FLUOR DE LYS® Green SIRT5 Fluorometric Drug Discovery Kit.

A *FLUOR DE LYS*® Green Fluorescent Assay System

Instruction Manual

BML-AK514

For research use only

✦ *FLUOR DE LYS*® **Green *SIRT5 Fluorometric Drug Discovery Kit*-BML-AK514**✦

⚜ **A *FLUOR DE LYS*® Green Fluorescent Assay System*** ⚜

BACKGROUND

Most sirtuin enzymes, also known as class III histone deactylases (class III HDACs), catalyze a reaction which couples deacetylation of protein N^{ε}-acetyllysine residues to the formation of *O*-acetyl-ADP-ribose and nicotinamide, from the oxidized form of nicotinamide adenine dinucleotide (NAD^+)[1-3]. Some sirtuins, notably human SIRT4 and SIRT6, are reported to catalyze an alternative reaction, the transfer of an ADP-ribosyl group from NAD^+ to proteins[4,5], although the physiological relevance of these reactions is in question[6]. Sirtuin homologs are found in all forms of life, including the archaea, the bacteria and both unicellular and multicellular eukaryotes[7-10]. The founding exemplar of the group, Sir2 from baker's yeast (*Saccharomyces cerevisiae*), was named for its role in gene-silencing (Silent information regulator 2)[11]. Transcriptional silencing by Sir2 is linked to its deacetylation of lysines in the N-terminal tails of the histones in chromatin, hence the classification as a class III HDAC. Lysine deacetylation by sirtuins, however, extends beyond histones. Targets of sirtuin regulatory deacetylation include mammalian transcription factors such as p53[12-14], the cytoskeletal protein, tubulin[15], the bacterial enzyme, acetyl-CoA synthetase[16,17] and its mammalian homologs[18].

SIRT5, along with two other mammalian sirtuins, SIRT3 and SIRT4, is localized to the mitochondria[19,20]. The human SIRT5 gene is located in a chromosomal region in which abnormalities are associated with malignancies, suggesting a possible SIRT5 role in cancer[21]. Thus far, the best studied of SIRT5's possible physiological roles is the deacetylation, and enhancement of the activity of the mitochondrial matrix enzyme carbamoyl phosphate synthase 1 (CPS1), the rate-limiting enzyme for urea synthesis in the urea cycle[20]. Increased urea synthesis is required for removal of nitrogenous waste (ammonia) during periods of increased amino acid catabolism, including calorie restriction, fasting and the consumption of a high protein diet. Nakagawa *et al.* report that under these conditions, SIRT5 deacetylation of CPS1 is increased, along with CPS1 activity[20]. At least in the instance of starvation, the increased SIRT5 activity may be attributed to increased levels of the sirtuin co-substrate NAD^+ in the mitochondria, which in turn is due to induction of the NAD^+ synthetic pathway enzyme nicotinamide phosphoribosyltransferase, (Nampt)[20]. It should be noted, however, that two proteomic studies of the mouse mitochondrial "acetylome" are in possible conflict with the CPS1 results of Nakagawa *et al*. One group observed that calorie restriction increased acetylation at 7 of 24 sites in CPS1, but did not lead to deacetylation at any sites[22]. A comparison of the acetylated proteins of fed and fasted mice found that fasting induced the addition of 4 acetylated sites to CPS1, while only one of five sites present in the fed condition disappeared upon fasting[23].

An alternative view of SIRT5's physiological function is that it may primarily involve catalysis of reactions other than deacetylation. SIRT5's deacetylase activity is detectable but weak with an acetylated histone H4 peptide[24] and with chemically acetylated histones or bovine serum albumin[25]. The catalytic efficiency of SIRT5 with an acetylated histone H3 peptide ($kcat/K_m$ = 3.5 s^{-1} M^{-1}) is orders of magnitude lower than several human and yeast sirtuins (SIRT1, SIRT2, Sir2, Hst2) and more than 20-fold lower than the next weakest deacetylase tested, human SIRT3[6]. Although there is a seeming conflict between the idea of SIRT5 as a non-deacetylase and its effects on CPS1, it should be noted that the rate of SIRT5 deacetylation of CPS1 has not been

quantified; the deacetylation was only shown in a qualitative way by western blotting with anti-acetyllysine[20]. Further, although SIRT5 performs an NAD^+-dependent activation of CPS1 and an NAD^+-dependent deacetylation of CPS1, no mechanistic link between the deacetylation and the activation has been established. The in vitro SIRT5/CPS1 activation experiments were performed with crude mitochondrial matrix lysates, from SIRT5 knockout mice, serving as the CPS1 source[20]. Conceivably, the CPS1 harbored another modification, in addition to acetylation, that SIRT5 reversed in an NAD^+-dependent reaction. Consistent with this possibility is recently presented evidence that mitochondrial proteins are lysine-succinylated and that SIRT5 can desuccinylate peptides with efficiencies similar to the deacetylation efficiencies of human SIRTs 1-3[26].

