

Background

Nucleosomes, which fold chromosomal DNA, contain two molecules each of the core histones H2A, H2B, H3, and H4. Almost two turns of DNA are wrapped around this octameric core, which represses transcription.¹ The histone amino termini extend from the core, where they can be modified post-translationally by acetylation, phosphorylation, ubiquitination, and methylation, affecting their charge and function. Acetylation of the ϵ -amino groups of specific histone lysines is catalyzed by histone acetyltransferases (HATs) and correlates with an open chromatin structure and gene activation. Histone deacetylases (HDACs) catalyze the hydrolytic removal of these acetyl groups from histone lysine residues and correlates with chromatin condensation and transcriptional repression.^{2,3} The sirtuins represent a distinct class of trichostatin A-insensitive lysyl-deacetylases (class III HDACs) and have been shown to catalyze a reaction that couples lysine deacetylation to the formation of nicotinamide and O-acetyl-ADP-ribose from NAD⁺ and the abstracted acetyl group.⁴⁻⁶ There are seven human sirtuins, which have been designated SIRT1-SIRT7.⁷ SIRT2 is a cytoplasmic protein responsible for the deacetylation of histone H4 at lysine-16 and lysine-40 of α -tubulin, a modification important for controlling the cell cycle.⁸⁻¹⁰ Specifically, SIRT2 co-localizes with HDAC6 and microtubules and functions as a mitotic checkpoint in preventing chromosomal instability that can lead to hyperpoloid cells. SIRT2 is found in many tissues, but is specifically enriched in skeletal muscle, heart, and in oligodendroglia cells in the brain.^{11,12} SIRT2 has been suggested to act as a tumor suppressor in human brain gliomas.¹³ Down-regulation of SIRT2 gene expression and/ or deletion of the chromosomal region containing the SIRT2 gene is frequently observed in gliomas. SIRT2 expression might serve as a potential diagnostic molecular marker for gliomas and modulation of its activity may therefore be of interest for the management of gliomas.

About This Assay

SIRT2 Direct Fluorescent Screening Assay Kit (Cat # KA1368) provides a convenient fluorescence-based method for screening SIRT2 inhibitors or activators. The procedure requires only two easy steps, both performed in the same microplate. In the first step, the substrate, which comprises the p53 sequence Gln-Pro-Lys-Lys(ε-acetyl)-AMC, is incubated with human recombinant SIRT2 along with its cosubstrate NAD⁺. Deacetylation sensitizes the substrate such that treatment with the developer in the second step releases a fluorescent product. The fluorophore can be analyzed with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm.

