SensoLyte® 520 FRET SIRT2 Assay Kit

Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72189**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect SIRT2 activity.
- ***Enhanced Value:*** It provides ample reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed in one hour
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	SIRT2 520 FRET substrate	1 mM, 50 µL
Component B	Deacetylated FRET reference substrate, Ex/Em=490 nm/520 nm after cleavage	1 mM, 20 µL
Component C	SIRT2, Human Recombinant	1 mg/mL, 10 µL
Component D	Assay Buffer	20 mL
Component E	NAD+	100 mM, 50 µL
Component F	Nicotinamide (Sirtuin Inhibitor)	30 mM, 0.5 mL
Component G	SIRT2 Developer (10X)	0.5 mL

Other Materials Required (but not provided)

- Microplate: Black, flat-bottom 96-well plate.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.

Storage and Handling

- Store all kit components at -20°C, except Component C.
- Store Component C at –80°C. Aliquot as needed to avoid freeze-thaw cycles.
- Protect Components A and B from light and from moisture.
- Component D can be stored at room temperature for convenience.

Introduction

Histone deacetylases (HDACs) act as transcriptional repressors of genes catalyzing the removal of acetyl groups from a ε-N-acetyl lysine of histone.[1] Sirtuins comprise a unique class of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases (class III HDACs) that target multiple protein substrates to execute diverse biological functions. Sirtuins catalyze a reaction that couples lysine deacetylation to NAD hydrolysis, yielding O-acetyl-ADP-ribose and nicotinamide.[2]

Sirtuin 2 (SIRT2) belongs to the family of Sir2 (Silent Information Regulator 2) proteins. Substrates for SIRT2 are not limited to histones but also include various transcription factors such as forkhead box (FOXO) transcription factors (FOXO1, FOXO3a) that modulate metabolic, cell cycle and cell death related pathways.[3, 4] Other prominent substrates include the tumor suppressor p53 and alpha-tubulin.[5, 6] Human SIRT2 is a cytoplasmic protein that increases in abundance during mitosis and regulates major events of cytokinesis.[7]

The SensoLyte® 520 FRET SIRT2 Assay Kit provides a homogeneous, two-step procedure for measuring sirtuin 2 activity. It contains a QXL™ 520/5-FAM FRET substrate. The sequence is derived from a human alpha-tubulin sequence surrounding the deacetylation site of SIRT2. In the first step an acetylated substrate is incubated with sirtuin-containing samples. In the second step, deacetylated substrate is cleaved by the sirtuin developer, resulting in an increase of fluorescence that can be detected with excitation at 490 nm and emission at 520 nm. Fluorescence produced is proportional to SIRT2 activity. The kit is ideal for high throughput screening of SIRT2 inhibitors and activators. The long wavelength fluorescence of 5-FAM is less interfered by the autofluorescence of cell components and test compounds.

Protocol

Note 1: For standard curve, please refer to the Appendix.
Note 2: Avoid protease inhibitors in the samples.

1. Prepare working solutions.

Note: Bring all kit components to room temperature before starting the experiment. Component C should be kept on ice after thawing.

1.1 SIRT2 FRET substrate solution: Dilute SIRT2 substrate (Component A) and NAD+ (Component E) 100-fold in assay buffer (Component D). For each experiment, prepare fresh substrate solution.

Table 1. SIRT2 FRET substrate solution for one 96-well plate (100 assays)

Components	Volume
SIRT2 FRET substrate (100X, Component A)	50 µL
NAD+ (100X, Component E)	50 µL
Assay buffer (Component D)	4.9 mL
Total volume	5 mL

1.2 SIRT2 diluent: Dilute SIRT (Component C) 40-fold in assay buffer (Component D).

Note 1: The amount of SIRT2 (Component C) provided in this kit is enough to serve only as a positive control. More human recombinant SIRT2 may be ordered from AnaSpec (Cat#72187).

Note 2: Prepare enzyme diluents immediately before use. Do not vortex the enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.3 1X developer: Dilute the developer (Component G) and the nicotinamide (Component F) in assay buffer (Component D). Both developer and nicotinamide should be diluted 10-fold in assay buffer. Each well requires 50 µL of developer solution.

Table 2. 1X developer solution for one 96-well plate (100 assays)

Components	Volume
SIRT2 developer (10X, Component G)	500 µL
Nicotinamide (Component F)	500 µL
Assay buffer (Component D)	4 mL
Total volume	5 mL

Note 1: The developer, containing nicotinamide, is a bi-functional buffer, which works as a stop solution for SIRT2 and initiates fluorescent signal releasing fluorophore.
Note 2: Prepare developer before use. Otherwise keep prepared solution on ice until use.

