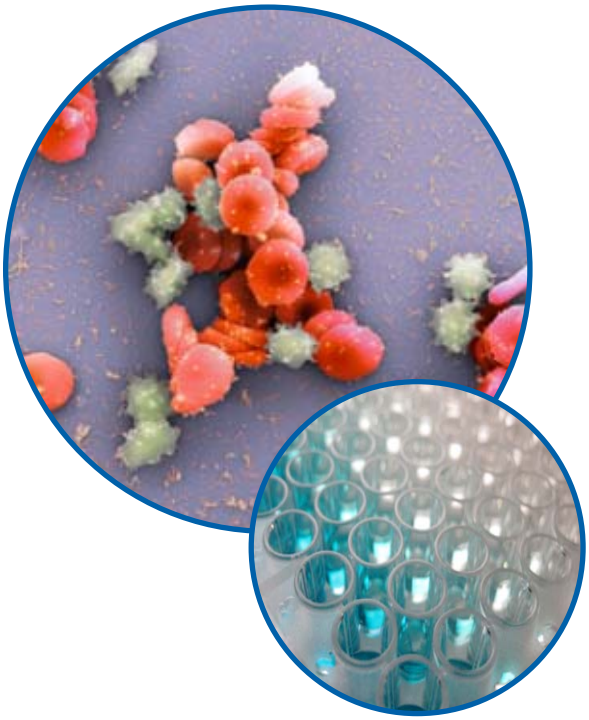


AdipoGen® LIFE SCIENCES

MANUAL

Sirtuin 1 (human) (Intracellular) ELISA Kit

For research use only. Not for diagnostic use.

Version 3 (04-May-2015)

Cat. No. AG-45A-0029YEK-KI01

Table of Contents

1. Intended Use

The Sirtuin 1 (human) (IntraCellular) ELISA Kit is to be used for the *in vitro* quantitative determination of human Sirtuin 1 in cell lysates or cell-based assays (screening). This ELISA Kit is for research use only.

2. Introduction

Sirtuin1 is the human ortholog for the yeast Sir2 (silent information regulator 2) protein regulating epigenetic gene silencing as a possible antiaging effect (1). Sirtuin1 is an NAD(+)-dependent histone deacetylase, which deacetylate lysines 9 and 14 of histone H3 and lysine-16 of histone H4, involved in various cellular functions such as transcription, energy sensing, and differentiation (2). Sirtuin1 plays an important role in regulating adipogenesis via repression of PPAR and the gluconeogenic/glycolytic pathways in liver in response to fasting signals through the transcriptional coactivator PGC1A deacetylated at specific lysine residues in an NAD(+)-dependent manner (3). It has been also demonstrated that AMPK controls the expression of genes involved in energy metabolism in mouse skeletal muscle by acting in coordination with Sirtuin1 (4). AMPK enhances Sirtuin1 activity by increasing cellular NAD+ levels, resulting in the deacetylation and modulation of the activity of downstream Sirtuin1 targets that include PGC1-alpha and the FOXO1A and FOXO3A transcription factors (5). This AMPK-induced Sirtuin1-mediated deacetylation of these targets explains many of the convergent biologic effects of AMPK and Sirtuin1 on energy metabolism. A line of small molecules that activate sirtuins, one of which is resveratrol, a potent activator as a polyphenol found in red wine, enhance Sirtuin1 activity, thereby increasing cell survival by stimulating Sirtuin1-dependent deacetylation of p53 (6). In diet-induced obese and genetically obese mice, these compounds improve insulin sensitivity, lower plasma glucose, and increase mitochondrial capacity (7). Therefore, Sirtuin1 is a central regulator of energy metabolism whose intracellular measurement provides valuable information on how this enzyme integrates various environmental or intracellular stimuli into metabolic regulation as well as a practical tool by which one can develop potential drugs leading to the transcriptional activation of Sirtuin1.