This latter view of SIRT5's role is supported by the far greater efficiency SIRT5 displays when assayed with a succinylated *FLUOR DE LYS®-Green* substrate as opposed to an acetylated one (Figure 2). Use of *FLUOR DE LYS®-Succinyl Green* (Cat. # BML-KI591) allows SIRT5 to be assayed at nM concentration, as opposed to the µM concentration typically required for assay with acetyllysine substrates. Quite aside from the savings on enzyme, this is a boon for drug discovery applications. Because a compound IC_{50} less than half the enzyme concentration cannot be determined, low enzyme concentration is desirable for both screening and subsequent characterization of "hits".

In addition to its uses in assays of purified SIRT5, the *FLUOR DE LYS®-Succinyl Green* substrate may be an important discovery tool in the area of oxidative stress responses. Although succinyl-CoA is an abundant metabolite, there is no known lysine succinyltransferase equivalent to the acetyltransferases (HATs). Succinylation of protein lysines does, however, occur via reaction with oxidation products of polyunsaturated fatty acids (PUFAs)[27] and cellular lysine desuccinylase activity apparently extends beyond SIRT5 (see Figs. 8 & 9).

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

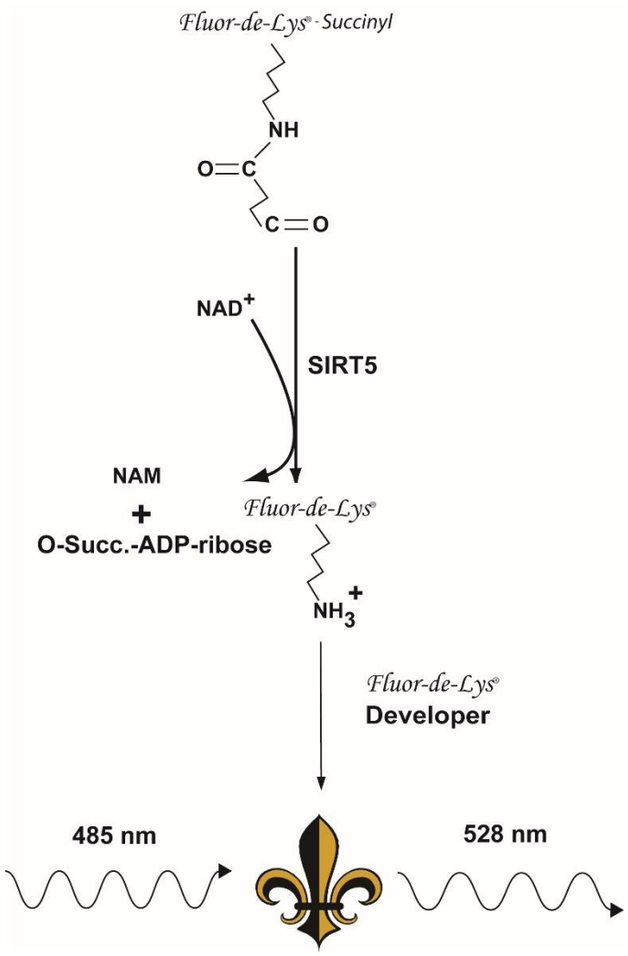

Figure 1. Reaction Scheme of the *SIRT5 Fluorescent Activity Assay*.* NAD^+-dependent desuccinylation of the substrate by recombinant human SIRT5 sensitizes it to Developer, which then generates a fluorophore (symbol). The fluorophore is excited with 485 nm light and the emitted light (528 nm) is detected on a fluorometric plate reader. NAD^+ is consumed in the reaction to produce nicotinamide (NAM) and O-succinyl-ADP-ribose.

*Patent Pending.

DESCRIPTION

The *SIRT5 Fluorometric Drug Discovery Kit* is a complete assay system designed to measure the lysyl desuccinylase activity of the recombinant human SIRT5 included in the kit. A black 96-well microplate is packaged with the kit, but it should be noted that reagents of the *FLUOR DE LYS*® system have also been successfully employed in other formats, including cuvettes and 384-well plates.

The *SIRT5 Fluorescent Activity Assay* is based on the unique *FLUOR DE LYS*®*-Succinyl Green* Substrate/Developer combination. The assay procedure has two steps (Figs. 1 & 3). The *FLUOR DE LYS*®*-Succinyl Green* Substrate, which comprises a lysine residue, N^{ε}-succinylated on its side-chains, is first incubated with human recombinant SIRT5 together with the cosubstrate NAD^+. Desuccinylation of *FLUOR DE LYS*®*-Succinyl Green* sensitizes it so that, in the second step, treatment with the *FLUOR DE LYS*® Developer produces a fluorophore. Use of a succinylated, rather than acetylated substrate with SIRT5 results in readily observed saturation kinetics and a greater than 1000-fold increase in assay sensitivity (Fig. 2).

