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Methods for studying human sirtuins with activity-based chemical probes

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Abstract

Sirtuins are unique posttranslational modification enzymes that utilize NAD⁺ as the co-substrate to remove acyl groups from lysine residues. The deacylation events result in profound biological consequences, from transcription silencing to metabolism regulation. This article focuses on a newly developed technology using activity-based chemical probes to report sirtuin functional state in various settings. These chemical probes, thioacyllysine peptides carrying photo-cross-linker as well as bioorthogonal functionality, target the active site of sirtuins to form stalled reaction intermediate. Subsequently, the probe forms covalent adduct with the protein through photocrosslinking. Ultimately, the active sirtuin can be visualized via “click” chemistry-mediated conjugation to a fluorescent tag. Here, we describe the labeling protocols on recombinant protein, whole cell lysate, and *in situ* labeling.

1. Introduction

Sirtuins, also called class III HDACs, are NAD⁺-dependent protein deacetylases found in diverse organisms from archaea to human (Blander & Guarente, 2004; Sauve, Wolberger, Schramm, & Boeke, 2006). Sirtuin activities are upregulated by calorie restriction (CR) in multiple species such as yeast, worms, flies and mice (Cohen et al., 2004; Lin, Ford, Haigis, Liszt, & Guarente, 2004; Rogina & Helfand, 2004; Tissenbaum & Guarente, 2001). More importantly CR causes extended lifespan and improved health profiles in the same species (Cohen et al., 2004; Lin et al., 2004; Rogina & Helfand, 2004; Tissenbaum & Guarente, 2001). The notion that sirtuin activity mediates the critical health and longevity benefits of CR has been supported by studies showing that increased gene dosage of sirtuins increases lifespan in model organisms. In addition, deletion of sirtuin activity abrogates the longevity effects provided by CR (Lee, Lee, Lee, & Min, 2019). Hence, sirtuins are sometimes referred as “longevity proteins” (Sack & Finkel, 2012).

In human, there are seven sirtuin isoforms (SIRT1–7) with diverse subcellular localizations and biological functions (Haigis & Sinclair, 2010). They have been implicated in the regulation of a myriad of cellular events including gene silencing (Brachmann et al., 1995), DNA repair (Ghosh, Liu, Wang, Hao, & Zhou, 2015), mitochondrial biogenesis (Yuan et al.,

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2016; Zhao et al., 2016) as well as cell cycle control (Zhang et al., 2019). Studies from the past few years revealed that sirtuins are complex enzymes with diverse catalytic activities. For example, in addition to being a weak deacetylase, SIRT5 also harbors robust desuccinylase, demalonylase, and deglutarylase activities (Du et al., 2011; Tan et al., 2014). Through these novel posttranslational modifications, SIRT5 regulates proteins and enzymes involved in carbohydrate and fatty acid metabolism, ammonia detoxification, and ketone body production (Kumar & Lombard, 2018). On the other hand, SIRT6 prefers to remove long-chain fatty acyl groups from lysines (Feldman, Baeza, & Denu, 2013; Jiang et al., 2013). These pleiotropic enzymatic activities give SIRT6 its far-reaching functions in genomic stability through the regulation of DNA repair (Mao et al., 2011; Van Meter et al., 2016) and telomere integrity (Tennen, Bua, Wright, & Chua, 2011) and in metabolism through the control of glucose and lipid homeostasis (Cui et al., 2017; Zhong et al., 2010). Due to their importance in various biological processes, human sirtuins have been intensely pursued as therapeutic targets for the treatment of metabolic disease (Kane & Sinclair, 2018), cancer (Carafa, Altucci, & Nebbioso, 2019) and other age-related disorders (Grabowska, Sikora, & Bielak-Zmijewska, 2017).

