



Enabling Discovery in Life Science®

SIRT3 Fluorometric Drug Discovery Kit

A Fluor de Lys® Fluorescent Assay System

Instruction Manual

BML-AK557

For research use only

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TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

A Fluor de Lys[®] Fluorescent Assay System **BACKGROUND**

Yeast Sir2 (Silent information regulator 2)¹ is the founding exemplar of the 'sirtuins', an apparently ancient group of enzymes which occurs in eukaryotes, the archaea and eubacteria². Originally described as a factor required for maintenance of silencing at telomeres and mating type loci, Sir2 was subsequently shown to be an enhancer of mother-cell replicative lifespan³. The sirtuins represent a distinct class of trichostatin A-insensitive protein-lysyl-deacetylases (class III HDACs) and have been shown to catalyze a reaction that couples lysine deacetylation to the formation of nicotinamide and O-acetyl-ADP-ribose from NAD⁺ and the abstracted acetyl group⁴⁻⁶. There are seven human sirtuins, which have been designated SIRT1-SIRT7⁷.

Like Sir2 and human SIRT1 and 2, SIRT3 is a class I sirtuin⁷. However, unlike SIRT1, which is located in nuclei⁸, or SIRT2, which is primarily cytoplasmic⁹⁻¹¹ with a lesser amount of nuclear localization^{10,12}, SIRT3 is synthesized as an inactive precursor protein¹³ whose mature, active form is located in the mitochondrial matrix^{13,14}. Although its substrates are as yet unidentified, SIRT3's mitochondrial localization suggests some interesting possibilities for SIRT3 function. NAD⁺/NADH are essential to mitochondrial electron transport and ATP production. Thus SIRT3 could, via its requirement for NAD⁺, act as a sensor and transducer of metabolic signals¹⁴. Alternatively, since mitochondrial NAD⁺ concentrations may be unresponsive to metabolic changes¹⁵, but depleted by opening of the mitochondrial permeability pore¹⁶, SIRT3 could be acting as a modulator of apoptotic signals that affect mitochondrial permeability¹³. In either case, SIRT3's rather high K_m for NAD⁺ (~2 mM, See Fig. 6) suggests that its activity may be regulated by NAD⁺ levels over a broad range of concentrations.

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PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

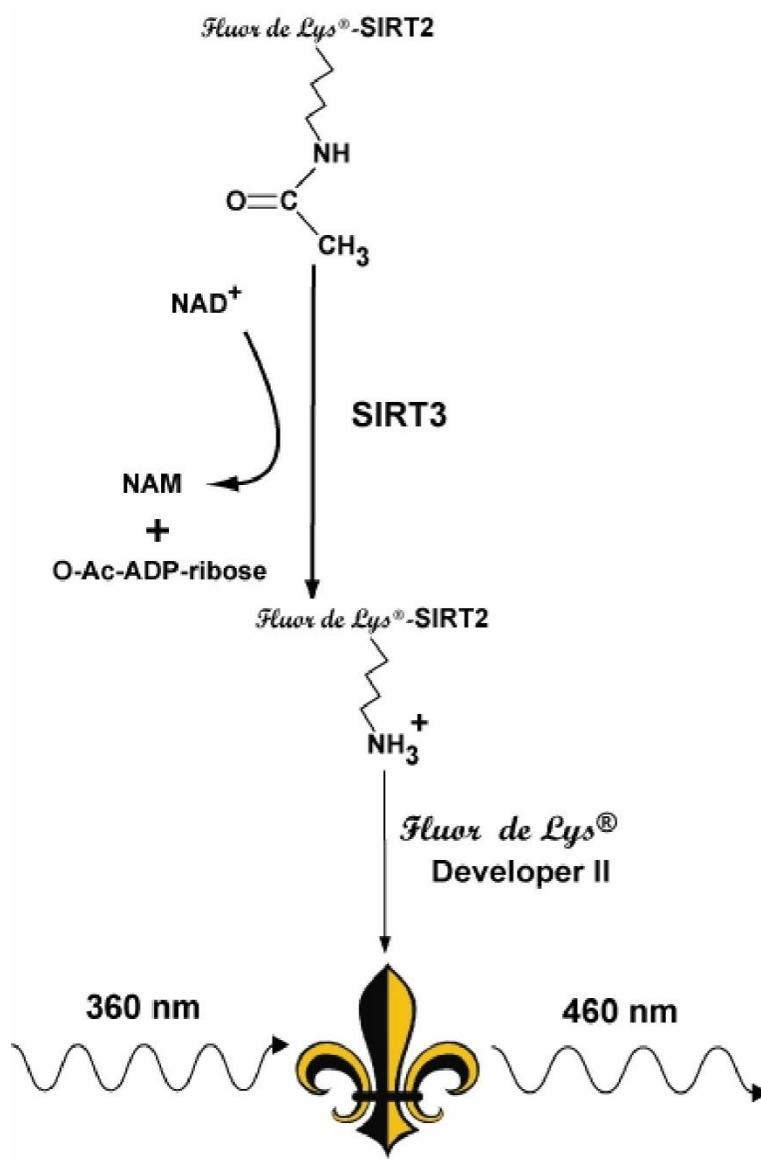


Figure 1. Reaction Scheme of the *SIRT3* Fluorescent Activity Assay*. NAD⁺-dependent deacetylation of the substrate by recombinant human SIRT3 sensitizes it to Developer II, which then generates a fluorophore (symbol). The fluorophore is excited with 360 nm light and the emitted light (460 nm) is detected on a fluorometric plate reader. NAD⁺ is consumed in the reaction to produce nicotinamide (NAM) and O-acetyl-ADP-ribose.

