Product Manual

HDAC3/NCOR1 Fluorometric Drug Discovery Kit

Catalog #: BML-AK531

A FLUOR DE LYS® *Fluorescent Assay System*

NOTE: This version contains a change to the assay buffer storage temperature.

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Product Manual

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Carefully note the handling and storage conditions of each kit component.

BACKGROUND

Histones form the core of nucleosomes, the DNA/protein complexes that are the subunits of eukaryotic chromatin. Histones' N-terminal "tails" are subject to a variety of post-translational modifications, including phosphorylation, methylation, ubiquitination, ADP-ribosylation and acetylation. These modifications have been proposed to constitute a 'histone code' with profound regulatory functions in gene transcription[1]. The best studied of these modifications, ε-amino acetylations of specific histone lysine residues, are catalyzed by histone acetyltransferases (HATs). Histone deacetylases (HDACs) are responsible for removal of these acetyl groups[2,3,4]. Histone hyperacetylation correlates with an open, decondensed chromatin structure and gene activation, while hypoacetylation correlates with chromatin condensation and transcriptional repression[1-7].

Eleven human class I and class II HDACs (hydrolytic deacetylases) have been identified, all trichostatin A-sensitive and homologs of either RPD3 (class I) or HDA1 (class II), yeast HDACs[8-17]. HDACs can associate with transcription repression complexes such as NuRD, Sin3A or N-CoR/SMRT[1-7,18].

HDAC3, although a class I HDAC[10], is found in co-repressor complexes with the SMRT/N-CoR proteins, complexes distinct from than those comprising other class I enzymes[19-21]. It has a unique domain structure, including both nuclear localization and nuclear export sequences[22] and has been assigned to its own phylogenetic subclass[23]. A key function of HDAC3-SMRT/NCOR complexes is repression of gene expression, mediated through interaction with unliganded nuclear receptors and deacetylation of histone tails (see reviews[24,25]). However, recent work on HDAC3 implicates it in deacetylation of non-histone targets and a wide array of regulatory roles. Inhibition of HDAC3 may be the determining factor in the anti-proliferative effects of HDAC inhibitors on cancer cells[28,29] and its caspase-dependent cleavage and relocation to the cytoplasm may be critical to the progression of apoptosis[28]. It has been shown that HDAC3, rather than the class II HDACs 4 and 5, is likely the direct deacetylater of MEF2[30], implying an important role in pathways affecting heart disease. HDAC3[31] and the HDAC3/NCOR1 interaction[32] are profoundly involved in circadian regulation, both via repression of the clock gene BMAL1[33] and via repression of CLOCK/BMAL1 driven transcription[31].

HDAC inhibitors have shown promise as anti-tumor agents and naturally this has stimulated interest in the screening of compounds for HDAC3 inhibition. The preparation provided with this kit, a complex of HDAC3 with the NCOR1 Deacetylase

Activation Domain (DAD), has over 100-fold greater specific activity than recombinant HDAC3 alone and more closely approximates the *in vivo* form of the enzyme. Another advantage of the complex for drug discovery purposes is the possibility of identifying small molecule disruptors of the HDAC3-DAD interaction. If found, these might provide the basis for highly specific HDAC3 inhibitors.

The original techniques for HDAC assay involved the use of [^{3}H]acetyl-histone or [^{3}H]acetyl-histone peptide substrates and a cumbersome acid/ethyl acetate extraction step prior to scintillation counting[8,34,35]. Enzo Life Sciences' *HDAC3/NCOR1 Fluorimetric Drug Discovery Kit* addresses these problems by providing a homogenous assay with just two simple mixing steps (Figures. 1 & 2).

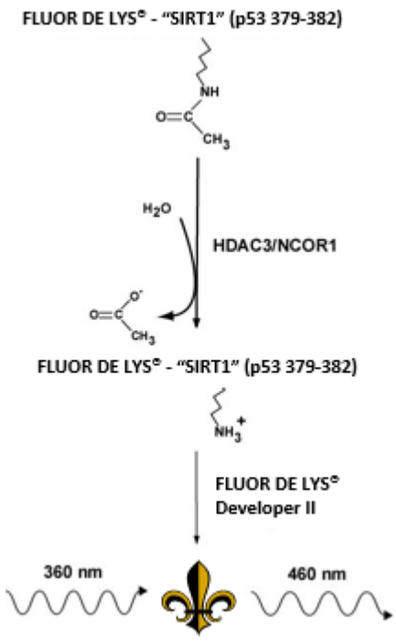

Figure 1. Reaction Scheme of the *HDAC3/NCOR1 Fluorometric Activity Assay*.* Deacetylation of the substrate sensitizes it to the Developer II, which then generates a fluorophore (symbol). The fluorophore is excited with 360 nm light and the emitted light (460 nm) is detected on a fluorometric plate reader.

