Enzo

Product Manual

SIRT1 Fluorometric Drug Discovery Kit

A FLUOR DE LYS® Fluorescent Assay System

Catalog # BML-AK555

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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 Support if necessary.

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INTRODUCTION

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 eubacteria[2]. Originally described as a factor required for maintenance of silencing at telomeres and matin-Gtype loci, Sir2 was subsequently shown to be an enhancer of mother-cell replicative lifespan[3]. 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 group[4-6]. There are seven human sirtuins, which have been designated SIRT1- SIRT7[7]. SIRT1, which is located in the nucleus, is the human sirtuin with the greatest homology to Sir2 and has been shown to exert a regulatory effect on p53 by deacetylation of lysine-382[8-10]. Sirtuins are inhibited by nicotinamide, a product of the deacetylation reaction[11]. In yeast this forms a basis for the regulation of Sir2 activity. Expression of the yeast nicotinamidase, PNC1, is upregulated by longevity-enhancing mild stresses including calorie restriction[12]. In yeast[3] and *C. elegans*[13], added copies of sirtuin genes extend lifespan and Sir2 is required for the lifespan extension conferred by caloric restriction in yeast[14].

Caloric restriction extends mammalian lifespan, although the connections between this effect and mammalian sirtuins have yet to be elucidated. Calorically restricted mammals exhibit lowered rates of age-related disorders including cancer, heart disease, diabetes and neurodegeneration[15, 16]. This has led to the hope that pharmacological agents that mimic the effects of caloric restriction, perhaps by way of sirtuin stimulation, might help prevent or ameliorate multiple age-related diseases.

Recently, a screen for modulators of SIRT1 activity yielded a number of small molecule activators, all of which were plant polyphenols. Several of these Sirtuin Activating Compounds (STACs) extended yeast lifespan in a way that mimicked caloric restriction. Resveratrol, the most potent of these STACs, activated SIRT1 in human cells and enhanced the survival rate of cells stressed by irradiation[17].

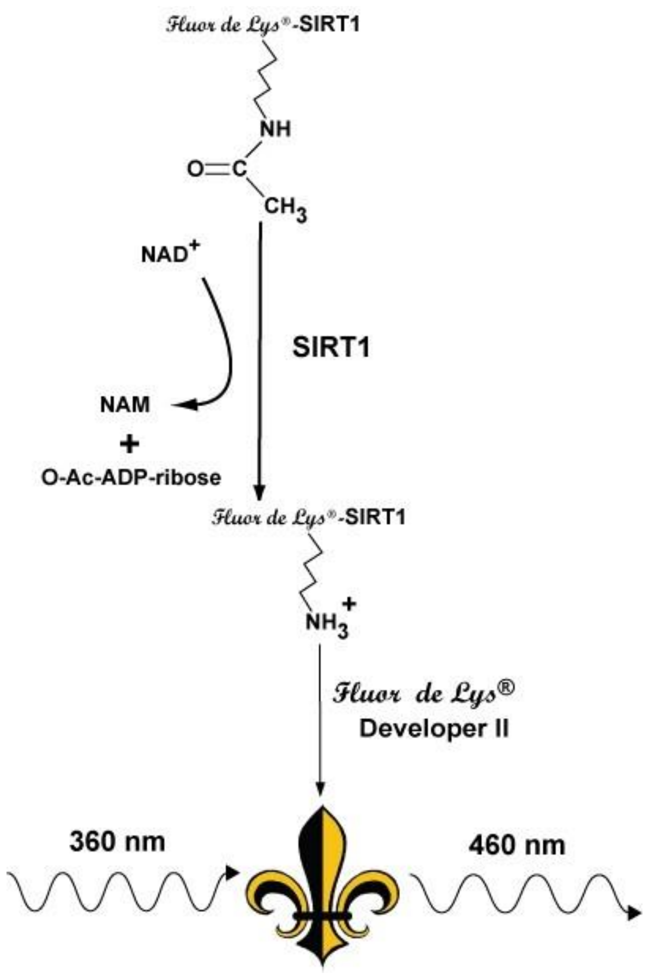

Figure 1. Reaction Scheme of the *SIRT1 Fluorescent Activity Assay.* NAD$^+$- dependent deacetylation of the substrate by recombinant human SIRT1 sensitizes it to Developer II, which then generates a fluorophore (symbol). The fluorophore is excited with 360 nm light and the emitted light (460 nm) is detected on a fluorometric plate reader. NAD+ is consumed in the reaction to produce nicotinamide (NAM) and O-acetyl-ADP-ribose.

