Subject: RevGenetics Resveratrol SIRT1 Test Information
From: Anthony Loera <anthony@revgenetics.com>
To: "galt@cems.umn.edu" <galt@cems.umn.edu>
Date Sent: Thursday, March 27, 2008 11:39:50 AM GMT-04:00
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Attachments: ak555.doc,RevGenetics X-100_SIRT1_assays_1-3-08.xls

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Hi,

the link below is the method and description of the test done with our
RevGenetics Resveratrol (It is in Word Format):
http://www.revgenetics.com/images/biomol/ak555.doc

The next link, is the actual data from the test that was performed and
compared using Biomol Research Resveratrol, and RevGenetics
Resveratrol.
http://www.revgenetics.com/images/biomol/RevGenetics%20X-100_SIRT1_assays_1-3-08.xls

The files are attached as well, in case the links don't work.

thank you
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(SIRT1 Fluorimetric Drug Discovery Kit* - AK-555(

image14.wmf **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. 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-3828-10.

Sirtuins are inhibited by nicotinamide, a product of the deacetylation reaction11. 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 restriction12. In yeast3 and *C. elegans*13, added copies of sirtuin genes extend lifespan and Sir2 is required for the lifespan extension conferred by caloric restriction in yeast14.

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 neurodegeneration15,16. 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 irradiation17.

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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 BIOMOL® TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

image1.wmf
image3.wmf

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.

*Patent Pending.

DESCRIPTION

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 activity8-10. 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').

image4.emf

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, SE-239), 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 (KI-101) 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 Arg-His-Lys-Lys(ε-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 (KI-284), a SIRT1 activator, and suramin sodium (KI-285), 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).

COMPONENTS OF AK-555

SE-239 SIRT1 (Sirtuin 1, hSir2SIRT1)(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: -70°C; AVOID FREEZE/THAW CYCLES!

QUANTITY: 100 U; One U=1 pmol/min at 37°C, 250 µM, Fluor de Lys™ Substrate (KI-104), 500 µM NAD+

KI-177 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 MgCl2

STORAGE: -70°C

QUANTITY: 100 µl

KI-176 Fluor de Lys® Developer II Concentrate (5x)

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

STORAGE: -70°C

QUANTITY: 5 x 250 µl

KI-282 NAD+ (Sirtuin Substrate)

FORM: 50 mM ß-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 µl

KI-283 Nicotinamide (Sirtuin Inhibitor)

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

STORAGE: -70°C

QUANTITY: 500 µl

KI-284 Resveratrol (Sirtuin Activator)

FORM: Solid

MW: 228.2

STORAGE: -70°C

QUANTITY: 10 mg;

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

KI-285 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)

KI-142 Fluor de Lys® Deacetylated Standard

FORM: 10 mM in DMSO (dimethylsulfoxide)

STORAGE: -70°C

QUANTITY: 30 µl

KI-286 Sirtuin Assay Buffer

(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

KI-101 1/2 VOLUME MICROPLATE

STORAGE: Room temperature.

KI-110 1/2 VOLUME WHITE MICROPLATE

STORAGE: Room temperature.

OTHER MATERIALS REQUIRED

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

ASSAY PROCEDURES

Notes On Storage: *Store all components except the microplates and instruction booklet at -70°C for the highest stability. The SIRT1 enzyme, SE-239, 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 (KI-176) 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 ul. The second stage, which is initiated by the addition of 50 ul of Developer II, including a SIRT1 inhibitor, stops SIRT1 activity and produces the fluorescent signal. See "Preparing Reagents For Assay" and Table 1 (p. 4).

Two types of ½-volume, 96-well microplates are provided with the kit. The signal obtained with the opaque, white plate (KI-110) can be ~5-fold greater than that obtained with the clear plate (KI-101). 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 (KI-101 or KI-110) 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 (KI-176). 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 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-Km 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 Km of SIRT1 for the **Fluor de Lys-SIRT1** Substrate has been measured at 64 µM at 3 mM NAD+ (Fig. 6). The Km for NAD+, determined at 1 mM **Fluor de Lys-SIRT1** Substrate, was 558 µM (Fig. 7). Use of substrate concentrations at or below Km will help avoid substrate competition effects, which could mask the effectiveness of competitive inhibitors or activators which act to lower substrate Km'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 (KI-142) 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 SIRT1 assay. This is also discussed in that section (pp. 4-5).

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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 (SE-239) and the 5x Developer II (KI-176). (See 'Notes on Storage', above.) All undiluted kit components are stable for several hours on ice.

2. Assuming 1 U of SIRT1 (SE-239) per assay, dilute a sufficient amount to 0.2 U/µl in Assay Buffer (KI-286) 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. 4).

3. Prepare dilution(s) of resveratrol, suramin, nicotinamide and/or Test Compounds in Assay Buffer (KI-286). 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** (KI-177; 5 mM) and NAD+ (KI-282, 50 mM), in Assay Buffer (KI-286), that will be 3.33x the desired final concentrations. For inhibitor screening, substrate concentrations at or below the Km 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.

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 NAD+ (3.33x)	SIRT1 plus
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 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.

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 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.

2. Add 25 µl of 0.04 U/µl SIRT1 or 15 µl of 0.067 U/µl SIRT1 plus 10 µl 5x Test Compound or 25 µl Assay Buffer to appropriate wells of the assay plate.

3. Warm the assay plate and 2x substrate solutions to 37°C.

4. 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.

5. 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).

6. 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. 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 (KI-142)**

Preparation of a Standard Curve:

1. The exact concentration range of the Fluor de Lys® Deacetylated Standard (KI-142) 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 0.2x 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 0.2x 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 0.2x 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. amount equivalent to 5 µM 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’.

image5.wmf

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 (KI-101) 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 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 Fluor de Lys® deAc. Standard (1.25x)	DEVELOPER II (0.2x).
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 SIRT1 assay. The

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.

APPLICATION EXAMPLES

image6.png

image7.wmf 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).

image8.emf **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).

image9.emf

DISCLAIMER

Figure 5. SIRT1 Kinetics and Signal/Noise Ratio at Low Substrate Concentration. SIRT1 (1 U/well) was incubated (37°C) with the indicated concentrations of peptide substrate and 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).

