Sirtuin Activity Assay Kit (Fluorometric)

Rev 04/19

(Catalog # K324-100; 100 assays; Store at -20°C)

I. Introduction:

Sirtuins are a class of proteins that possess either histone deacetylase or mono-ribosyltransferase activity. Sirtuins are localized in the cytoplasm, nucleus, nucleolus as well as mitochondria. They are associated with aging, cellular protection, sugar metabolism and cell cycle regulation. Unlike other known protein deacetylases, which simply hydrolyze acetyl-lysine residues, the sirtuin-mediated deacetylation reaction hydrolyzes acetyl-lysine and NAD. This hydrolysis yields the deacetylated substrate, O-acetyl-ADP-ribose and nicotinamide, itself an inhibitor of sirtuin activity. Studies suggest that the human sirtuins may function as intracellular regulatory proteins with mono-ADP-ribosyltransferase activity. In BioVision's Sirtuin Activity Assay Kit, the acetylated p53-AFC substrate is deacetylated by Sirtuins in the presence of NAD^+ to generate the deacetylated p53-AFC substrate, nicotinamide and O-Acetyl-ADP Ribose. Cleavage of the deacetylated p53-AFC substrate by the Developer releases the fluorescent group, which is detected fluorometrically at Ex/Em = 400/505 nm. HDAC's also deacetylate the acetylated p53-AFC substrate. Trichostatin A is added to the reaction to specifically inhibit HDAC's in samples. This kit provides a rapid, simple, sensitive, and reliable test to measure Sirtuin Activity in a variety of samples. The limit of quantification of the assay is 0.06 µU of recombinant human SIRT6.

Acetylated p53-AFC substrate + NAD^+ $\xrightarrow{\text{Sirtuin}}$ Deacetylated p53-AFC substrate + Nicotinamide + O-Acetyl-ADP Ribose

Deacetylated p53-AFC substrate $\xrightarrow{\text{Developer}}$ Deacetylated p53-substrate + AFC (Ex/Em = 400/505 nm)

II. Application:

- Detection of Sirtuin Activity in variety of samples

III. Sample Type:

- Purified recombinant protein
- Cell and tissue lysate
- Nuclear Extract
- Mitochondria
- Immunoprecipitated samples

IV. Kit Contents:

Components	K324-100	Cap Code	Part Number
Sirtuin Assay Buffer	25 ml	WM	K324-100-1
Homogenization Buffer	100 ml	NM	K324-100-2
1M DTT	0.4 ml	Green	K324-100-3
Substrate (in DMSO)	0.2 ml	Red	K324-100-4
NAD	1 vial	Purple	K324-100-5
Positive Control	20 µl	Amber	K324-100-6
Trichostatin A (in DMSO)	50 µl	Blue	K324-100-7
Developer	1 ml	Orange	K324-100-8
AFC Standard (in DMSO) (1 mM)	0.1 ml	Yellow	K324-100-9

V. User Supplied Reagents and Equipment:

- 96-well plate with flat bottom. White plate is preferred for this assay.
- Fluorescence microplate reader
- Protease Inhibitor Cocktail (Cat. # K271 or its equivalent)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **Sirtuin Assay Buffer:** Store at 4°C or -20°C. Warm to 37°C and add DTT to final concentration of 2 mM just before use. Make fresh as needed.
- **Homogenization Buffer**: Thaw at room temperature and keep on ice while in use.
- **Substrate:** Aliquot and Store at -20°C. Avoid repeated freeze/thaw. Use fresh tip each time.
- **NAD:** Reconstitute with 220 µl deionized water. Aliquot and store at -80°C after reconstituting. Avoid repeated freeze/thaw.
- **Positive Control:** Store at -80°C. Avoid repeated freeze/thaw.
- **Trichostatin A**: Store at -20°C. Use fresh tip each time.
- **Developer**: Aliquot and store at -20°C. Avoid repeated freeze/thaw. Keep on ice while in use.
- **AFC Standard**: Store at -20°C

VII. Sirtuin Activity Assay protocol:

1. **Sample Preparation:** Add DTT to Homogenization Buffer to a final concentration of 2 mM. Make fresh as needed. Rinse tissue and transfer ~100 mg of fresh or frozen tissue (stored at -80°C) to a prechilled tube. Add 600 µl cold Homogenization Buffer containing protease inhibitor cocktail (not provided) and thoroughly homogenize tissue on ice. Transfer the tissue homogenate to a cold microfuge tube. To prepare cell extract, add 150-300 µl cold Homogenization Buffer containing protease inhibitor cocktail (not provided) to 1-5 x 10^6 fresh or frozen cells and homogenize cells on ice. Transfer the cell homogenate including cell debris to a cold microfuge tube and agitate on a rotary shaker at 4°C for 15 min. Centrifuge the tissue or cell homogenate at 16,000 X g for 20 min. at 4°C. Transfer the clarified

supernatant to a fresh pre-chilled tube and keep on ice. Use lysates immediately to assay for Sirtuin Activity. Mitochondria can be isolated using Mitochondria Isolation Kit For Tissue & Cultured Cells (Cat. # K288 or equivalent) or Mitochondria/Cytosol Fractionation Kit (Cat. # K256 or equivalent). Nuclear extracts can be prepared using Nuclear/Cytosol Fractionation Kit (Cat. # K266 or equivalent).

Note: Lysates can be aliquoted and snap frozen in liquid nitrogen before storing at -80°C. Avoid freeze/thaw.