3. General References

(1) Characterization of two genes required for the position-effect control of yeast mating-type genes: D. Shore, et al.; EMBO J. **3,** 2817 (1984)

(2) Phosphorylation of HuR by Chk2 regulates SIRT1 expression: K. Abdelmohsen, et al.; Molec. Cell. **25,** 543 (2007)

(3) Nutrient control of glucose homeostasis through a complex of PGC-1-alpha and SIRT1: J. Rodgers, et al.; Nature **434,** 113 (2005)

(4) AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity: C. Canto, et al.; Nature **458,** 1056 (2009)

(5) Nutrient availability regulates SIRT1 through a forkhead-dependent pathway: S. Nemoto, et al.; Science **306,** 2105 (2004)

(6) Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan: K.T. Howitz, et al.; Nature **425,** 191 (2003)

(7) Small molecular activators of SIRT1 as therapeutics for the treatment of type 2 diabetes: J.C. Milne, et al.; Nature **450,** 712 (2007)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human Sirtuin 1 in cells. A monoclonal antibody specific for Sirtuin 1 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, Sirtuin 1 is recognized by the addition of a purified polyclonal antibody specific for Sirtuin 1 (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-rabbit IgG (HRP) 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 Sirtuin 1 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 Sirtuin 1 Antibody	(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 bottle Lysis Buffer 10X	(12 ml)	(LYSIS Buffer)
1 vial Detection Antibody	(70 µl)	(DET)
1 vial HRP 100X (HRP Conjugated anti-rabbit IgG)	(150 µl)	(HRP 100X)
1 vial human Sirtuin 1 Standard (lyophilized)	(4 ng)	(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
- 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.

- **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.

- **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.

- **Detection Antibody (DET)** has to be diluted to 1:200 in ELISA Buffer 1X
  (50 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.*

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

  - This reconstitution produces a stock solution of 4 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) (**4 ng/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.

  - Suggested standard points are:

    2 , 1 , 0.5 , 0.25 , 0.125 , 0.063 , 0.031 and 0 ng/ml.

Dilute further for the standard curve:

To obtain	Add	Into
2 ng/ml	300 µl of Sirtuin 1 (4 ng/ml)	300 µl of ELISA Buffer 1X
1 ng/ml	300 µl of Sirtuin 1 (2 ng/ml)	300 µl of ELISA Buffer 1X
0.5 ng/ml	300 µl of Sirtuin 1 (1 ng/ml)	300 µl of ELISA Buffer 1X
0.25 ng/ml	300 µl of Sirtuin 1 (0.5 ng/ml)	300 µl of ELISA Buffer 1X
0.125 ng/ml	300 µl of Sirtuin 1 (0.25 ng/ml)	300 µl of ELISA Buffer 1X
0.063 ng/ml	300 µl of Sirtuin 1 (0.125 ng/ml)	300 µl of ELISA Buffer 1X
0.031 ng/ml	300 µl of Sirtuin 1 (0.063 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 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 **Lysis Buffer 1X** 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 ELISA Buffer 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 sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

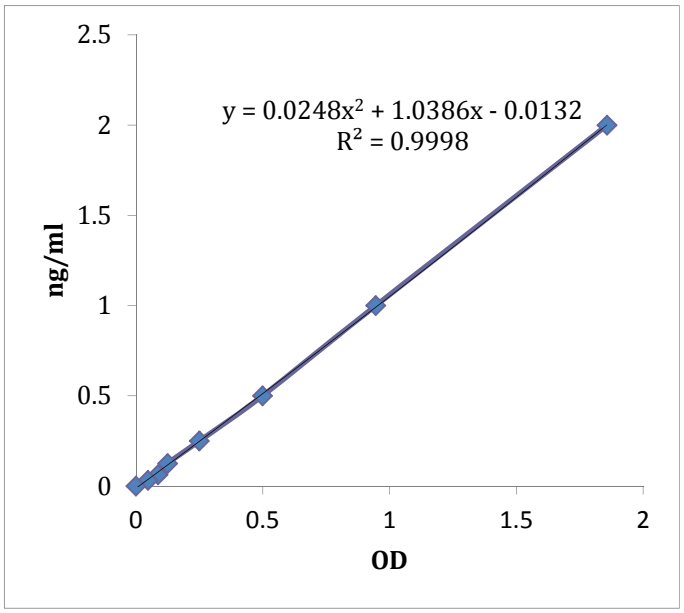
☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 16-well strips coated with Sirtuin 1 antibody when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted cell 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 **(DET)**. **(see 8.1. Preparation and Storage of Reagents)**.
☐	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 **HRP (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 **(TMB)**.
☐	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 **(STOP)**. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution **(STOP)** 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, control 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 Sirtuin 1 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the Sirtuin 1 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 Sirtuin 1 in the samples.

