Published in final edited form as:

Methods Mol Biol. 2013 ; 1077: 167–177. doi:10.1007/978-1-62703-637-5_11.

Measurement of Sirtuin Enzyme Activity Using a Substrate-Agnostic Fluorometric Nicotinamide Assay

Basil P. Hubbard and David A. Sinclair

Abstract

The sirtuins are NAD⁺-dependent, multifunctional lysine deacylases that play key roles in cellular homeostasis. They are increasingly being found to target a variety of substrates including acetyl-, butyryl-, malonyl-, and succinyl-lysines. Early assays for measuring sirtuin activity in vitro were criticized for their use of fluorophores on the peptide substrates used, which may alter the results obtained and not be representative of the in vivo situation. We describe a new protocol for the measurement of sirtuin activity by detecting the production of nicotinamide (NAM). The assay is amenable to any substrate and any modification removed by sirtuins. The assay may also be used to measure glycohydrolase (e.g., CD38) and ADP-ribosyltransferase activity (e.g., mARTs and PARPs).

Keywords

Sirtuin assay; SIRT1; HDAC; Deacetylation; Aging; Desuccinylation; Depropionylation; Debutyrylation; Demalonylation; Glycohydrolase assay; PARP assay; Nicotinamide assay; yPnc1; Ortho-phthalaldehyde (OPT); Fluorometric detection of ammonia

1 Introduction

The sirtuin family of NAD⁺-dependent histone deacetylases has been implicated in numerous cellular processes in yeast, worms, flies, and mammals involved in aging and organismal health [1]. For example, yeast Sir2 modulates budding yeast replicative life span by suppressing recombination within the rDNA array [2 – 4], while mammalian SIRT1 regulates DNA damage and apoptosis [5, 6], metabolism, and mitochondrial biogenesis [7 – 9]. Sirtuins catalyze the removal of acetyl groups from lysine residues via a multistep reaction using β -NAD as a cofactor [10 – 14]. The products of the reaction are the deacetylated protein substrate, nicotinamide (NAM), and 2' O-acetyl-adenosine diphosphate-ribose (OAADPR) [10 – 14]. A schematic outlining the mechanism of deacetylation may be found in Fig. 1 [12]. First, the acetyl oxygen of the substrate initiates a nucleophilic addition on the 1'-carbon of nicotinamide ribose to form a C1'-O-alkylamidate intermediate [10 – 14]. This step is accompanied by release of free NAM [10 – 14]. Next, the 2' hydroxyl group on the ribose ring is activated by a conserved catalytic histidine residue (His-135 in Hst2) and attacks the C1'-O-alkylamidate intermediate yielding a 1'2'-cyclic intermediate [11, 15]. This intermediate is then cleaved by a base-activated water molecule, resulting in the release of the deacetylated substrate and 2'-OAADPr [11, 16, 17].

Subsequently, nonenzymatic conversion of 2'OAADPr into 3'OAADPr may take place [11, 16]. In addition to catalyzing lysine deacetylation, specific sirtuins have been shown to possess depropionylase, deubutyrylase [18, 19], desuccinylase, demalonylase [20], and ADP-ribosyltransferase activities [21, 22].

Traditional techniques used to measure sirtuin deacetylase activity include isotopic assays such as the charcoal binding assay, which employs a ^3H -labeled acetyl-lysine substrate to measure acetate release from hydrolyzed OAADPr [17], and [^{14}C] NAM release assays [23]. More commonly, two-step commercial assays such as the Fluor de Lys assayTM (BIOMOL/Enzo), which measures deacetylation of an acetyl-lysine peptide conjugated to aminomethylcoumarin (AMC), have been used [24]. This assay takes advantage of the property that while free AMC fluoresces, its peptide conjugate does not. Thus, following the deacetylation step, trypsin is subsequently added to cleave and release free AMC molecules from deacetylated peptides [24]. Importantly, this assay has been the subject of intense controversy due to its use of non-physiological peptides, and its potential to yield artifacts in drug screening assays [23, 25]. Assays employing electrophoresis [26], fluorometric detection of β -NAD [27], and mass spectrometry [28, 29] have also been reported.

