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Sirtuin 6 (SIRT6) Activity Assays

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Abstract

SIRT6 has been shown to possess weak deacetylation, mono-ADP-ribosyltransferase activity, and deacylation activity in vitro. SIRT6 selectively deacetylates H3K9Ac and H3K56Ac. Several SIRT6 assays have been developed including HPLC assays, fluorogenic assays, FRET, magnetic beads, in silico, and bioaffinity chromatography assays. Herein, we describe detailed protocols for the HPLC based activity/inhibition assays, magnetic beads deacetylation assays, bioaffinity chromatographic assays as well as fluorogenic and in silico assays.

Keywords

Deacetylation assays; Activation assays; Inhibition assays; Bioaffinity chromatography; In silico screening

1 Introduction

Sirtuins are NAD+-dependent histone deacetylase enzymes (HDACs) that function as regulators of many cellular processes [1], and are evolutionarily conserved. To date, seven sirtuins have been identified (SIRT1-7), with SIRT1-3 being the most studied. However, SIRT6 has gained interest in age-associated diseases due to its role genomic stability, oxidative stress, and glucose metabolism [2]. While other SIRTuins have been shown to be less selective in which acetylated peptides they deacetylate, SIRT6 has been shown to only deacetylate H3K9Ac and H3K56Ac. SIRT6 has also been shown to possess mono-ADP-ribosyltransferase activity [3] and deacylation activity [4]. In fact, SIRT6 preferentially hydrolyzes long-chain fatty acyl groups from lysines with much higher activity than its deacetylation activity in vitro [4].

Due to the increased interest in SIRT6, several assays have been developed as seen in Table 1, including HPLC assays [5, 6], fluorogenic assays [7 - 9], FRET [10], magnetic beads [11], in silico and bioaffinity chromatography [12]. Of these assays, the SIRT6 deacetylation assays coupled to HPLC-MS are the most commonly used. These assays have the added advantage in that they have been demonstrated to be robust in determining both the inhibition and activation activity of the tested compounds for SIRT6 and are discussed in greater detail below [6, 13]. The HPLC deacetylation assays, predominantly use H3K9Ac as the peptide substrate, and the activity is determined based on the quantification of the deacetylated peptide, H3K9 (Fig. 1). In order to study the deacylation activity of SIRT6, two different substrates were used TNFα (EALPK (Myr)TGGPQWW) [5] or H3K9myr [14]

containing the acyl lysine modifications. While these assays are equally important, they are not discussed here and we refer the readers to Ref. [7] and Ref. [13] for experimental details.

Fluorogenic assays for SIRT6 differ from the HPLC assays as they produce a fluorescent signal after deacetylation/deacylation-coupled proteolytic cleavage of the substrate. A potential disadvantage with this method may arise with the use of complex mixtures, as it may result in the assay becoming susceptible to autofluorescence, fluorescence quenching, or anti-proteolytic properties of unknown compounds in the complex mixture [10]. Kokkonen et al. (2014) profiled a collection of fluorogenic substrates using SIRT6 fluorogenic deacetylation assay for the identification of novel SIRT6 inhibitors, and this is discussed in greater detail below [9]. Currently, there are two commercially available kits for measuring SIRT6 activity: Biovision and Cayman. Biovision provides a fluorometric SIRT6 inhibitor screening kit, where the substrate is deacetylated, and fluoresces after cleavage of the peptide by a proteolytic enzyme following deacetylation (please see http://www.biovision.com). Cayman's SIRT6 Direct Fluorescent Screening Assay measures both SIRT6 activation and inhibition using a fluorescence-based method where a p53 sequence Arg-His-Lys-Lys(ε-acetyl)-AMC is incubated with SIRT6 and NAD+. Deacetylation sensitizes the substrate such that subsequent treatment with the developer releases a fluorescent product (please see www.caymanchemicals.com for more details).