DESCRIPTION

The *SIRT3* Fluorescent Activity Assay/Drug Discovery Kit is a complete assay system designed to measure the lysyl deacetylase activity of SIRT3. The mature, active form of recombinant human SIRT3, (residues 102-399, minus the mitochondrial transit peptide) is included in the kit. For convenience, two types of 96-well microplates come packaged with the kit, but it should be noted that the reagents have also been successfully employed in other formats, including cuvettes and 384-well plates.

The *SIRT3* Fluorescent Activity Assay is based on the unique *Fluor de Lys*[®]-SIRT2 Substrate/Developer II combination. The *Fluor de Lys*[®]-SIRT2 Substrate is a peptide comprising amino acids 317-320 of human p53 (Gln-Pro-Lys-Lys(Ac)). The assay's fluorescence signal is generated in proportion to the amount of deacetylated substrate produced by SIRT3 action. *Fluor de Lys*[®]-SIRT2 was the substrate deacetylated most efficiently by SIRT3, from among a panel of substrates patterned on p53, p65, histone H3 and histone H4 acetylation sites (See Fig. 2, *Fluor de Lys*[®]-SIRT2 is labeled 'p53-320').

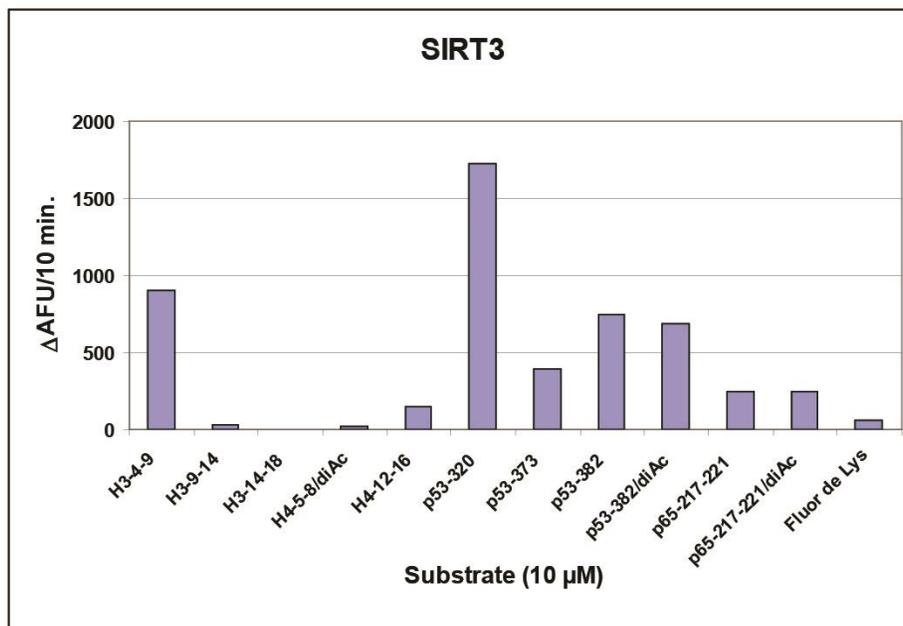


Figure 2. SIRT3 Peptide Substrate Preferences. Initial rates of deacetylation were determined for a series of fluorogenic acetylated peptide substrates. Based on native acetylation sites in human histones H3 and H4, and the transcription factors p53 and p65 sequence. Recombinant human SIRT3 (BML-SE270), was incubated for 10 min at 37°C with 10 μM of the indicated fluorogenic acetylated peptide substrate and 500 μM NAD⁺. Reactions were stopped by the addition of Developer II/2 mM nicotinamide and the deacetylation-dependent fluorescent signal was allowed to develop for 45 min. at 37°C. Fluorescence was then measured in the wells of a white microplate (BML-KI110) with a CytoFluor™ II fluorescence plate reader at 5 min intervals until development reached a plateau (Perseptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85). The substrate labeled 'p53-320' is *Fluor de Lys*[®]-SIRT2 (BML-KI179).

The assay procedure has two steps (Fig. 1). First, the *Fluor de Lys*[®]-SIRT2 Substrate, which comprises the p53 Sequence Gln-Pro-Lys-Lys(ε-acetyl), is incubated with human recombinant SIRT3 together with the cosubstrate NAD⁺. Deacetylation of *Fluor de Lys*[®]-SIRT2 sensitizes it so that, in the second step, treatment with the *Fluor de Lys*[®] Developer II produces a fluorophore.

The protocols and application examples described below emphasize conditions suitable for the screening of potential inhibitors of SIRT3. Nicotinamide (BML-KI283) and suramin sodium (BML-KI285), are included as positive controls for SIRT3 inhibition (See Fig. 4). Although inhibitor screens are typically done at relatively low substrate concentrations, the kit does include enough substrate to perform kinetic studies over a full range of relevant concentrations (See Figures 5 & 6).

COMPONENTS OF BML-AK557

BML-SE270-0500 SIRT3 (Sirtuin 3)(human, recombinant)

FORM: Recombinant enzyme dissolved in 25 mM Tris, pH 7.5, 100 mM NaCl, 5 mM dithiothreitol and 10% glycerol. See vial label for activity and protein concentrations.

STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!

QUANTITY: 500 U; One U=1 pmol/min at 37°C, 500 μ M, *Fluor de Lys*[®]-SIRT2 Substrate (BML-KI179), 500 μ M NAD⁺

BML-KI179-0005 *Fluor de Lys*[®]-SIRT2, Deacetylase Substrate

FORM: 5 mM solution in 25 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂

STORAGE: -70°C

QUANTITY: 100 μ L

BML-KI176-1250 *Fluor de Lys*[®] Developer II Concentrate (5x)

FORM: 5x Stock Solution; Dilute in Assay Buffer before use.

STORAGE: -70°C

QUANTITY: 5 x 250 μ L

BML-KI282-0500 NAD⁺ (Sirtuin Substrate)

FORM: 50 mM β -Nicotinamide adenine dinucleotide (oxidized form) in 50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂.

STORAGE: -70°C

QUANTITY: 500 μ L

BML-KI283-0500 Nicotinamide (Sirtuin Inhibitor)

FORM: 50 mM Nicotinamide in 25 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂.

STORAGE: -70°C

QUANTITY: 500 μ L

BML-KI285-0010 Suramin sodium (Sirtuin Inhibitor)

FORM: Solid

MW: 1429.2

STORAGE: -70°C

QUANTITY: 10 mg

SOLUBILITY: Water or Assay Buffer to 25 mM (10 mg in 0.27 mL)

BML-KI142-0030 *Fluor de Lys*[®] Deacetylated Standard

FORM: 10 mM in DMSO (dimethylsulfoxide)

STORAGE: -70°C

QUANTITY: 30 μ L

BML-KI286-0020 Sirtuin Assay Buffer

FORM: (50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mg/mL BSA)

STORAGE: -70°C

QUANTITY: 20 mL

80-2407 1/2 Volume Microplates

FORM: 1 clear and 1 white, 96-well

STORAGE: Room temperature.

OTHER MATERIALS REQUIRED

- Microplate-reading fluorometer capable of excitation at a wavelength in the range 350-380 nm and detection of emitted light in the range 450-480 nm.
- Pipetman or multi-channel pipetman capable of pipetting 2-100 µL accurately
- Ice bucket to keep reagents cold until use.
- Microplate warmer or other temperature control device

ASSAY PROCEDURES

Notes On Storage: Store all components except the microtiter plate at -70°C for the highest stability. Components with storage temperatures other than -70°C can be stored at the temperature listed OR at -70°C . The SIRT3 enzyme, BML-SE270, must be handled with particular care in order to retain maximum enzymatic activity. Defrost it quickly in a RT water bath or by rubbing between fingers, then immediately store on an ice bath. The remaining unused extract should be refrozen quickly, by placing at -70°C . If possible, snap freeze in liquid nitrogen or a dry ice/ethanol bath. To minimize the number of freeze/thaw cycles, aliquot into separate tubes and store at -70°C . The 5x Developer II (BML-KI176) can be prone to precipitation if thawed too slowly. It is best to thaw this reagent in a room temperature water bath and, once thawed, transfer immediately onto ice.

Some Things To Consider When Planning Assays:

1. The assay is performed in two stages. The first stage, during which the SIRT3 acts on the Substrate, is done in a total volume of 50 μL . The second stage, which is initiated by the addition of 50 μL of Developer II, including a SIRT3 inhibitor, stops SIRT3 activity and produces the fluorescent signal. See “Preparing Reagents For Assay” and Table 1 (p. 4).

Two types of $\frac{1}{2}$ -volume, 96-well microplates are provided with the kit. The signal obtained with the opaque, white plate can be ~5-fold greater than that obtained with the clear plate. As long as the fluorometer to be used is configured so that excitation and emission detection occur from above the well, the white plate should significantly increase assay sensitivity.

Should it be necessary, for convenience in adding or mixing reagents, there is some leeway for change in the reaction volumes. The wells of the microplates provided (80-2407) can readily accommodate 150 μL . If planning a change to the volume of the Developer, it should be noted that it is important to keep two factors constant: 1) concentration of SIRT3 inhibitor (1 mM nicotinamide) in the final mix; 2) 10 μL /well amount of Developer II Concentrate (BML-KI176). See “Preparing Reagents For Assay”, Step #5, (p. 4).

2. Experimental samples should be compared to a “Time Zero” (sample for which 1x Developer II/2 mM nicotinamide is added immediately before mixing of the SIRT3 with substrate) and/or a negative control (no enzyme).
3. For many applications, including inhibitor screening, a signal approximately proportional to the initial enzyme rate is desirable. Particularly if a sub- K_m substrate concentration is chosen (See point 4. below) the rate will immediately begin to decline as substrate is used up. In the case of SIRT3, inhibition by one of the reaction products, nicotinamide, will also contribute to this effect. A preliminary time course experiment will aid in the selection of an incubation time, which yields a signal that is both sufficiently large and proportional to enzyme rate.
4. The K_m of SIRT3 for the *Fluor de Lys*[®]-SIRT2 Substrate has been measured at 32 μM at 5 mM NAD^+ (Fig. 5). The K_m for NAD^+ , determined at 1 mM *Fluor de Lys*[®]-SIRT2 Substrate, was 2 mM (Fig. 6). USE of substrate concentrations at or below K_m will help avoid substrate competition effects, which could mask the effectiveness of competitive inhibitors.