2. Set up the enzymatic reaction.

2.1 Add test compounds and SIRT2 diluent to the microplate wells. For one well of a 96-well plate, the suggested volume of enzyme solution is 40 µL and 10 µL of test compound.

2.2 Establish the following control wells at the same time, as deemed necessary:

- Positive control contains SIRT2 enzyme without test compound.
- Inhibitor/activator control contains SIRT2 enzyme and SIRT2 inhibitor/activator.
- Vehicle control contains SIRT2 enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer (Component D) and test compound. Some test compounds may themselves be fluorescent and thereby give false results.

Note: Test compound can be additionally tested for interference with fluorophore (see Appendix).

- Substrate control contains assay buffer (Component D).

2.3 Using the assay buffer (Component D), bring the total volume of all controls to 50 µL.

2.4 Pre-incubate the plate for 10 min at 37°C.

3. Detect SIRT2 activity.

3.1 Add 50 µL of the prepared SIRT2 substrate solution into each well, except the test compound control wells. Mix the reagents completely by shaking the plate gently for no more than 30 sec.

3.2 Incubate the plate for 30-60 minutes at 37°C.

3.3 Add 50 µL of the prepared developer solution and mix thoroughly.

3.4 Incubate the plate an additional 10 min at 37°C.

3.5 Measure fluorescence signal at Ex/Em=490 nm/520 nm.

3.6 Data analysis:

- The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).
- Plot data as RFU versus concentration of test compounds.
- A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

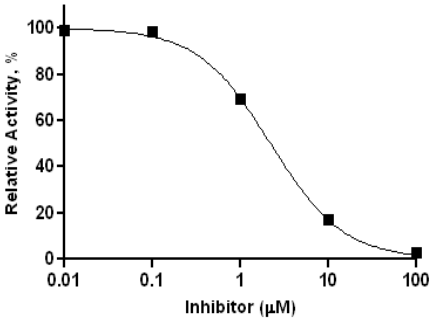

Figure 1. Inhibition of SIRT2 activity by Ro-31-8220 as measured with SensoLyte® 520 FRET SIRT2 Assay Kit.

Appendix: Instrument Calibration

- Deacetylated reference substrate: Dilute 1 mM (Component B) to 10 μM in assay buffer (Component D). Do 2-fold serial dilutions to get concentrations of 5, 2.5, 1.25, 0.625, 0.313, and 0.156, include an assay buffer blank. Add 50 μL/well of these serially diluted deacetylated 520 reference solutions.
- Add 50 μL/well of the diluted SIRT2 substrate solution (refer to Protocol, step 1.1 for preparation).

  Note: The addition of SIRT2 substrate solution to the reference standard is to correct for the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- For proper comparison, add 50 μL/well of developer solution (refer to Protocol, Step 1.3 for preparation). Mix the reagents by shaking the plate gently for 3 to 5 sec.
- Incubate the plate for an additional 10 min at 37°C.
- Measure the fluorescence intensity of the reference substrate and substrate control wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the deacetylated FRET fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.

Note: The final concentrations of reference substrate are 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078 and 0 μM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

- If testing compounds for interference with developer solution, use deacetylated substrate at concentration that provides signal comparable to positive control. After incubation of deacetylated substrate with assay buffer or test compound, proceed with the addition of developer solution. This will allow discrimination between SIRT2 inhibition/activation versus interference with the developer.

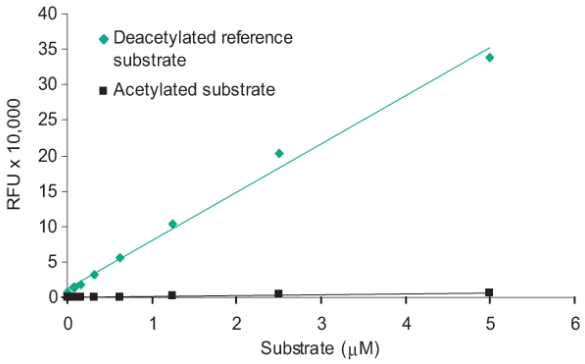

Figure 2. Deacetylated FRET reference substrate. Deacetylated substrate was serially diluted with assay buffer containing acetylated FRET SIRT2 substrate, and after a 10 min incubation with developer, fluorescence was recorded at Ex/Em=490/520 nm (Flexstation 384II, Molecular Devices). Acetylated FRET SIRT2 substrate was also titrated separately and incubated with developer.

References:

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