Recently, two non-isotopic enzyme-coupled NAM assays have been described, PNC1-GDH [29, 30] and PNC1-OPT [29]. These assays are relatively convenient to perform, and overcome many of the shortcomings of classical deacetylase detection methods in that they are safe, cost-effective, versatile (any substrate may be used), and do not require the use of any specialized equipment. In both assays, the nicotinamidase enzyme from budding yeast, yPnc1 [31], is added to the reaction to convert the NAM produced by the sirtuin deacetylation reaction into ammonia. Ammonia may be either quantified spectrophotometrically via coupled reaction with glutamate dehydrogenase (GDH) [30, 32] or detected fluorometrically in a second step via chemical reaction with ortho-phthalaldehyde (OPT) [33] to produce 1-alkylthio-substituted isoindoles [29, 33]. While the PNC1-GDH assay has the advantage of being continuous rather than fixed-point, the PNC1-OPT assay offers a higher degree of sensitivity and is better suited for drug discovery since it requires the use of only two enzymes rather than three. Here we outline a detailed protocol for using the PNC1-OPT assay (schematic in Fig. 2) to measure SIRT1 enzyme activity. We have previously used this assay to measure SIRT1 enzyme kinetics with several different natural amino acid peptides, and to study the effects of several SIRT1 inhibitors and activators in vitro [29]. Importantly, this technique is also applicable to the assay of different sirtuin isoforms, as well as other NAD-cleaving enzymes such as glycohydrolases (e.g., CD38) [34] and PARPs [35].

2 Materials

All solutions should be prepared using distilled or ultrapure water with analytical grade reagents.

2.1 Enzymes and Substrates

1. Recombinant SIRT1 and yPnc1 enzymes should be expressed and purified to >85 % using previously described protocols [29, 36, 37], aliquoted, and stored at -20°C

°C until use. Typically the final storage buffer consists of a 1:1 dilution of glycerol with elution buffer (250 mM imidazole, 40 mM Tris pH 8.0, 3 mM BME in distilled water). Repeated freeze–thaw cycles may decrease enzymatic activity.

2. Custom-acetylated peptide substrates may be purchased from commercial vendors or synthesized in house (>85 % purity), and should typically be dissolved in distilled water. In general, peptides should be between 5 and 15 amino acids with the acetylated lysine near the middle of the sequence, and should be flanked by an N-terminal acetyl group and a C-terminal NH₂ group. For example, an appropriate peptide substrate corresponding to the well-known SIRT1 substrate H3K9ac would be: Ac-TARK(ac)STG-NH₂ [29]. Sequence considerations should be taken into account in order to minimize substrate background fluorescence (*see* Note 1).

2.2 Buffers and Solutions

1. Reaction Buffer: Phosphate-buffered saline (PBS): 1 mM KH₂PO₄, 155 mM NaCl, 3 mM Na₂HPO₄·7H₂O, pH 7.4 supplemented with 1 mM dithiothreitol (DTT) (add fresh from a frozen stock before each experiment). Depending on the enzyme being studied, alternative buffers may be used (*see* Note 2).
2. OPT Developer Reagent: A solution of 10 mM OPT and 10 mM DTT in 30 % ethanol/70 % PBS (pH 7.4) (*see* Note 3). Dissolve OPT in pure ethanol first (vortex on the highest setting) and then add this to a PBS/DTT solution. Cover with aluminum foil in order to minimize exposure to light and store at –20 °C until use. This solution will freeze and must be warmed at 37–42 °C prior to use; if precipitation of DTT is observed, vortex the mixture on the highest setting until no particulates remain.
3. β-NAD aliquots: 100 mM β-NAD dissolved in distilled water, aliquoted, and frozen at –20 °C. Thaw and dilute tenfold in distilled water prior to assay. Use one aliquot per experiment and discard any remainder; avoid freeze–thaw cycles.
4. Stock solution of NAM for standard curve preparation (e.g., 100 mM). Aliquot and store at –20 °C.

2.3 Other Assay Equipment/Supplies

1. Aluminum foil.
2. 37 °C incubator and 37 or 42 °C water bath.
3. Vortex mixer.
4. Orbital shaker.

¹The amount of background fluorescence observed in the assay is significantly affected by the choice of peptide substrate used. In general, shorter peptides elicit less background, as do peptides with fewer aromatic groups.