5. The effects of some enzyme modulators, such as covalent inhibitors, may be time-dependent. In other cases, time dependence may be indicative of artifacts such as the formation of aggregates. Two schemes for order of reagent mixing are outlined in the notes under Table 1. One includes a preincubation of enzyme and test compound. The other presents substrates and test compound to the enzyme simultaneously.
6. It is conceivable that some compounds being screened for modulation of SIRT3 activity may interfere with the action of the *Fluor de Lys*[®] Developer II. It is therefore important to confirm that apparent “hits” are in fact acting only via SIRT3 effects. One approach to this involves retesting the candidate compound in a reaction with the *Fluor de Lys*[®] Deacetylated Standard (BML-KI142) plus the *Fluor de Lys*[®] Developer II. A detailed retesting procedure is described below, in the section “USES Of The *Fluor de Lys*[®] Deacetylated Standard” (p. 4). In some cases, it may be possible to avoid this retesting by means of measurements taken during the fluorescence development phase of the initial SIRT3 assay. This is also discussed in that Section (pp. 4-5).

Preparing Reagents For Assay:

1. Defrost all kit components and keep these, and all dilutions described below, on ice until use. Note that it is best to rapidly thaw both the SIRT3 enzyme (BML-SE270) and the 5x Developer II (BML-KI176). (See ‘Notes on Storage’, above.) All undiluted kit components are stable for several hours on ice.
2. Assuming 5 U of SIRT3 (BML-SE270) per assay, dilute a sufficient amount to 1 U/ μ L in Assay Buffer (BML-KI286) to provide for the assays to be performed (slightly more than # of wells x 5 μ L). Subsequent dilutions of five-fold to 0.2 U/ μ L or three-fold to 0.33 U/ μ L will be made, depending on whether test compounds will be added with substrate or preincubated with the enzyme (See Performing the Assay and Table 1, p. 4).
3. Prepare dilution(s) of suramin, nicotinamide and/or Test Compounds in Assay Buffer (BML-KI286). Since 10 μ L will be used per well (Table 1), and since the final volume of the SIRT3 reaction is 50 μ L, these inhibitor dilutions will be 5x their final concentration. Suramin sodium is soluble in both water and Assay Buffer (10 mg in 0.27 mL = 25 mM). High concentrations of both ethanol and DMSO affect SIRT3 activity and appropriate solvent controls should always be included.
4. Prepare a dilution of the substrates, *Fluor de Lys*[®]-SIRT2 (BML-KI179; 5 mM) and NAD⁺ (BML-KI282, 50 mM), in Assay Buffer (BML-KI286), that will be 3.33x the desired final concentrations. For inhibitor screening, substrate concentrations at or below the K_m are recommended. This 3.33x stock will constitute 60% of a 2x substrate stock, prepared either with or without added test compounds (See Performing the Assay and Table 1, below).
5. Shortly before use (<30 min.), prepare sufficient 1x *Fluor de Lys*[®] Developer II plus nicotinamide (2 mM) for the assays to be performed (50 μ L per well). One ml will contain 760 μ L Assay Buffer, 200 μ L 5x Developer II and 40 μ L 50 mM nicotinamide. Addition of nicotinamide to the Developer II insures that SIRT3 activity stops when the Developer II is added. Keep diluted Developer II on ice until use.

Performing the Assay:

- Table 1 gives examples of solutions and volumes for use in various types of SIRT3 assays. These are mixtures for the first, deacetylation, phase of the assay. The SIRT3 reaction is initiated by mixing 25 μL of a 2x substrate solution with 25 μL containing the enzyme. The notes below Table 1 (\ddagger) describe schemes for mixing the stock solutions prepared above (Preparing Reagents for Assay) so that the test compounds are added as part of the 2x substrate solution (1) or are preincubated with the enzyme (2).

TABLE 1. COMPOSITION OF EXAMPLE ASSAY MIXTURES (PER WELL VOLUMES)

Sample	Assay Buffer	SIRT3 (1 U/ μL)	Test Cmpd.or Solvent Control (5x)	Substrates <i>Fluor de Lys</i> [®] -SIRT2 plus NAD (3.33x)
Blank (No Enzyme)	25 μL	0	10 μL	15 μL
Time Zero [⊛]	10 μL + 10 μL [‡]	5 μL	10 μL	15 μL [⊛]
Control	10 μL + 10 μL [‡]	5 μL	10 μL	15 μL
Suramin	10 μL + 10 μL [‡]	5 μL	10 μL	15 μL
Test Sample	10 μL + 10 μL [‡]	5 μL	10 μL	15 μL

[‡] The Assay Buffer amount is written as a split “10 μL + 10 μL ” in reference to two possibilities for the order in which test compounds are mixed with the SIRT3 enzyme:

- If substrate and test compound are to be mixed with the enzyme simultaneously, then the entire 20 μL would be mixed with 5 μL of enzyme or a master mix consisting of 0.2 U/ μL SIRT3 in Assay Buffer could be aliquoted at 25 μL per well. In this case, substrates plus test compound (25 μL) could be added from a mother plate in which the wells contain a mixture of 40% 5x Test Compound and 60% 3.33x Substrates.
- If the test compound is to be preincubated with enzyme prior to substrate addition, 15 μL of an enzyme master mix consisting of 0.33 U/ μL SIRT3 in Assay Buffer could be aliquoted per well and then mixed with 10 μL of 5x Test Compound. The reaction would then be initiated by addition of 25 μL of 2x Substrates in Assay Buffer (40% Assay Buffer, 60% 3.33x Substrates).

