

Supporting Information

An improved fluorogenic assay for SIRT1, SIRT2 and SIRT3

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A. General methods and materials.

The SIRT2 direct fluorescent assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI). All peptides were synthesized by solid phase peptide synthesis using standard Fmoc-chemistry on FOCUS peptide synthesizer (aapptec). LC-MS experiments were carried out on a Shimadzu HPLC LC20-AD and Thermo Scientific LCQ Fleet with a Kinetex 5u EVO C18 100A column (30 × 2.1 mm, 5 μm, Phenomenex) monitoring at 215 and 260 nm with positive mode for detection of mass-to-charge ratio of ions. Solvents for LC-MS were water with 0.1% acetic acid (solvent A) and acetonitrile with 0.1% acetic acid (solvent B) at a flow rate of 0.3 mL/min. Analytic HPLC analysis was carried out on a SHIMADZU LC with Kinetex 5u EVO C18 100A column (100 mm × 4.60 mm, 5 μm, Phenomenex) monitoring at 215 nm and 326 nm. Solvents for analytical HPLC were water with 0.1% trifluoroacetic acid (TFA) as solvent A and acetonitrile with 0.1% TFA as solvent B. Compounds were analyzed at a flow rate of 0.5 mL/min. Preparative HPLC experiments were done on SHIMADZU LC with a Targa™ Prep C18 column (250 × 20 mm, 10 μm, Higgins Analytical, Inc.) monitoring at 215 and 260 nm. Solvents for preparative HPLC were water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). Compounds were eluted at a flow rate of 8.0 mL/min. Fluorescence assay was recorded by Fluoroskan Ascent FL microplate fluorometer (fluorometric mode, excitation at the wavelength of 355 nm and emission at the wavelength of 460 nm, Thermo Labsystems).

Expression and purification of SIRT1, SIRT2, and SIRT3. Human SIRT1 and SIRT2 were expressed as previously described.^[1] SIRT3 (101-399 aa) was expressed in BL21 cells using a pET28a-based expression vector containing an N-terminal hexahistidine (His₆) tag. Cells were grown in Luria-Bertani (LB) broth supplemented with Kanamycin (50 µg/mL) and Chloramphenicol (20 µg/mL) at 37 °C until OD 600 reached ~0.6. Protein overexpression was induced with 0.2 mM isopropyl 1-β-D-galactopyranoside (IPTG) and incubated at 16 °C for 16 h. Cells were harvested by centrifugation and lysed using EmulsiFlex-C3 High Pressure Homogenizer (AVESTIN, Inc.) in lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2% glycerol). His₆-tagged SIRT3 was purified using Ni-NTA affinity chromatography. Ni-NTA resin and the cell lysate containing SIRT3 were incubated at 4 °C for 1 h. The resin was washed with 20 mM Tris-HCl, 500 mM NaCl, 2% glycerol, and 20 mM imidazole, pH 8.0. The bound SIRT3 was eluted with 20 mM Tris-HCl, 500 mM NaCl, and 2% glycerol, 250 mM imidazole, pH 8.0. Eluted samples were analyzed by SDS-PAGE to reveal a 36 kDa band corresponding to SIRT3 with purity > 90%. Combined samples were dialyzed in buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2% glycerol) at 4 °C. Aliquots were frozen at -80 °C for further use.