Ratios of the 60 min. signals to background (0 min.) for [Fdl-SIRT1]/[NAD+]'s of 5 µM/25 µM, 25 µM/25 µM and 100 µM/100 µM were 3.7, 6.0 and 15 respectively.

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.

image10.emf

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).

image11.emf

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. # 2820).

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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PRODUCT	**CATALOG #**
SIRT1 Fluorescent Activity Assay	AK-555
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Fluor de Lys® Developer	KI-105
Fluor de Lys-SIRT1 Substrate	KI-177
Fluor de Lys-SIRT2 Substrate	KI-179
Fluor de Lys-H4-AcK16 Substrate	KI-174
Fluor de Lys-HDAC8 Substrate	KI-178
Fluor de Lys® Developer II	KI-176
HeLa Nuclear Extract	KI-140
HDAC8 (recombinant, human)	SE-145
SIRT1 (recombinant, human)	SE-239
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SIRT3 (recombinant, human)	SE-270
Resveratrol (SIRT1 Activator)	FR-104
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Chart1

50441
27207
13444
5558
2913
372
Slope = 1260 AFU/µM
[Fluor de Lysä Deacetylated Standard], µM
AFU

time course

time (min)	500 µM	100 µM	20 µM			
0	0	0	0	-446	-1014	-2654
5	1093	714	396	-50	-300	-1561
10	2365	1485	628	182	471	-289
15	3135	2044	844	398	1030	481

time course

0 0 0
0 0 0
0 0 0
0 0 0
500 µM 213 AFU/min

100 µM 138 AFU/min
20 µM 55 AFU/min
500 µM
100 µM
20 µM
time (min)
DAFU

AMC std curve

AFU	[AMC], µM				
+++	200				
+++	100				
+++	50		40	50441	40
50441	20		20	27207	20
27207	10		10	13444	10
13444	5		4	5558	4
5558	2		2	2913	2
2913	1		0	372	0
372	0				

AMC std curve

AMC
[AMC], (µM)
AFU
AMC Standard Curve in TBS/1mM MgCl2/1µM trichostatin A

IP

Slope = 1260 AFU/µM
[Fluor de Lysä Deacetylated Standard], µM
AFU

trich.A inhibition

time (min)	10 µg	cont.
0	401	365
10	1995	390
20	7045	587
40	17085	959

trich.A inhibition

0 0
0 0
0 0
0 0

10 µg anti-HDAC1
Non-immune Control Serum
10 µg anti-HDAC1 (SA-401)
Rabbit serum control
time (min)
AFU

	0 nM	0.5 nM	5 nM	50 nM	500 nM
rate (AFU/min	116.4	100.6	50.8	12.6	7.3
	100	86.4261168385	43.6426116838	10.824742268	6.2714776632

[Trichostatin A]
% activity

1308

Sirt1 0.5ug/well 1/3/08 360/460/Gain 70	25uM p53-382 w/25uM NAD [µM]	w/o Compound	0 min	0 min	20 min	20 min	20 min	Dif. AFU (0, 20min)	spiked with 5 µl of 30µM AMC (final of 3µM) 0 min	spiked with 5 µl of 30µM AMC (final of 3µM) 0 min	spiked with 5 µl of 30µM AMC (final of 3µM) 20 min	spiked with 5 µl of 30µM AMC (final of 3µM) 20 min	spiked with 5 µl of 30µM AMC (final of 3µM) 20 min	Mean AFU Increase from 3 µM AMC	Mean AFU increase from 3 µM AMC in Controls	pmol/AFU (Controls)	Quench Correction Factor	Mean Dif. AFU (0, 20min) Quench Corrected	Mean 0 min.	STD. DEV. Dif. AFU (0, 20min)	STD. DEV. Dif. AFU (0, 20min) Quench Corrected	Mean Specific Activity pmol/min/µg	STD. DEV. Specific Activity pmol/min/µg
Resveratrol	0		226	235	459	533	516	272.2	4876	5339	5832	5875	5899	4087.2	4242.5	0.0353565115	1.0379966725	282.5080943759	230.5	38.7599449604	40.2326938967	0.9988497439	0.1422487242
Biomol FR-104	5		224	233	825	864	936	646.5	5080	5236	6788	5726	6268	4223.0			1.0046175704	649.4852592943	228.5	56.3116329012	56.5716558331	2.296352557	0.2000175749
	10		222	225	971	1120	960	793.5	4622	5171	6501	6484	6180	3989.4			1.0634431243	843.8421191157	223.5	89.3700173436	95.0399304608	2.9835303885	0.3360279301
	15		226	247	1324	1653	1969	1412.2	4653	4582	6640	6891	7404	3836.8			1.1057391576	1561.4879804351	236.5	322.5218338862	356.6250209191	5.520874978	1.2609012552
	20		227	223	1395	2098	2180	1666.0	4766	4695	6985	7505	7646	3872.8			1.0954606486	1825.0374406114	225	431.5008690605	472.6922219039	6.4526936269	1.6712742544
	50		239	245	2585	3476	4084	3139.7	4518	4362	7473	8076	9434	3548.6			1.1955419038	3753.6030641192	242	753.9392106353	901.3659192696	13.2714266737	3.1869144125
	100		270	268	3302	3347	3905	3249.0	4633	4467	7495	8105	9014	3563.8			1.1904427858	3867.7486110332	269	335.9062369174	399.8771564403	13.6750053766	1.4138256682
	500		313	321	2927	2923	3630	2843.0	3262	2851	6344	6661	7929	2639.8			1.607129328	4569.0686794454	317	407.0368533683	654.1608646167	16.1546276765	2.312883861
Rev Genetic X-100 Batch 4070928	0		248	238	517	555	556	299.7	5466	5484	6747	6186	5889	4397.8			0.9646868889	289.0845043734	243	22.2336081942	21.4484703179	1.0221016279	0.0758342841
	5		231	231	854	823	899	627.7	4705	4989	6467	5956	5804	3956.2			1.0723674233	673.0892860152	231	38.2143079662	40.9797789663	2.3798081341	0.1448901555