²In situations where PBS is incompatible with the enzyme being studied, alternative buffers such as Tris may be used: 25 mM Tris pH = 8, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂. The pH of the reaction must be regulated since the subsequent development step is pH-sensitive; *see* ref. [33] for additional information on buffer selection.

³Depending on the desired sensitivity and kinetics of the development reaction, the amount of ethanol in the developer solution may be adjusted. For example, a solution of 10 mM OPT and 10 mM DTT in 100 % ethanol may be used, although this will typically result in a decreased signal intensity/unit time.

5. Black opaque bottom 96-well plates suitable for fluorometry.
6. Fluorometer capable of exciting at ~420 nm and measuring emission at ~460 nm.

3 Methods

While the coupled SIRT1 deacetylation/yPnc1 conversion reaction is typically carried out at 37 °C, all other steps may be performed at room temperature unless specified otherwise. In general, samples should be assayed in triplicate.

3.1 Nicotinamide (NAM) Standard Curve Preparation

1. Thaw the NAM standard solution on ice and perform serial dilutions in Eppendorf tubes to obtain concentrations that are 100× of the desired final concentration. Example final concentrations are 0, 5, 10, 20, 30, 40, and 50 µM NAM. Pipette 1 µL of each of these into the bottom of an Eppendorf tube (will be diluted 100-fold in the next step).
2. Thaw yPnc1 enzyme on ice and chill Reaction Buffer. Prepare a master-mix corresponding to the total number of sample reactions to be performed in a 15 mL BD Falcon tube (or similar tube). For each sample mix 1 µg of purified yPnc1 with 100 µL Reaction Buffer. Mix by pipetting up and down, and vortexing gently (~setting 4 for 5 s).
3. Next, pipette 100 µL of the master-mix into each NAM standard sample tube, mix up and down by pipetting, seal each tube, and incubate samples in a holder rack at 37 °C for 1 h. During this incubation period NAM will be converted into ammonia by yPnc1 (*see* Note 4).
4. During the incubation period, thaw the OPT Developer Reagent by incubating at 37 or 42 °C for about 15 min (keep covered in aluminum foil). Subsequently, vortex on the highest setting until completely mixed; ensure that no DTT precipitate is present.
5. Following the incubation period, remove the samples from the 37 °C incubator. Under dimly lit conditions (dark) (*see* Note 5) add 100 µL of OPT Developer Reagent to each tube as quickly as possible to ensure consistency (*see* Note 6). Close tubes and vortex all samples immediately on the highest setting for 5 s.
6. Return the samples to the holder rack. Cover all the samples (the entire holder rack) with aluminum foil. Once covered, ambient lighting may be restored. Incubate the reactions on an orbital shaker for 1 h at room temperature (*see* Note 7).

⁴If the doses of NAM selected for the standard curve are very high, the length of this incubation period may need to be increased.

⁵This reaction is light-sensitive; *see* ref. [33] for more details.

⁶If available, a multichannel pipetman may be used to simultaneously add the developer reagent to all samples. The timing and consistency of the addition of developer, and subsequent mixing, are critical to minimizing sample variance.

⁷Depending on the concentrations of NAM present and the signal sensitivity desired, this time may need to be adjusted. A 1h incubation period has been chosen since it yields a substantial signal, and produces a linear readout of NAM over the ranges of concentrations suggested in this protocol. Importantly, the reaction kinetics are affected by both buffer composition and temperature. The development reaction may not reach completion within a 1-h time window, and the fluorometric signal may continue to increase in a linear fashion until a plateau signal is reached. The conditions outlined in [33] result in reaction completion in about 60–90 min. Therefore, it may be desirable to allow the incubation period to proceed for a longer time period. However, consistency in the length of this incubation period is critical in order to minimize sample variance.

7. Transfer samples to a 96-well plate for reading (do this under dim light). Read the fluorescence on a fluorometer with filters set to excitation at $\sim 420 \pm 10$ nm and emission at $\sim 460 \pm 10$ nm (*see* Note 8). A 0.1 or 1 s read is generally recommended.
8. A standard curve may be produced by plotting fluorescence intensity (y-axis) versus NAM concentration (x-axis), as depicted in Fig. 3.