Synthesis of substrate 4. A peptide Ac-Gln(Trt)-Pro-Lys(Boc)-Lys(Myristoyl)-OH was synthesized by solid phase peptide synthesis using a myristoyl lysine and protected amino acids on a 2-chlorotrityl resin. The protected peptide was then cleaved from the resin using a mild acidic solution (AcOH: CF₃CH₂OH: CH₂Cl₂, 1:1:8, v/v/v).^[2] To a solution of Ac-Gln(Trt)-Pro-Lys(Boc)-Lys(Myristoyl)-OH (175 mg, 0.16 mmol) and *N*-methylmorpholine (NMM, 0.035 mL, 0.32 mmol) in dry CH₂Cl₂ (2.7 mL) was added isobutylchloroformate (0.033 mL, 0.24 mmol) dropwisely at 0 °C. The reaction mixture was stirred for 20 min at 0 °C. 7-Amino-4-methyl-coumarin (56 mg, 0.32 mmol) in DMF (0.3 mL) was then added at 0 °C and the reaction mixture was stirred for 16 h at room temperature, followed by evaporation and partitioning between EtOAc and water. The aqueous phase was extracted with EtOAc (3 × 15 mL). The combined organic phase was washed with brine, dried over anhydrous Na₂SO₄, and then evaporated to dryness. The obtained residue was deprotected with 1% triisopropylsilane in TFA (1 mL) for 1 h, and the reaction mixture was then evaporated to dryness. The resulting residue was purified using preparative HPLC. The gradient was: 100% solvent A for 10 min, 0% to 80% solvent B over 30 min, then 80% to 95% solvent B for 5 min. After lyophilization, the

desired substrate **4** with the sequence Ac-Gln-Pro-Lys-Lys(Myr)-AMC was obtained as a white solid. LCMS (ESI) calcd. For $C_{36}H_{52}N_8O_9$ $[M+H]^+$ 741.4, obsd. 741.4.

Kinetics of SIRT1, SIRT2, and SIRT3 on the substrates 3,4 , and 5. For substrates **3**, the reactions containing various concentration of substrate (5.2, 10.4, 20.8, 41.6, 83.2, 166.5, 333 μ M) and 1 mM NAD in buffer (20 mM Tris-HCl, pH 8.0, 1 mM DTT) were started by adding 0.5 μ M sirtuin enzymes (SIRT1, SIRT2, and SIRT3) and incubated at 37 °C for indicated time (9 min for SIRT1, 45 min for SIRT2 and SIRT3). For substrate **4** and **5**, the reactions containing various concentration of substrate (0.5, 1, 2, 4, 8, 16, 32, and 64 μ M) and 1 mM NAD in buffer (20 mM Tris-HCl, pH 8.0, 1 mM DTT) were started by adding the sirtuin enzymes (0.5 μ M for SIRT1, 0.1 μ M for SIRT2, 1 μ M for SIRT3) and incubated for 8 min at 37 °C. The conversion of each reaction was less than 20%. Each reaction was stopped using equal volume of 0.5 N HCl in 50% methanol. The reaction mixtures were centrifuged at 17000g for 10 min and analyzed on an analytical HPLC with Kinetex 5u EVO C18 100A column (100 mm \times 4.60 mm, 5.0 μ m, Phenomenex). The gradient was: 0% B for 2 min, 0 to 30% B in 13 min, and then 30% to 100% in 10 min at a flow rate of 0.5 mL/min. All reactions were done in duplicate. The product and the substrate peaks were quantified using absorbance at 326 nm. The calculated initial velocity V_0 was then plotted against the peptide concentrations [S], and the data were fitted to the Michaelis–Menten equation using GraphPad Prism software.

Comparing substrate 3, 4 and 5 in sirtuin fluorogenic assays. End point assays were performed by incubating 125 μ M or 10 μ M substrate (**3**, **4**, and **5**) and 3 mM NAD, in assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 60 μ L final volume) with or without 1 μ M sirtuin (SIRT1, SIRT2, and SIRT3) at 37 °C for 45 min. The reactions were then quenched with the stop/developing solution (60 μ L) containing trypsin and 2 mM nicotinamide. The resulting reaction mixtures were incubated for 30 min at room temperature and then transferred to a 96-well plate. The fluorescence was analyzed using Fluoroskan Ascent FL microplate fluorometer with excitation at 355 nm and emission at 460 nm. All reactions were done in duplicate.