[⊛] **NOTE:** In a ‘Time Zero’ sample, the substrate addition is made after the addition of 1x Developer II/2 mM nicotinamide.

- Add 25 μL of 0.2 U/ μL SIRT3 or 15 μL of 0.33 U/ μL SIRT3 plus 10 μL 5x Test Compound or 25 μL Assay Buffer to appropriate wells of the assay plate.
- Warm the assay plate and 2x substrate solutions to 37°C.
- Initiate SIRT3 reactions by adding 25 μL 2x substrate solutions to the assay wells and thoroughly mixing. **DO NOT ADD SUBSTRATE TO “TIME ZERO” WELLS.**
- Allow SIRT3 reactions to proceed for desired length of time and then stop by addition of 1x Developer II/2 mM nicotinamide (50 μL). Add 25 μL of 2x

Substrate solution to “Time Zero” samples. Incubate plate at room temperature for at least 45 min. Signal development can be accelerated by higher temperature (30-37°C).

6. Read samples in a microplate-reading fluorometer capable of excitation at a wavelength in the range 350-380 nm and detection of emitted light in the range 450-480 nm. Completion of signal development can be assessed by taking fluorescence readings at 5 min. intervals. The Developer reaction is complete when the fluorescence readings reach a maximum and plateau. Signals are stable for at least 60 min. beyond this time.

USES OF THE *Fluor de Lys*[®] DEACETYLATED STANDARD (BML-KI142)

Preparation of a Standard Curve:

1. The exact concentration range of the *Fluor de Lys*[®] Deacetylated Standard (BML-KI142) that will be useful for preparing a standard curve will vary depending on the fluorometer model, the gain setting and the exact excitation and emission wavelengths used. We recommend diluting some of the standard to a relatively low concentration with Assay Buffer (1 to 5 μM). The fluorescence signal should then be determined, as described below, after mixing 50 μL of the diluted standard with 50 μL of 1x Developer II. The estimate of AFU(arbitrary fluorescence units)/μM obtained with this measurement, together with the observed range of values obtained in the enzyme assays can then be used to plan an appropriate series of dilutions for a standard curve. Provided the same wavelength and gain settings are used each time, there should be no need to prepare a standard curve more than once.
2. After ascertaining an appropriate concentration range, prepare, in Assay Buffer, a series of *Fluor de Lys*[®] Deacetylated Standard dilutions that span this range. Pipet 50 μL of each of these dilutions, and 50 μL of Assay Buffer as a ‘zero’, to a set of wells on the microplate.
3. Prepare enough of a 1x dilution of *Fluor de Lys*[®] Developer II in Assay Buffer for addition of 50 μL to each of the standard wells.
4. Mix 50 μL of the 1x Developer II with the 50 μL in each standard well and incubate 5-10 min. at room temperature (25°C).
5. Read samples in a microplate-reading fluorometer capable of excitation at a wavelength in the range 350-380 nm and detection of emitted light in the range 450-480 nm.
6. Plot fluorescence signal (y-axis) versus concentration of the *Fluor de Lys*[®] Deacetylated Standard (x-axis). Determine slope as AFU /μM. See example in Fig. 3.

Testing of Potential SIRT3 Inhibitors for Interference with the *Fluor de Lys*[®] Developer II or the Fluorescence Signal:

1. The *Fluor de Lys*[®] Developer is formulated so that, under normal circumstances, the reaction goes to completion in less than 30 min. at 25°C. That, together with the recommended 45 min. reaction time, should help insure that in most cases, even when some retardation of the development reaction occurs, the signal will fully develop prior to the reading of the plate.
2. A convenient step to control for substances that interfere with the Developer reaction or the fluorescence signal itself may be built directly into an inhibitor

screening protocol. After waiting for the signal from the SIRT3 reaction to fully develop and stabilize (usually less than 45 min., See 1. above), the fluorescence is recorded and a 'spike' of *Fluor de Lys*[®] Deacetylated Standard is added (e.g. amount equivalent to 5 μM in the 50 μL SIRT3 reaction). Sufficient Developer reactivity should remain to produce a full signal from this 'spike'. When the new, increased fluorescence level has fully developed (<15 min.), the fluorescence is read and the difference between this reading and the first one can provide an internal standard, in terms of AFU/ μM , for appropriate quantitation of each well. This is particularly useful in cases where the development reaction itself is not compromised but the fluorescence signal is diminished. Highly colored test compounds, for example, may have such an effect. As discussed further below (See 3.), interference with the development reaction *per SE* will be reflected in the kinetics of signal development, both that due to the initial SIRT3 reaction and that due to a subsequent Deacetylated Standard 'spike'.