3.2 Measurement of SIRT1 Deacetylase Activity

1. Thaw aliquoted solutions of β -NAD, acetylated peptide, and SIRT1 and yPnc1 enzyme preparations on ice. Prepare a master-mix in a 15 mL BD Falcon tube (or similar tube) corresponding to *twice* the total number of samples to be assayed. One set of reactions will be performed in the presence of β -NAD to measure SIRT1 deacetylase activity, while a second set of parallel reactions will be run in the absence of β -NAD to account for background fluorescence (*see* Note 9). For each single reaction, mix the following on ice in the order presented: Reaction Buffer (100 μ L—volume of other components), native peptide substrate (typically 10–30 μ M final concentration), purified yPnc1 enzyme (~ 1 –2 μ g), and purified SIRT1 enzyme (~ 1 –2 μ g) (*see* Note 10). Mix the master-mix by pipetting up and down, and gently vortex (setting 5 or 6 for 5 s).
2. Divide the master-mix into two tubes, and add either β -NAD (typically 75–200 μ M final concentration), or an equal volume of distilled water to each tube. Mix again by pipetting and gentle vortexing.
3. Array a series of labeled Eppendorf tubes in a tube holder rack, corresponding to each experimental reaction and its corresponding background control reaction. If test compounds (e.g., activators or inhibitors) are to be added, this should be performed at this stage (*see* Note 11).
4. Next, add 100 μ L of master-mix into the appropriate Eppendorf tubes, close them, and return them to the tube holder rack. If a test compound is present, first mix by pipetting up and down. Incubate the reactions at 37 °C for 1 h (*see* Note 12).

⁸Excitation and emission wavelengths of 420 and 460 nm are recommended for filter-based fluorometers. However, the optimal wavelengths for measurement are described in [33]. If a monochromator is available, excitation and emission wavelengths may be set to 413 and 476 nm, respectively.

⁹In addition to inherent background from the plate/fluorometer, the buffer, peptide substrate, β -NAD⁺ (at high doses), and resulting complexes formed in the presence of OPT and DTT may contribute to background fluorescence in the assay. In order to account for this, appropriate controls must be performed. When using concentrations of β -NAD less than the K_M value for SIRT1 (~ 200 μ M), background fluorescence due to β -NAD is typically negligible. Therefore, performing parallel reactions in the presence or absence of β -NAD and subtracting the two values yield a fluorescence value proportional to the actual amount of NAM produced ($F_{\text{corrected}} = F_{+\text{NAD}} - F_{-\text{NAD control}}$). This control also eliminates noise due to nonenzymatic deacetylation of the substrate, or deacetylation by contaminating, non-NAD⁺-dependent enzymes. For reactions involving high doses of β -NAD, performing parallel reactions in the presence or absence of SIRT1 enzyme may be more appropriate. Performing parallel reactions using either an acetylated peptide or the corresponding non-acetylated peptide is an alternative approach. Similarly, the quantity of fluorescence corresponding to NAM production may be obtained through calculation: $F_{\text{corrected}} = F_{\text{acetyl substrate}} - F_{\text{non-acetyl substrate}}$.

¹⁰The amount of recombinant yPnc1 and SIRT1 enzymes which need to be added to obtain a strong signal will vary depending on the specific activity of the preparation.

¹¹Test compounds such as small molecule activators or inhibitors should first be tested in the context of a NAM standard curve to ensure that they do not alter yPnc1 activity, or quench the fluorescence signal. If they do, an alternative assay should be used. However, slight background fluorescence from test compounds is not prohibitive as this may be corrected for by using the corresponding reaction in the absence of β -NAD.