It is essential to elucidate the deacetylation landscape and to assess the functional state of each individual sirtuin for a better understanding of their physiological and pathological roles. Recently, we developed an assay to directly characterize sirtuin functions in biological samples using activity-based chemical probes (Graham, Rymarchyk, Wood, & Cen, 2018). The chemical structures of the first-generation probes are shown in Fig. 1. As a common feature, these probes carry the thioacyl groups which can target specifically the active site of sirtuins, forming stalled thioimide intermediates (Fig. 2). The presence of a photocrosslinkable group, benzophenone, allows the formation of a covalent adduct upon photo irradiation. The terminal alkyne group, a bioorthogonal functionality, enables the conjugation of the protein-probe complex to an azido-fluorophore via copper (I)-catalyzed [3+2]-cycloaddition (also known as the “click” reaction) for in-gel visualization (Fig. 2). This labeling protocol can accurately report the active sirtuin content in complex proteomes. The detection and profiling of enzyme activities can be accomplished in one setup that cannot be directly assayed by other existing methods for activity screenings. This workflow represents a valuable new approach for functional genomics study under physiologically relevant conditions.

In this chapter, we first detail the expression and purification of several recombinant human sirtuins. The labeling procedures for recombinant enzymes are then described. We also describe the preparation of whole cell lysate and the subsequent labeling with the chemical probe. Finally, the *in situ* labeling of SIRT2 is introduced in which the enzymatic activity is directly interrogated in live cells in response to NAD⁺ concentration change, a factor that is known to alter sirtuin activity.

2. Recombinant sirtuin expression and purification

Plasmids for SUMO-SIRT2 (38–356), SIRT5 (34–302), SIRT6 (1–314) and ULP-1 are generous gifts from Dr. Hening Lin at Cornell University. The identity of the protein is

further confirmed by trypsin digestion followed by LC-MS/MS analysis. Protein concentrations are determined by Bradford assay.

2.1 SIRT2

2.1.1 Procedures for SUMO-SIRT2 expression

1. Transform the pET28a-SUMO-SIRT2 plasmid into Rosetta™ (DE3) competent cells. Plate the cells on LB plates with 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol.
2. Select single colony to inoculate overnight starter culture with 10mL of LB media containing 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol in a 37°C shaker.
3. Inoculate 2L of LB media containing 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol with the overnight starter culture in a 37°C shaker until OD₆₀₀ reaches 0.6.
4. Induce protein expression with 20 µM of IPTG. Incubate cells in a shaker at 15°C and 200rpm for 20h.
5. Harvest the cells by centrifugation at 4°C and 8000rpm for 5min. Re-suspend the cell pellet in 40mL of lysis buffer (20mM Tris-HCl, 500mM NaCl, 5mM imidazole, 10mM MgCl₂, 5% glycerol at pH 7.5) with protease inhibitor (without EDTA).
6. Add lysozyme (50mg) to the cell suspension and incubate it on ice for 30min. Sonicate the solution on ice for 30s, 4 times, with 1min cool-down on ice in between the sonication steps.
7. Remove cell debris by centrifugation at 4°C and 13,000rpm for 60min. Incubate the supernatant with Ni-NTA agarose beads (1.5mL per 2L cells, pre-equilibrated with lysis buffer) at 4°C for 2h.
8. Load the protein bound Ni-NTA beads onto an Econo-column® and wash twice with 20mL of lysis buffer containing 25mM imidazole.
9. Elute the protein with elution buffers (50, 100, 150, 200, 250, 300, 350 and 500mM imidazole in the lysis buffer, 3mL each).
10. Dialyze the purified protein against 20mM Tris-HCl, 500mM NaCl and 5% glycerol at pH 7.5. Concentrate the protein using Amicon® Ultra-4 centrifugal filter unit.

2.1.2 Procedures for ULP-1 expression

1. Transform the pET28a-ULP-1 plasmid into Rosetta™ (DE3) competent cells. Plate the cells on LB plates with 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol.