#070928

10	232	227	1003	1094	1045	817.8	5283	4979	6744	5856	6149	4111.4	1.0318869485	843.9115427024	229.5	45.5448496905	46.9971359664	2.983775846	0.1661654238
15	234	229	1724	1729	1640	1466.2	5038	4553	6962	6588	6466	3837.4	1.1055662688	1620.9444111464	231.5	50.0033332222	55.2819985395	5.7310921073	0.1954577981
20	218	225	1965	2191	1958	1816.5	4463	4460	7304	7135	6656	3658.2	1.1597233612	2106.6374856487	221.5	132.5481044753	153.7191332449	7.4483328261	0.5434970535
50	243	248	3818	3948	3572	3533.8	4310	4634	9396	9076	8539	3773.6	1.124258003	3972.940406155	245.5	190.9589833795	214.687165303	14.046926747	0.759058676
100	269	272	4333	3868	3643	3677.5	4215	4243	9378	8738	8394	3635.6	1.1669325558	4291.3944740896	270.5	351.8877661983	410.6292903774	15.1728688723	1.4518414505
500	322	299	3557	3658	4446	3576.5	3749	3458	7776	7800	8900	3031.6	1.3994260457	5005.047252276	310.5	486.735040859	681.1496935098	17.6960953175	2.4083069139

[Resveratrol] µM	Biomol Resveratrol Cat. # FR-104 Mean Specific Activity pmol/min/µg	RevGenetics X-100 Resveratrol Batch #070928 Mean Specific Activity pmol/min/µg	Biomol Resveratrol Cat. # FR-104 Standard Deviation pmol/min/µg	RevGenetics X-100 Resveratrol Batch #070928 Standard Deviation pmol/min/µg
0	0.9988497439	1.0221016279	0.1422487242	0.0758342841
5	2.296352557	2.3798081341	0.2000175749	0.1448901555
10	2.9835303885	2.983775846	0.3360279301	0.1661654238
15	5.520874978	5.7310921073	1.2609012552	0.1954577981
20	6.4526936269	7.4483328261	1.6712742544	0.5434970535
50	13.2714266737	14.046926747	3.1869144125	0.759058676
100	13.6750053766	15.1728688723	1.4138256682	1.4518414505
500	16.1546276765	17.6960953175	2.312883861	2.4083069139

[Resveratrol] µM	Biomol Resveratrol Cat. # FR-104 Fold Activation	RevGenetics X-100 Resveratrol Batch #070928 Fold Activation	Biomol Resveratrol Cat. # FR-104 Standard Deviation Fold Activation	RevGenetics X-100 Resveratrol Batch #070928 Standard Deviation Fold Activation
5	2.2989969924	2.3283478562	0.2002479113	0.141757093
20	6.4601244246	7.2872722466	1.6731988616	0.5317446315
100	13.6907532493	14.8447751753	1.4154538026	1.4204472539

1308

0.1422487242	0.1422487242	0.0758342841	0.0758342841
0.2000175749	0.2000175749	0.1448901555	0.1448901555
0.3360279301	0.3360279301	0.1661654238	0.1661654238
1.2609012552	1.2609012552	0.1954577981	0.1954577981
1.6712742544	1.6712742544	0.5434970535	0.5434970535
3.1869144125	3.1869144125	0.759058676	0.759058676
1.4138256682	1.4138256682	1.4518414505	1.4518414505
2.312883861	2.312883861	2.4083069139	2.4083069139

BIOMOL FR-104
RevGenetics X-100
[Resveratrol], µM
SIRT1 Rate, pmol/min./µg

0.2002479113	0.2002479113	0.141757093	0.141757093
1.6731988616	1.6731988616	0.5317446315	0.5317446315
1.4154538026	1.4154538026	1.4204472539	1.4204472539

5
20
100
BIOMOL, FR-104
RevGenetics X-100
[Resveratrol], µM
SIRT1 Activity, Fold Activation

✦ SIRT1 Fluorimetric Drug Discovery Kit* - AK-555✦

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 eubacteria[2]. 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 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].

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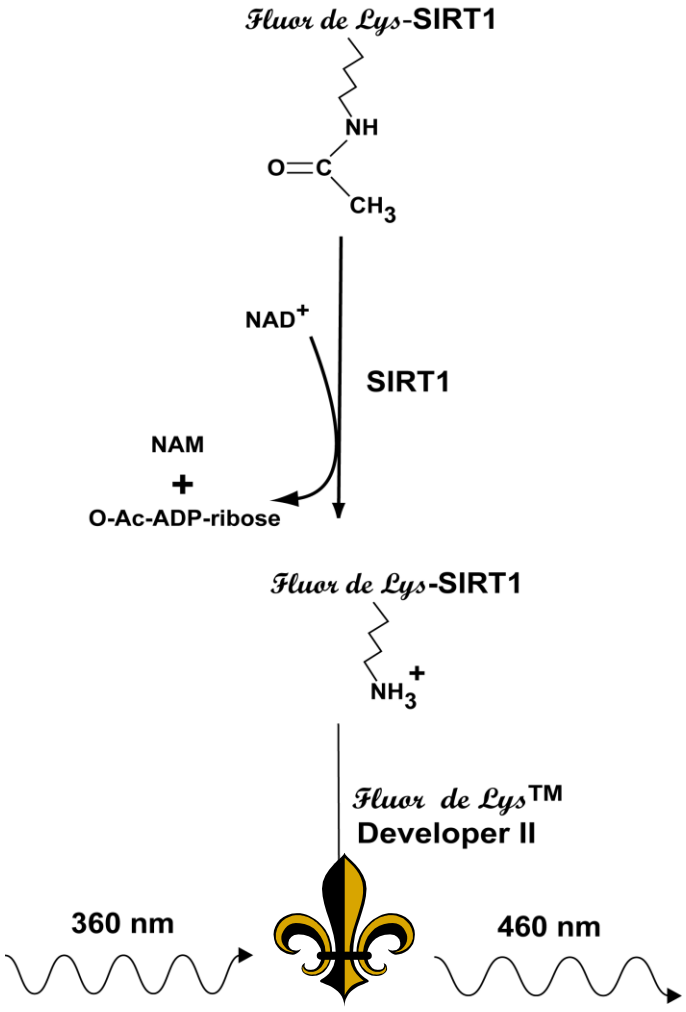

Figure 1. Reaction Scheme of the *SIRT1 Fluorescent Activity Assay.** NAD^+-dependent deacetylation of the substrate by recombinant human SIRT1 sensitizes it to Developer II, which then generates a fluorophore (symbol). The fluorophore is excited with 360 nm light and the emitted light (460 nm) is detected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

*Patent Pending.