In another approach, SIRT6 was immobilized onto the surface of magnetic beads and the deacetylation activity was determined by measuring the production of the deacetylated H3K9Ac peptide [11]. An added advantage of this method lies in its reusability and its use in ligand fishing from complex matrices including botanical extracts [15]. In order to study the binding affinities of tested compounds for SIRT6, the SIRT6 protein was subsequently immobilized onto the surface of an open tubular capillary [15]. The immobilized SIRT6 was well characterized using frontal affinity chromatography and it was demonstrated that the SIRT6-OT column correctly predicted a compounds deacetylation activity with a single chromatographic run [16].

More recently, an in silico method for the screening of SIRT6 inhibitors has also been developed [7]. The availability of the experimentally identified ligands for SIRT6 opened up the possibility to use ligand-based approaches for virtual screening. Ligand-based virtual screening methods can be an efficient approach to find novel regulators. Usually they can be used for screening large databases including millions of compounds, which cannot be done with structure-based methods. Thus ligand-based methods are widely used for identifying novel regulators. Ligand-based virtual screening usually produces chemically and structurally similar hits than already known hits. Therefore, the main drawback of ligand-based methods is its capability to identify structurally diverse scaffolds. This method has also been discussed in greater detail below.

2 Materials

2.1 SIRT6 Deacetylation Activation Assay [13]

1. Tris buffered saline (TBS) [25 mM, pH 8.0] containing 137 mM NaCl, 1 mM $MgCl_2$.

2. 10 mM NAD+ (NAD) in TBS (*see* Note 1).
3. 0.96 mM ARTKQTARK(Ac)STGGKAPRKQLA [H3K9Ac: residues 1–21].
4. GST-Tag SIRT6 (SIRT6-GST) (*see* Note 2): The human SIRT6 expression vector hSIRT6-pGEX-6P3 was kindly provided by Prof. Katrin Chua (Stanford, USA). The recombinant GST-tagged SIRT6 was produced by fermentation in *Escherichia coli* BL21(DE3)-pRARE. The production was done at +16 °C with 0.1 mM IPTG for 20 h and the soluble overexpressed protein was affinity purified on glutathione agarose.
5. Active His-tagged SIRT6 (SIRT6-HIS), human recombinant: SIRT6 protein is a solution of 1 mg/mL in Tris buffer [50 mM, pH 8.0] containing 100 mM NaCl and 20 % glycerol.
6. Zorbax Eclipse XDB-C18 column (4.6 mm × 50 mm, 1.8 μm).
7. HPLC: Shimadzu prominence system consisting of a CBM-20A, LC-20 AB binary pumps, an SIL-20 AC-HT autosampler and a DGU-20A3 degassing unit.
8. MS/MS: 5500 QTRAP from Applied Biosystems/MDS Sciex equipped with Turbo V electrospray ionization source (TIS)®.

2.2 SIRT6 Deacetylation Fluorogenic Assay [9]

1. Tris buffered saline (TBS) [50 mM, pH 8] containing 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$.
2. 50 mM NAD+ (NAD) in TBS.
3. 10 mM RYQK(Ac)-AMC (AMC) in TBS was purchased from CASLO ApS.
4. Developer solution (6 μg/μl trypsin, 4 mM NAD).
5. (SIRT6-GST): Please see **item 4** of Subheading 2.1.
6. Black 1/2 AREAPLATE-96 F.

2.3 SIRT6-Magnetic Beads Deacetylation Assay [11]

1. BcMag amine-terminated magnetic beads (50 mg/mL, 1 μm).
2. The manual magnetic separator Dynal MPC-S.
3. 2-(*N*-morpholino)ethanesulfonic acid buffer (MES) [100 mM, pH 5.5].
4. N-Hydroxysulfosuccinimide (Sulfo-NHS).
5. 1-ethyl-3-(3-methylaminopropyl)carbodiimide (EDC).