Optimizing the fluorogenic assay with various concentration of substrate 4. SIRT1, 2, or 3 (1 μ M) was incubated with various concentrations of substrate **4** (2.5, 5, 10, and 20 μ M) and NAD (1 mM) in buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM

KCl, 1 mM MgCl₂, 60 μL final volume, final volume 60 μL) for 45 min at 37 °C. Then 60 μL stop/developing solution was added and the reactions were incubated for 30 min at room temperature. The mixture (100 μL) was transferred to a 96-well plate and the fluorescence was recorded by a microplate fluorometer. All reactions were done in duplicate. Signal-to-background (S/B) ratio and Z'-factor are determined according to the following equations.^[3]

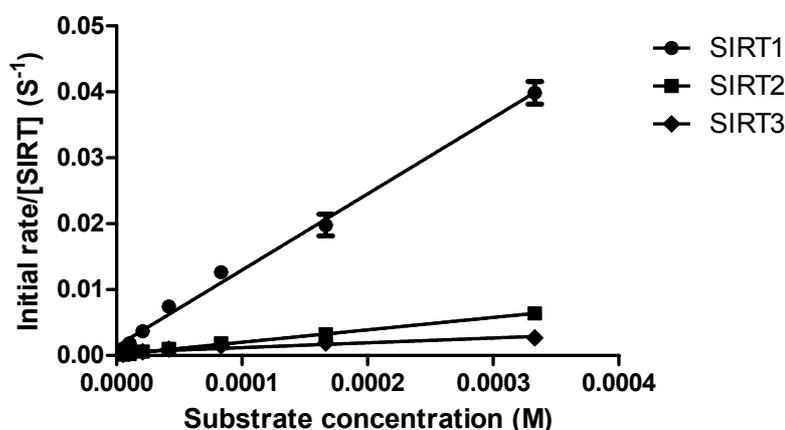
$$\frac{S}{B} = \frac{\text{mean signal}}{\text{mean background}}$$

$$Z' = 1 - \frac{3(\text{SD of positive control} + \text{SD of negative control})}{|\text{mean of positive control} - \text{mean of negative control}|}$$

Measuring the IC₅₀ of nicotinamide using the fluorogenic assay. SIRT1, 2, or 3 (1 μM) was mixed with 1 mM NAD, 5 μM substrate **4**, and the inhibitor nicotinamide at various concentrations (0.25, 12.5, 25, 50, 100, 200, 400, and 5000 μM) in assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM Mg₂Cl, 60 μL final volume). The mixture was incubated at 37 °C for 45 min. The reaction was then stopped by the addition of stop/developing solution and the mixture was then incubated for 30 min at room temperature. The mixture (100 μL) was transferred to a 96-well plate and the fluorescence was recorded by a microplate fluorometer. The amount of inhibition was determined with respect to the control mixture without nicotinamide. IC₅₀ values were determined with GraphPad Prism software.

B. Supplementary Figures

a.



b.

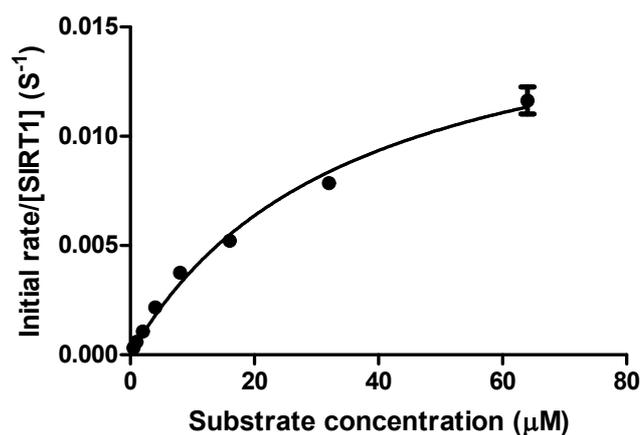
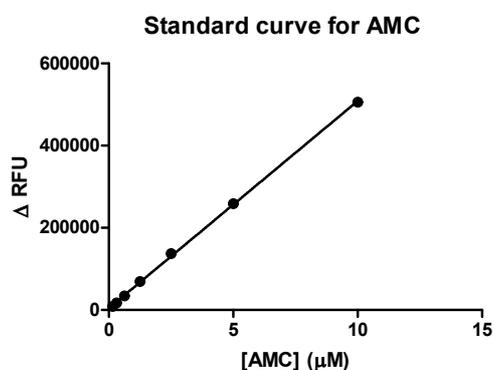