5. During the incubation period, thaw the OPT Developer Reagent by incubating at 37 or 42 °C for about 15 min (keep covered in aluminum foil). Vortex on the highest setting until the solution is completely mixed; ensure that no DTT precipitate is present.
6. Following the incubation period, remove the samples from the 37 °C incubator, and under dimly lit or dark conditions, add 100 µL of OPT Developer Reagent to each tube as quickly as possible to ensure consistency (*see* Note 6). Close tubes and vortex all samples immediately on the highest setting for 5 s.
7. Return the samples to the holder rack and cover all the samples (the entire holder rack) with aluminum foil. Once covered, ambient lighting may be restored. Incubate the reactions on an orbital shaker for 1 h at room temperature (*see* Note 7).
8. Under dim light, transfer samples to a 96-well plate for reading. Read the fluorescence on a fluorometer with filters set to excitation at $\sim 420 \pm 10$ nm and emission at $\sim 460 \pm 10$ nm (*see* Note 8). A 0.1 or 1 s read is generally recommended.
9. Using the arbitrary fluorescence unit (AFU) readings for each reaction, calculate the net fluorescence for each sample by subtracting the fluorescence of the background control reaction (no NAD) from the experimental reaction (with NAD), $F_{\text{corrected}} = F_{+\text{NAD}} - F_{-\text{NADcontrol}}$. The resulting value is proportional to the amount of NAM produced during the deacetylation reaction.
10. Rates of deacetylation may be calculated using a linear equation obtained from the NAM standard curve.

Acknowledgments

This work was supported by an NSERC PGS-D Fellowship to B.P.H., the Paul F. Glenn Foundation for Medical Research, the United Mitochondrial Disease Foundation, The Juvenile Diabetes Research Foundation, and NIA/NIH grants to D.A.S. B.P.H. and D.A.S. are inventors on a patent licensed to Millipore. D.A.S. is a consultant to GlaxoSmithKline.

References

1. North BJ, Verdin E. Sirtuins: Sir2-related NAD-dependent protein deacetylases. *Genome Biol.* 2004; 5(5):224. doi:10.1186/gb-2004-5-5-224 [pii]. [PubMed: 15128440]
2. Gottlieb S, Esposito RE. A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. *Cell.* 1989; 56(5):771–776. doi:0092-8674(89)90681-8 [pii]. [PubMed: 2647300]
3. Gotta M, Strahl-Bolsinger S, Renauld H, Laroche T, Kennedy BK, Grunstein M, Gasser SM. Localization of Sir2p: the nucleolus as a compartment for silent information regulators. *EMBO J.* 1997; 16(11):3243–3255. doi:10.1093/emboj/16.11.3243 [PubMed: 9214640]
4. Kaerberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev.* 1999; 13(19):2570–2580. [PubMed: 10521401]

¹²Longer incubation times may be used if lower amounts of SIRT1 enzyme are being used, or if activity on the substrate being assayed is weak.