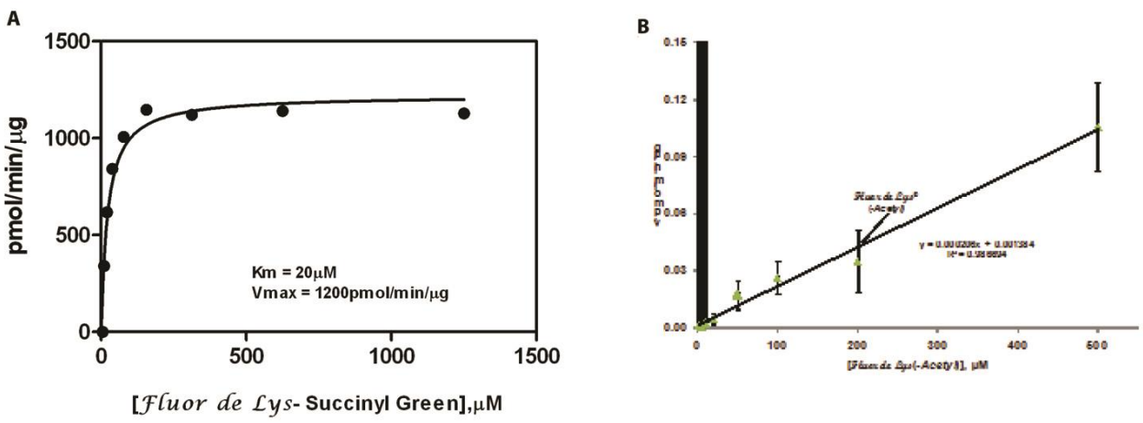

Figure 2. SIRT5 Kinetics with Succinylated and Acetylated *FLUOR DE LYS*®-Substrates. Initial desuccinylation/deacetylation rates were determined at the indicated concentrations of *FLUOR DE LYS*®-Succinyl Green (Cat. # BML-KI591) or *FLUOR DE LYS*®- (Cat. # BML-KI104; "*FLUOR DE LYS*(*-Acetyl*)") and 1 mM NAD^+. Desuccinylation reactions (**A**) contained 10 ng SIRT5 and deacetylation reactions (**A** & **B**) 5 µg SIRT5. Both sets of reactions proceeded for 20 min. at 37°C and were stopped by addition of Developer/2 mM nicotinamide. Signal was allowed to develop for 15 min. and fluorescence was read in a microplate reader at 485 nm (excitation)/528 nm (emission), Data represent the mean of the differences of two determinations with enzyme from the mean of two no-enzyme samples for each substrate concentration. Error bars shown are standard deviations. Fluorescence differences were converted to specific activities (pmol/min/µg) by reference to a product standard curve (see Fig. 4). Scales are significantly different in (A) and (B).