DESCRIPTION

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').

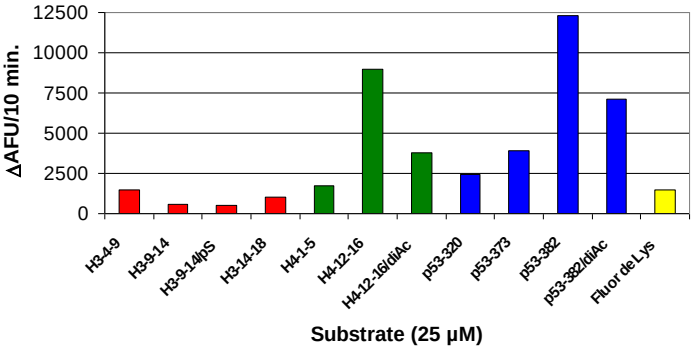

Figure 2. SIRT1 Peptide Substrate Preferences. Initial rates of deacetylation were determined for a series of fluorogenic acetylated peptide substrates based on short stretches of human histone H3, H4 and p53 sequence. Recombinant human SIRT1 (1 U, SE-239), was incubated for 10 min at 37°C with 25 µM 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 (KI-101) 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 Arg-His-Lys-Lys(ε-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 (KI-284), a SIRT1 activator, and suramin sodium (KI-285), 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).

COMPONENTS OF AK-555

SE-239 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: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 100 U; One U=1 pmol/min at 37°C, 250 µM, ***Fluor de Lys***™ Substrate (KI-104), 500 µM NAD^+

KI-177 ***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: -70°C
QUANTITY: 100 µl

KI-176 ***Fluor de Lys***™ Developer II Concentrate (5x)
FORM: 5x Stock Solution; Dilute in Assay Buffer before use.
STORAGE: -70°C
QUANTITY: 5 x 250 µl

KI-282 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: -70°C
QUANTITY: 500 µl

KI-283 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: -70°C
QUANTITY: 500 µl

KI-284 Resveratrol (Sirtuin Activator)
FORM: Solid
MW: 228.2
STORAGE: -70°C
QUANTITY: 10 mg;
SOLUBILITY: DMSO or EtOH to 100 mM (10 mg in 0.44 ml)

KI-285 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)

KI-142 ***Fluor de Lys***™ Deacetylated Standard
FORM: 10 mM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 30 µl

KI-286 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: -70°C
QUANTITY: 20 ml

KI-101 1/2 VOLUME MICROPLATE
STORAGE: Room temperature.

KI-110 1/2 VOLUME WHITE MICROPLATE
STORAGE: Room temperature.

OTHER MATERIALS REQUIRED

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

ASSAY PROCEDURES

Notes On Storage: *Store all components except the microplates and instruction booklet at -70°C for the highest stability. The SIRT1 enzyme, SE-239, 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 (KI-176) 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 ul. The second stage, which is initiated by the addition of 50 ul of Developer II, including a SIRT1 inhibitor, stops SIRT1 activity and produces the fluorescent signal. See "Preparing Reagents For Assay" and Table 1 (p. 4).

   Two types of ½-volume, 96-well microplates are provided with the kit. The signal obtained with the opaque, white plate (KI-110) can be ~5-fold greater than that obtained with the clear plate (KI-101). 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 (KI-101 or KI-110) 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 (KI-176). 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 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 (KI-142) 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 SIRT1 assay. This is also discussed in that section (pp. 4-5).

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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 (SE-239) and the 5x Developer II (KI-176). (See 'Notes on Storage', above.) All undiluted kit components are stable for several hours on ice.
2. Assuming 1 U of SIRT1 (SE-239) per assay, dilute a sufficient amount to 0.2 U/µl in Assay Buffer (KI-286) 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. 4).
3. Prepare dilution(s) of resveratrol, suramin, nicotinamide and/or Test Compounds in Assay Buffer (KI-286). 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** (KI-177; 5 mM) and NAD^+ (KI-282, 50 mM), in Assay Buffer (KI-286), 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 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.
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 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.

2. Add 25 µl of 0.04 U/µl SIRT1 or 15 µl of 0.067 U/µl SIRT1 plus 10 µl 5x Test Compound or 25 µl Assay Buffer to appropriate wells of the assay plate.
3. Warm the assay plate and 2x substrate solutions to 37°C.
4. 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.
5. 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).
6. 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. 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 (KI-142)

Preparation of a Standard Curve:

1. The exact concentration range of the ***Fluor de Lys***™ Deacetylated Standard (KI-142) 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 0.2x 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 0.2x 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 0.2x 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. amount equivalent to 5 µM 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'.

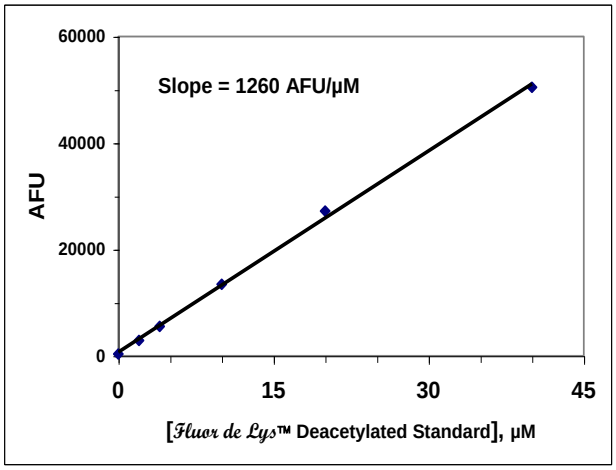

Figure 3. Fluorescence Standard Curve. Fifty µl aliquots of *Fluor de Lys*™ Deacetylated Standard, in Assay Buffer at the indicated concentrations, were mixed with 50 µl 0.2x Developer II and incubated 15 min., 25°C. Fluorescence was then measured in the wells of the clear microplate (KI-101) with a 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 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)	Diluted[6] *Fluor de Lys*™ deAc. Standard (1.25x)	DEVELOPER II (0.2x).
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.