2. Select single colony to inoculate overnight starter culture with 10mL of LB media containing 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol in a 37°C shaker.
3. Inoculate 2L of LB media containing 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol with the overnight starter culture in a 37°C shaker until OD₆₀₀ reaches 0.6.
4. Induce protein expression with 20 µM of IPTG and incubate cells in a shaker at 15°C and 200rpm for 20h.
5. Harvest the cells by centrifugation at 4°C and 8000rpm for 5min. Re-suspend the cell pellet in 40mL of lysis buffer (20mM Tris-HCl, 500mM NaCl, 5mM imidazole, 10mM MgCl₂, 5% glycerol at pH 7.5) with protease inhibitor (without EDTA).
6. Add lysozyme (50mg) to the cell suspension and incubate it on ice for 30min. Sonicate the solution on ice for 30s, 4 times, with 1min cool-down on ice in between the sonication steps.
7. Remove cell debris by centrifugation at 4°C and 13,000rpm for 60min. Incubate the supernatant with pre-equilibrated Ni-NTA agarose beads (1.5mL per 2L cells) at 4°C for 2h.
8. Load the protein bound Ni-NTA beads onto an Econo-column® and wash twice with 20mL of lysis buffer containing 25mM imidazole. Elute the protein with elution buffers (50, 100, 150, 200, 250, 300, 350 and 500mM imidazole in the lysis buffer, 3mL each).
9. Dialyze the purified protein against 20mM Tris-HCl, 500mM NaCl and 5% glycerol at pH 7.5. Concentrate the protein using Amicon® Ultra-4 centrifugal filter unit.

2.1.3 Procedures for SIRT2 purification—The His₆-SUMO tag of SIRT2 is removed by ULP-1.

1. Incubate purified SUMO-SIRT2 with ULP-1 in 20mM Tris-HCl, 500mM NaCl and 5% glycerol at pH 7.5 for 2h at 4°C.
2. Incubate the protein mixture with pre-equilibrated Ni-NTA resins at 4°C for 2h. Load the resins onto an Econo-column® and collect the flow-through (contains tag-removed SIRT2).

2.2 SIRT5

Procedures for SIRT5 expression and purification

1. Transform the pET28a-SIRT5 plasmid into Rosetta™ (DE3) competent cells. Plate the cells on LB plates with 100 µg/mL Ampicillin and 20 µg/mL Chloramphenicol.

2. Select single colony to inoculate overnight starter culture with 10mL of LB media containing 100 µg/mL Ampicillin and 20 µg/mL Chloramphenicol in a 37°C incubator.
3. Inoculate 2L of LB media containing 100 µg/mL Ampicillin and 20 µg/mL Chloramphenicol with the overnight starter culture in a 37°C shaker until OD₆₀₀ reaches 0.6. Induce protein expression with 100 µM of IPTG and incubate cells in a shaker at 15°C and 200rpm for 20h.
4. Harvest the cells by centrifugation at 4°C and 8000rpm for 5min. Re-suspend the cell pellet in 40mL of lysis buffer (20mM Tris-HCl, 500mM NaCl, 5mM imidazole, 10mM MgCl₂, 5% glycerol at pH 8) with EDTA-free protease inhibitor.
5. Add lysozyme (50mg) to the cell suspension and incubate it on ice for 30min. Sonicate the solution on ice for 30s, 4 times, with 1min cool-down on ice in between the sonication steps.
6. Remove cell debris by centrifugation at 4°C and 13,000rpm for 60min. Incubate the supernatant with Ni-NTA agarose beads pre-equilibrated with lysis buffer at 4°C for 2h.
7. Load the Ni-NTA beads onto an Econo-column® and wash twice with 20mL of lysis buffer containing 25mM imidazole. Elute the protein with elution buffers (50, 100, 150, 200, 250, 300, 350 and 500mM imidazole in the lysis buffer, 3mL each).
8. Dialyze the purified protein against 20mM Tris-HCl, 500mM NaCl and 5% glycerol at pH 8. Concentrate the protein using Amicon® Ultra-4 centrifugal filter unit.

2.3 SIRT6

Procedures for SIRT6 expression and purification

1. Transform the pET28a-SIRT6 plasmid into Rosetta™ (DE3) competent cells. Plate the cells on LB plates with 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol.
2. Select single colony to inoculate overnight starter culture with 10mL of LB media containing 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol in a 37°C shaker.
3. Inoculate 2L of LB media containing 50 µg/mL Kanamycin and 20 µg/mL Chloramphenicol with the overnight starter culture in a 37°C shaker until OD₆₀₀ reaches 0.6. Induce protein expression with 200 µM of IPTG and incubate cells in a shaker at 16°C and 200rpm for 20h.
4. Harvest the cells by centrifugation at 4°C and 8000rpm for 5min. Re-suspend the cell pellet in 40mL of lysis buffer (20mM Tris-HCl, 500mM NaCl, 5mM imidazole, 10mM MgCl₂, 5% glycerol at pH 7.2) with EDTA-free protease inhibitor.