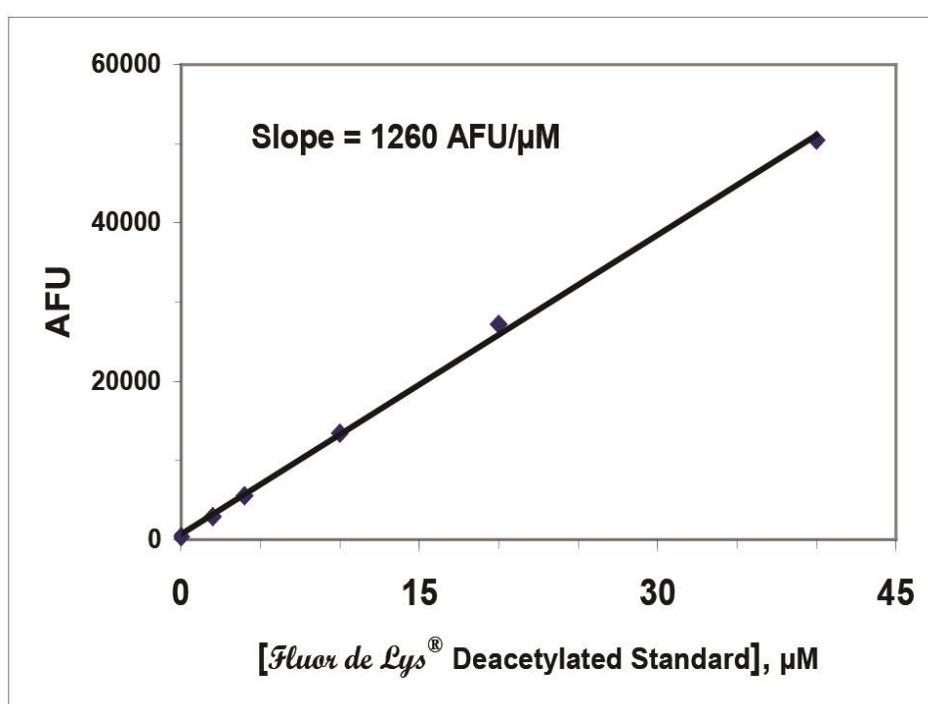


Figure 3. Fluorescence Standard Curve. Fifty μl aliquots of *Fluor de Lys*[®] Deacetylated Standard, in Assay Buffer at the indicated concentrations, were mixed with 50 μL 1x Developer II and incubated 15 min., 25°C. Fluorescence was then measured in the wells of the clear $\frac{1}{2}$ volume microplate with a CytoFluor[™] II fluorescence plate reader (Perseptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85)

3. It should be possible to identify many cases in which there is interference with the development reaction by taking a series of fluorescence readings immediately following addition of the *Fluor De Lys*[®] Developer (e.g. readings at 5 min. intervals for 45 min.). The fluorescence of control samples (no inhibitor) will change very little after the third or fourth reading. Samples containing compounds which inhibit SIRT3, but which do not interfere with Developer II, will display similarly rapid kinetics, although a lower final fluorescence. Nicotinamide (100 μM) provides a good model of this behavior. Any sample in which the approach to the final fluorescence is substantially slower than in the above examples should be suspected of interference with the development reaction. For samples in which little or no fluorescence has developed, it may be impossible to assess the development kinetics.
4. Absolute certainty regarding interference with the Developer II reaction can only be obtained through an assay in which the compound in question is tested

for its effect on the reaction of *Fluor de Lys*[®] Deacetylated Standard with the Developer. Using a standard curve such as that described in the previous Section, determine the concentration of Deacetylated Standard that will yield a signal similar to that produced after development of a control (no inhibitor) SIRT3 reaction. Mix 40 µl of the diluted Standard with 10 µL inhibitor or 10 µL Assay Buffer (See Table 2). Initiate development by adding 50 µL of 0.2x Developer II to each well. Follow fluorescence development by reading at 1 or 2 min. intervals for 30 min. If a test inhibitor sample reaches its final fluorescence significantly more slowly than the control then there may be interference with the Developer II reaction. Compounds that decrease the final fluorescence signal without slowing the kinetics of its development may be quenching the fluorescence signal rather than interfering with the Developer II reaction (See point 2. above).

5. Once it is determined that a particular substance does interfere with the Developer reaction, it may be possible to adjust reaction conditions to eliminate this effect. In cases where the same final fluorescence is achieved, but more slowly than the control, simply extending the incubation time after addition of the Developer II would be sufficient. Other possible adjustments include increasing the volume of Developer II used per well (e.g. to 100 µL). Both approaches may be used separately or in combination.

TABLE 2. ASSAY MIXTURES FOR TEST COMPOUND RETESTING WITH *Fluor de Lys*[®] DEACETYLATED STANDARD

Sample	Test Compound or Solvent Control (5x)	Diluted⁶<i>Fluor de Lys</i>[®] deAc. Standard (1.25x)	DEVELOPER II (1x)
Control	10 µL	40 µL	50 µL
Test Compound	10 µL	40 µL	50 µL

⁶The appropriate dilution of the *Fluor de Lys*[®] Deacetylated Standard, in Assay Buffer may be determined from the standard curve and should be the concentration producing a fluorescent signal equal to that produced by control (no Test Compound) samples in the SIRT3 assay. The dilution in Assay Buffer is prepared at 1.25x this concentration to compensate for the 4/5 dilution due to addition of 10 µL of Assay Buffer or 5x Test Compound.

APPLICATION EXAMPLES

The *SIRT3 Fluorescent Activity Assay/Drug Discovery Kit* has been used for investigating SIRT3 kinetics as a function of the concentrations of *Fluor de Lys*[®]-SIRT2 Substrate and NAD⁺ (Figures 5 & 6) as well as for the discovery and characterization of activators and inhibitors of the enzyme (Figure 4).