The *SIRT1 Fluorescent Activity Assay/Drug Discovery Kit* is a complete assay system designed to measure the lysyl deacetylase activity of the recombinant human SIRT1 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 *SIRT1 Fluorescent Activity Assay* is based on the unique FLUOR DE LYS® SIRT1 Substrate/Developer II combination. The FLUOR DE LYS® SIRT1 Substrate is a peptide comprising amino acids 379-382 of human p53 (Arg-His-Lys-

Lys(Ac)). The assay's fluorescence signal is generated in proportion to the amount of deacetylation of the lysine corresponding to Lys-382, a known *in vivo* target of SIRT1 activity[8-10]. FLUOR DE LYS® SIRT1 was the substrate deacetylated most efficiently by SIRT1 from among a panel of substrates patterned on p53, histone H3 and histone H4 acetylation sites (see Fig. 2, FLUOR DE LYS® SIRT1 is labeled 'p53-382').

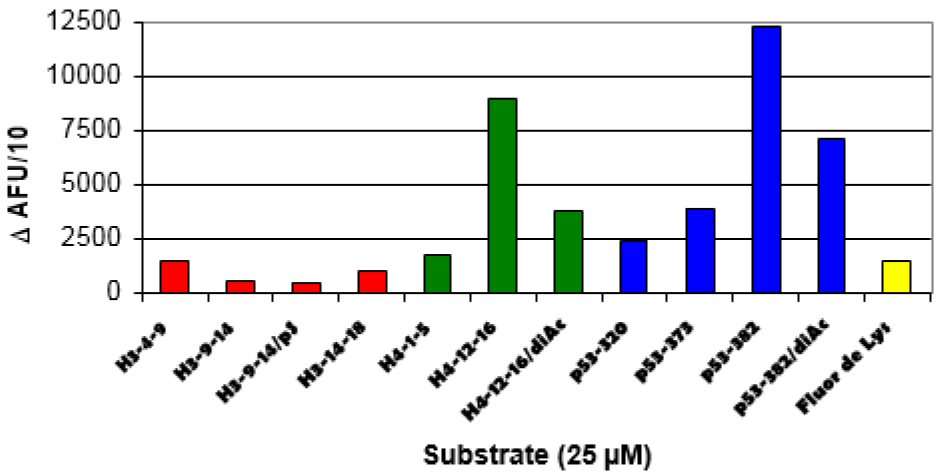

Figure 2. SIRT1 Peptide Substrate Preferences. Initial rates of deacetylation were determined for a series of fluorogenic acetylated peptide substrates based on short stretches of human histone H3, H4 and p53 sequence. Recombinant human SIRT1 (1 U, BML-SE239), was incubated for 10 min at 37°C with 25 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. Fluorescence was then measured in the wells of a clear microplate with a CytoFluor™ II fluorescence plate reader (PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85).

The assay procedure has two steps (Fig. 1). First, the FLUOR DE LYS® SIRT1 Substrate, which comprises the p53 sequence ArBML-GHis-Lys-Lys(Ɛ-acetyl), is incubated with human recombinant SIRT1 together with the cosubstrate NAD^+. Deacetylation of FLUOR DE LYS® SIRT1 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 or activators of SIRT1. Resveratrol (BML-KI284), a SIRT1 activator, and suramin sodium (BML-KI285), an inhibitor, are included as positive controls for these two types of activity modulation (see Figures 8 & 9). Although modulator screens are typically done at relatively low substrate concentration, the kit does include enough substrate to perform kinetic studies over a full range of relevant concentrations (see Figures 6 & 7).

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MATERIALS PROVIDED

BML-SE239-0100 SIRT1 (Sirtuin 1, hSir2^{SIRT1})(human, recombinant)

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

STORAGE: -80°C; AVOID FREEZE/THAW CYCLES!

