SIRT1 Activity Assay Kit (Fluorometric)

Cat. No. KITZ-001 **Lot. No.** (See product label)

SPECIFICATION

Product Overview	SIRT1 Activity Assay Kit (Fluorometric) detects SIRT1 activity in lysates. Primarily, the SIRT1 Activity Assay Kit is designed for the rapid and sensitive evaluation of SIRT1 inhibitors or activators using crude SIRT1 fraction or purified SIRT1. Additionally, any cultured primary cell, cell line, or tissue homogenate can be assayed for SIRT1 activity with the SIRT1 Activity Assay Kit if the appropriate antibody directed against SIRT1 (Anti-SIRT1 antibody) is used for immunoprecipitation. The kit has been shown to detect the activity of Sirtuins, at least SIRT1 in Human or animal cell lysates or in column fractions. The assay shows good linearity of sample response. The assay may be used to follow the purification of Sirtuins or may be used to detect the presence of Sirtuins in cell lysates.
Applications	1.Monitoring the purification of SIRT1. 2.Screening inhibitors or activators of SIRT1. 3.Detecting the effects of pharmacological agents on SIRT1.
Storage	Store the developer and recombinant SIRT1 at -80°C, all other kit reagents should be stored below -20°C.
Kit Components	1.SIRT1 Assay Buffer, 2 x 1 mL, -20°C 2.Fluoro-Substrate Peptide (0.2 mM), 500 µ, -20°C 3.Fluoro-Deacetylated Peptide (0.2 mM), 100 µ, -20°C 4.NAD (2 mM), 500 µ, -20°C 5.Developer, 500 µ, -80°C 6.Recombinant SIRT1, 500 µ, -80°C

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	7.Stop Solution, 2 x 1 mL, 20°C
Materials Required but Not Supplied	1.96 well plate – black wells 2.MilliQ water or other type of double distilled water (ddH2O) 3.Microcentrifuge 4.Pipettes and pipette tips 5.Microplate reader capable of measuring fluorescence at Ex/Em = 340-360/ 440-460 nm 6.Orbital shaker 7.500 or 1000 mL graduated cylinder 8.Reagent reservoirs For SIRT1 Activity in an Immunoprecipitate: 1.Cell Lysis Buffer (1X): 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT. 2.Protein A Agarose Beads
Detection method	Fluorescent reader (Ex/ Em = 350/ 460 nm)
Compatible Sample Types	Cell culture extracts, Tissue Extracts
Assay Protocol	SIRT1 Activity Assay Kit (Fluorometric) can measure the enzyme activity of SIRT1 with a homogeneous method. In this method, the reaction is initiated and the fluorescence intensity is measured by mixing simultaneously fluorescence-labeled acetylated peptide, which is substrate, SIRT1, trichostatin A, NAD and lysylendopeptidase. Since the reaction is not stopped, it is necessary to measure fluorescence intensity at regular intervals after the reaction is initiated, and to determine reaction velocity. Alternatively, within a time in which the reaction velocity is kept constant, it is also possible to stop the reaction by adding 2X stop solution and to measure fluorescence intensity.

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Assay for Quantification of SIRT1 Activity:

1.Following the table below and in duplicate, add ddH2O, SIRT1 Assay Buffer, Fluoro-Substrate Peptide and NAD to microtiter plate wells.

2.Add Developer to each well of the microtiter plate and mix well.

3.Initiate reactions by adding 5 µ of your Enzyme Sample or Buffer of Enzyme Sample or Recombinant SIRT1 to each well and mixing thoroughly at room temperature.

NOTE: Although the volume of addition of Enzyme Sample or Buffer of Enzyme Sample or Recombinant SIRT1 is set to 5 µ in the previous table, it may be changed to a volume up to 20 µ at your discretion. In that case, please reduce the volume of Distilled water to set the final reaction volume of 50 µ.