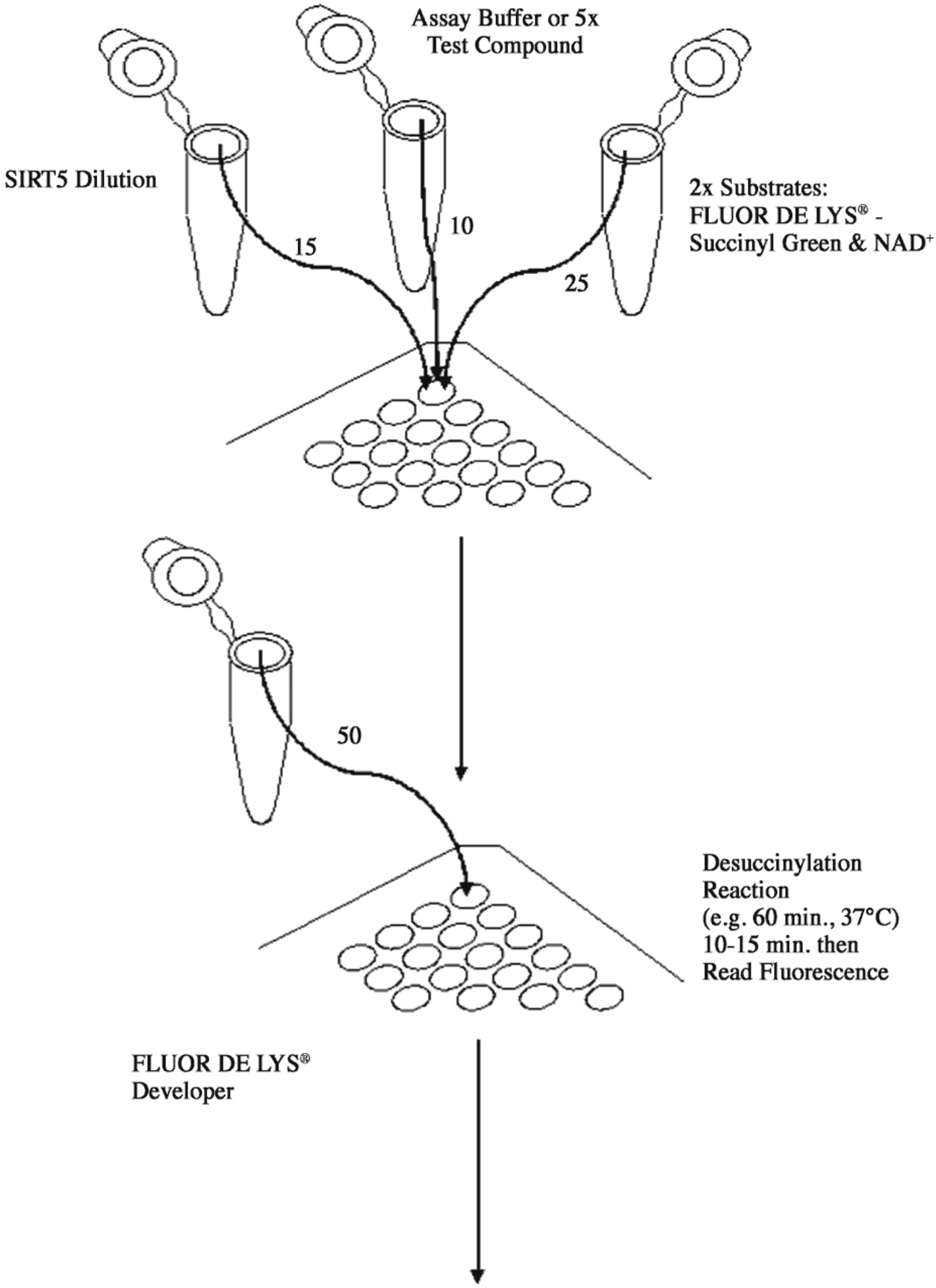

Figure 3. Performing the *FLUOR DE LYS®-Succinyl Green* SIRT5 Activity Assay. The procedure is done in two stages. First, the components of the desuccinylation reaction (SIRT5, buffer or test compound, substrates) are mixed. Following an incubation in which substrate desuccinylation takes place, Developer is added and mixed. This stops the desuccinylation and produces the fluorescent signal. The fluorescent signal develops and can be read in less than 15 min. The scheme depicts mixes for "Control" or "Test Sample" reactions; see Table 1 and text for other sample types and more details.

COMPONENTS OF BML-AK514

BML-SE555-9090 SIRT5 (Sirtuin 5)(human, recombinant)

FORM: Dissolved in 25 mM Tris, pH 7.5, 100 mM NaCl, 5 mM DTT, 1 mg/mL BSA and 10% glycerol.

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

QUANTITY: 1200 U; See vial label for specific activity and protein concentration. One U= 1 pmol/min at 37°C, 250 µM *FLUOR DE LYS*® Succinyl, Desuccinylase (BML-KI590), 2000 µM NAD+.

BML-KI591-0050 *FLUOR DE LYS*®*–Succinyl Green*, Desuccinylase Substrate

FORM: 5 mM solution in DMSO

STORAGE: -70°C

QUANTITY: 50 µL

BML-KI105-0300 *FLUOR DE LYS*® Developer Concentrate (20x)

FORM: 20x Stock Solution; Dilute in Assay Buffer before use.

STORAGE: -70°C

QUANTITY: 300 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: -70°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: -70°C

QUANTITY: 500 µL

BML-KI285-0010 Suramin sodium (Sirtuin Inhibitor)

FORM: Solid

MW: 1429.2

STORAGE: -70°C

QUANTITY: 10 mg

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

BML-KI605-0030 *FLUOR DE LYS*®*-Green* Desuccinylated Standard

FORM: 1 mM in DMSO

STORAGE: -70°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: -70°C

QUANTITY: 20 mL

80-2409 1/2 VOLUME BLACK NBS MICROPLATE

STORAGE: Room temperature.

OTHER MATERIALS REQUIRED

- Microplate reading fluorometer capable of excitation at a wavelength in the range 470-500 nm and detection of emitted light in the range 520-550 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: *For the highest stability, store all components except the microplate at -70°C. The SIRT5 enzyme, BML-SE555, 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 -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 20x Developer (BML-KI105) 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. As with the SIRT5, it is best to refreeze unused portion in liquid nitrogen or a dry ice/ethanol bath.*

Some Things To Consider When Planning Assays:

1. The assay is performed in two stages. The first stage, during which the SIRT5 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, including a SIRT5 inhibitor, stops SIRT5 activity and produces the fluorescent signal. See "Preparing Reagents For Assay" and Table 1 (pp. 9-10).

   A black NBS ½-volume, 96-well microplate is provided with the kit. Opaque white plates can also be used with this kit, though background fluorescence, relative to signal, will likely be lower with the black plate.

   Should it be necessary, 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 in the volume of the Developer, it should be noted that it is important to try and maintain the ratios of the components as closely to those outlined in this manual. See "Preparing Reagents For Assay", Step #5, (p. 10).

2. Experimental samples should be compared to a “Time Zero” (sample for which 1x Developer/2 mM nicotinamide is added to the SIRT5 immediately before substrate) and/or a negative control (no enzyme). Desuccinylation is proportional to the ***increase*** in fluorescence (Exp. Sample fluorescence minus “time 0” or “no enzyme”) relative to these controls.

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 SIRT5, 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 that yields a signal that is both sufficiently large and proportional to enzyme rate.

4. The K_m of SIRT5 for the *FLUOR DE LYS®-Succinyl Green* Substrate has been measured at 20 µM at 1 mM NAD^+ (Fig. 2). The K_m for NAD^+, determined at 0.5 mM *FLUOR DE LYS®-Succinyl Green* Substrate, was 700 µM (Fig. 5). 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.