QUANTITY: 100 U; One U=1 pmol/min at 37°C, 250 µM, FLUOR DE LYS® Substrate (BML-KI104), 500 µM NAD+

BML-KI177-0005 FLUOR DE LYS® SIRT1, Deacetylase Substrate

FORM: 5 mM solution in 50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$

STORAGE: -80°C QUANTITY: 100 µL

BML-KI176-1250 FLUOR DE LYS® Developer II Concentrate (5x)

FORM: 5x Stock Solution; Dilute in Assay Buffer before use. STORAGE: -80°C

QUANTITY: 5 x 250 µL

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 $MgCl_2$.

STORAGE: -80°C QUANTITY: 500 µL

BML-KI283-0500 Nicotinamide (Sirtuin Inhibitor)

FORM: 50 mM Nicotinamide in 50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$.

STORAGE: -80°C QUANTITY: 500 µL

BML-KI284-0010 Resveratrol (Sirtuin Activator)

FORM: Solid

MW: 228.2 STORAGE: -80°C QUANTITY: 10 mg;

SOLUBILITY: DMSO or EtOH to 100 mM (10 mg in 0.44 mL)

BML-KI285-0010 Suramin sodium (Sirtuin Inhibitor)

FORM: Solid

MW: 1429.2 STORAGE: -80°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: -80°C QUANTITY: 30 µL

BML-KI286-0020 Sirtuin Assay Buffer

(50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/ml BSA)

STORAGE: -80°C QUANTITY: 20 mL

80-2407 ½ Volume Microplates

1 clear and 1 white, 96-well

STORAGE: Room temperature.

OTHER MATERIALS NEEDED

- Microplate reading fluorimeter 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

PROTOCOL

Notes on Storage: *Store all components except the microtiter plate at -80°C for the highest stability. Components with storage temperatures other than -80°C can be stored at the temperature listed OR at -80°C. The SIRT1 enzyme,* ***BML-SE239****, 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 enzyme should be refrozen quickly, by placing at -80°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 -80°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 SIRT1 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 SIRT1 inhibitor, stops SIRT1 activity and produces the fluorescent signal. See "Preparing Reagents For Assay" and Table 1 (p. 13).

   Two types of ½-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 fluorimeter 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 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 SIRT1 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. 13).

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 SIRT1 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 SIRT1, 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 (Fig. 4).
4. The K_m of SIRT1 for the FLUOR DE LYS® SIRT1 Substrate has been measured at 64 µM at 3 mM NAD^+ (Fig. 6). The K_m for NAD^+, determined at 1 mM FLUOR DE LYS® SIRT1 Substrate, was 558 µM (Fig. 7). Use of substrate concentrations at or below K_m will help avoid substrate competition effects, which could mask the effectiveness of competitive inhibitors or activators which act to lower substrate K_m’s. Examples of reactions run at several low substrate concentrations and the signals generated at various incubation times are shown in Fig. 5.
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 SIRT1 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 SIRT1 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. 15). In some cases, it may be possible to avoid this retesting by means of measurements taken during the fluorescence development phase of the initial SIRT1 assay.

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 SIRT1 enzyme (BML-SE239) 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 1 U of SIRT1 (BML-SE239) per assay, dilute a sufficient amount to 0.2 U/µL in Assay Buffer (BML-KI286) to provide for the assays to be performed (slightly more than # of wells x 5 µL). Subsequent dilutions of five-fold to 0.04 U/µL or three fold to 0.067 U/µL 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. 13).

3. Prepare dilution(s) of resveratrol, suramin, nicotinamide and/or Test Compounds in Assay Buffer (BML-KI286). Since 10 µL will be used per well (Table 1), and since the final volume of the SIRT1 reaction is 50 µl, these inhibitor dilutions will be 5x their final concentration. A concentrated resveratrol stock may be prepared in either ethanol or DMSO (10 mg in 0.44 mL = 100 mM) and 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 SIRT1 activity and appropriate solvent controls should always be included.

4. Prepare a dilution of the substrates, FLUOR DE LYS®-SIRT1 (BML-KI177; 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 µL per well). One ml will contain 760 µL Assay Buffer, 200 µL 5x Developer II and 40 µL 50 mM nicotinamide. Addition of nicotinamide to the Developer II insures that SIRT1 activity stops when the Developer II is added. Keep diluted Developer II on ice until use.