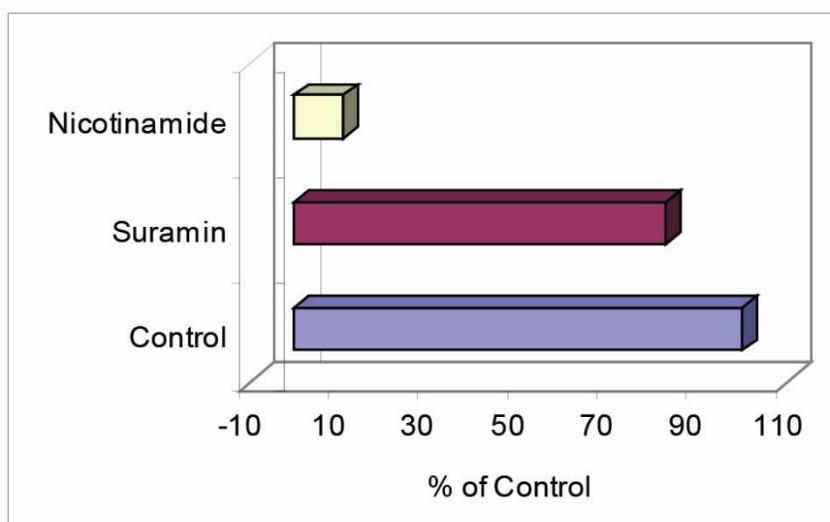


Figure 4. Inhibitors of SIRT3. Initial deacetylation rates of SIRT3 were determined at 10 μM *Fluor de Lys*[®]-SIRT2, 1 mM NAD⁺ (37°C) in the presence of 100 μM suramin or nicotinamide. Reactions were stopped with *Fluor de Lys*[®] Developer II/2 mM nicotinamide and fluorescence measured (CytoFluor[™] II, Perceptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85).

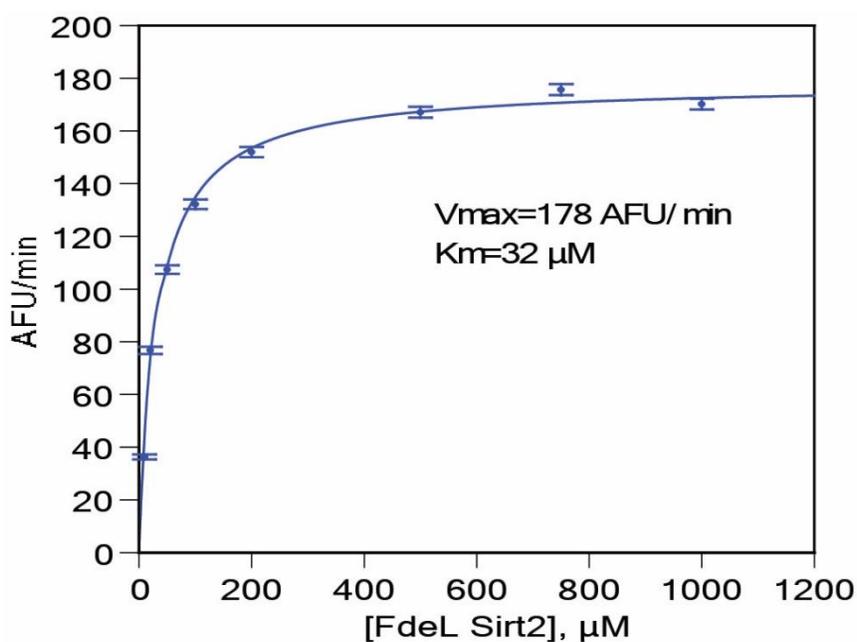


Fig. 5 Dependence of SIRT3 Kinetics on the Concentration of *Fluor de Lys*[®]-SIRT2. Initial deacetylation rates of SIRT3 were determined with 40 min. incubations (37°C) in the presence of 5 mM NAD⁺. Reactions were stopped with *Fluor de Lys*[®] Developer II/2 mM nicotinamide and fluorescence measured (CytoFluor[™] II, Perceptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85). Each point represents the mean of three determinations and the error bars are standard errors. The line is a non-linear least squares fit to the Michaelis-Menten equation. The K_m for *Fluor de Lys*[®]-SIRT2 was 32 μM and the V_{max} was 178 AFU/min.