APPLICATION EXAMPLES

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).

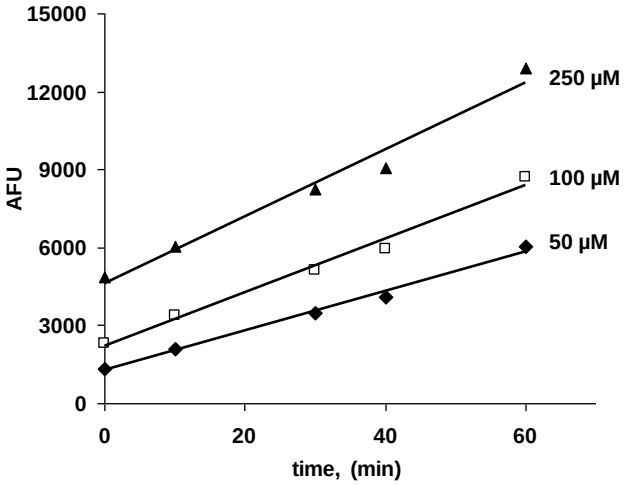

Figure 4. Time Courses of *Fluor de Lys*–SIRT1 Deacetylation by Recombinant SIRT1. SIRT1 (2 U/well) was incubated (37°C) with the indicated concentrations of peptide substrate and 500 µM NAD^+. Reactions were stopped at indicated times with ***Fluor de Lys***™ Developer II/2 mM nicotinamide and fluorescence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

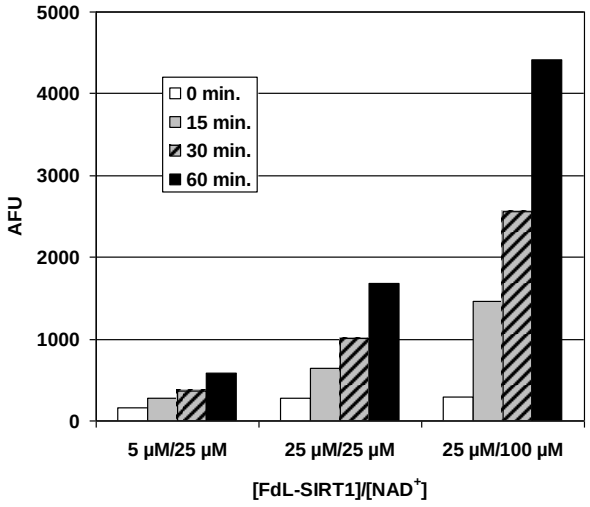

Figure 5. SIRT1 Kinetics and Signal/Noise Ratio at Low Substrate Concentration. SIRT1 (1 U/well) was incubated (37°C) with the indicated concentrations of peptide substrate and NAD^+. Reactions were stopped at indicated times with ***Fluor de Lys***™ Developer II/2 mM nicotinamide and fluorescence measured. (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85).
Ratios of the 60 min. signals to background (0 min.) for [FdL-SIRT1]/[NAD^+]'s of 5 µM/25 µM, 25 µM/25 µM and 100 µM/100 µM were 3.7, 6.0 and 15 respectively.

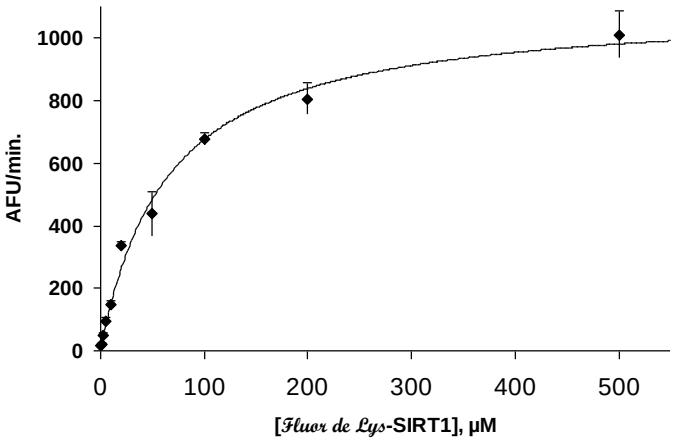

Fig. 6 Dependence of SIRT1 Kinetics on the Concentration of *Fluor de Lys*–SIRT1. Initial deacetylation rates of SIRT1 were determined with 5 min. incubations (37°C) in the presence of 3 mM NAD^+. Reactions were stopped with ***Fluor de Lys***™ Developer II/2 mM nicotinamide and fluorescence measured (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 K_m for ***Fluor de Lys*–SIRT1** was 64 µM and the V_{max} was 1107 AFU/min.

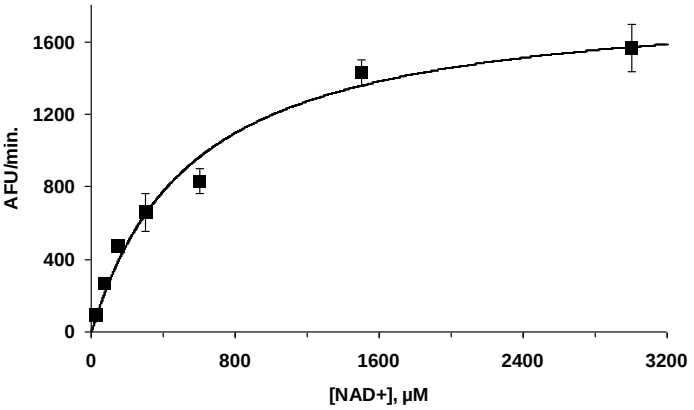

Fig. 7 Dependence of SIRT1 Kinetics on the Concentration of NAD^+. Initial deacetylation rates of SIRT1 were determined with 5 min. incubations (37°C) in the presence of 1 mM ***Fluor de Lys*–SIRT1**. Reactions were stopped with ***Fluor de Lys***™ Developer II/2 mM nicotinamide and fluorescence measured (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 K_m for NAD^+ was 558 µM and the V_{max} was 1863 AFU/min.