[1]NAD+ solution should be prepared fresh for each experiment.
[2]Avoid repeated cycles of freezing and thawing of GST-Tag SIRT6

6. Storage Buffer (SB): phosphate buffer [10 mM, pH 7.4] containing 150 mM NaCl and 0.02 % sodium azide.
7. HDAC assay buffer (Tris–HCl, [50 mM,pH 8.0] containing 150 mM NaCl, 1 mM DTT, and 0.2 mM NAD+).
8. ARTKQTARK(Ac)STGGKAPRKQLA [H3K9Ac: residues 1–21] in TBS.
9. Zorbax Eclipse XDB-C18 column (4.6 mm × 50 mm, 1.8 μm).
10. HPLC: Shimadzu prominence system consisting of a CBM-20A, LC-20 AB binary pumps, an SIL-20 AC-HT autosampler and a DGU-20A3 degassing unit.
11. 5500 QTRAP from Applied Biosystems/MDS Sciex equipped with Turbo V electrospray ionization source (TIS)®.
12. Mobile phase: Eluent A: water containing 0.02 % formic acid; Eluent B: acetonitrile containing 0.02 % formic acid.
13. SIRT6 protein [11]: The expression plasmid pMBP-SIRT6 encodes a maltose-binding protein (MBP)-SIRT6 fusion protein. It was created by amplifying the full-length chicken SIRT6 cDNA from DT40 cDNA using primers SIRT6F (5′-CGCGGATCCATGGCGGTGAATTACGC-3′)/ SIRT6R(5′CGCTCGAGTCAGGT GAGGAGAGGCTC-3′), digesting the PCR product with BamHI and XhoI, and ligating is into the BamHI/XhoI sites of pMH6.

2.4 SIRT6 Frontal Chromatographic Analysis [15]

1. Reagents similar to Subheading 2.3 unless otherwise specified.
2. Open tubular capillary (30 cm × 100 μm i.d.).
3. Rabbit peristaltic pump.
4. Frontal mobile phase: ammonium acetate [10 mM, pH 7.4]: methanol (90:10 v/v) containing 0.2 mM NAD+.(*see* Notes 3 and 4)
5. Agilent HPLC-MS: Series 1100 Liquid Chromatography/Mass Selective Detector equipped with a vacuum de-gasser (G1322 A), a binary pump (1312 A), an autosampler (G1313 A) with a 20 μL injection loop, a mass selective detector (G1946 B) supplied with atmospheric pressure ionization electrospra and an online nitrogen generation system.

2.5 SIRT6 In Silico-Screen [7]

1. The homology model for SIRT6 was constructed using the crystal structure of SIRT3 complexed with a substrate (PDB id: 3glr) as a template. The sequence identity between SIRT3 and SIRT6 was about

[3]Addition of NAD to the mobile phase to study the SIRT6-OT is necessary for correct ranking
[4]The ranking of a series of compounds on the SIRT6-OT column can only be determined by looking at the change in retention volume of a marker ligand, for example, quercetin, and not based on retention of the compound tested.

34 %. The homology model for SIRT6 was constructed with ORCHESTRAR tool in SYBYL-X version 1.2.

2. The protein structure was prepared for the molecular docking with protein preparation wizard in Schrödinger's Maestro (Schrödinger's Release 2011 Maestro version 9.2 LLC, New York, 2011). This procedure includes a short minimization (with convergence criteria of RSMD 0.30 Å) for heavy atoms with OPLS_2005 force field.

3. Several known SIRT1 and SIRT2 peptidic and pseudopeptidic inhibitors were selected for docking studies. The 3D structure of compounds were constructed with Discovery Studio (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2.5, San Diego: Dassault Systèmes, 2010) and MOE (Molecular Operating Environment (MOE) version 2010.10; Chemical Computing Group Inc., Canada, 2010) and refined with Ligprep (Schrödinger release 2011: Schrödinger, LLC, New York, 2011). All tautomeric states were determined at pH 7 ± 2.

3 Methods

3.1 SIRT6 Deacetylation Assay [13]

3.1.1 Acitvation Assay

1. 43.3 μL TBS is added to 3.6 μL NAD (final 0.6 mM), 6 μL 10 mM DTT, and 2.5 μL 0.96 mM H3K9Ac (final 40 μM).