4.Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minutes intervals using microtiter plate fluorometer with excitation at 340-360 nm and emission at 440-460 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Note: More information on Sample preparation in Sample Preparation Section

Alternative procedure:

1.Follow procedure described above till step 4.
2.While the reaction rate is kept constant, add 20 µ of Stop Solution to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader capable of excitation at a wavelength in the range 340-360 nm and detection of emitted light in the range 440-460 nm.
NOTE: During the time in which SIRT1 reaction rate is maintained, the difference in fluorescence intensity between Enzyme Sample Assay and No Enzyme Control Assay indicates the SIRT1 activity of your Enzyme Sample.
If enzyme samples contain some protease/peptidase able to break down Fluoro-Substrate Peptide, resulting in an increase of fluorescence intensity in No NAD Control Assay, the SIRT1 activity in the samples cannot be evaluated correctly.
If enzyme samples contain inhibitors for protease/peptidase, precise SIRT1 enzyme activity cannot be measured. Since protease/peptidase inhibitors used in the usual protein purification process strongly inhibit the peptidase activity in the development reaction, please avoid using any protease/peptidase inhibitors during the process of protein purification.
If enzyme samples have an inhibitory effect on the peptidase in the development reaction, the final fluorescence intensity will not increase. Please use Fluoro-Deacetylated Peptide instead of Fluoro-Substrate Peptide, and conduct a control experiment.
Assay for SIRT1 Inhibitor/Activator Screening:
1.Following the table on the next page and in duplicate, add ddH2O, SIRT1 Assay Buffer, Fluoro-Substrate Peptide or Fluoro-Deacetylated Peptide and NAD to microtiter plate wells.
2.Add Test Compound (inhibitor compound to test) or just the Solvent in which compound is dissolved (control) or Control Compound (not provided) to each well of the microtiter plate and mix.
3.Add Developer to each well of the microtiter plate and mix well.
4.Initiate reactions by adding 5 µ of your Recombinant SIRT1 or your Enzyme Sample to each well and mix thoroughly at RT.

NOTE: Although the volume of addition of Recombinant SIRT1 or your Enzyme Sample is set to 5 µ in above tables, it may be changed to a volume up to 20 µ at your discretion. In that case, please reduce the volume of ddH2O to set the final reaction volume of 50 µ.

5.Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minutes intervals using microtiter plate fluorometer with excitation at 340-360 nm and emission at 440-460 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Note: More information on Sample preparation in Sample Preparation Section

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1.Follow procedure described above till step 4.
2.While the reaction rate is kept constant, add 20 µ of Stop Solution to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader at Ex/Em = 350-380 nm/440-460 nm.
NOTE: Although the above tables indicate the volume of addition of Test Compound or Solvent of Test Compound or Control Compound (not provided) as 5 µ, the concentration and the volume of the reagents to add can be changed so that the concentration of test compounds becomes the setting concentration. For example, since the final volume of reaction is 50 µ here, it is also possible to add 10 µ of Test Compound or Solvent of Test Compound or Control Compound (not provided). In this case, please reduce the volume of Distilled water to set the final reaction volume of 50 µ.
During the time in which SIRT1 reaction rate is maintained, the difference in fluorescence intensity between Solvent Control Assay and No Enzyme Control Assay indicates the SIRT1 activity.
In order to estimate the active or inhibitory effect on SIRT1 activity by the test compounds correctly, it is necessary to conduct the control experiment of Solvent Control Assay at least once for every experiment and Control Compound Assay at least once for the first experiment, in addition to Test Compound Assay as indicated in the Table.2. When test compounds cause an active or inhibitory effect on SIRT1 activity, the level of increase of fluorescence intensity is strengthened or weakened as compared with Solvent Control Assay.
The efficacy of the test compounds on the SIRT1 activity is the difference in fluorescence intensity between Test Compound Assay minus No Enzyme Control Assay and Solvent Control Assay minus No Enzyme Control Assay.
If test compounds have an inhibitory effect on protease/peptidase, resulting that the increase in fluorescence intensity is not or a little observed in Development Control Assay, the effect on SIRT1 activity cannot be evaluated correctly.
SIRT1 Activity in An Immunoprecipitate:
Reagents Required:

1.Cell Lysis Buffer (1X): 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT.
2.Protein A Agarose Beads: Add 5 mL of 1X PBS to 1.5 g of Protein A Agarose Beads. Shake 2 hours at 4°C; spin down. Wash the pellet twice with PBS. Re-suspend beads in 1 volume of PBS (can be stored for 2 weeks at 4°C).