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. Some compounds being screened for modulation of SIRT5 activity may interfere with the action of the *FLUOR DE LYS®* Developer. This is, however, unlikely unless the concentration of the screening compound is well in excess of 100 µM. One approach to confirm that apparent “hits” are acting only via SIRT5 effects involves retesting the candidate compound in a reaction with the *FLUOR DE LYS®-Green* Desuccinylated Standard (BML-KI605) plus the *FLUOR DE LYS®* Developer. A detailed retesting procedure is described below, in the section “Uses Of The *FLUOR DE LYS®-Green* Desuccinylated Standard” (p. 11). In some cases, it may be possible to avoid this retesting by means of measurements taken during the fluorescence development phase of the initial SIRT5 assay. This is also discussed in the same section (pp. 11-12).

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 SIRT5 enzyme (BML-SE555) and the 20x Developer (BML-KI105). (See ‘Notes on Storage’, p. 8). All undiluted kit components are stable for several hours (1-4hrs) on ice.

2. Assuming, for example, 12 U of SIRT5 (BML-SE555) per assay (6 nM in 50 µL desuccinylation reaction at a 1200 U/µg Spec. Act.), dilute a sufficient amount to 2.4 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 0.48 U/µL or three fold to 0.8 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. 10).

3. Prepare dilution(s) of 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 SIRT5 reaction is 50 µL, these inhibitor dilutions will be 5x their final concentration. Suramin sodium is soluble in both water and Assay Buffer (10 mg in 0.28 mL = 25 mM) and nicotinamide (BML-KI283) comes supplied in buffer at 50 mM. When working with test compound stocks prepared in non-aqueous solvents, it should be noted that high concentrations of, for example, ethanol and DMSO affect SIRT5 activity and appropriate solvent controls should always be included.
4. Prepare a dilution of the substrates, *FLUOR DE LYS®-Succinyl Green* (BML-KI591; 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, final substrate concentrations at or below the K_m are recommended. We recommend a final concentration of 10 µM *FLUOR DE LYS®-Succinyl Green* and 500 µM NAD^+. This 3.33x stock would then be 33.3 µM *FLUOR DE LYS®-Succinyl Green* and 1667 µM NAD^+ (see Performing the Assay and Table 1, below).
5. Shortly before use (<30 min.), prepare sufficient 1x *FLUOR DE LYS®* Developer plus nicotinamide (2 mM) for the assays to be performed (50 µL per well plus some excess). One mL will contain 910 µL Assay Buffer, 50 µL 20x Developer and 40 µL 50 mM nicotinamide. Addition of nicotinamide to the Developer insures that SIRT5 activity stops when the Developer is added. Keep diluted Developer on ice until use.

Performing the Assay:

1. Table 1 gives examples of solutions and volumes for use in various types of SIRT5 assays. These are mixtures for the first, desuccinylation phase of the assay. The SIRT5 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	SIRT5 (2.4 U/µL)	Test Cmpd.or Solvent Control (5x)	Substrates: *FLUOR DE LYS®–SucGr* 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
Nicotinamide	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 SIRT5 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 0.48 U/µL SIRT5 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 10 µL 5x Test Compound and 15µL 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 0.8 U/µL SIRT5 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 (10 µL Assay Buffer, 15 µL 3.33x Substrates).

⌘ **NOTE:** In a 'Time Zero' sample, the substrate addition is made after the addition of 1x Developer/2 mM nicotinamide.

1. Add 25 µL of 0.48 U/µL SIRT5 or 15 µL of 0.8 U/µL SIRT5 plus 10 µL 5x Test Compound or 25 µL Assay Buffer to appropriate wells of the assay plate.
2. Warm the assay plate and 2x substrate solutions to 37°C.
3. Initiate SIRT5 reactions by adding 25 µL 2x substrate solutions to the assay wells and thoroughly mixing. DO NOT ADD SUBSTRATE TO "TIME ZERO" WELLS.
4. Allow SIRT5 reactions to proceed for desired length of time and then stop by addition of 1x Developer/2 mM nicotinamide (50 µL). Add 25 µL of 2x Substrate solution to "Time Zero" samples. Incubate plate at room temperature for at least 10-15 min. Signal development can be accelerated by higher temperature (30-37°C).
5. Read samples in a microplate-reading fluorometer capable of excitation at a wavelength in the range 470-500 nm and detection of emitted light in the range 520-550 nm. Completion of signal development can be assessed by taking fluorescence readings at timed intervals (e.g. 2 or 5 min.). 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®-Green* DESUCCINYLATED STANDARD (BML-KI605)

Preparation of a Standard Curve:

1. The exact concentration range of the *FLUOR DE LYS®-Green* Desuccinylated Standard (BML-KI605) that will be useful for preparing a standard curve will vary depending on the fluorometer model, the gain setting and the exact excitation and emission wavelengths used. We recommend diluting some of the standard to a relatively low concentration with Assay Buffer (1 to 5 µM). The fluorescence signal should then be determined, as described below, after mixing 50 µL of the diluted standard with 50 µL of 1x Developer. 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®-Green* Desuccinylated 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 in Assay Buffer for addition of 50 µL to each of the standard wells.
4. Mix 50 µL of the 1x Developer with the 50 µL in each standard well and incubate 10-15 min. at room temperature (25°C).
5. Read samples in a microplate-reading fluorometer capable of excitation at a wavelength in the range 470-500 nm and detection of emitted light in the range 520-550 nm.
6. Plot fluorescence signal (y-axis) versus concentration of the *FLUOR DE LYS®-Green* Desuccinylated Standard (x-axis). Determine slope as AFU /µM. See example in Fig. 4.