Performing the Assay:

1. Table 1 gives examples of solutions and volumes for use in various types of SIRT1 assays. These are mixtures for the first, deacetylation phase, of the assay. The SIRT1 reaction is initiated by mixing 25 µL of a 2x substrate solution with 25 µL containing the enzyme. The notes below Table 1 (‡) 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	SIRT1 (0.2 U/µL))	Test Cmpd.or Solvent Control (5x)	Substrates FLUOR DE LYS® SIRT1 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
Resveratrol	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 SIRT1 enzyme:

1) 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 0.04 U/µL SIRT1 in Assay Buffer could be dispensed 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.
2) If the test compound is to be preincubated with enzyme prior to substrate addition, 15 µL of an enzyme master mix consisting of 0.067 U/µL SIRT1 in Assay Buffer could be dispensed 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 <u>after</u> the addition of 1x Developer II/2 mM nicotinamide.

3) Add 25 µL of 0.04 U/µl SIRT1 <u>or</u> 15 µL of 0.067 U/µL SIRT1 plus 10 µL 5x Test Compound <u>or</u> 25 µL Assay Buffer to appropriate wells of the assay plate.
4) Warm the assay plate and 2x substrate solutions to 37°C.
5) Initiate SIRT1 reactions by adding 25 µL 2x substrate solutions to the assay wells and thoroughly mixing. DO NOT ADD SUBSTRATE TO "TIME ZERO" WELLS.
6) Allow SIRT1 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).

<u>Preparation of a Standard Curve:</u>

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 fluorimeter 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 fluorimeter 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 SIRT1 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 SIRT1 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. 5 µL of 20 µM Deacetylated Standard (BML-KI142) in Assay Buffer; i.e. amount equivalent to 2 µM/100 pmol in the 50 µL SIRT1 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 SIRT1 reaction and that due to a subsequent Deacetylated Standard 'spike'.

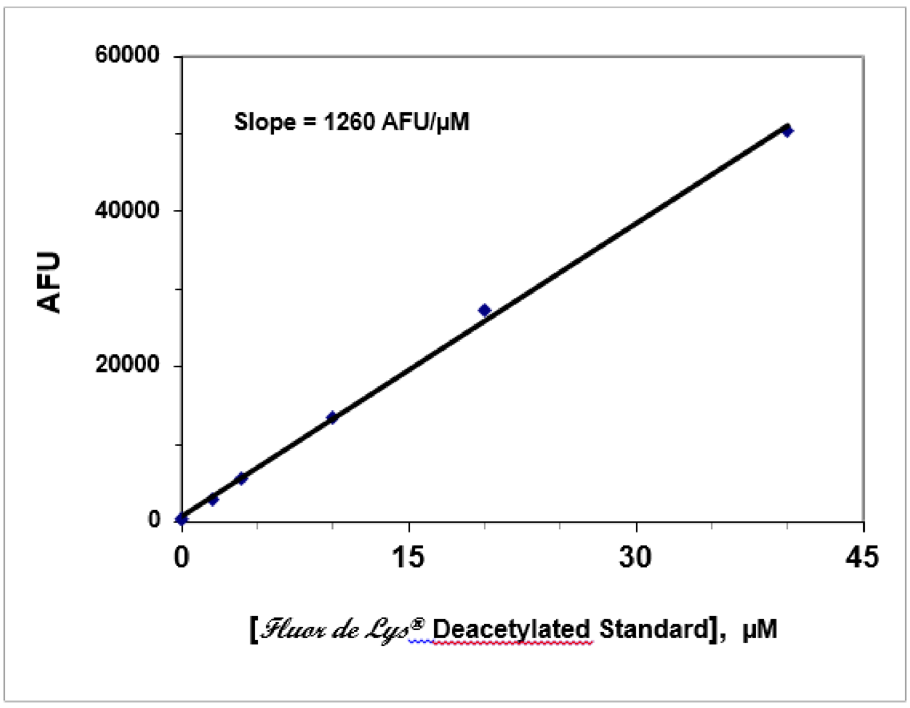

Figure 3. Fluorescence Standard Curve. Fifty µL aliquots of FLUOR DE LYS® Deacetylated Standard, in Assay Buffer at the indicated concentrations, were mixed with 50 µL 0.2x Developer II and incubated 15 min., 25°C. Fluorescence was then measured in the wells of the clear microplate with a CytoFluor™ 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 SIRT1, 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) SIRT1 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 1x 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)	Diluted[6] FLUOR DE LYS® deAc. Standard (1.25x)	DEVELOPER II (1x).
Control	10 µL	40 µL	50 µL
Test Compound	10 µL	40 µL	50 µL

[6]The 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 SIRT1 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.