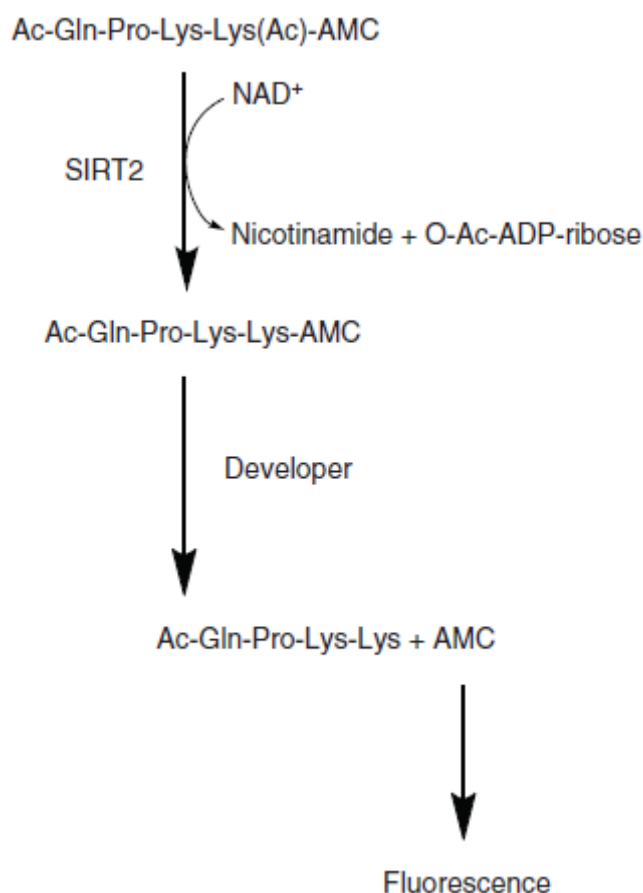


Figure 1. Assay scheme

Material Supplied

<u>Item</u>	<u>Quantity</u>	<u>Storage</u>
SIRT2 Direct Assay Buffer (10X)	1 vial	-20 °C
SIRT2 (human recombinant)	2 vials	-80 °C
SIRT2 Direct Peptide	2 vials	-20 °C
SIRT2 Direct NAD ⁺	1 vial	-20 °C
SIRT2 Direct Nicotinamide	1 vial	-20 °C
SIRT2 Direct Developer	1 vial	-20 °C
SIRT2 Direct Fluorophore	1 vial	-20 °C
Half-Volume 96-Well Plate (White)	1 plate	Room temperature
96-Well Cover Sheet	1 cover	Room temperature

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Materials Needed But Not Supplied

- ✓ A fluorometer with the capacity to measure fluorescence using excitation wavelength of 350-360 nm and emission wavelength of 450-465 nm
- ✓ Adjustable pipettes and a repeat pipettor
- ✓ A source of pure water; glass distilled water or HPLC-grade water is acceptable

Storage and Stability

This kit will perform as specified if stored as specified in the **Materials Supplied** section and used before the expiration date indicated on the outside of the box.

Reagent Preparation

- ✓ SIRT2 Direct Assay Buffer (10X) - Dilute 3 ml of Assay Buffer (10X) with 27 ml of HPLC-grade water. The final Buffer (50 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl₂) should be used in the assay and for diluting reagents. When stored at 4 °C, this diluted buffer is stable for at least six months.
- ✓ SIRT2 (human recombinant) - Each vial contains 30 µl of human recombinant SIRT2. Thaw the enzyme on ice, add 270 µl of diluted assay buffer to the vial, and vortex. The diluted enzyme is stable for four hours on ice. One vial of enzyme is enough SIRT2 to assay 60 wells. Use the additional vial if assaying the entire plate.
- ✓ SIRT2 Direct Peptide - Each vial contains 100 µl of a 5 mM peptide solution comprising amino acids 317-320 of human p53 conjugated to aminomethylcoumarin (AMC). It is ready to use to make the Substrate Solution. One vial of peptide will make enough Substrate to assay wells. Use the additional vial if assaying the entire plate.

- ✓ SIRT2 Direct NAD⁺ - The vial contains 500 µl of a 50 mM solution of NAD⁺. It is ready to use to make the Substrate Solution.
- ✓ SIRT2 Direct Nicotinamide - The vial contains 500 µl of a 50 mM solution of nicotinamide, a sirtuin inhibitor. It is ready to use to make the Stop/Developing Solution.
- ✓ SIRT2 Direct Developer - The vial contains 100 mg of the SIRT2 Developer.
- ✓ SIRT2 Direct Fluorophore - The vial contains 50 µl of 10 mM 7-amino-4-methylcoumarin in DMSO. The fluorophore can be used to assay for interference.

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background wells. A typical layout of samples and compounds to be measured in triplicate is given below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells

A - 100% Initial Activity Wells

1-30 - Inhibitor Wells

Figure 2. Sample plate format

Pipetting Hints

- ✓ It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- ✓ Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- ✓ Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- ✓ The final volume of the assay is 100 μ l in all the wells.
- ✓ Use the diluted assay buffer in the assay.
- ✓ All reagents except SIRT2 and Stop/Developing Solution must be equilibrated to room temperature before beginning the assay.
- ✓ It is not necessary to use all the wells on the plate at one time.
- ✓ If the appropriate inhibitor/activator concentration is not known, it may be necessary to assay at several dilutions.
- ✓ We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- ✓ Thirty inhibitor/activator samples can be assayed in triplicate or 46 in duplicate.
- ✓ The assay temperature is 37°C.
- ✓ Monitor the fluorescence with an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm.