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DESCRIPTION

The HDAC3/NCOR1 Fluorometric Drug Discovery Kit is based on the FLUOR DE LYS®-"SIRT1" (p53 379-382) Substrate (Cat. # BML-KI177) and Developer II (BML-KI176) combination. The FLUOR DE LYS® system (Fluorogenic Histone deAcetylase Lysyl Substrate/Developer) is a highly sensitive and convenient alternative to radiolabeled, acetylated histones or peptide/HPLC methods for the assay of histone deacetylases. The assay procedure has two steps (Figure 1). First, the FLUOR DE LYS® - SIRT1" Substrate, which comprises an acetylated lysine side chain, is incubated with recombinant HDAC3/NCOR1 (BML-KI574). Deacetylation of the substrate sensitizes the substrate so that, in the second step, treatment with the FLUOR DE LYS® Developer II produces a fluorophore. Despite the Substrate's name (it was first developed as a SIRT1 substrate), FLUOR DE LYS®-"SIRT1" (BML-KI177) is an excellent substrate for HDAC3/NCOR1 (Km= 3.2 µM, see Figure 4).

MATERIALS SUPPLIED

BML-KI574-0030 HDAC3/NCOR1 Complex (human, recombinant)

FORM: 30 ng/µl in 50 mM Tris, pH 8.0, 138 mM NaCl, 10% glycerol, 1 mg/ml BSA

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

QUANTITY: 67 µl

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

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

STORAGE: -80°C

QUANTITY: 100 µl

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

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

STORAGE: -80°C

QUANTITY: 5 x 250 µl

BML-GR309-9090 Trichostatin A (HDAC Inhibitor)

FORM: 0.2 mM in DMSO

STORAGE: -80°C

QUANTITY: 100 µl

BML-KI142-0030 FLUOR DE LYS® Deacetylated Standard

FORM: 10 mM in DMSO

STORAGE: -80°C

QUANTITY: 30 µl

BML-KI422-0020 HDAC ASSAY BUFFER II

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

STORAGE: -20°C

QUANTITY: 20 ml

BML-KI143-0020 HDAC ASSAY BUFFER

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

STORAGE: -20°C

QUANTITY: 20 ml

80-2407 ½ Volume NBS Microplates

1 clear and 1 white, 96-well, non-binding

STORAGE: Ambient

Storage temp

STORAGE

Store all components except the microplate at -80°C for the highest stability. The HDAC3/NCOR1 Complex, BML-KI574, must be handled with particular care in order to retain maximum enzymatic activity. Defrost it quickly in a RT water bath or by rubbing between fingers, then immediately store on an ice bath. The remaining unused enzyme should be refrozen quickly, by placing at -80°C. If possible, snap freeze in liquid nitrogen or a dry ice/ethanol bath. To minimize the number of freeze/thaw cycles, aliquot the enzyme into separate tubes and store at -80°C.

ADDITIONAL MATERIALS NEEDED

- Microplate reading fluorimeter capable of excitation at a wavelength in the range 350-380 nm and detection of emitted light in the range 440-480 nm.
- Pipetter or multi-channel pipetter capable of pipetting 2-100 µl accurately
- Ice bucket to keep reagents cold until use.
- Microplate warmer and/or other temperature control device (optional)

SAFETY WARNINGS & PRECAUTIONS

Important/ Warning

1. Wear appropriate personnel protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately. Consult a physician if required.
2. Use a safety pipetting device for all pipetting. Never pipet by mouth.
3. Interpretation of the results is the sole responsibility of the user.

PROCEDURE

Some Things To Consider When Planning Assays:

1. The assay is performed in two stages. The first stage, during which the HDAC3/NCOR1 acts on the Substrate, is done in a total volume of 50 µl. The second stage, which is initiated by the addition of 50 µl of Developer II, stops HDAC activity and produces the fluorescent signal. See “Preparing Reagents For Assay”, Figure 2 and Table 1.

   Two types of ½-volume, 96-well microplates are provided with the kit. The signal obtained with the opaque, white plate can be ~5-fold greater than that obtained with the clear plate. As long as the fluorimeter to be used is configured so that excitation and emission detection occur from above the well, the white plate should significantly increase assay sensitivity.