5. Das C, Lucia MS, Hansen KC, Tyler JK. CBP/p300-mediated acetylation of histone H3 on lysine 56. *Nature*. 2009; 459(7243):113–117. doi:nature07861 [pii] 10.1038/nature07861. [PubMed: 19270680]
6. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science*. 2004; 305(5682):390–392. doi:10.1126/science.10991961099196 [pii]. [PubMed: 15205477]
7. Rodgers JT, Lerin C, Gerhart-Hines Z, Puigserver P. Metabolic adaptations through the PGC-1 α and SIRT1 pathways. *FEBS Lett*. 2008; 582(1):46–53. doi:S0014-5793(07)01178-7 [pii] 10.1016/j.febslet.2007.11.034. [PubMed: 18036349]
8. Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z, Puigserver P. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1 α . *EMBO J*. 2007; 26(7):1913–1923. doi:10.1038/sj.emboj.7601633 [PubMed: 17347648]
9. Bernier M, Paul RK, Martin-Montalvo A, Scheibye-Knudsen M, Song S, He HJ, Armour SM, Hubbard BP, Bohr VA, Wang L, Zong Y, Sinclair DA, de Cabo R. Negative regulation of STAT3 protein-mediated cellular respiration by SIRT1 protein. *J Biol Chem*. 2011; 286(22):19270–19279. doi:M110.200311 [pii] 10.1074/jbc.M110.200311. [PubMed: 21467030]
10. Dittenhafer-Reed KE, Feldman JL, Denu JM. Catalysis and mechanistic insights into sirtuin activation. *Chembiochem*. 2011; 12(2):281–289. doi:10.1002/cbic.201000434 [PubMed: 21243715]
11. Sauve AA, Celic I, Avalos J, Deng H, Boeke JD, Schramm VL. Chemistry of gene silencing: the mechanism of NAD $^{+}$ -dependent deacetylation reactions. *Biochemistry*. 2001; 40(51):15456–15463. doi:bi011858j [pii]. [PubMed: 11747420]
12. Smith BC, Denu JM. Sir2 protein deacetylases: evidence for chemical intermediates and functions of a conserved histidine. *Biochemistry*. 2006; 45(1):272–282. doi:10.1021/bi052014t [PubMed: 16388603]
13. Jackson MD, Schmidt MT, Oppenheimer NJ, Denu JM. Mechanism of nicotinamide inhibition and transglycosidation by Sir2 histone/protein deacetylases. *J Biol Chem*. 2003; 278(51):50985–50998. doi:10.1074/jbc.M306552200M306552200 [pii]. [PubMed: 14522996]
14. Smith BC, Denu JM. Mechanism-based inhibition of Sir2 deacetylases by thioacetyl-lysine peptide. *Biochemistry*. 2007; 46(50):14478–14486. doi:10.1021/bi7013294 [PubMed: 18027980]
15. Sauve AA, Wolberger C, Schramm VL, Boeke JD. The biochemistry of sirtuins. *Annu Rev Biochem*. 2006; 75:435–465. doi:10.1146/annurev.biochem.74.082803.133500 [PubMed: 16756498]
16. Jackson MD, Denu JM. Structural identification of 2'- and 3'-O-acetyl-ADP-ribose as novel metabolites derived from the Sir2 family of beta -NAD $^{+}$ -dependent histone/protein deacetylases. *J Biol Chem*. 2002; 277(21):18535–18544. doi:10.1074/jbc.M200671200M200671200 [pii]. [PubMed: 11893743]
17. Borra MT, Langer MR, Slama JT, Denu JM. Substrate specificity and kinetic mechanism of the Sir2 family of NAD $^{+}$ -dependent histone/protein deacetylases. *Biochemistry*. 2004; 43(30):9877–9887. doi:10.1021/bi049592e [PubMed: 15274642]
18. Smith BC, Denu JM. Acetyl-lysine analog peptides as mechanistic probes of protein deacetylases. *J Biol Chem*. 2007; 282(51):37256–37265. doi:M707878200 [pii] 10.1074/jbc.M707878200. [PubMed: 17951578]
19. Garrity J, Gardner JG, Hawse W, Wolberger C, Escalante-Semerena JC. N-lysine propionylation controls the activity of propionyl-CoA synthetase. *J Biol Chem*. 2007; 282(41):30239–30245. doi:M704409200 [pii] 10.1074/jbc.M704409200. [PubMed: 17684016]
20. Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, Kim J, Woo J, Kim JH, Choi BH, He B, Chen W, Zhang S, Cerione RA, Auwerx J, Hao Q, Lin H. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. *Science*. 2011; 334(6057):806–809. doi:10.1126/science.1207861334/6057/806 [pii]. [PubMed: 22076378]
21. Kowieski TM, Lee S, Denu JM. Acetylation-dependent ADP-ribosylation by *Trypanosoma brucei* Sir2. *J Biol Chem*. 2008; 283(9):5317–5326. doi:M707613200 [pii] 10.1074/jbc.M707613200. [PubMed: 18165239]

