User Manual

Universal Continuous Sirtuin Assay Kit

Universal Substrate for Sirtuin 1-6 Activity Assays

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Please read the entire Manual before starting your Experiments!
Carefully note the handling and storage conditions.
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1 Introduction

1.1 Kit Contents

Component	Quantity	Format
Sirtuin substrate	3*100µg (200 µM)	TNFα-derived peptide with fluorescently labeled lysine acyl residue

1.2 Storage

Store in a cool (approx. 4°C / 39°F) and dry environment.

1.3 Additional Materials Required

- **Microplate reader** for fluorescence measurements at $\lambda_{excitation}$=320nm and $\lambda_{emission}$=408nm
- **96-well microtiter plates** (black, flat bottom)
- **Assay buffer**
  20 mM TRIS/HCl (pH=7.8), 150 mM NaCl, 5 mM $MgCl_2$
- **NAD+** (oxidized nicotinamide adenine dinucleotide)
- **NAM** (nicotinamide (positive control))

1.4 Sample Material

Depending on your experimental design/ aims of the study: purified sirtuin(s) or lysates of cellular/ tissue samples containing intact sirtuins.

1.5 Product Specifications

This assay enables discovery and screening of potential SIRT1-6 modulators by measuring kinetic properties of the sirtuin-mediated deacylation reaction. The corresponding inhibitory constants (Ki) can be determined recording the initial reaction rates under NAD+ saturation in the presence of varying substrate concentration (0-100 µM) and in combination with different amounts of a potential modulator (at least three concentrations are recommended).
The assay protocol includes the following steps: assay calibration, sirtuin activity measurement and data analysis.

1.6 Principle and Procedure

Background

Silent information regulator 2 (SIR2) proteins, or sirtuins, are found in organisms ranging from bacteria to humans and represent a family of enzymes with NAD+-dependent lysine deacylase activity [1].
The seven mammalian sirtuins, SIRT1-7, share a highly conserved NAD+-binding catalytic core domain although they exhibit distinct expression patterns, catalytic activities, and biological functions [2]. SIRT1 and -2 have a huge variety of targets in the cytoplasm and nucleus, whereas SIRT3, -4 and -5 deacylate proteins only in mitochondria [3]. SIRT6 and -7 are deacylases localized in the nucleus [4,5].
Sirtuins catalyze the removal of acetyl and different acyl moieties from lysine side chains including propionylations, succinylations, malonylations, glutarylations, crotonylations, butyrylations and myristoylations [2].
By deacylating different substrate proteins, including histones, transcription factors, and metabolic enzymes, sirtuins regulate various biological processes, such as transcription, cell survival, longevity, DNA damage and repair [6]. Sirtuins play an important role in many diseases, among them diabetes, cancer and neurodegeneration [2] that makes these enzymes attractive targets for drug discovery.
This assay was developed in the laboratory of Prof. Mike Schutkowski, Department of Enzymology, Institute of Biochemistry and Biotechnology, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Strasse 3, 0610 Halle (Saale), Germany [7].

References

1. North, B. et al. (2004) "Sirtuins: Sir2-related NAD-dependent protein deacetylases", Genome Biol 5: 224.
2. Anderson, K. A. et al. (2014) "SnapShot: Mammalian Sirtuins", Cell 159: 956–956.
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4. Jiang, H. et al. (2013) "Sirt6 Regulates TNFα Secretion via Hydrolysis of Long Chain Fatty Acyl Lysine" Nature 496: 110–113.
5. Barber, M. F. et al. (2012) "SIRT7 Links H3K18 Deacetylation to Maintenance of Oncogenic Transformation", Nature 487: 114–118.
6. Hu, J. et al. (2014) "Sirtuin Inhibitors as Anticancer Agents", Future medicinal chemistry 6: 945–966.
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Assay Principle

A universal substrate is designed for a systematic discovery of sirtuin 1-6 modulators and enables monitoring effects of pharmacological compounds on SIRT1-6 activity or enzyme purity analysis. The assay allows for a direct and continuous quantification of the deacylase activity of recombinant human sirtuins 1-6 utilizing a fluorescent signal readout.
The provided substrate is a novel TNFα-derived peptide containing a fluorescently labeled acyl residue at the lysine side chain (Figure 1) as a well-recognized universal substrate for human sirtuins 1-6. More information can be found in [7].