2. 0.6 μL of DMSO is used as a control or 0.6 μL of tested compounds is dissolved in DMSO (*see* Note 5).

3. The reaction is started by the addition of 4 μl of SIRT6 protein (final 0.05 μg/μL) (SIRT6-GST or SIRT6-HIS).

4. The solution is incubated for 30 min at 37 °C.

5. The reaction is terminated with the addition of 6 μL cold (100 %) formic acid (final concentration 10 %).

6. The resulting solution is centrifuged at 16,100 × *g* on Eppendorf Microcentrifuge 5424 (SoCal BioMedical) for 15 min.

7. The samples are then collected and analyzed using a HPLC coupled to a 5500 QTRAP.

8. The chromatographic separation of H3K9 and acetylated H3K9 is achieved on a Zorbax Eclipse XDB-C18 column (4.6 mm × 50 mm, 1.8 μm) at room temperature. The mobile phase consists of Eluent A and B with the following gradient: 0–2.0 min, 0 % B; 2.0–10 min, 0—8 % B; 10–10.10 min, 8—80 % B; 10.10–12 min, 80 %; 12–12.1 min 80–0 % B;

[5]The SIRT6-magnetic beads should be tested periodically (once a week) to confirm that the SIRT6 activity is maintained at similar levels

15 min, 0 % B at 0.9 mL/min. The total run time is 15 min and the injection volume per sample is 20 μl.

9. Positive electrospray ionization data are acquired using multiple reaction monitoring (MRM). The TIS instrumental source settings for temperature, curtain gas, ion source gas 1 (nebulizer), ion source gas 2 (turbo ion spray), entrance potential, and ion spray voltage were 550 °C, 20 psi, 60 psi, 50 psi, 10 V, and 5500 V, respectively. The TIS compound parameter settings for declustering potential, collision energy, and collision cell exit potential were 231 V, 45 V, and 12 V, respectively, for H3K9Ac and 36 V, 43 V, and 12 V, respectively, for H3K9. The standards are characterized using the following MRM ion transitions: H3K9Ac (*m/z* 766.339 → 760.690) and H3K9 (*m/z* 752.198 → 746.717).

3.1.2 Inhibition Assay [13]

1. 36.4 μL of TBS is mixed with 3.6 μL NAD (final 0.6 mM), 6 μL 10 mM DTT, and 9.4 μL 0.96 mM H3K9Ac (final 150 μM) in a 1.5 mL Eppendorf tube.
2. 0.6 μL of DMSO is added to the control tubes and 0.6 μL of varying concentrations of tested compounds in DMSO is added to the eppendorf tube (*see* Note 5).
3. The reaction is initiated by the addition of 4 μL SIRT6 protein (GST or His-tagged) (final 0.05 μg/μL) and the solution is incubated for 60 min at 37 °C with 300 rpm rotation on the Thermo shaker incubator (Hangzhou Allsheng Instruments Co., Ltd).
4. The reaction is terminated by the addition of 6 μL 100 % cold formic acid (final concentration 10 %).
5. The samples are centrifuged at 16,100 × *g* for 15 min in Eppendorf Microcentrifuge 5424 (SoCal BioMedical) and the supernatant is analyzed as described above in **steps 7 - 9**.

3.2 SIRT6 Deacetylation Fluorogenic Assay [9]

1. 3.2 μL NAD (final 3.2 mM), 1.6 μL AMC (final 320 μM), and 2.5 μL inhibitor solution/DMSO are added to 38.7 μL of TBS, in a well plate (*see* Note 6).
2. 4 μL the SIRT6-GST is added to start the reaction.
3. The solution is incubated for 90 min at 37 °C.
4. 50 μL of developer is added to the solution for 30 min at room temperature to terminate the reaction.