22. Hawse WF, Wolberger C. Structure-based mechanism of ADP-ribosylation by sirtuins. *J Biol Chem.* 2009; 284(48):33654–33661. doi:M109.024521 [pii] 10.1074/jbc.M109.024521. [PubMed: 19801667]
23. Kaeberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, Napper A, Curtis R, DiStefano PS, Fields S, Bedalov A, Kennedy BK. Substrate-specific activation of sirtuins by resveratrol. *J Biol Chem.* 2005; 280(17):17038–17045. doi:M500655200 [pii] 10.1074/jbc.M500655200. [PubMed: 15684413]
24. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature.* 2003; 425(6954):191–196. doi:10.1038/nature01960 [pii]. [PubMed: 12939617]
25. Borra MT, Smith BC, Denu JM. Mechanism of human SIRT1 activation by resveratrol. *J Biol Chem.* 2005; 280(17):17187–17195. doi:M501250200 [pii] 10.1074/jbc.M501250200. [PubMed: 15749705]
26. Fan Y, Hense M, Ludewig R, Weisgerber C, Scriba GK. Capillary electrophoresis-based sirtuin assay using non-peptide substrates. *J Pharm Biomed Anal.* 2011; 54(4):772–778. doi:S0731-7085(10)00605-9 [pii] 10.1016/j.jpba.2010.10.010. [PubMed: 21074959]
27. Feng Y, Wu J, Chen L, Luo C, Shen X, Chen K, Jiang H, Liu D. A fluorometric assay of SIRT1 deacetylation activity through quantification of nicotinamide adenine dinucleotide. *Anal Biochem.* 2009; 395(2):205–210. doi:S0003-2697(09)00559-4 [pii] 10.1016/j.ab.2009.08.011. [PubMed: 19682970]
28. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, Bemis JE, Xie R, Disch JS, Ng PY, Nunes JJ, Lynch AV, Yang H, Galonek H, Israelian K, Choy W, Iffland A, Lavu S, Medvedik O, Sinclair DA, Olefsky JM, Jirousek MR, Elliott PJ, Westphal CH. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature.* 2007; 450(7170):712–716. doi:nature06261 [pii] 10.1038/nature06261. [PubMed: 18046409]
29. Hubbard BP, Gomes AP, Dai H, Li J, Case AW, Considine T, Riera TV, Lee JE, E SY, Lamming DW, Pentelute BL, Schuman ER, Stevens LA, Ling AJ, Armour SM, Michan S, Zhao H, Jiang Y, Sweitzer SM, Blum CA, Disch JS, Ng PY, Howitz KT, Rolo AP, Hamuro Y, Moss J, Perni RB, Ellis JL, Vlasuk GP, Sinclair DA. Evidence for a common mechanism of SIRT1 regulation by allosteric activators. *Science.* 2013; 339(6124):1216–1219. doi:10.1126/science.1231097339/6124/1216 [pii]. [PubMed: 23471411]
30. Smith BC, Hallows WC, Denu JM. A continuous microplate assay for sirtuins and nicotinamide-producing enzymes. *Anal Biochem.* 2009; 394(1):101–109. doi:S0003-2697(09)00493-X [pii] 10.1016/j.ab.2009.07.019. [PubMed: 19615966]
31. Ghislain M, Talla E, Francois JM. Identification and functional analysis of the *Saccharomyces cerevisiae* nicotinamidase gene, PNC1. *Yeast.* 2002; 19(3):215–224. doi:10.1002/yea.810 [pii] 10.1002/yea.810. [PubMed: 11816029]
32. Mondzac A, Ehrlich GE, Seegmiller JE. An enzymatic determination of ammonia in biological fluids. *J Lab Clin Med.* 1965; 66(3):526–531. [PubMed: 4284306]
33. Sugawara K, Oyama F. Fluorogenic reaction and specific microdetermination of ammonia. *J Biochem.* 1981; 89(3):771–774. [PubMed: 7287639]
34. Nishina H, Inageda K, Takahashi K, Hoshino S, Ikeda K, Katada T. Cell surface antigen CD38 identified as ectoenzyme of NAD glycohydrolase has hyaluronate-binding activity. *Biochem Biophys Res Commun.* 1994; 203(2):1318–1323. doi:S0006-291X(84)72326-6 [pii] 10.1006/bbrc.1994.2326. [PubMed: 8093047]
35. Burkle A. Poly(ADP-ribose). The most elaborate metabolite of NAD⁺ FEBS J. 2005; 272(18): 4576–4589. doi:EJB4864 [pii] 10.1111/j.1742-4658.2005.04864.x. [PubMed: 16156780]
36. Schneider B, Xu YW, Janin J, Veron M, Deville-Bonne D. 3'-Phosphorylated nucleotides are tight binding inhibitors of nucleoside diphosphate kinase activity. *J Biol Chem.* 1998; 273(44):28773–28778. [PubMed: 9786875]
37. Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature.* 2004; 430(7000):686–689. doi:10.1038/nature02789 [pii]. [PubMed: 15254550]

Fig. 1.
Schematic outlining the biochemical mechanism of sirtuin deacetylation

Fig. 2.
Diagram illustrating the main steps in the PNC1-OPT assay

Fig. 3. Example nicotinamide standard curve produced using the PNC1-OPT assay. Each point represents mean $\pm$ s.d. ($n = 3$)