5. Add lysozyme (50mg) to the cell suspension and incubate it on ice for 30min. Sonicate the solution on ice for 30s, 4 times, with 1min cool-down on ice in between the sonication steps.
6. Remove cell debris by centrifugation at 4°C and 13,000rpm for 60min. Incubate the supernatant with Ni-NTA agarose beads pre-equilibrated with lysis buffer at 4°C for 2h.
7. Load the Ni-NTA beads onto an Econo-column® and wash twice with 20mL of lysis buffer containing 25mM imidazole. Elute the protein with elution buffers (50, 100, 150, 200, 250, 300, 350 and 500mM imidazole in the lysis buffer, 3mL each).
8. Dialyze the purified protein against 20mM Tris-HCl, 500mM NaCl and 5% glycerol at pH 7.2. Concentrate the protein using Amicon® Ultra-4 centrifugal filter unit.

3. Activity-based labeling of recombinant sirtuin

The following is a standard protocol for labeling recombinant human sirtuins with activity-based chemical probes. The exogenously expressed and purified sirtuins are incubated with NAD⁺ and the probe. Following photo irradiation and copper-mediated “click” conjugation to a fluorophore, the active sirtuin content can be visualized on a SDS-PAGE gel via fluorescence scanning. This protocol can be applied to either time-dependent (Graham et al., 2018) or concentration-dependent (Fig. 3) labeling. In the latter case, the labeling efficiency should directly correlate with the concentrations of the probe. No appreciable labeling is detected for the sample with no probe. The optimal labeling is achieved by using the minimum concentration of the probe that reaches the signal plateau.

1. Add the protein and other reagents in a 0.7mL Eppendorf tube: purified recombinant sirtuin (final concentration: 10 µM), NAD⁺ (final concentration: 500 µM) and the activity-based probe (probe 1 for SIRT2, probe 2 for SIRT5 and probe 3 for SIRT6). Add 100mM phosphate buffer at pH 7.5 to make the final volume of the reaction 20 µL. Incubate the sample at 37°C for 10min, allowing the formation of the thioimide intermediate.
2. Transfer the sample to a clear-bottom 96-well plate. Place the plate on ice, and irradiate the sample with a hand-held UV lamp at 365nm for 1h at 4°C.
3. At the meantime, prepare the “click” reaction stocks:
 - a. CuSO₄ (50mM in distilled water, freshly prepared)
 - b. Tris(2-carboxyethyl)phosphine (TCEP, 100mM in distilled water, freshly prepared)
 - c. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 30mM in DMSO/*t*BuOH (3/1, v/v))
 - d. TAMRA-azide (5mM in DMSO)

4. Transfer the sample back to the Eppendorf tube. Add the ingredients of the “click” reaction to the sample to the following final concentrations: CuSO₄ 2mM, TCEP 4mM, TBTA 1.25mM, TAMRA-azide 200 μM. Incubate the sample on a microshaker at 250rpm and room temperature in the dark for 30min.
5. Add 8 μL of 4× loading dye to the reaction and boil it at 95°C for 10min.
6. Spin down the sample briefly and load it onto a 10% SDS-PAGE gel submerged in 1× Tris-Glycine SDS running buffer. Place the electrophoresis cell in the dark to minimize fluorophore decomposition. Run the gel for 1h at 120V.
7. Destain the gel for 4h at room temperature in methanol/distilled water/acetic acid (4/5/1, v/v/v).
8. Scan the gel using Biorad Versa Doc 4000 MP with excitation 532nm, 580nm cutoff filter, and 30nm band-pass.
9. After the desired fluorescent images are acquired, stain the gel with Coomassie blue for loading control.

4. Labeling sirtuin in whole cell lysate

The activity-based chemical probes selectively interact with the active enzyme, leading to efficient labeling of the target protein. The probes we developed also demonstrate isoform selectivity with probe 1 prefers SIRT2, probe 2 is specific for SIRT5, and probe 3 is selective for SIRT6. This enables the labeling of one particular sirtuin isotype in a complex proteome such as whole cell lysate. The cell lysate can be incubated with the probe, followed by photocrosslinking and “click” coupling with azido-containing fluorophores. The labeling pattern and efficiency can then be easily evaluated by in-gel fluorescence scanning.

