SensoLyte® Green SIRT1 Assay Kit

Fluorimetric

Revision Number: 1.2 Last updated November 18-2014

Catalog #	72156
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect SIRT1 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	SIRT1 substrate	1 mM, 50 µL
Component B	Deacetylated standard	1 mM, 20 µL
Component C	SIRT1, Human Recombinant	0.025 mg/mL, 400 µL
Component D	Assay Buffer	20 mL
Component E	NAD+	20 mM, 100 µL
Component F	Nicotinamide (Sirtuin Inhibitor)	30 mM, 0.5 mL
Component G	SIRT1 Developer (10X)	0.5 mL

Other Materials Required (but not provided)

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

Storage and Handling

- Store all kit components, except Component C, at -20°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 1 (SIRT1), the human homolog of yeast Sir2 (Silent Information Regulator 2), is the most studied of the seven members of sirtuin family. SIRT1 have been implicated in several important cellular processes, including genomic stability and DNA repair,[3,4] p53-mediated apoptosis,[5] adipogenesis,[6] and aging.[7, 8]

The SensoLyte® Green SIRT1 Assay Kit provides a convenient, two-step homogeneous procedure for measuring sirtuin 1 activity and screening of enzyme inhibitors and activators. The fluorogenic peptide substrate in this kit is derived from p53 sequence. In the first step, an acetylated substrate is incubated with sirtuin-containing samples. Deacetylation of substrate sensitizes it to the sirtuin developer, which, in the second step, releases the green fluorophore. Fluorescence produced is proportional to SIRT1 activity and can be detected with excitation at 490 nm and emission at 520 nm.

Protocol

Note 1: For standard curve, please refer to 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 SIRT1 substrate solution: Dilute SIRT1 substrate (Component A) and NAD+ (Component E) in assay buffer (Component D). Both SIRT1 substrate and NAD+ should be diluted in assay buffer 100-fold. For each experiment, prepare fresh substrate solution.

Table 1. SIRT1 substrate solution for one 96-well plate (100 assays)

Components	Volume
SIRT1 substrate (100X, Component A)	50 µL
NAD+ (Component E)	50 µL
Assay buffer (Component D)	4.9 mL
Total volume	5 mL

1.2 SIRT1, human recombinant enzyme (Component C): Ready to use. For positive control, use 40 µL Component C per well.

Note 1: The amount of SIRT1 (Component C) provided in this kit is enough to serve only as a positive control. More human recombinant SIRT1 may be ordered from AnaSpec (Cat#72212).
Note 2: Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the 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 assay requires 50 µL of developer solution.

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

Components	Volume
SIRT1 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 SIRT1 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 SIRT1 enzyme to the microplate wells. For one assay in 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 SIRT1 enzyme without test compound.
- Inhibitor/activator control contains SIRT1 enzyme and SIRT1 inhibitor/activator.
- Vehicle control contains SIRT1 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 developer solution (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 SIRT1 activity.

3.1 Add 50 µL of the prepared SIRT1 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.

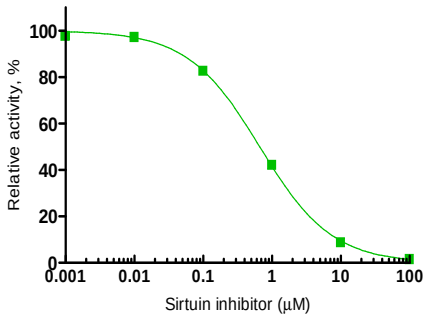

Figure 1. Inhibition of SIRT1 by Ro-31-8220.

Appendix: Instrument Calibration

- Deacetylated standard: Dilute 1 mM of deacetylated standard (Component B) to 10 µM in assay buffer (Component D). Do 1:2 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 standard solutions.
- Add 50 µL/well of the diluted SIRT1 substrate solution (refer to Protocol, step 1.1 for preparation).
- Add 50 µL of developer solution (refer to Protocol, step 1.3) to each well. 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 standard 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 reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.

Note: The concentration of deacetylated reference standard solutions 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 enzymatic reaction final product.

- If testing compounds for interference with developer solution, use deacetylated standard at concentration that provides signal comparable to positive control. After incubation of deacetylated substrate with assay buffer or test compound, proceed to the addition of developer solution. This will allow discrimination between SIRT1 inhibition/activation versus interference with the developer.

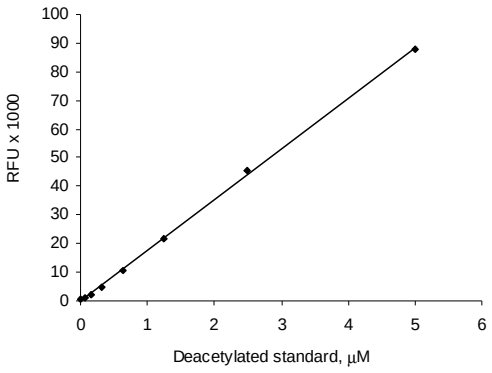

Figure 2. Deacetylated reference standard. Deacetylated standard was serially diluted with assay buffer containing SIRT1 substrate, and after 10 min incubation with developer, fluorescence was recorded at Ex/Em=490/520 nm. (Flexstation 384II, Molecular Devices).

References:

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