression curve formula as shown above in a form of a 4-parameter logistic equation.
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of irisin in the samples.

10. Typical Data

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



Standard Irisin (µg/ml)	Optical Density (mean)
5	0.219
2.5	0.428
1	0.803
0.5	1.161
0.25	1.409
0.1	1.631
0.01	1.781
0.001	1.882

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of irisin that can be detected by this assay is 1 ng/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.001 µg/ml – 5 µg/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant irisin in human samples. It should also work in mouse, rat and monkey biological samples. It does not cross-react with FNDC4, human adiponectin, human Nampt, human RBP4, human clusterin, human leptin, human vaspin, human GPX3, human resistin, human ACE2, human lipocalin-2, human ANGPTL3, human ANGPTL6, human DNER, human DLK1, human calreticulin, human IL-33, mouse Nampt, mouse clusterin, mouse vaspin, mouse resistin.

D. Intra-assay precision:

Six human samples of known concentrations of irisin were assayed in replicates 8 times to test precision within an assay.

Samples	Means (µg/ml)	SD	CV (%)	n
1	0.678	0.033	4.863	8
2	0.878	0.072	8.193	8
3	1.370	0.105	7.635	8
4	0.437	0.035	7.980	8
5	0.440	0.027	6.036	8
6	1.539	0.104	6.748	8

E. Inter-assay precision:

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

Samples	Means (µg/ml)	SD	CV (%)	n
1	0.532	0.051	9.673	5
2	1.145	0.092	8.027	5
3	0.725	0.060	8.254	5
4	0.731	0.071	9.656	5
5	0.696	0.068	9.719	5

F. Recovery:

When human samples (serum) are spiked with known concentrations of irisin, the recovery averages 109% (range from 85% to 120%).

Samples	Average recovery (%)	Range (%)
1	109.897	85-115
2	119.475	95-125
3	99.577	85-110

G. Linearity:

Different human serum samples containing irisin were diluted several fold (1/2 to 1/4) and the measured recoveries ranged from 90% to 107%.

Samples	Sample Dilution	Expected (µg/ml)	Observed (µg/ml)	% of Expected
1	1 : 2	0.229	0.229	100
	1 : 4	0.115	0.121	105.677
2	1 : 2	0.346	0.346	100
	1 : 4	0.173	0.175	101.156
3	1 : 2	0.232	0.232	100
	1 : 4	0.116	0.107	92.241
4	1 : 2	0.282	0.282	100
	1 : 4	0.141	0.145	102.837

H. Expected values:

Irisin levels range in human plasma and serum from **0.2 to 2 µg/ml**.

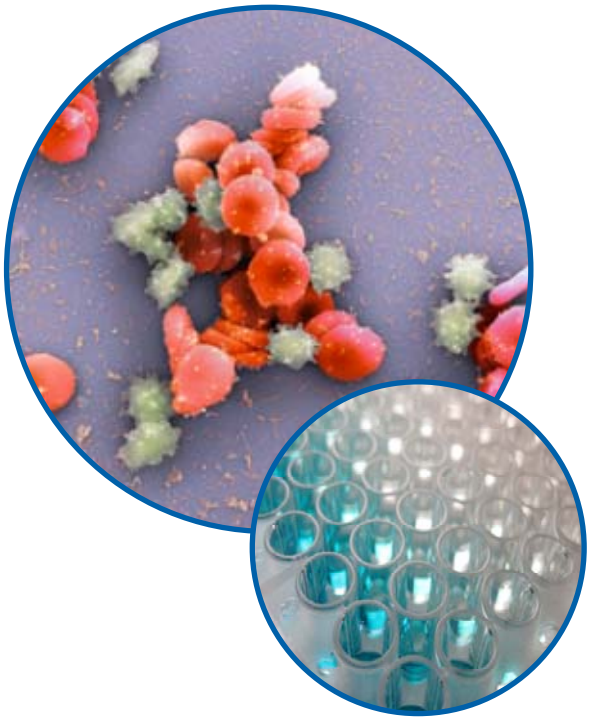
12. Technical Hints and Limitations

- It is recommended that all standards, controls 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 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution (TMB) protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution (STOP) 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 HRP 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. Notes



Product Specific References:

1. E.A. Lawson, et al.; J. Clin. Endocrinol. Metab. **99,** E881 (2014)
2. H.Y. Choi, et al.; J. Clin. Endocrinol. Metab. **99,** 2778 (2014)
3. V. Singhal, et al.; PLoS One **9,** e100218 (2014)
4. T. Klangjareonchai, et al.; Int. J. Endocrinol. **2014,** ID261545 (2014)

For more References please visit www.adipogen.com!

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