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

2. Experimental samples should be compared to a “time zero” (sample for which Developer II with Trichostatin is added immediately before mixing of the HDAC with substrate) and/or a negative control (no enzyme).

3. The K_m of HDAC3/NCOR1 for the FLUOR DE LYS®-SIRT1 Substrate has been measured at 3.2 µM (Figure 5). Use of substrate concentrations around or below K_m will help avoid substrate competition effects, which could mask the effectiveness of competitive inhibitors. For inhibition studies a final substrate concentration of 3-5 µM would be appropriate (see Figure 6).

4. Best results will be obtained by adding the chilled, undiluted enzyme directly to pre-warmed buffer and proceeding immediately to the addition of pre-warmed substrate. Plan the timing of the preparation and warming of enzyme dilutions, 2x substrate solutions and inhibitor solutions accordingly. (See "Preparing Reagents for Assay".)

5. Two buffers are provided with the kit—HDAC Assay Buffer II (BML-KI422) and HDAC Assay Buffer (BML-KI143). The first of these, BML-KI422, is for running the first phase of the assay, the HDAC3/NCOR1 deacetylation reaction itself. It should therefore be used for preparing all working dilutions of HDAC3/NCOR1 (BML-KI574), substrate (BML-KI177) and any compounds being screened for effects on HDAC3/NCOR1. The other buffer, BML-KI143, should be used for diluting the Developer II Concentrate (BML-KI176) and Trichostatin A in preparation of 1x Developer.

6. It is conceivable that some compounds being screened for inhibition of HDACs may interfere with the action of the FLUOR DE LYS® - Developer II. It is therefore important to confirm that apparent HDAC3/NCOR1 inhibitor "hits" are in fact acting only via HDAC3/NCOR1 inhibition. One approach to this involves retesting the candidate inhibitor in a reaction with the FLUOR DE LYS® - Deacetylated Standard (BML-KI142) plus the FLUOR DE LYS® - Developer II. A detailed retesting procedure is described below, in the section "PREPARATION OF A STANDARD CURVE (USES OF THE FLUOR DE LYS®-DEACTYLATED STANDARD (BML-KI242)". In some cases, it may be possible to avoid this retesting by means of measurements taken during the fluorescence development phase of the initial HDAC3/NCOR1 assay. This is also discussed in that section.