[6]The immobilization of SIRT6-GST is not recommended for ligand fishing experiments, as the GST fusion protein may result in retention of a significant amount of compounds that have no affinity for the SIRT6 protein.

5. The activity is determined by measuring the fluorescence with excitation and emission wavelengths of 380 and 440 nm using EnVision 2104 Multilabel Reader.

3.3 SIRT6-Magnetic Beads Deacetylation Assay [11]

1. 0.5 mL (25 mg) of BcMag beads is rinsed with 1 mL of MES buffer.
2. After magnetic separation, the supernatant is discarded, and the BcMag beads are suspended in 300 μL of MES buffer containing 260 μg of SIRT6 protein.
3. 50 μL of a mixture of 10 mg of EDC and 15 mg of sulfo-NHS in 1 mL of water is added to the reaction mixture and vortexed for 5 min and left for 3 h at 4 °C with gentle rotation.
4. 20 μL of 1 M hydroxylamine is added for 30 min at 4 °C with gentle rotation.
5. The supernatant is discarded and the SIRT6 (CT)-MB is rinsed three times with 1 mL of SB.(*see* Notes 7 and 8)
6. SIRT6 (CT)-MB is incubated with 50 μL of HDAC assay buffer containing 5 μg of H3K9Ac for 4 h at 37 °C.
7. After magnetic separation, the supernatant is collected and analyzed using a HPLC coupled to a 5500 QTRAP.
8. The chromatographic separation of H3K9 and acetylated H3K9 is achieved as described in Subheading 3.1, **steps 8** and **9**.

3.4 SIRT6 Frontal Chromatographic Analysis [15]

1. An open tubular capillary (30 cm × 100 μm i.d.) is washed with MES buffer for 20 min using a Rabbit peristaltic pump with a setting of 85.
2. A 1 mL solution of MES containing 700 μL of SIRT6 (44 μg/mL) with 100 μl of EDC (500 mg/mL) and 50 μl of Sulpho-NHS (340 mg/mL) is passed through the column.
3. Both tips of the capillary are submerged into the solution for 18 h at 4 °C. After which MES buffer is passed through for 10 min. The column is now ready for analysis.
4. The SIRT6(CT)-OT column is connected to the Agilent HPLC-MS system.
5. Frontal mobile phase containing a series of concentrations of the tested compounds is delivered at 0.05 μL/min at room temperature for determination of the binding affinity (Kd).

[7]DMSO should be kept below 2 %.
[8]The use of black well plate is recommended for fluorogenic assay.

6. In order to study the quercetin binding site, a series of concentrations of the tested compounds is placed in the frontal mobile phase containing 5 μM Quercetin (Ki). Quercetin is monitored in the negative ion mode using single ion monitoring at *m/z* = 301.00 [MW-H], with the capillary voltage at 3000 V, the nebulizer pressure at 35 psi, and the drying gas flow at 11 L/min at a temperature of 350 °C.

3.5 SIRT6 In Silico-Screen [7]

A [7]

1. The homology model for SIRT6 is used for molecular docking studies.
2. The centroid of the grid box defining the docking region is determined with Trp187 and Pro219. The constraint for hydrogen bonding is set with carbonyl oxygen of Leu184 in order to ensure the correct binding pose for acetylated lysine. Molecular docking is carried out with Schrödinger Glide SP (Standard Precision) version (Small-Molecule Drug Discovery Suite 2011: Glide, version 5.7, Schrödinger, LLC, New York, 2011).
3. Docking poses are visually inspected.

B [17]

1. The modified crystal structure of SIRT6 is utilized for docking.
2. The centroid of grid box is set based on ADP-ribose ligand present in the crystal structure and all water is removed.
3. Molecular docking is performed with SP (Standard Precision) protocol of Schrödinger Glide version (Small-Molecule Drug Discovery Suite 2012: Glide, version 5.8, Schrödinger, LLC, New York, 2012) for screening.
4. Based on Glide score top 1500 compounds are further docked with Glide's XP (Extra Precision) and top 500 molecules of them are visually inspected and further manually filtered.
5. Pan Assay Interference Compounds (PAINS) using FAF-Drugs2 is used to filter the final active compounds.