Preparation of a Standard Curve:

The *SIRT1 Fluorescent Activity Assay/Drug Discovery Kit* has been used for investigating SIRT1 kinetics as a function of the concentrations of FLUOR DE LYS® SIRT1 Substrate and NAD+ (Figures 4-7) as well as for the discovery and characterization of activators and inhibitors of the enzyme (Figures 8 & 9).

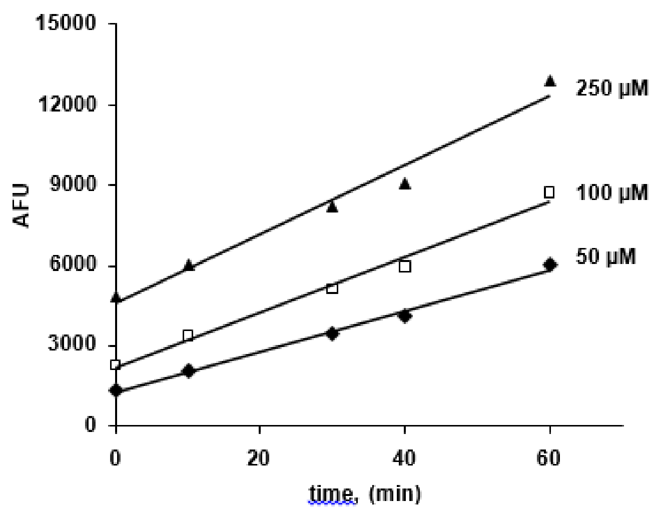

Figure 4. Time Courses of FLUOR DE LYS® SIRT1 Deacetylation by Recombinant SIRT1. SIRT1 (2 U/well) was incubated (37°C) with the indicated concentrations of peptide substrate and 500 µM NAD+. Reactions were stopped at indicated times with FLUOR DE LYS® Developer II/2 mM nicotinamide and fluorescence measured. (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85).