Performing the Assay

1. **Preparation of Substrate Solution** - To one of the thawed SIRT2 Direct Peptide vials, add 160 μ l of NAD⁺ Solution, and 930 μ l of diluted Assay Buffer. One vial of peptide will make enough Substrate Solution for 79 wells. The Substrate Solution is stable for six hours. The addition of 15 μ l to the assay yields a final concentration of 125 μ M peptide and 2 mM NAD⁺. *NOTE: the K_m values for the peptide and NAD⁺ are 51 and 213 μ M, respectively.*
2. **100% Initial Activity Wells** - add 25 μ l of diluted Assay Buffer, 5 μ l of diluted SIRT2 (human recombinant), and 5 μ l of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. **Background Wells** - add 30 μ l of diluted Assay Buffer and 5 μ l of solvent (the same solvent used to dissolve the inhibitor) to three wells.
4. **Inhibitor/Activator Wells** - add 25 μ l of diluted Assay Buffer, 5 μ l of diluted SIRT2 (human recombinant), and 5 μ l of Inhibitor/Activator* to three wells.

*Inhibitors/activators can be dissolved in Assay Buffer, methanol, ethanol, or DMSO and should be added to the assay in a final volume of 5 μ l. In the event that the appropriate concentration of Inhibitor/Activator needed for SIRT2 inhibition or activation is completely unknown, we recommend that several dilutions of the Inhibitor/Activator be assayed.

	Assay Buffer (μ l)	Solvent (μ l)	SIRT2 (μ l)	Test Compound (μ l)
100% Initial Activity	25	5	5	-
Background	30	5	-	-
Inhibitor/Activator	25	-	5	5

Table 1. Pipetting summary

5. Initiate the reactions by adding 15 μ l of Substrate Solution to all the wells being used.
6. Cover the plate with the plate cover and incubate on a shaker for 45 minutes at 37°C.

7. **Preparation of Stop/Developing Solution** - Weigh 30 mg of Developer into a vial that will hold 5 ml then add 200 μ l of Nicotinamide and 4.8 ml of diluted assay buffer. Vortex until the Developer is into solution. This is enough Stop/Developing Solution for the entire plate. The Stop/Developing solution is stable for four hours on ice.
8. Remove the plate cover and add 50 μ l of Stop/Developing Solution. Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
9. Remove the plate cover and read the plate using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples. The development is stable for 30 minutes.

Calculations

1. Determine the average fluorescence of each sample.
2. Subtract the fluorescence of the Background wells from the fluorescence of the 100% Initial Activity and the Inhibitor/Activator wells.
3. Determine the percent Inhibition/Activation for each sample. To do this, subtract each Inhibitor/Activator sample value from the 100% Initial Activity sample value. Divide the result by the 100% Initial Activity value and then multiply by 100 to give the percent Inhibition/Activation.
4. Either graph the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). An example of SIRT2 inhibition by nicotinamide, a sirtuin-specific inhibitor.

$$\% \text{ Inhibition/Activation} = \left[\frac{\text{Initial Activity} - \text{Sample}}{\text{Initial Activity}} \right] \times 100$$

$$\% \text{ Inhibition Activity} = \left[\frac{\text{Sample Activity}}{\text{Initial Activity}} \right] \times 100$$