2. **AFC Standard:** Dilute AFC Standard to 10 µM by adding 10 µl of 1 mM AFC Standard to 990 µl of Sirtuin Assay Buffer (with DTT). Add 0, 20, 40, 60, 80, and 100 µl of diluted 10 µM AFC Standard into individual wells in a 96-well white plate and adjust the volume to 100 µl/well with Sirtuin Assay Buffer (with DTT) to generate 0, 200, 400, 600, 800, and 1000 pmol/well of AFC Standard respectively. Mix well.
3. **Sirtuin Activity Assay:** Add 2-50 µl of cell/tissue homogenate/nuclear extract/mitochondria or purified protein into desired wells in a 96-well plate. For positive control, add 2 µl of Positive Control in desired well. Make up the volume of samples and Positive Control to 50 µl/well with Sirtuin Assay Buffer (with DTT).

Notes:

a. For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.

b. For samples having HDAC activity, it is recommended using two wells for each sample, one with (1µl) and the other without Trichostatin A. You can measure the Sirtuin Activity and HDAC + Sirtuin Activity respectively.

c. Use heat inactivated sample as sample background control. For Positive Control Background, use Sirtuin Assay Buffer (with DTT) as background control. Make up the volume to 50 µl/well with Sirtuin Assay Buffer (with DTT).

4. **Reaction Mix:** Prepare enough reagents for the number of assays to be performed. Make 40 µl Reaction Mix for each well containing:

	Reaction Mix
Sirtuin Assay Buffer (with DTT)	36 µl
Substrate	2 µl
NAD	2 µl

Add 40 µl of Reaction Mix into each Sample, Positive Control and Background Control wells. Mix well. Incubate at 37°C for 30-60 min. After incubation, add 10 µl of Developer to each well except Standards and mix the contents. Incubate for 10-15 min. at 37°C

5. **Measurement:** Measure fluorescence (Ex/Em = 400/505 nm) in end point mode.
6. **Calculations:** Subtract 0 Standard reading from all Standard readings and plot the AFC Standard Curve. Subtract sample background control reading from Sample reading. Apply the corrected sample reading to the AFC Standard Curve to get B pmol of AFC generated by sirtuin activity in the sample wells.

Sample Sirtuin/HDAC activity = B/TS = pmol/min/µg = µU/µg

Where: **B** = AFC amount in the sample well from Standard Curve (pmol)
T = reaction time in min. (30-60 min.)
S = sample amount (µg)

Sample Sirtuin Activity can also be expressed as mU/mg (nmoles/min. deacetylated peptide generated per mg of protein).

Unit Definition: One unit of Sirtuin activity is the amount of enzyme that hydrolyzes the substrate to yield 1.0 µmol of AFC/min. at 37°C.

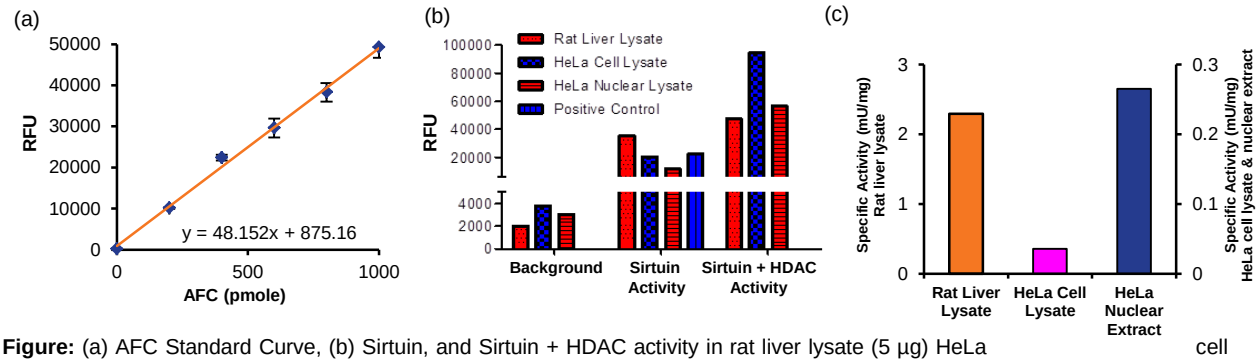

Figure: (a) AFC Standard Curve, (b) Sirtuin, and Sirtuin + HDAC activity in rat liver lysate (5 µg) HeLa cell lysate (160 µg), HeLa nuclear extract (11.5 µg) and Positive Control (2 µl). (c) Sirtuin Specific Activity in Samples used in (b). Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS

Sirtuin 1 (human intracellular) ELISA Kit (K4923)
Sirtuin 2 (human intracellular) ELISA Kit (K4924)
SIRT2 Inhibitor Screening Assay Kit (Fluorometric) (K322)
SIRT6 Inhibitor Screening Assay Kit (Fluorometric) (K323)
HDAC Fluorometric Activity Assay Kit (K330)
HDAC Colorimetric Activity Assay Kit (K331)
InSitu HDAC Activity Fluorometric Assay Kit (K339)
Sirtuin 2, human recombinant (7632)
SIRT4 (GST-tagged), Human recombinant (7673)
SIRT5 (GST-tagged), Human recombinant (7674)
SIRT1 (193-747 aa) (GST-tagged) (7264)
Sirtuin 6, human recombinant (7578)
SIRT7 (2-400 aa) (His-tagged), Human recombinant (7675)
Sirtinol (2062)

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