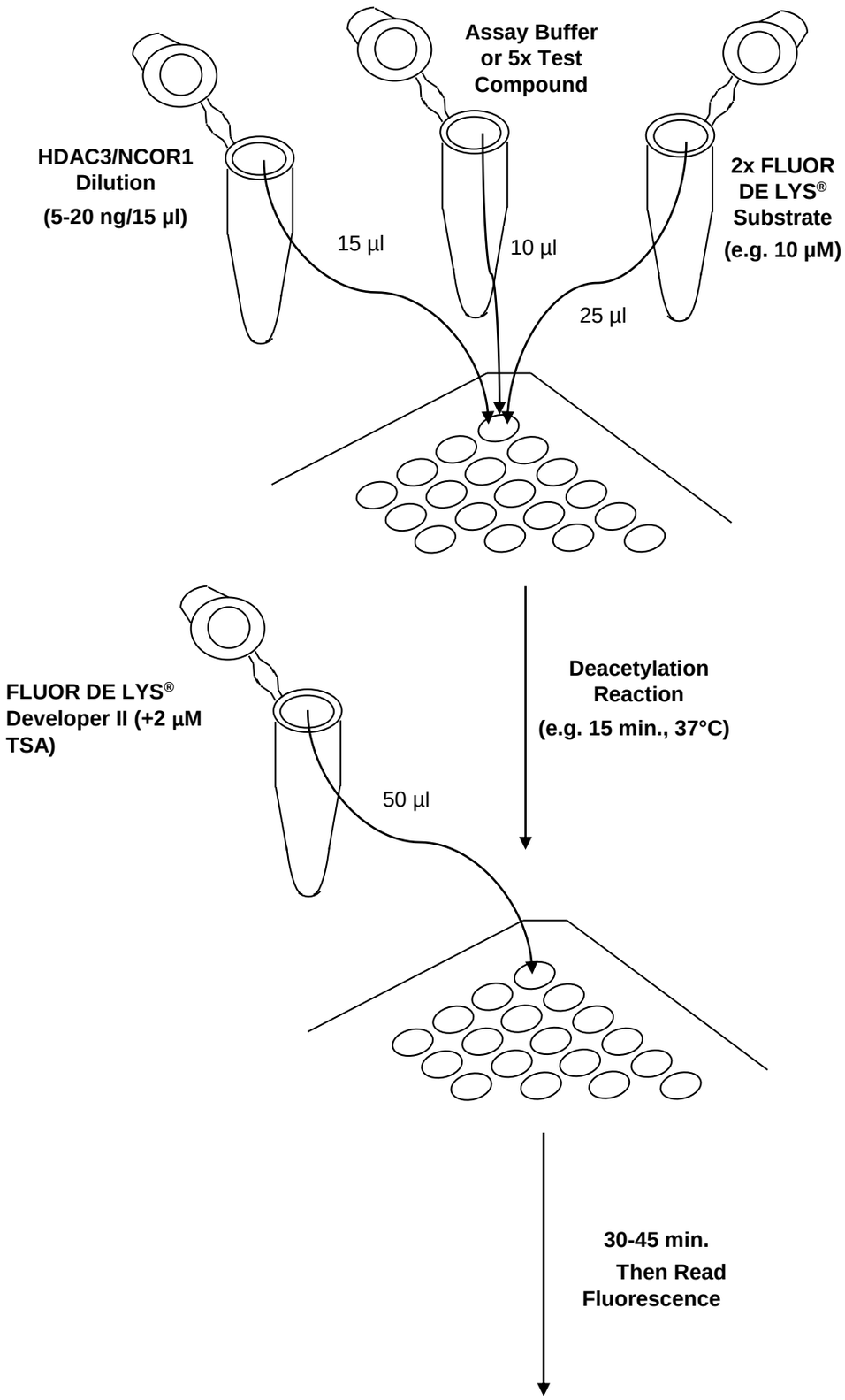

Figure 2. Performing the FLUOR DE LYS® - HDAC3/NCOR1 Activity Assay. The procedure is done in two stages. First, the components of the deacetylation reaction (HDAC3/NCOR1, buffer or test compound, substrate) are mixed. Following an incubation in which substrate deacetylation takes place, Developer II is added and mixed. This stops the deacetylation and produces the fluorescent signal. The fluorescent signal develops and can be read in less than 45 min. or less. The scheme depicts mixes for "Control" or "Test Sample" reactions; see Table 1and text for other sample types and more details.

PREPARING REAGENTS FOR ASSAY

1. Defrost all kit components and keep these, and all dilutions described below, on ice until use. With the exception of the HDAC3/NCOR1, undiluted kit components are stable for several hours on ice. The enzyme is stable on ice for the time typically required to set up an experiment (30-60 min.), but may lose activity with dilution and/or prolonged storage on ice. It is recommended that the enzyme be thawed and placed on ice as shortly before its use as practical.
2. The HDAC3/NCOR1 Complex (BML-KI574) will be diluted in HDAC Assay Buffer II (BML-KI422). Dilutions of the HDAC1 in which 15 µl (volume used per well) contains 5-20-ng of the enzyme are appropriate (see Table 1, Figures. 2-5). Volume of diluted enzyme required to provide for the assays to be performed = # of wells x 15 µl. Prewarm the buffer to assay temperature, add chilled, undiluted enzyme and proceed immediately to the aliquoting of enzyme to assay wells and the addition of substrate.
3. Prepare dilution(s) of Trichostatin A and/or Test Inhibitors in HDAC Assay Buffer II (BML-KI422). Since 10 µl will be used per well (Table 1), and since the final volume of the HDAC reaction is 50 µl, these inhibitor dilutions will be 5x their final concentration.
4. Prepare dilution(s) of the FLUOR DE LYS® - SIRT1 Substrate (BML-KI177; 5 mM) in HDAC Assay Buffer II (BML-KI422) that will be 2x the desired final concentration(s). For inhibitor screening, final substrate concentrations in the range of 3 µM-5 µM are recommended. 25 µl will be used per well (Table 1).
5. Shortly before use (<30 min.), prepare sufficient FLUOR DE LYS® Developer II for the assays to be performed (50 µl per well). First, dilute the FLUOR DE LYS® Developer II Concentrate 5-fold (e.g. 250 µl plus 1000 µl Assay Buffer) in cold HDAC Assay Buffer (BML-KI143). Second, dilute the 0.2 mM Trichostatin A (BML-GR309-9090) 100-fold in the 1x Developer II just prepared (e.g. 12.5 µl in 1.25 ml; final Trichostatin A concentration in the 1x Developer II = 2 µM; final concentration after addition to HDAC/Substrate reaction = 1 µM). Addition of Trichostatin A to the Developer II insures that HDAC activity stops when the Developer II is added. Keep Developer II on ice until use.