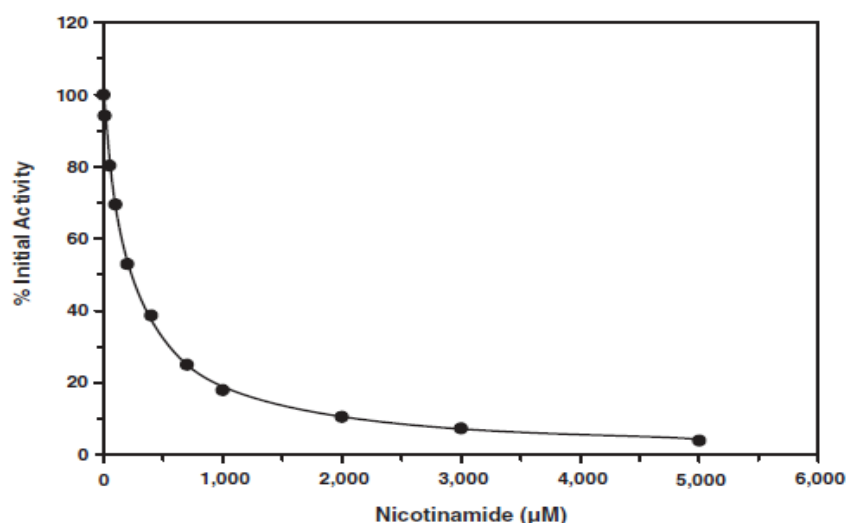


Figure 3. Inhibition of SIRT2 by Nicotinamide ($IC_{50} = 250 \mu$ M)

Precision

When a series of 16 SIRT2 measurements were performed on the same day, the intra-assay coefficient of variation was 3.8%. When a series of 16 SIRT2 measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 3.9%.

Interferences

It is possible that a compound tested for SIRT2 inhibition/activation will interfere with the development of the assay or interfere with the fluorophore. Potential fluorophore interference can be tested by assaying the compound in question with the fluorophore. A procedure is outlined below.

Testing for fluorophore interference

1. Dilute 5 μ l of fluorophore with 995 μ l of diluted assay buffer.
2. Fluorophore wells - add 5 μ l of diluted fluorophore, 5 μ l of solvent (the same solvent used to dissolve the compound), and 90 μ l of diluted assay buffer to three wells.
3. Compound wells - add 5 μ l of diluted fluorophore, 5 μ l of compound, and 90 μ l of diluted assay buffer to three wells.
4. Cover the plate with the plate cover and incubate for 10 minutes at room temperature.
5. Remove the plate cover and read the plate using an excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Calculating the percent fluorophore interference

1. Determine the average fluorescence of each sample.
2. Determine the percent interference for the compound. To do this, subtract each compound value from the fluorophore value. Divide the result by the fluorophore value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be not affecting the fluorophore.

Testing for developer interference

1. **SIRT2 wells** - add 25 μ l of assay buffer and 5 μ l of diluted SIRT2 to three wells.
2. **Compound wells** - add 25 μ l of assay buffer and 5 μ l of diluted SIRT2 to three wells.
3. Initiate the reactions by adding 15 μ l of Substrate Solution to all the wells being used.
4. Cover the plate with the plate cover and incubate on a shaker for 45 minutes at 37 °C.
5. Remove the plate cover and add 50 μ l of Stop/Developing Solution to the SIRT2 and Compound wells.
6. Add 5 μ l of compound to the Compound wells and 5 μ l of solvent (the same solvent used to dissolve the compound) to the SIRT2 wells.
7. Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
8. Remove the plate cover and read the plate using an excitation wavelength of 350-360 nm and an

emission wavelength of 450-465 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Calculating the percent developer interference

1. Determine the average fluorescence of each sample.
2. Determine the percent interference for the compound. To do this, subtract each compound value from the SIRT2 value. Divide the result by the SIRT2 value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be not affecting the developer.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in any of the wells	Either SIRT2 or Stop Solution was not added to the wells	Make sure to add all the components to the wells and re-assay
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
No inhibition/activation seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor/activator of the enzyme	Increase the compound concentration and re-assay

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