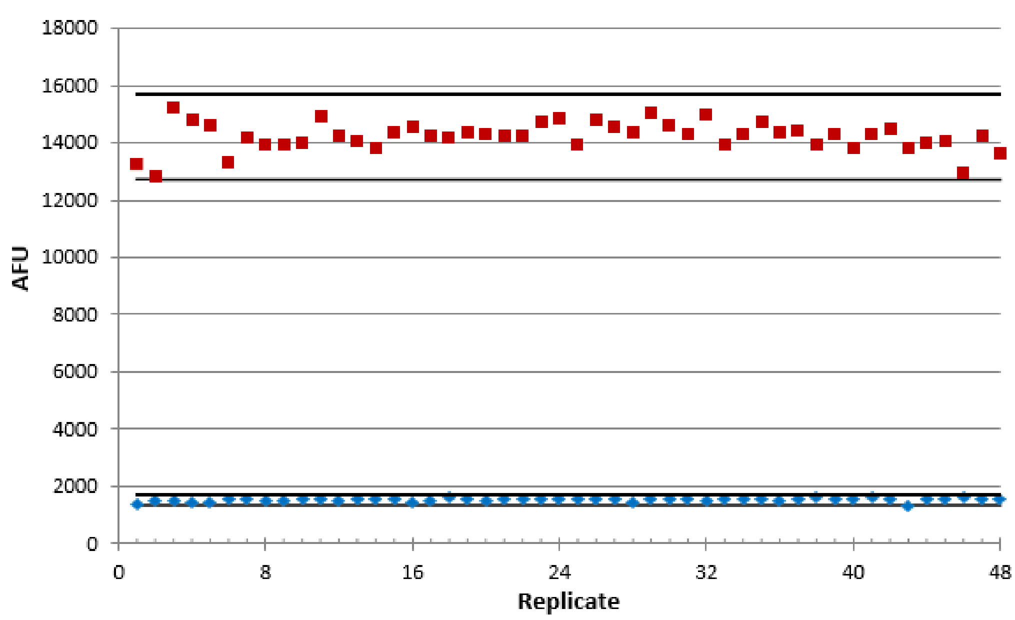

Figure 5. **Z-factor analysis**. SIRT 1 (1U/well) (red squares) or buffer (blue diamonds) was incubated for 60 minutes at 37°C with 55 µM FLUOR DE LYS® SIRT1, Deacetylase Substrate and 600 µM NAD+. Reactions were stopped with FLUOR DE LYS® Developer II/2 mM nicotinamide, The Z' factor for this assay was 0.902, (Z-factor = 1-((3 x SD positive + 3 x SD negative)/(mean positive – mean negative)). Dashed lines indicate the 3 x Standard deviation range.

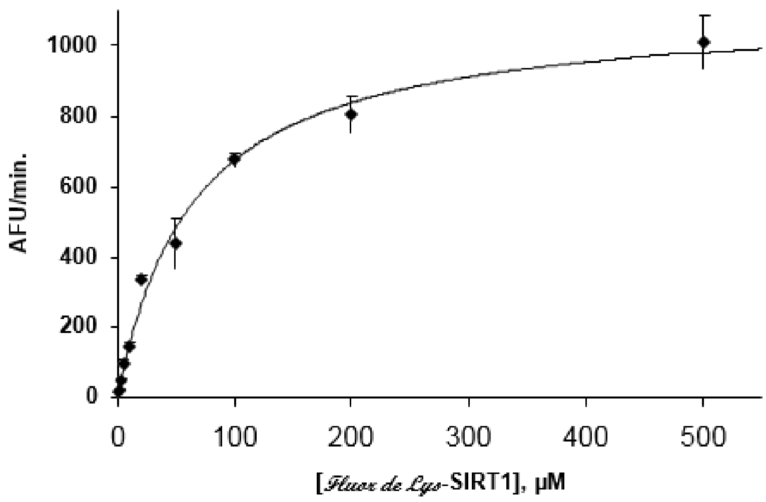

Fig. 6 Dependence of SIRT1 Kinetics on the Concentration of FLUOR DE LYS® SIRT1. Initial deacetylation rates of SIRT1 were determined with 5 min. incubations (37°C) in the presence of 3 mM NAD+. Reactions were stopped with FLUOR DE LYS® Developer II/2 mM nicotinamide and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85). Each point represents the mean of four determinations and the error bars are standard errors. The line is a non-linear least squares fit to the Michaelis-Menten equation. The Km for FLUOR DE LYS® SIRT1 was 64 µM and the Vmax was 1107 AFU/min.

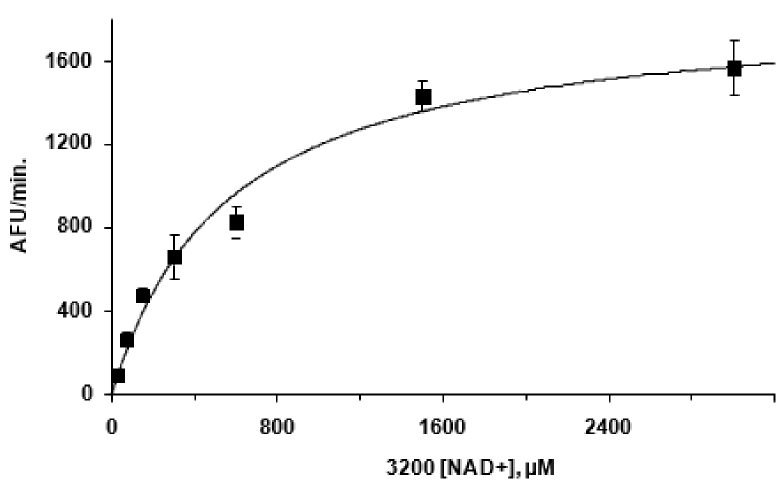

Fig. 7 Dependence of SIRT1 Kinetics on the Concentration of NAD+. Initial deacetylation rates of SIRT1 were determined with 5 min. incubations (37°C) in the presence of 1 mM *FLUOR DE LYS*®SIRT1. Reactions were stopped with *Fluor de Lys*® Developer II/2 mM nicotinamide and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85). Each point represents the mean of four

determinations and the error bars are standard errors. The line is a non-linear least squares fit to the Michaelis-Menten equation. The Km for NAD+ was 558 µM and the Vmax was 1863 AFU/min.