4.1 Cell culturing and lysis

1. Grow human embryonic kidney (HEK293) cells in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, and 100mg/mL streptomycin in a 150cm² cell culture flask to 70–80% confluency.
2. Harvest cells by repetitively pipetting up and down, and then centrifuge at 4°C and 1000×g for 10min.
3. Remove the supernatant, and re-suspend the cells in PBS followed by centrifuging at 4°C and 1500×g for 10min. Repeat this step three times.
4. Remove the supernatant and re-suspend the cell pellet in 4mL of RIPA lysis buffer supplemented with protease inhibitor cocktail. Gently shake the tube and incubate on ice for 15min.
5. Centrifuge the lysed cells at 4°C and 14,000×g for 15min.
6. Recover the supernatant, and quantify the protein concentration by Bradford assay. Dilute the cell lysate with 50mM Tris-HCl buffer to a final concentration of 2mg protein/mL.

4.2 Labeling whole cell lysate

1. Add the cell lysate and other ingredients to a 0.7 mL Eppendorf tube
 - a. 7.5 μ L of cell lysate (15 μ g of protein)
 - b. 1 μ L of 10mM NAD⁺ stock (final concentration: 500 μ M)
 - c. 0.2 μ L of 1mM probe stock (final concentration: 10 μ M)
2. Add 100mM phosphate buffer at pH 7.5 to make the final volume of the reaction 20 μ L. Incubate the sample at 37°C for 10min.
3. Transfer the sample to a clear-bottom 96-well plate. Place the plate on ice, and irradiate the sample with a hand-held UV lamp at 365nm for 1h at 4°C.
4. Transfer the sample back to the Eppendorf tube. Add the “click” reaction cocktail to the sample (CuSO₄ 2mM, TCEP 4mM, TBTA 1.25mM, TAMRA-azide 200 μ M). Gently agitate the sample on a microshaker at 250rpm and room temperature in the dark for 30min.
5. Add 8 μ L of 4 $\times$ loading dye to the reaction and boil it at 95°C
6. Spin down the sample briefly and load it onto a 10% SDS-PAGE gel submerged in 1 $\times$ Tris-Glycine SDS running buffer. Place the electrophoresis cell in the dark to minimize fluorophore decomposition. Run the gel for 1h at 120V.
7. Destain the gel for 4h at room temperature in methanol/distilled water/acetic acid (4/5/1, v/v/v).
8. Scan the gel using Biorad Versa Doc 4000 MP with excitation 532nm, 580nm cutoff filter, and 30nm band-pass.
9. After the desired fluorescent images are acquired, stain the gel with Coomassie blue for loading control.

5. *In situ* labeling of human SIRT2

Our recent study (Graham et al., 2018) indicates that probe 1 (Fig. 1) is a cell permeable sirtuin inhibitor, a feature allows it to be used in *in situ* labeling. The dynamic change of enzyme activity under different cellular conditions can thus be captured and compared. Ample literature precedent has suggested that NAD⁺ concentration change can impact sirtuin activity (Skoge, Dolle, & Ziegler, 2014; Yang et al., 2007), but not sirtuin protein abundance. The intracellular NAD⁺ level can be significantly reduced by the pharmacological inhibition of NAD⁺ biosynthetic pathways. Subsequently, sirtuin activity is reduced as reflected by the elevated acetylation level of endogenous sirtuin substrates (Skoge et al., 2014; Van Gool et al., 2009). This activity change in response to NAD⁺ availability can then be directly assessed through *in situ* labeling using probe 1 (Graham et al., 2018). Indeed, in the NAD⁺ depletion scenario (Fig. 4, FK866 treatment), the labeling of SIRT2 is drastically decreased compared to the control sample despite the fact that SIRT2 protein level remains constant as determined by Western blot (Fig. 4, WB).

5.1 Transfection and FK866 treatment

SIRT2 Flag was a generous gift from Eric Verdin (Addgene plasmid no. 13813) (North, Marshall, Borra, Denu, & Verdin, 2003). FK866 is a nanomolar inhibitor of nicotinamide phosphoribosyltransferase (NAMPT) (Hasmann & Schemainda, 2003), the rate-limiting enzyme of the NAD⁺ salvage pathway (Fig. 5). Intracellular NAD⁺ level is decreased upon FK866 treatment (Graham et al., 2018; Skoge et al., 2014). The following protocol is used in a 6-well plate format. All the amounts and numbers are given on a per well basis.