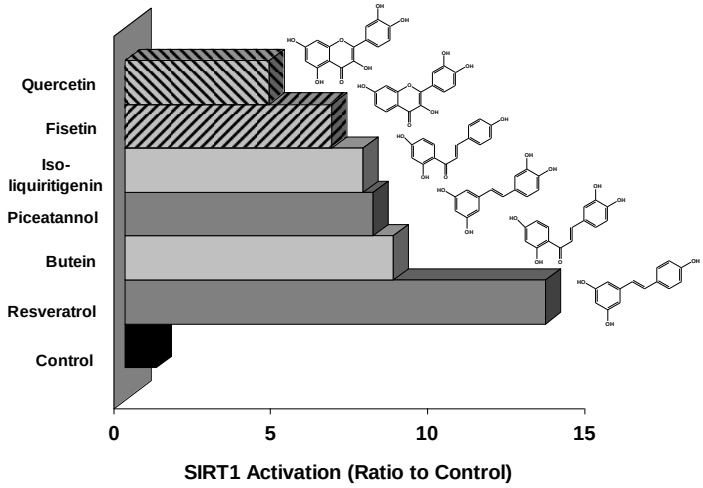

Fig. 8 Polyphenol Activators of SIRT1. Initial deacetylation rates of SIRT1 were determined at 25 µM ***Fluor de Lys*–SIRT1,** 25 µM NAD^+ (37°C) in the absence (Control) or presence of 100 µM of the indicated compound. Reactions were stopped with ***Fluor de Lys***™ Developer II/2 mM nicotinamide and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

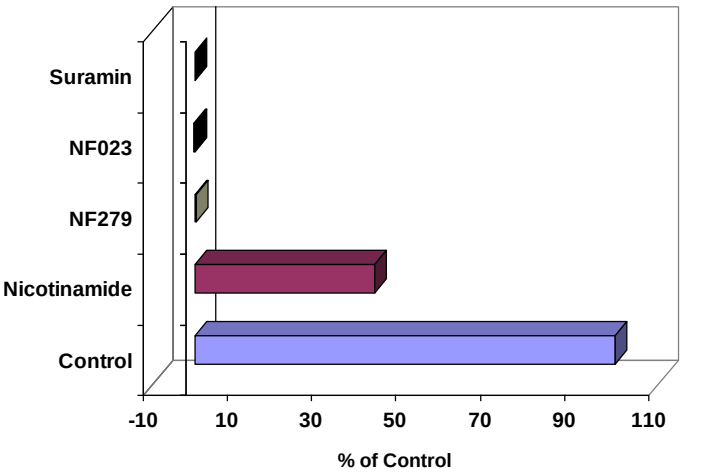

Fig. 9 Inhibitors of SIRT1. Initial deacetylation rates of SIRT1 were determined at 25 µM ***Fluor de Lys***–SIRT1, 25 µM NAD^+ (37°C) in the absence (Control) or presence of 100 µM of the indicated compound. Reactions were stopped with ***Fluor de Lys***™ Developer II/2 mM nicotinamide and fluorescence 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. # 2820).

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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ALSO AVAILABLE …

PRODUCT	CATALOG #
SIRT1 Fluorescent Activity Assay	AK-555
HDAC Fluorescent Activity Assay	AK-500
HDAC Colorimetric Assay Kit	AK-501
Fluor de Lys™ Substrate	KI-104
Fluor de Lys™ Developer	KI-105
Fluor de Lys-SIRT1 Substrate	KI-177
Fluor de Lys-SIRT2 Substrate	KI-179
Fluor de Lys-H4-AcK16 Substrate	KI-174
Fluor de Lys-HDAC8 Substrate	KI-178
Fluor de Lys™ Developer II	KI-176
HeLa Nuclear Extract	KI-140
HDAC8 (recombinant, human)	SE-145
SIRT1 (recombinant, human)	SE-239
SIRT2 (recombinant, human)	SE-251
SIRT3 (recombinant, human)	SE-270
Resveratrol (SIRT1 Activator)	FR-104
Piceatannol (SIRT1 Activator)	GR-323
Suramin sodium (SIRT1 Inhibitor)	G-430
Trichostatin A (Class I/II HDAC Inhibitor)	GR-309
Anti-HDAC1 (polyclonal Ab)	SA-401
Anti-HDAC2 (polyclonal Ab)	SA-402
Anti-HDAC3 (polyclonal Ab)	SA-403
Anti-HDAC4 (polyclonal Ab)	SA-404