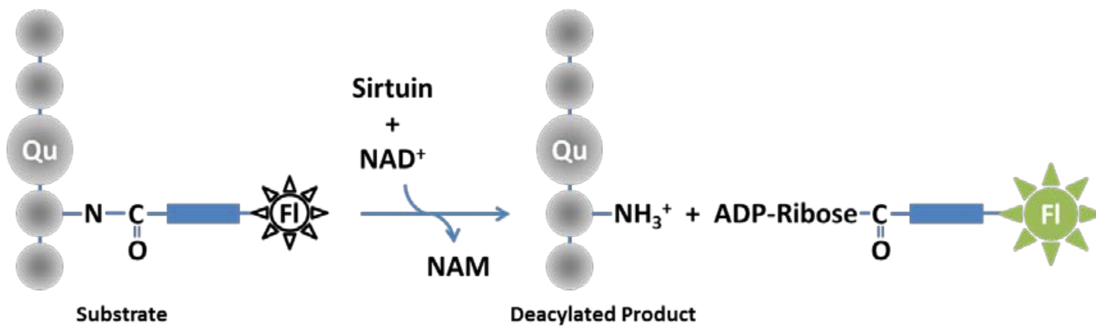


Figure 1. Assay principle: a sirtuin-mediated deacylation reaction removes a fluorophore from the vicinity of a quenching moiety leading to an increase of fluorescence signal. Qu - quencher, Fl - fluorophore, NAM - nicotinamide.

The substrate emits no fluorescence while the fluorophore is quenched by a quenching moiety within the substrate. In the presence of oxidized nicotinamide adenine dinucleotide (NAD+), a sirtuin-mediated deacylation reaction transfers fluorescently labeled acyl residue from lysine side chain to ADP-ribose and generates increase in fluorescent signal proportionally to the amount of deacylated reaction product.

2 Experimental Protocols

2.1 Assay calibration

Calibration lines provide a relationship between the amounts of deacylated reaction product and the fluorescence intensity measured.

1. To obtain a 200µM substrate stock solution, dissolve the content of one substrate vial (100µg) in 375 µl assay buffer.
2. Prepare the reaction mixture in assay buffer as shown in the table below:

Component	Add from stock [µl]	Working concentration [µM]
Assay buffer	15	-
SIRT	40	2
Sirtuin substrate	60	100
NAD^+	5	500

Table 1: *Assay components, exemplary volumes and respective concentrations.*

The enzymatic reaction is initiated by adding *NAD^+*.

3. Let the reaction proceed for 30 min at 37°C in the dark until the complete turnover of the peptide substrate.
4. Prepare six 1:4 serial dilutions (recommended; other dilution factors can be applied) of the reaction mixture in *Assay buffer* and transfer them into a 96-well microtiter plate. The resulting concentration of the deacylated product will be 100, 25, 6.25, 1.56, 0.39 and 0.098 µM, respectively. Use *Assay buffer* only as a blank sample.
5. Measure fluorescence intensity by microplate reader at $\lambda_{excitation}$=320nm and $\lambda_{emission}$=408nm.
6. Plot the calibration line as measured fluorescence intensity (arbitrary units) versus the applied product concentration as depicted in Figure 2.