1. Culture HEK293 cells in DMEM/10% FBS without antibiotics to 90–95% confluency.
2. One hour before the transfection, replace the growth medium with fresh DMEM alone (be careful not to disturb the cells).
3. Dilute 4 µg of plasmid into 250 µL of Opti-MEM medium, mix gently.
4. Dilute 10 µL of Lipofectamine 2000 into 250 µL of Opti-MEM medium. Incubate at room temperature for 5min.
5. Combine the diluted plasmid with diluted Lipofectamine. Mix gently and incubate at room temperature for 20min.
6. Add the plasmid mixture dropwise into the well, mix gently.
7. Incubate cells at 37°C for 24h.
8. Add either vehicle (DMSO) or FK866 to a final concentration of 40nM and culture for additional 6h.

5.2 *In situ* labeling of SIRT2

After being cultured in the presence or absence of FK866, the SIRT2-overexpressing cells are treated with probe 1, followed by in cell photocrosslinking, cell lysis and the “click” conjugation to the fluorescent reporter.

1. Remove the medium. Add fresh DMEM and mix gently by rocking the plate back and forth. Repeat this step three times.
2. Remove the medium. Add fresh DMEM supplemented with 10 µM of probe 1.
3. Incubate the cells at 37°C for 1h.
4. Place the cell culture plate on ice, and irradiate the sample with a hand-held UV lamp at 365nm for 1h at 4°C.
5. Harvest cells by repetitively pipetting up and down, and then centrifuge at 4°C and 1000×g for 10min.
6. Remove the supernatant, and re-suspend the cells in PBS followed by centrifuging at 4°C and 1500×g for 10min. Repeat this step three times.
7. Remove the supernatant and re-suspend the cell pellet in 100 µL of RIPA lysis buffer supplemented with protease inhibitor cocktail. Gently shake the tube and incubate on ice for 15min.

8. Centrifuge the lysed cells at 4°C and 14,000×g for 15min.
9. Recover the supernatant, and quantify the protein concentration by Bradford assay.
10. Add the cell lysate (15 µg of protein) to a 0.7mL Eppendorf tube. Add 100mM phosphate buffer at pH 7.5 to make the final volume of the reaction 20 µL.
11. Add the “click” reaction cocktail to the sample (CuSO₄ 2mM, TCEP 4mM, TBTA 1.25mM, TAMRA-azide 200 µM). Gently agitate the sample on a microshaker at 250rpm and room temperature in the dark for 30min.
12. Add 8 µL of 4× loading dye to the reaction and boil it at 95°C for 10min.
13. Load the sample onto a 10% SDS-PAGE gel. Run the gel in the dark at 120V for 1h.
14. Destain the gel for 4h at room temperature in methanol/distilled water/acetic acid (4/5/1, v/v/v).
15. Acquire the fluorescence images using a Biorad Versa Doc 4000 MP imager.
16. Determine SIRT2 protein level by western blot using anti-SIRT2 anti-body from Santa Cruz Biotechnology.

5.3 Determination of NAD⁺ concentration

The cellular NAD⁺ concentration can be assessed right after the FK866 treatment using a continuous and sensitive fluorescence intensity assay as described previously (Fulco et al., 2008). In this assay, the concentration of NAD⁺ is measured using a coupled enzyme system with lactate dehydrogenase (LDH) and diaphorase (Fig. 6).

5.3.1 Procedures for sample preparation

1. Harvest cells by repetitively pipetting up and down, and then centrifuge at 4°C and 1000×g for 10min.
2. Remove the supernatant, and re-suspend the cells in PBS followed by centrifuging at 4°C and 1500×g for 10min. Repeat this step three times.
3. Remove the supernatant and re-suspend the cell pellet in 30 µL of ice-cold 7% perchloric acid.
4. Vortex the sample for 30s and then sonicate on ice for 5min. Repeat this step three times.
5. Centrifuge the sample at 14,000×g and 4°C for 3min.
6. Transfer the clear supernatant to another Eppendorf tube, and neutralize it to pH 7 with 3M of NaOH and 1M phosphate buffer (pH 9). Neutralized sample can be used in the following steps for NAD⁺ measurement.