10. Typical Data

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



Standard hSirtuin 1 (ng/ml)	Optical Density (mean)
2	1.85
1	0.95
0.5	0.49
0.25	0.25
0.125	0.125
0.0625	0.087
0.03125	0.047
0	0

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of Sirtuin 1 that can be detected by this assay is 30 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: 0.031 ng/ml – 2 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human Sirtuin 1. It does not cross-react with human Sirtuin 2, human Sirtuin 5, human Sirtuin 6, human adiponectin, human resistin, human RBP4, human vaspin, human progranulin, human GPX3, human FTO, human Nampt, human leptin, mouse FTO, mouse Nampt.

D. Intra-assay precision:

Three samples of known concentrations of human Sirtuin 1 were assayed in replicates 4 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
293E cells	30.765	0.742	2.413	4
HT-29 cells	7.980	0.645	8.082	4
Molt4 cells	10.502	0.748	7.121	4

E. Inter-assay precision:

Three samples of known concentrations of human Sirtuin 1 were assayed in 6 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
293E cells	30.778	2.326	7.558	6
HT-29 cells	7.675	0.687	8.951	6
Molt4 cells	10.655	0.430	4.037	6

F. Recovery:

When samples (cell lysates) are spiked with known concentrations of human Sirtuin 1, the recovery averages 98% (range from 90% to 110%).

Samples	Average recovery (%)	Range (%)
293E cells	92.703	90-100
HT-29 cells	98.497	95-105
Molt4 cells	104.333	100-110

G. Linearity:

Different human cell lysates samples containing Sirtuin 1 were diluted several fold (1/10 to 1/40) and the measured recoveries ranged from 85% to 113%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
293E cells	1 : 10	28.88	28.88	100
	1 : 20	14.44	16.31	112.96
	1 : 40	7.22	7.77	107.60
HT-29 cells	1 : 10	7.09	7.09	100
	1 : 20	3.54	3.12	88.07
	1 : 40	1.77	1.76	99.42
Molt4 cells	1 : 10	8.43	8.43	100
	1 : 20	4.21	3.69	87.48
	1 : 40	2.11	1.81	85.93

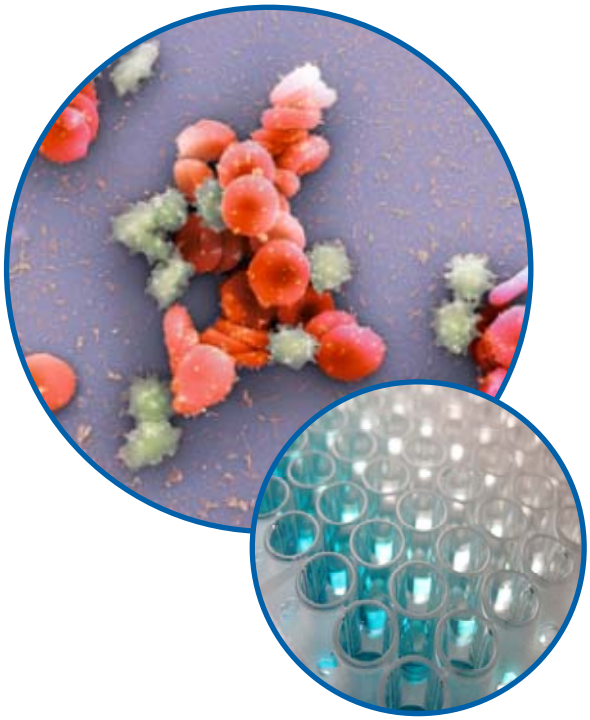
12. Technical Hints and Limitations

- 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



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