USE OF PRODUCT

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

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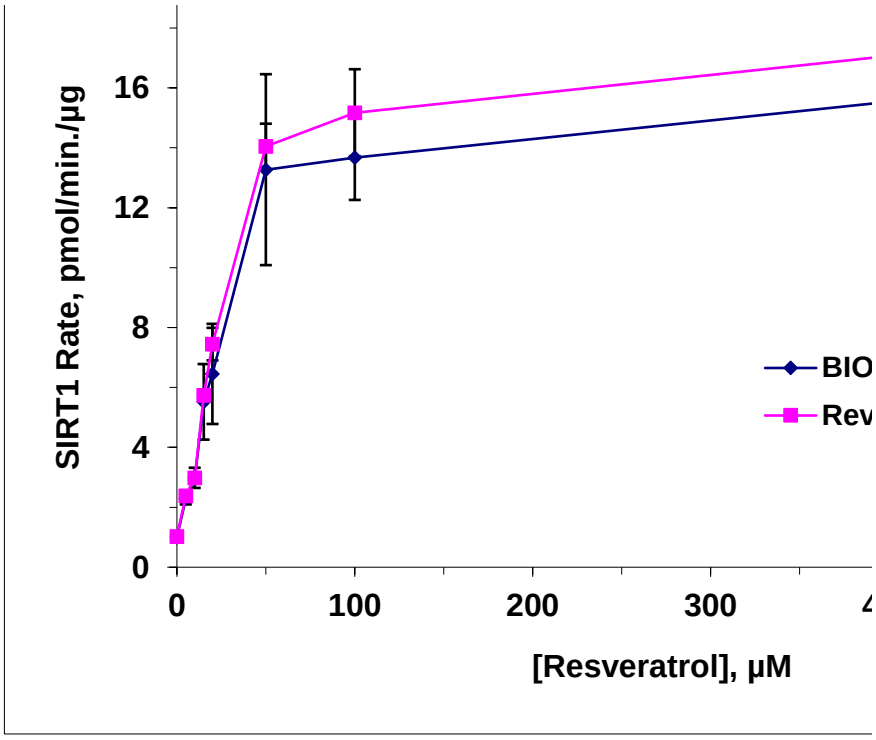
Sirt1 0.5ug/well 1/3/08 360/460/Gain 70	25uM p53-382 w/25uM NADw/ [µM]Compound	0 min	0 min	20 min	20 min
Resveratrol	**0**	226	235	459	533
Biomol FR-104	**5**	224	233	825	864
	10	222	225	971	1120
	15	226	247	1324	1653
	20	227	223	1395	2098
	50	239	245	2585	3476
	100	270	268	3302	3347
	500	313	321	2927	2923
Rev Genetic X-100	**0**	248	238	517	555
Batch #070928	**5**	231	231	854	823
	10	232	227	1003	1094
	15	234	229	1724	1729
	20	218	225	1965	2191
	50	243	248	3818	3948
	100	269	272	4333	3868
	500	322	299	3557	3658

[Resveratrol] µM	Biomol Resveratrol Cat. # FR-104 Mean Specific Activity pmol/min/µg	RevGenetics X-100 Resveratrol Batch #070928 Mean Specific Activity pmol/min/µg	Biomol Resveratrol Cat. # FR-104 Standard Deviation pmol/min/µg
0	0.998849744	1.022101628	0.142248724
5	2.296352557	2.379808134	0.200017575
10	2.983530388	2.983775846	0.33602793
15	5.520874978	5.731092107	1.260901255
20	6.452693627	7.448332826	1.671274254
50	13.27142667	14.04692675	3.186914412
100	13.67500538	15.17286887	1.413825668
500	16.15462768	17.69609532	2.312883861

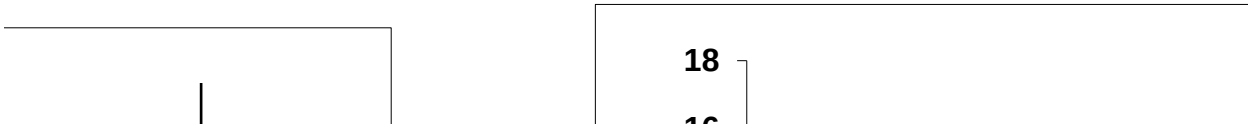
20

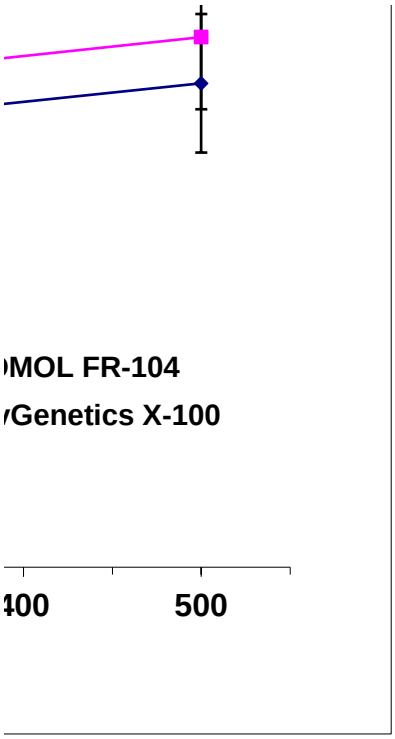

SIRT1 Rate, pmol/min./µg
16
12
8
4
0
0
100
200
300
[Resveratrol], µM
BIO
Rev

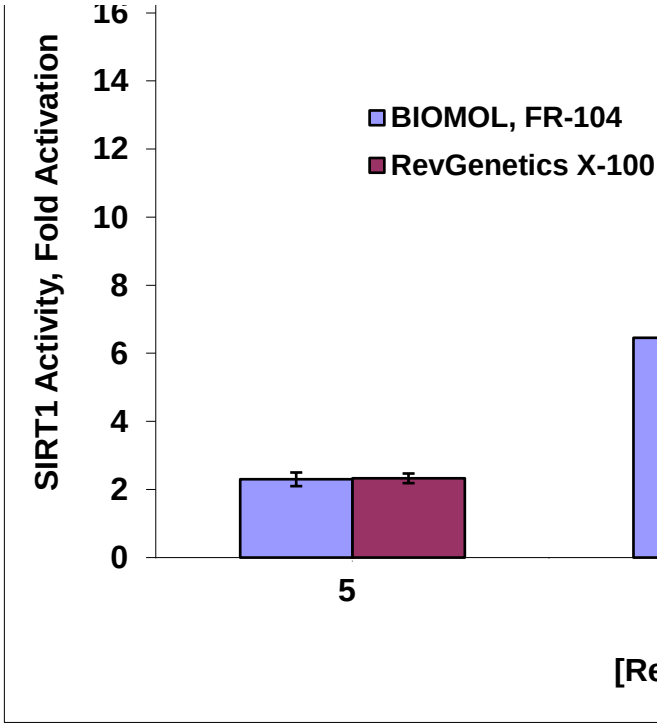
20 min	Dif. AFU (0, 20min)	spiked with 5 µl of 30µM AMC (final of 3µM) 0 min	spiked with 5 µl of 30µM AMC (final of 3µM) 0 min	spiked with 5 µl of 30µM AMC (final of 3µM) 20 min	spiked with 5 µl of 30µM AMC (final of 3µM) 20 min	spiked with 5 µl of 30µM AMC (final of 3µM) 20 min
516	**272.2**	4876	5339	5832	5875	5899
936	**646.5**	5080	5236	6788	5726	6268
960	**793.5**	4622	5171	6501	6484	6180
1969	**1412.2**	4653	4582	6640	6891	7404
2180	**1666.0**	4766	4695	6985	7505	7646
4084	**3139.7**	4518	4362	7473	8076	9434
3905	**3249.0**	4633	4467	7495	8105	9014
3630	**2843.0**	3262	2851	6344	6661	7929
556	**299.7**	5466	5484	6747	6186	5889
899	**627.7**	4705	4989	6467	5956	5804
1045	**817.8**	5283	4979	6744	5856	6149
1640	**1466.2**	5038	4553	6962	6588	6466
1958	**1816.5**	4463	4460	7304	7135	6656
3572	**3533.8**	4310	4634	9396	9076	8539
3643	**3677.5**	4215	4243	9378	8738	8394
4446	**3576.5**	3749	3458	7776	7800	8900