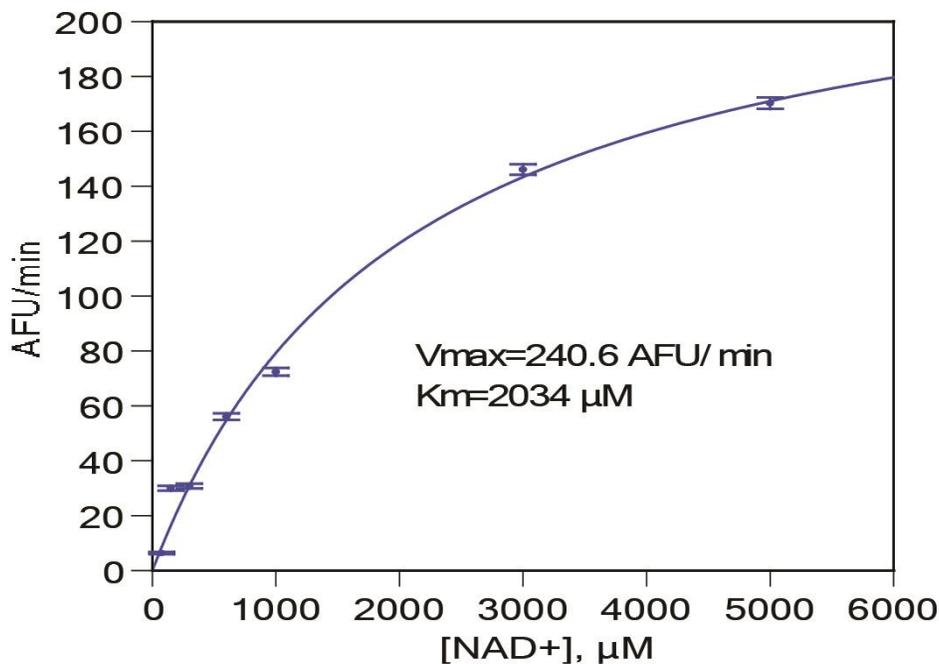


Fig. 6 Dependence of SIRT3 Kinetics on the Concentration of NAD⁺. Initial deacetylation rates of SIRT3 were determined with 40 min. incubations (37°C) in the presence of 1 mM *Fluor de Lys*[®]-SIRT2. Reactions were stopped with *Fluor de Lys*[®] Developer II/2 mM nicotinamide and fluorescence measured (CytoFluor™ II, Perceptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85). Each point represents the mean of three determinations and the error bars are standard errors. The line is a non-linear least squares fit to the Michaelis-Menten equation. The K_m for NAD⁺ was 2034 μM and the V_{max} was 240.6 AFU/min.

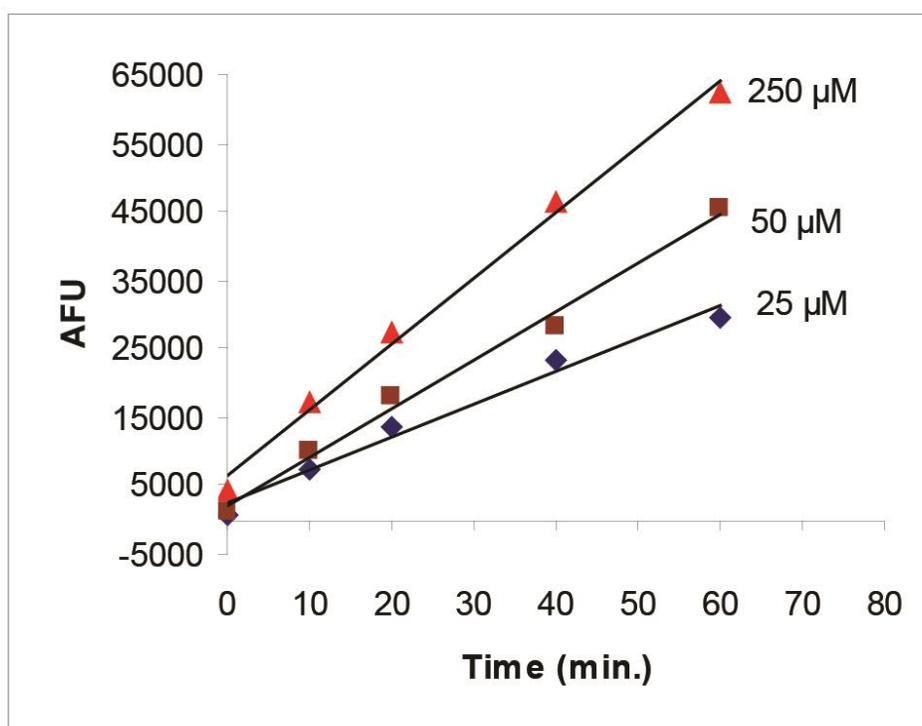


Fig. 7 Time Courses of *Fluor de Lys*[®]-SIRT2 Deacetylation by Recombinant SIRT3. SIRT3 (5 U/well) was incubated (37°C) with the indicated concentrations of peptide substrate and 500 μM NAD⁺. Reactions were stopped with *Fluor de Lys*[®] Developer II/2 mM nicotinamide and fluorescence measured (CytoFluor™ II, Perceptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85).

NOTE: THE APPLICATION EXAMPLES, DESCRIBED HEREIN, ARE INTENDED ONLY AS GUIDELINES. THE OPTIMAL CONCENTRATIONS OF SUBSTRATES AND INHIBITORS, ASSAY VOLUMES, BUFFER COMPOSITION, AND OTHER EXPERIMENTAL CONDITIONS MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PARTICULAR RESULTS, THROUGH THE USE OF THESE PROCEDURES, IS MADE OR IMPLIED.

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HDAC Fluorescent Activity Assay	BML-AK500
HDAC Colorimetric Assay Kit	BML-AK501
<i>Fluor de Lys</i> [®] Substrate	BML-KI104
<i>Fluor de Lys</i> [®] Developer	BML-KI105
<i>Fluor de Lys</i> [®] -SIRT1 Substrate	BML-KI177
<i>Fluor de Lys</i> [®] -SIRT2 Substrate	BML-KI179
<i>Fluor de Lys</i> [®] -H4-AcK16 Substrate	BML-KI174
<i>Fluor de Lys</i> [®] -HDAC8 Substrate	BML-KI178
<i>Fluor de Lys</i> [®] Developer II	BML-KI176
HeLa Nuclear Extract	BML-KI140
HDAC8 (recombinant, human)	BML-SE145
SIRT1 (recombinant, human)	BML-SE239
SIRT2 (recombinant, human)	BML-SE251
SIRT3 (recombinant, human)	BML-SE270
Resveratrol (SIRT1 Activator)	BML-FR104
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