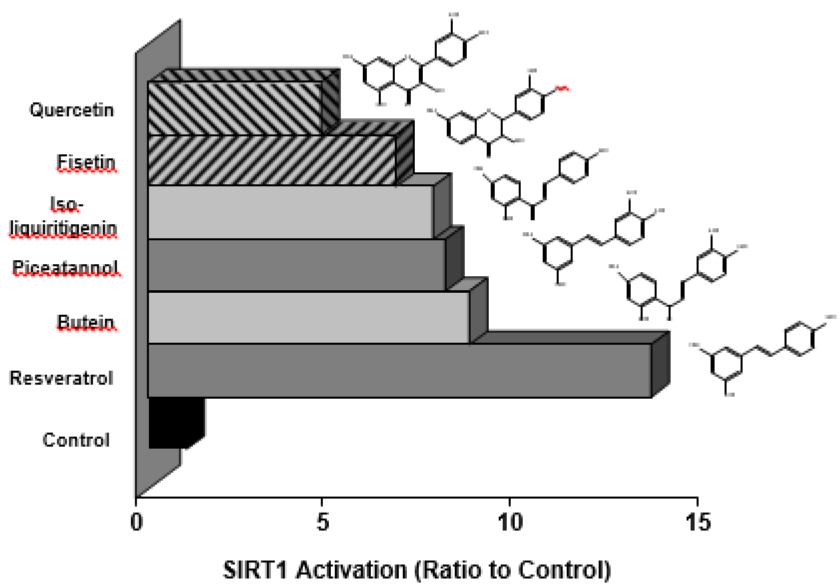

Fig. 8 Polyphenol Activators of SIRT1. Initial deacetylation rates of SIRT1 were determined at 25 µM *Fluor de Lys*®-SIRT1, 25 µM NAD+ (37°C) in the absence (Control) or presence of 100 µM of the indicated compound. Reactions were stopped with FLUOR DE LYS® Developer II/2 mM nicotinamide and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85).

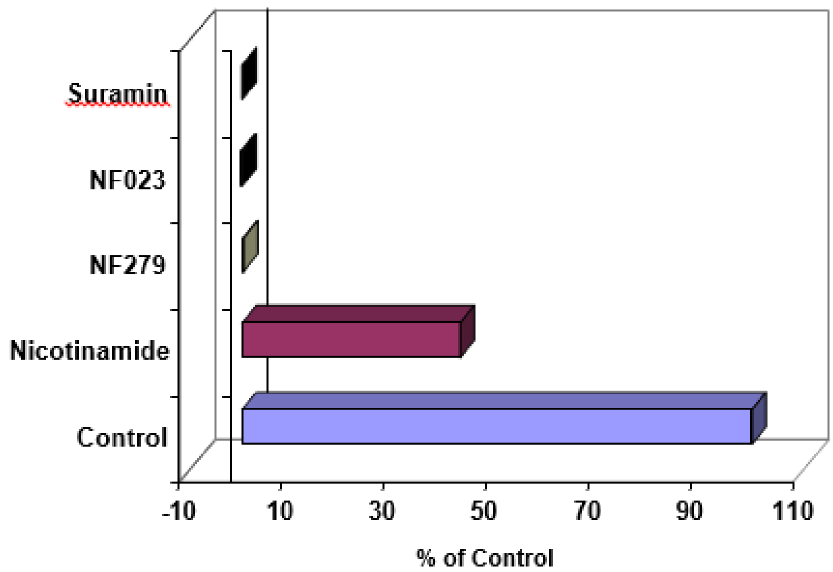

Fig. 9 Inhibitors of SIRT1. Initial deacetylation rates of SIRT1 were determined at 25 µM FLUOR DE LYS® SIRT1, 25 µM NAD^+ (37°C) in the absence (Control) or presence of 100 µM of the indicated compound. Reactions were stopped with FLUOR DE LYS® Developer II/2 mM nicotinamide and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85). NF023 and NF279 are structural relatives of suramin available as part of the Purinergic Ligand Library (Cat.# BML-2820).

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 GUARENTEE OF PARTICULAR RESULTS, THROUGH THE USEOF THESE PROCEDURES, IS MADE OR IMPLIED.

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