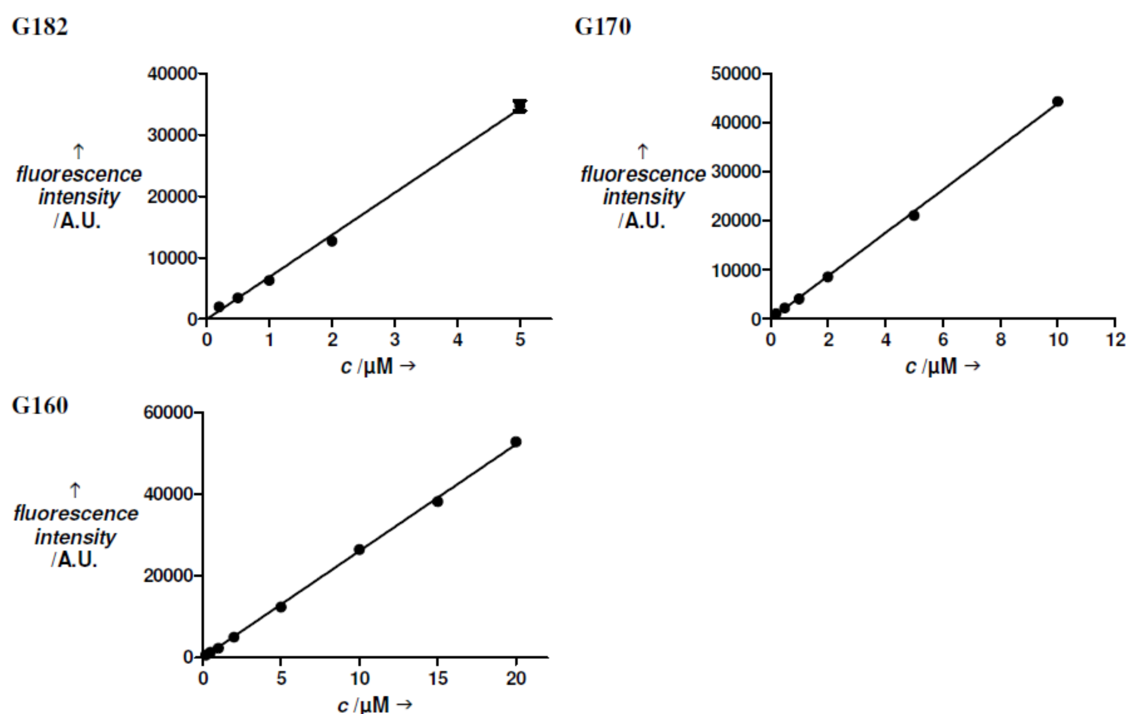


Figure 2. *Calibration lines generated using serial dilutions of the reaction mixture (2 µM SIRT2, 500 µM NAD^+ and 100 µM Sirtuin substrate) after the complete substrate turnover. Fluorescence measurements were performed by Tecan infinite M200 microplate reader at $\lambda_{excitation}$=320nm and $\lambda_{emission}$=408nm (lag time 9 µs, integration time 20 µM, gain 182 (G182), 170 (G170) and 160 (G160)).*

2.2 Sirtuin Activity Measurement

For drug discovery efforts, to analyze the inhibitory potency of sirtuin modulators different approaches can be followed. Frequently, dose-response measurements to calculate the inhibitor concentration that generates half-maximum inhibitory effect (IC50) are performed. However, the obtained IC50 value depends on substrates used and in many cases (e.g. tight-binding inhibitors) does not reflect the real inhibitory potency of enzyme modulators. This protocol allows for estimating the inhibition constant Ki as an intrinsic molecular measure of the inhibitory potency by calculating initial rates of enzymatic reaction.
The assay can be performed as screening experiment to discover potential sirtuin modulators. In this case, the inhibitory potency of up to four compounds can be investigated in parallel on the same microtiter plate. For more precise estimation of the inhibition constant Ki, a higher number of serial dilutions of the test substance can be applied. A recommended experimental design to generate required reaction conditions is shown in Figure 3.

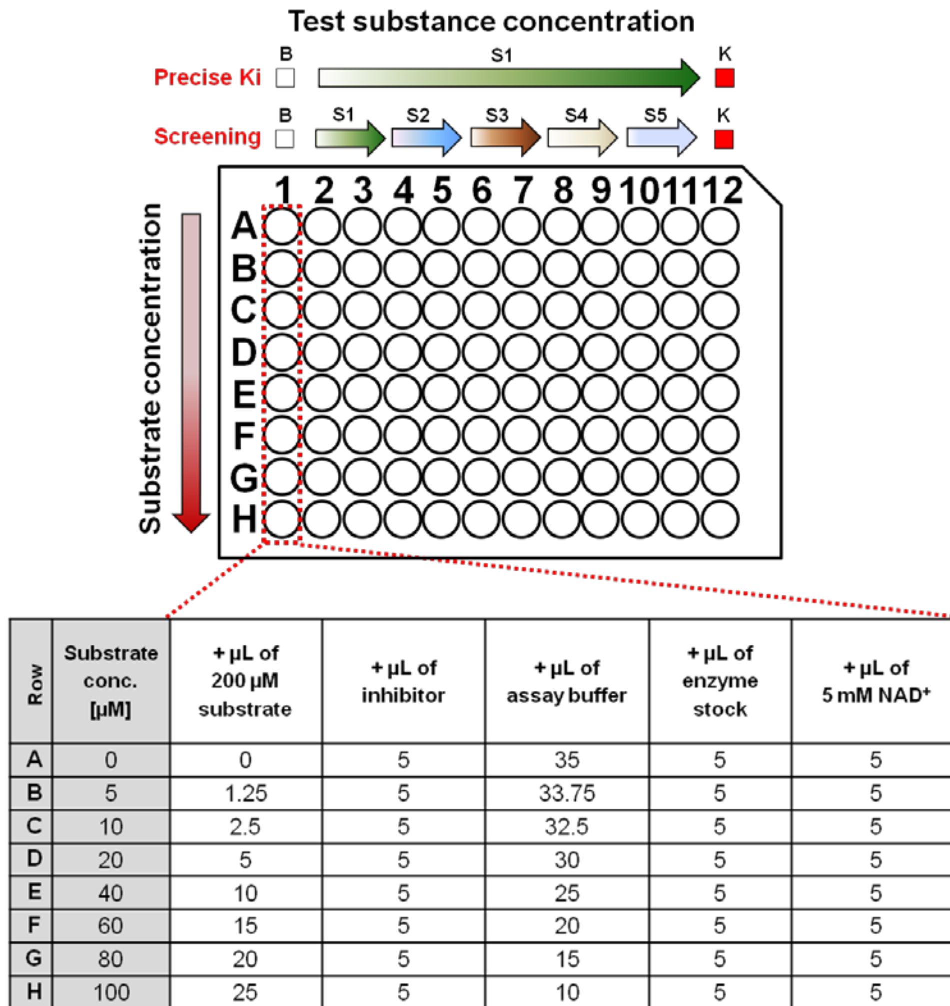
1. Prepare serially diluted 10-fold stock solutions of your test substance(s) in Assay Buffer. Use Assay Buffer only for a zero concentration of the test substance. Prepare 50 µl of a 6 mM NAM or other positive control of your choice.
2. Prepare stock solutions of sirtuin(s). The proposed working concentrations are shown in table 2:

Enzyme	Working concentration [µM]	Stock solution [µM]
SIRT1	0.5	5
SIRT2	0.01	0.1
SIRT3	0.1	1
SIRT4	1	10
SIRT5	0.5	5
SIRT6	0.5	5

3. Add assay components to the microtiter plate wells using the recommended volumes and order shown in Figure 3. Enzymatic reaction will be initiated by adding *NAD*$^+$. The total volume of each reaction mixture is 50 µl.
4. Perform a kinetic measurement of the fluorescence intensity by microplate reader at $\lambda_{excitation}$=320nm and $\lambda_{emission}$=408nm for at least 10 minutes with 1 minute-steps.

2.3 Data Analysis

1. Plot the reaction progress curve (fluorescence versus time) and ascertain the linear steady-state region.
2. Using linear regression calculate the initial reaction rates for all reaction conditions as [fluorescence intensity a.u./sec].
3. Convert [fluorescence intensity a.u./sec] into [µmol/sec] using assay calibration lines (see Figure 2).
4. Plot the calculated initial reaction rates for each test substance concentration versus the substrate concentration applied.
5. By non-linear curve fitting identify the appropriate model (case) of enzyme inhibition for each test substance. Using the model equation calculate the inhibitory constant Ki.



Row	Substrate conc. [µM]	+ µL of 200 µM substrate	+ µL of inhibitor	+ µL of assay buffer	+ µL of enzyme stock	+ µL of 5 mM NAD⁺
A	0	0	5	35	5	5
B	5	1.25	5	33.75	5	5
C	10	2.5	5	32.5	5	5
D	20	5	5	30	5	5
E	40	10	5	25	5	5
F	60	15	5	20	5	5
G	80	20	5	15	5	5
H	100	25	5	10	5	5

Figure 3. *Example of assay mixture composition. B - blank sample (no inhibitor), S - test substance, K - positive control (NAM).*

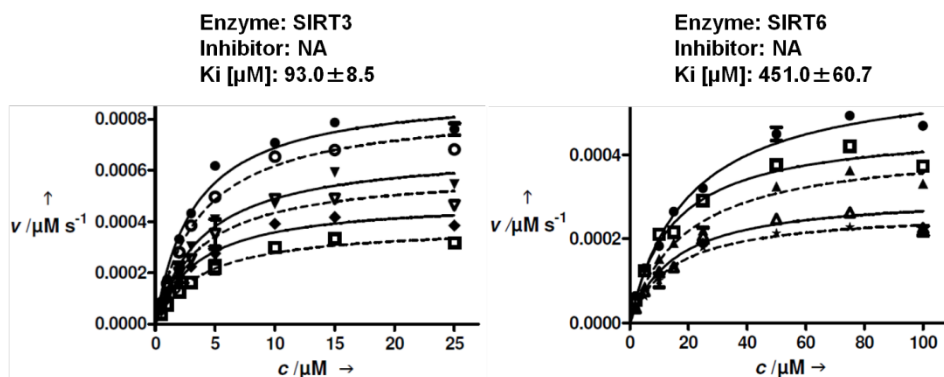


Figure 4.

Examples of K_i calculation for a specific inhibition of SIRT3 and SIRT6 by nicotinamide: Kinetic characteristics for non-competitive sirtuin inhibitor nicotinamide (NA). The reactions were performed with 0.1 µM Sirt 3 or 0.5 µM Sirt6, 500 µM NAD^+ and varying concentrations of sirtuin substrate (0.5-100 µM). K_i values were determined using different inhibitor concentrations (0, 25, 50, 75, 100, 125, 150, 300, 450, and 600 µM).

3 Contact Us

Technical Support	Address
Customer Support +49-30-322980-7878 peptide@jpt.com **Technical Support Europe** +49-30-322980-7830 peptide@jpt.com **Technical Support North America** 1.888.JPT.COM0 (1.888.578.2660) us-bd@jpt.com	JPT Peptide Technologies GmbH Hermann-Dorner-Allee 23 12489 Berlin Germany Visit our Website!

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