Preparing Cell Lysates

1.Aspirate media. Treat cells by adding fresh media containing test compound for desired time.
2.To harvest cells under non-denaturing conditions, remove media and rinse cells once with ice-cold PBS.
3.Remove PBS and add 0.5 mL 1X ice-cold Cell Lysis Buffer to each plate (10 cm dish) and incubate the plate on ice for 5 minutes.
4.Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5.Sonicate 4 times for 5 seconds each on ice.
6.Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at –80°C.

Immunoprecipitation

1.Take 200 µ cell lysate and incubate with a suitable anti-SIRT1 antibody according to the manufacturer's instructions.
2.Add Protein A agarose beads (20 µ of 50% bead slurry). Incubate with gentle rocking for 1-3 hours at 4°C.
3.Microcentrifuge for 30 seconds at 4°C. Wash pellet 3 times with 500 µ of 1X Cell Lysis Buffer and once with 500 µ of SIRT1 assay buffer (50 mM Tris-HCl (pH 8.8), 0.5 mM DTT). Keep on ice during washes.
4.After immunoprecipitation, add reaction mixture containing Fluoro-Substrate peptide solution to Protein A agarose beads as an "Enzyme Sample" and measure NAD dependent deacetylase activity according to the procedure in Section 8.1 1. Assay for Quantification of SIRT1 Activity.

Sample Preparation:

Numerous extraction and purification methods can be used to isolate SIRT1. The

following protocols have been shown to work with number of different cells and enzyme sources and are provided as examples of suitable methods. Crude samples can frequently be used without dilution while more concentrated or highly purified SIRT1 should be diluted.

It is strongly advised that the user always perform an initial experiment to determine the proper dilution to be used in subsequent experiments. This need not be any more than a single time point assay using serial dilutions of the crude extract, cell lysate or sample fraction taken prior to a purification step. All sample preparation should be performed at 4°C and recovered fractions should be kept at -80°C to prevent loss of enzymatic activity.

A.Buffer Preparation

B.Isolation of Nuclei

1.Resuspend 1x 107 cells into 1 mL of lysis buffer.

2.Vortex for 10 second.

3.Keep on ice for 15 min.

4.Spin the cells through 4 ml of sucrose cushion at 1,300 x g for 10 min at +4°C.

5.Discard the supernatant.

6.Wash the nuclei pellet once with cold 10 mM Tris HCl (pH7.5), 10 mM NaCl.

C.Extraction of Nuclei

1.Resuspend the isolated nuclei in 50-100 µ of extraction buffer.

2.Sonicate for 30 seconds.

3.Stand on ice for 30 min.
4.Centrifuge at 20,000 x g for 10 min.
5.Take supernatant (the crude nuclear extract).
6.Determine protein concentration by Bradford method or equivalent.
7.Store the crude nuclear extract at -80°C until use.

Troubleshooting:

1.When chemicals that have an inhibitory effect on the peptidase are mixed in a crude SIRT1 fraction purified from various cells or the immunoprecipitate using a specific antibody against SIRT1 or other proteins, precise SIRT1 enzyme activity cannot be measured. Since the protease/peptidase inhibitors used in the usual protein purification process inhibit the peptidase activity strongly, please avoid the use of any protease/peptidase inhibitors during the protein purification process.
2.Final fluorescence intensity will not increase, both when test chemicals have an inhibitory effect on SIRT1, also when there is an inhibitory effect on the peptidase.
3.If the test reagents themselves emit fluorescence at excitation wavelength: 340-360 nm and fluorescence wavelength: 440-460 nm, the inhibitory effect of the test assay cannot be evaluated correctly.
4.The recombinant SIRT1 should be run in duplicate, using the protocol described in the Assay Protocol. Incubation times or temperatures significantly different from those specified may give erroneous results.
5.The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to 29 non-linearity of the curve, as can assay kinetics that are other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.
6.Poor duplicates indicate inaccurate dispensing. If all instructions in the Assay Protocol were followed accurately, such results indicate a need for multi-channel pipettor maintenance.

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