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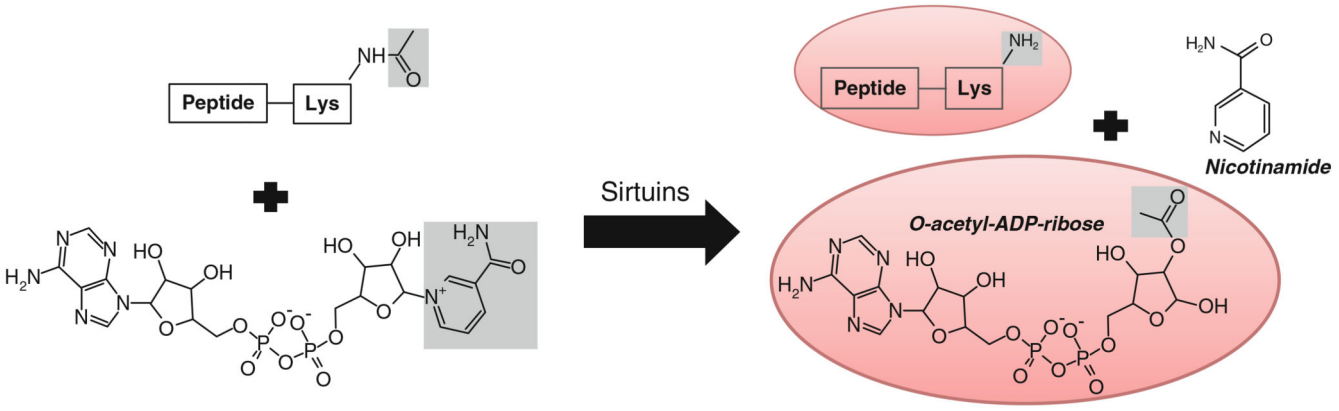

Fig. 1.

Sirtuin mediated deacetylation. In most cases HPLC detection is based on the quantification of deacetylated substrate or O-acetyl-ADP-ribose (*red oval*)

Table 1
The reaction conditions of different SIRT6 in vitro assays

Activation	Peptide substrate	NAD (mM)	SIRT6	Reaction volume (μL)	Inc. time (min)	Ex-/Em-wl
HPLC	**QTARK(Ac)STGG**: 70 μM [6]	0.5	4 μM		60	NA
	ARTKQTARK(Ac)STGGK APRKQLA 40 μM [13]	0.5	0.05 μg/μL	60	30	NA
HPLC	**QTARK(Ac)STGGAc**: 300 μM[14]	2	4 μM	40	360	–
	KQTARK(Ac)STGGWW: 50 μM [5]	1	10 μM	30	60	–
	KQTARK(Myr)STGGWW: 50 μM [5]	0.5	1 μM	30	15	–
	EALPK(Myr)TGGPQWW: 50 μM [5]	0.5	1 μM	30	15	–
	ARTKQTARK(Ac)STGGK APRKQLA 150 μM [13]	0.5	0.05 μg/μL	60	60	NA
Frontal chrom.						
Magnetic beads	**ARTKQTARK(Ac)STGGK APRKQLA** 43 μM [11]	0.2	–	50	240	–
	RYQK(Ac)-AMC[a]: 320 μM [9]	3	0.09 μg/μL	50	90	370/430 nm
Fluorogenic	**EALPK(Myr)**-AMC[a]: 10 μM[8]	1	1 μM	60	120	360/460 nm
	RHKK(Ac)-AMC[a, b] 400 μM	3	0.09 μg/μL	50	45–90	355–370/ 430–460 nm
FRET	(DABCYL)-**ISGASE(Myr)DIVHSE**-(EDANS)G: 10 μM [10]	1	0.5 μM	60	60	336/490

[a] 7-amino-4-methylcoumarin

[b] Cayman commercial kit