5.3.2 Procedures for NAD⁺ cycling assay

1. Prepare the following stock solutions for the measurement:

- a. 100mM Tris-HCl, pH 7.5
 - b. 250mM MgCl₂
 - c. 466.7mM KCl
 - d. 27mM resazurin
 - e. 0.1U/μL LDH
 - f. 100mM lactate
2. Combine the ingredients of the cycling assay buffer. For each 150 μL reaction, combine 37.5 μL of Tris-HCl, 3 μL of MgCl₂, 16.1 μL of KCl, 0.3 μL of resazurin, 0.6 μL of LDH, 3.4 μL of lactate, and 59.1 μL of distilled water.
 3. Load the neutralized samples and NAD⁺ standards in a black 96-well plate. Load 2 and 4 μL of the sample. Load NAD⁺ standard to the following final concentrations (for 150 μL reaction): 0, 50nM, 100nM, 200nM, 400nM, 500nM, 1 μM and 2 μM. Add distilled water to all the samples to bring the total volume up to 10 μL.
 4. Add 120 μL of the cycling assay buffer to each well.
 5. Add 20 μL of 0.035U/μL of diaphorase to each well using a multi-channel pipette to initiate the cycling reactions.
 6. Record the fluorescence intensity change using a microplate reader with excitation at 530nm and emission at 580nm at 27s intervals for 15min.
 7. Plot the readings at different time points to acquire slopes. Create the NAD⁺ standard curve by plotting slopes as a function of their concentrations.
 8. Compare the slope of the sample with that of the NAD⁺ standard curve to determine the NAD⁺ concentration in each well. Adjust the final concentration of the sample by multiplying by the dilution factor. The final NAD⁺ concentration is expressed as pmol/10⁶ cells or pmol/mg protein.

6. Conclusion

A set of powerful small molecules that can directly interrogate the functional state of sirtuins in their native matrix has been developed. They demonstrate good labeling efficiency and isoform selectivity. They label individual recombinant sirtuin, one particular sirtuin isotype in protein mixture, and also report the sirtuin activity change in complex cellular setting when subjected to different stimuli. In this chapter, we have discussed the expression and isolation of recombinant sirtuins, cell culturing and cell lysate preparation. More importantly, we have highlighted the labeling assays for different biological samples ranging from recombinant protein to live cell labeling. Application of the above-mentioned chemical probes and associated methods will facilitate the elucidation of sirtuin activity in specific physiological and pathological processes.

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Fig. 1.
Chemical structure of the probes.

Fig. 2.
General scheme of the labeling protocol.

Fig. 3.

Concentration-dependent labeling of recombinant SIRT2 with probe 1. SIRT2 (10 μM) was incubated with 500 μM NAD^+ and various concentrations of probe 1 (0, 2.5, 5, 7.5, 10, 20 and 50 μM) at 37°C for 10min. The samples were then subjected to the UV irradiation and “click” conjugation process as described in Section 3. SIRT2 can be robustly labeled by probe 1 in a dose-dependent manner. Coomassie staining serves as a loading control.

Fig. 4.

In situ labeling of SIRT2. HEK293 cells were transfected to transiently overexpress human SIRT2. Cells were treated with either vehicle (DMSO) or 40nM FK866 for 6h. The medium was removed. After thorough rinsing, the culture was replenished with fresh medium supplemented with 10 μM probe 1. The cells were irradiated on ice at 365nm for 1h before the cell lysate was isolated and “click” conjugated to a fluorophore as described in Section 5.2.

Fig. 5.

FK866 inhibits NAD⁺ biosynthesis. In mammalian cells, NAD⁺ can be broken down to nicotinamide (NAM) by NAD⁺-utilizing enzymes such as sirtuins, CD38 and PARP. NAM is recycled to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme of the NAD⁺ salvage pathway. Ultimately, NMN is fully incorporated into NAD⁺ by NMN adenylyltransferase (NMNAT). FK866, a potent and selective NAMPT inhibitor, can significantly reduce intracellular NAD⁺ level.

Fig. 6.
Cycling assay for NAD⁺ measurement.