Testing of Potential SIRT5 Inhibitors for Interference with the *FLUOR DE LYS®* Developer 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 5 min. at 25°C. That, together with the recommended 10-15 min. reaction time (Fig. 3), 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 SIRT5 reaction to fully develop and stabilize (usually less than 15 min., see 1. above), the fluorescence is recorded and a 'spike' of *FLUOR DE LYS®-Green* Desuccinylated Standard is added [e.g. 5 µL of 20 µM Desuccinylated Standard (BML-KI605) in Assay Buffer; i.e. amount equivalent to 2 µM/100 pmol in the 50 µL SIRT5 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. 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.

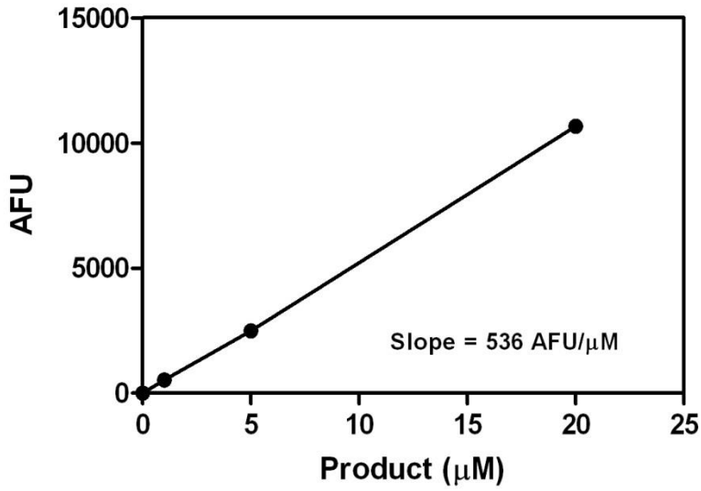

Figure 4. Fluorescence Standard Curves. Fifty microliter aliquots of *FLUOR DE LYS®-Green* Desuccinylated Standard (BML-KI605), in Assay Buffer, at the indicated concentrations, were mixed with 50 µL 1x Developer and incubated 15 min., 37 ºC. Fluorescence was measured at Ex. 485 nm, Em. 528 nm.

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 1 min. intervals for 15 min.). The fluorescence of control samples (no inhibitor) will change very little after the third or fourth reading. Samples containing compounds which inhibit SIRT5, but which do not interfere with Developer, 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 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®-Green* Desuccinylated Standard with the Developer. Using a standard curve such as that described in the previous section, determine the concentration of Desuccinylated Standard that will yield a signal similar to that produced after development of a control (no inhibitor) SIRT5 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 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 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 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 would be sufficient. Other possible adjustments include increasing the volume of Developer used per well (e.g. to 100 µL). Both approaches may be used separately or in combination.

TABLE 2. ASSAY MIXTURES FOR TEST COMPOUND REASSAY WITH DEVELOPER AND *FLUOR DE LYS®-Green* DESUCCINYLATED STANDARD

Sample	Test Compound or Solvent Control (5x)	Diluted[6] *FLUOR DE LYS®* GrdeAc. Standard (1.25x)	DEVELOPER (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®-Green* Desuccinylated 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 SIRT5 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 *SIRT5 Fluorometric Drug Discovery Kit* has been used for investigating SIRT5 kinetics as a function of the concentrations of *FLUOR DE LYS®-Succinyl Green* Substrate and NAD^+ (Figures 2 & 5), for characterization of inhibitors of the enzyme (Figure 8), for investigation of lysine desuccinylation activity by recombinant HDACs and sirtuins (Figure 8-9) and for the investigation of lysine desuccinylation activity in cell extracts (Figure 9). The assay is linear for at least 60 minutes (Figure 6) and is suitable for high-throughput screening, as demonstrated by its Z-factor of 0.8 (Figure 7).

No other recombinant human sirtuin had measurable activity with the *FLUOR DE LYS®-Succinyl Green* Substrate (not shown), although some activity was observed with HDAC2, the HDAC3/NCOR1 Complex and a HeLa nuclear extract (Figure 9B).