RevGenetics X-100 Resveratrol Batch #070928 Standard Deviation pmol/min/µg
0.075834284
0.144890156
0.166165424
0.195457798
0.543497053
0.759058676
1.451841451
2.408306914

[Resveratrol] µM	Biomol Resveratrol Cat. # FR-104 Fold Activation	RevGenetics X-100 Resveratrol Batch #070928 Fold Activation
5	2.298996992	2.328347856
20	6.460124425	7.287272247
100	13.69075325	14.84477518

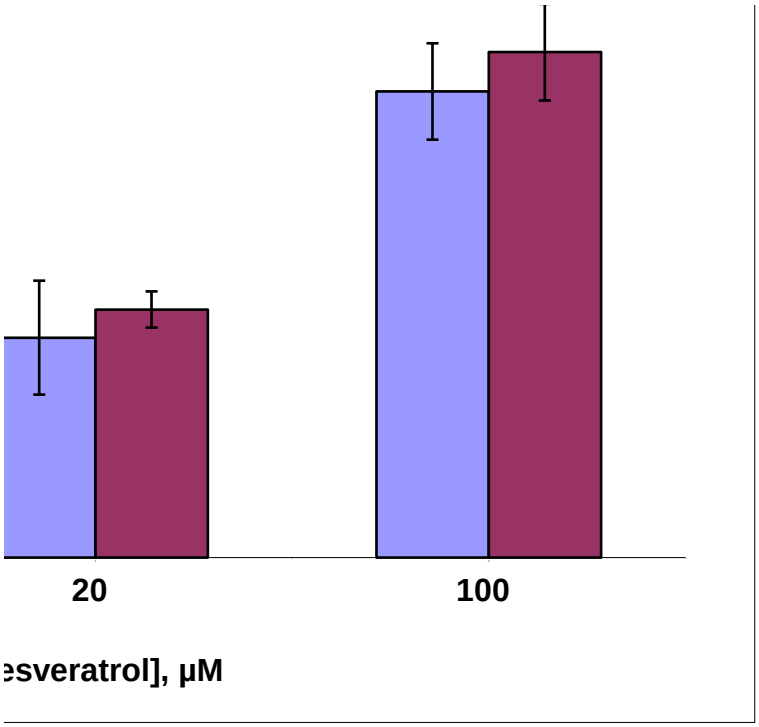



)MOL FR-104
/Genetics X-100
400
500


SIRT1 Activity, Fold Activation
16
14
12
10
8
6
4
2
0
BIOMOL, FR-104
RevGenetics X-100
5
[Re

Mean AFU Increase from 3 µM AMC	Mean AFU Increase from 3 µM AMC in Controls	pmol/AFU (Controls)	Quench Correction Factor	Mean Dif. AFU (0, 20min) Quench Corrected	Mean 0 min.	STD. DEV. Dif. AFU (0, 20min)
4087.2	4242.5	0.035356511	1.03799667	282.50809	230.5	38.75994
4223.0			1.00461757	649.48526	228.5	56.31163
3989.4			1.06344312	843.84212	223.5	89.37002
3836.8			1.10573916	1561.488	236.5	322.5218
3872.8			1.09546065	1825.0374	225	431.5009
3548.6			1.1955419	3753.6031	242	753.9392
3563.8			1.19044279	3867.7486	269	335.9062
2639.8			1.60712933	4569.0687	317	407.0369
4397.8			0.96468689	289.0845	243	22.23361
3956.2			1.07236742	673.08929	231	38.21431
4111.4			1.03188695	843.91154	229.5	45.54485
3837.4			1.10556627	1620.9444	231.5	50.00333
3658.2			1.15972336	2106.6375	221.5	132.5481
3773.6			1.124258	3972.9404	245.5	190.959
3635.6			1.16693256	4291.3945	270.5	351.8878
3031.6			1.39942605	5005.0473	310.5	486.735

Biomol Resveratrol Cat. # FR-104 Standard Deviation Fold Activation	RevGenetics X-100 Resveratrol Batch #070928 Standard Deviation Fold Activation
0.200247911	0.141757093
1.673198862	0.531744631
1.415453803	1.420447254


20
100
esveratrol], μM

STD. DEV. Dif. AFU (0, 20min) Quench Corrected	Mean Specific Activity pmol/min/µg	STD. DEV. Specific Activity pmol/min/µg
40.232694	0.998849744	0.142248724
56.571656	2.296352557	0.200017575
95.03993	2.983530388	0.33602793
356.62502	5.520874978	1.260901255
472.69222	6.452693627	1.671274254
901.36592	13.27142667	3.186914412
399.87716	13.67500538	1.413825668
654.16086	16.15462768	2.312883861
21.44847	1.022101628	0.075834284
40.979779	2.379808134	0.144890156
46.997136	2.983775846	0.166165424
55.281999	5.731092107	0.195457798
153.71913	7.448332826	0.543497053
214.68717	14.04692675	0.759058676
410.62929	15.17286887	1.451841451
681.14969	17.69609532	2.408306914