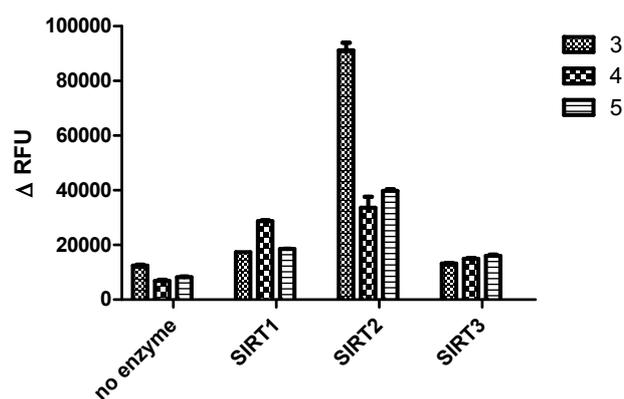
Figure S1. a. Linear plot of sirtuins against substrate **3**. In the reactions, 0.5 μM sirtuin enzymes (SIRT1, SIRT2, and SIRT3) was incubated with various concentration of substrate **3** (5.2, 10.4, 20.8, 41.6, 83.2, 166.5, 333 μM) and 1 mM NAD in buffer (20 mM Tris-HCl, pH 8.0, 1 mM DTT) at 37 $^{\circ}\text{C}$ for indicated time (9 min for SIRT1, 45 min for SIRT2 and SIRT3). The K_m and k_{cat} value could not be determined due to the linear relationship at the concentration range, which means that K_m is higher than 333 μM . However, k_{cat}/K_m can be obtained from the slope of the linear plot. b. Michaelis-Menten plot of SIRT1 against substrate **4**. In the reaction, 0.5 μM SIRT1 was incubated with 1 mM NAD and various concentration of peptide **4** (0.5, 1, 2, 4, 8, 16, 32, and 64 μM) in buffer (20 mM Tris-HCl pH 8.0, 1 mM DTT) for 8 min at 37 $^{\circ}\text{C}$. All reactions were done

in duplicate. The k_{cat} is $0.017 \pm 0.0011 \text{ s}^{-1}$ and K_m is $35.3 \pm 4.5 \text{ }\mu\text{M}$ for SIRT1 against substrate 4.

a.



b.



c.

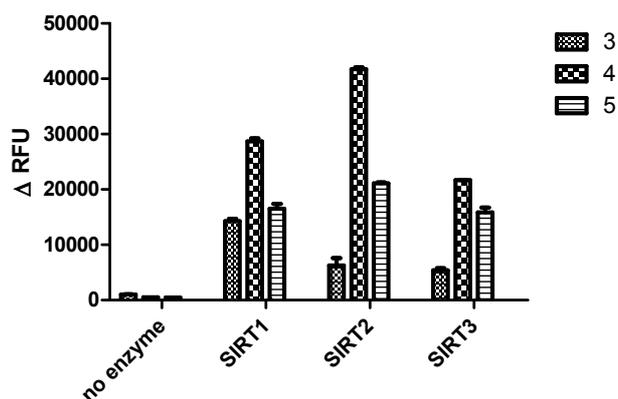


Figure S2. Comparison of the fluorescence increase with different fluorogenic substrates. **a.** Standard curve of relative fluorescence unit (ΔRFU) for the AMC fluorophore. **b.** Fluorescence increase with different substrates at $125 \text{ }\mu\text{M}$. **c.** Fluorescence increase with different substrates at $10 \text{ }\mu\text{M}$. The fluorogenic substrate (3, 4, and 5) was incubated with

or without 1 μ M SIRT1, 2, and 3 at 37 °C for 45 min and then with stop/developing solution for 30 minutes at room temperature. The data were obtained as end point readings in duplicate.

C. References

- [1] Y. B. Teng, H. Jing, P. Aramsangtienchai, B. He, S. Khan, J. Hu, H. Lin, Q. Hao, *Sci. Rep.* **2015**, 5:8529.
- [2] J. Hu, B. He, S. Bhargava, H. Lin, *Org. Biomol. Chem.* **2013**, 11, 5213-5216.
- [3] J. H. Zhang, T. D. Y. Chung, K. R. Oldenburg, *J. Biomol. Screen.* **1999**, 4, 67-73.