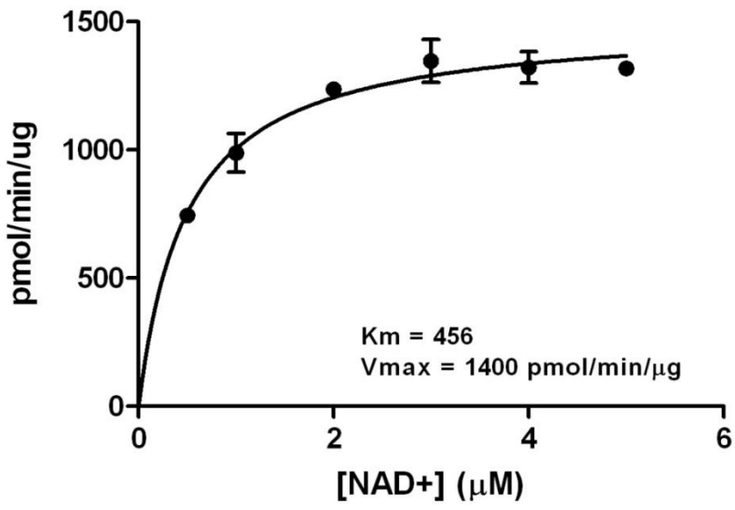

Fig. 5 Dependence of SIRT5 Kinetics on the Concentration of NAD^+. Initial desuccinylation rates of SIRT5 (12U) were determined with 20 min. incubations (37°C) in the presence of 0.5 mM *FLUOR DE LYS®–Succinyl* Green and the indicated concentrations of NAD^+. Reactions were stopped with *FLUOR DE LYS®* Developer/2 mM nicotinamide and fluorescence measured (Ex. 485 nm, Em. 528 nm). Each point represents the mean of two determinations and the error bars are standard deviations.

The K_m for NAD^+ was 1400 µM and the V_{max} was 456 pmol/min./µg.

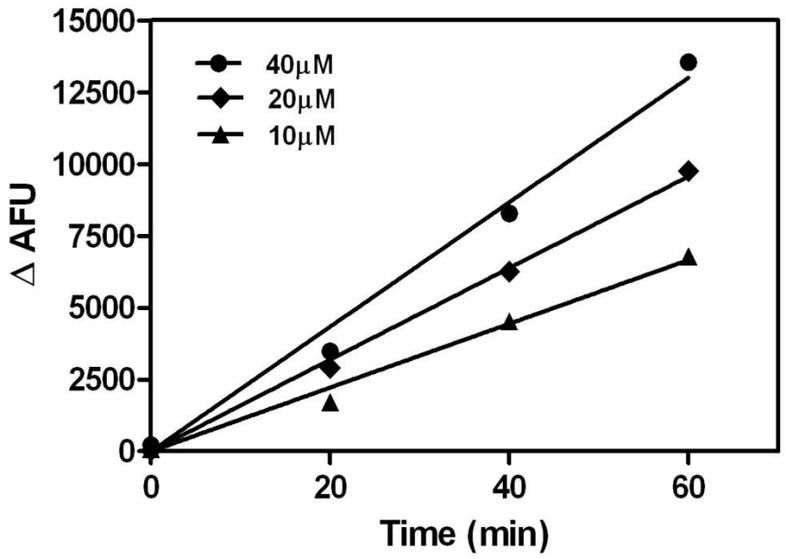

Fig. 6 Time Course of *FLUOR DE LYS®–Succinyl Green* Desuccinylation by SIRT5. SIRT5 (12 U) was incubated for varying times at 37 °C and 10, 20, or 40 µM *FLUOR DE LYS®–Succinyl* Green with 2 mM NAD^+. Reactions were stopped with *FLUOR DE LYS®* Developer/2 mM nicotinamide and fluorescence measured (Ex. 485 nm, Em. 528 nm). Each point represents the mean of duplicate determinations.

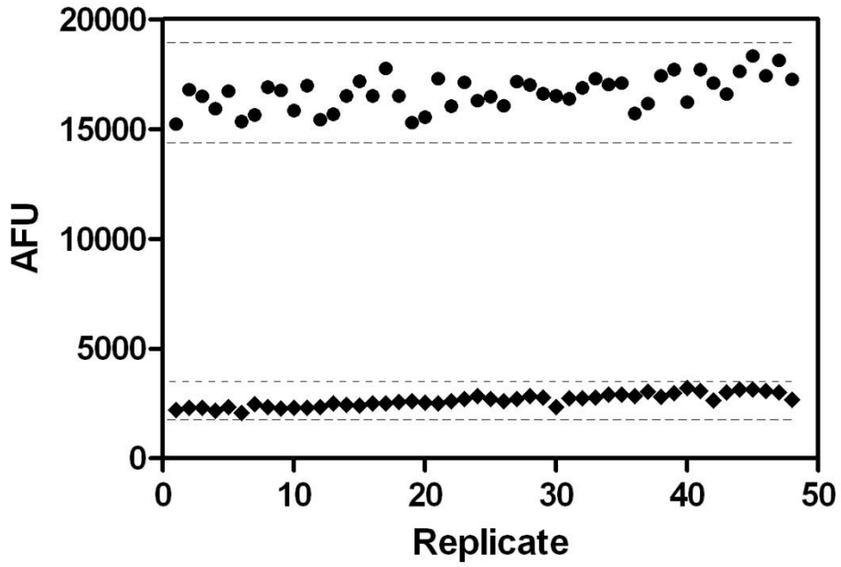

Fig. 7 SIRT5 Z - factor analysis. SIRT5 (12 U) (●) or buffer (◆) was incubated for 60 mintues at 37 °C with 10 µM *FLUOR DE LYS®–Succinyl* Green and 500 µM NAD^+. Reactions were stopped with *FLUOR DE LYS®* Developer/2 mM nicotinamide and fluorescence measured (Ex. 485 nm, Em. 528 nm). The Z' factor for this assay was 0.8, (Z-factor = $1-((3SD^{positive} + 3SD^{negative})/(mean^{positive} - mean^{negative}))$. Dashed lines indicate the 3*Standard deviation range.

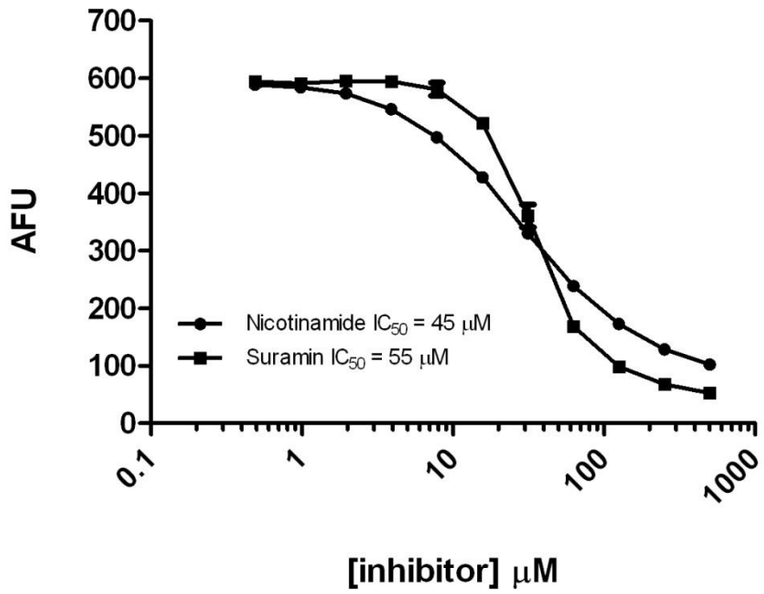

Fig. 8 Suramin and Nicotinamide Inhibition of SIRT5 Desuccinylase Activity. Initial desuccinylation rates were determined with 60 min. incubations at 37 °C, the indicated concentrations of suramin (ENZO Life Sciences Cat. # ALX-430-022) and nicotinamide (BML-KI283) at constant concentrations of 10 µM *FLUOR DE LYS®–**Succinyl***, 500 µM NAD^+ and 12 U SIRT5. Reactions were stopped with *FLUOR DE LYS®* Developer/2 mM nicotinamide and fluorescence measured (Synergy 2, BioTek, Ex. 360 nm, Em. 460 nm). Each point represents the mean of two determinations and the error bars are standard deviations.

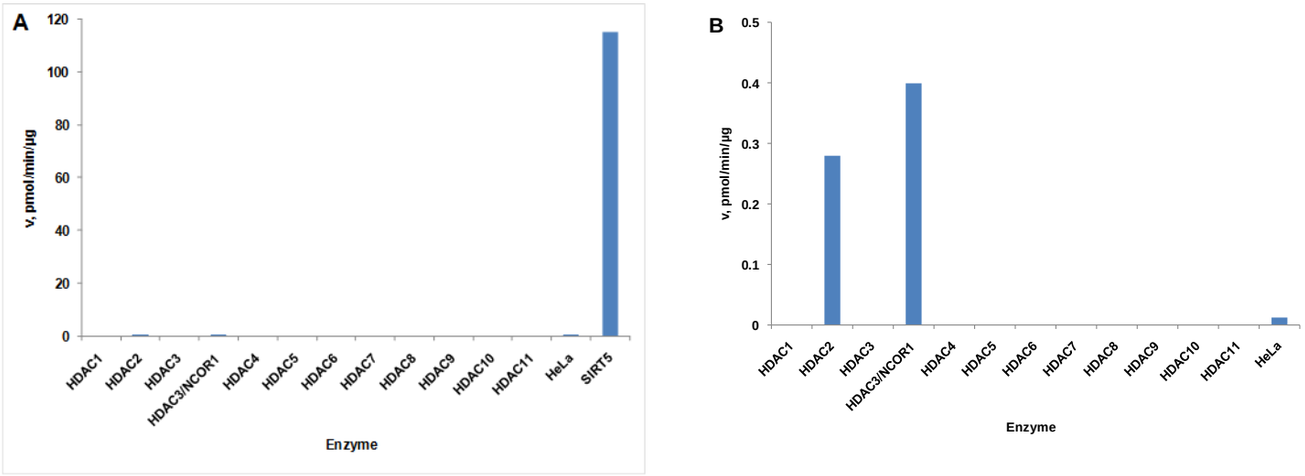

Fig. 9 SIRT5 Specific Activity with *FLUOR DE LYS®–Succinyl* Is Much Higher than that of Other Human Recombinant HDACs and Sirtuins. Initial rate activities of the indicated enzymes, and SIRTs 1-4, 6 & 7, were determined with 50 µM *FLUOR DE LYS®–Succinyl* for HDACs 1-11 and HeLa nuclear extract and with 50 µM *FLUOR DE LYS®–Succinyl* plus 500 µM NAD^+ for SIRTs. No activity was detected for SIRTs 1-4, 6 and 7, which are omitted from the figure. Panels **A** and **B** present the same data, but with the SIRT5 bar omitted from **B** in order to display the remaining bars at a 240-fold expanded scale.

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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