Performing the Assay

1. Add HDAC Assay Buffer II, diluted Trichostatin A or Test Inhibitor to appropriate wells of the microplate. Table 1 lists examples of various assay types and the additions required for each. Figure 2 depicts the basic assay procedure.
2. Warm 2x Substrate solution and the HDAC Assay Buffer II for diluting the enzyme to assay temperature. Add chilled, undiluted HDAC3/NCOR1 to the warmed buffer.
3. Add diluted HDAC3/NCOR1 to all wells except those that are to be "No Enzyme Controls."
4. Initiate HDAC3/NCOR1 reactions by adding diluted substrate (25 µl) to each well and mixing thoroughly.
5. Allow HDAC3/NCOR1 reactions to proceed for desired length of time and then stop them by addition of FLUOR DE LYS® Developer II (50 µl) prepared in step #5 of "Preparing Reagents for Assay". Incubate plate at room temperature (or 30°C) for 30- 45 min. Signal is stable for at least 60 min. beyond this time.
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 440-480 nm.

Table 1. ASSAY MIXTURE EXAMPLES[6]

Sample	HDAC Assay Buffer II	HDAC3/ NCOR1 (Dilution)	Inhibitor (5x)	FLUOR DE LYS® - SIRT1 Substrate (BML-KI177) (2x)
BLANK (No Enzyme)	25 µl	0	0	25 µl
Control	10 µl	15 µl	0	25 µl
Trichostatin A‡	0	15 µl	10 µl‡	25 µl
Test Sample⌘	0	15 µl	10 µl⌘	25 µl

[6]HDAC reaction mixtures, prior to addition of FLUOR DE LYS® Developer II.

‡Refers to dilution of Trichostatin A in HDAC Assay Buffer II, which will be 5x the final concentration. Examples: 1) As a measure of non-HDAC background, 10 µM would produce final 2 µM concentration and essentially complete HDAC1 inhibition; 2) As a model inhibitor "hit", 10 nM would produce final 2 nM and ~50% inhibition.

⌘Refers to dilution of potential inhibitor in HDAC Assay Buffer II, which will be 5x its final concentration.

PREPARATION OF A STANDARD CURVE (USES OF THE FLUOR DE LYS® DEACTYLATED STANDARD (BML-KI142)

1. The exact concentration range of the FLUOR DE LYS® Deacetylated Standard (BML-KI142) that will be useful for preparing a standard curve will vary depending on the fluorimeter model, the gain setting and the exact excitation and emission wavelengths used. We recommend diluting some of the standard to a relatively low concentration with HDAC Assay Buffer II (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 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 microplate type, 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 HDAC Assay Buffer II as a ‘zero’, to a set of wells on the microtiter plate.

3. Prepare, as described in “Preparing Reagents For Assay”, step #5, sufficient FLUOR DE LYS® Developer II for the standard wells (50 µl per well).

4. Mix 50 µl of the Developer II with the 50 µl in each standard well and incubate 5-10 min. at room temperature (or 30 °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 440-460 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 Figure 2.

Testing of Potential HDAC3/NCOR1 Inhibitors for Interference with the FLUOR DE LYS® Developer II or the Fluorescence Signal:

1. The FLUOR DE LYS® Developer II is formulated so that, under normal circumstances, the reaction goes to completion in less than 5 min. at 30 °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 II reaction or the fluorescence signal itself may be built directly into an inhibitor screening protocol. After waiting for the signal from the HDAC3/NCOR1 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 HDAC3/NCOR1 reaction). Sufficient Developer II 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, for example with highly colored potential inhibitors, where the development reaction itself is not compromised but the fluorescence signal is diminished. As discussed further below (see 3.), interference with the development reaction *per se* will be reflected in the kinetics of signal development, both that of the initial HDAC3/NCOR1 reaction and that of the Deacetylated Standard 'spike'.

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 II (e.g. readings at 5 min. intervals for 60 min.). The fluorescence of control samples (no inhibitor) will change very

little after the first or second reading. Samples containing compounds which inhibit HDAC3/NCOR1, but which do not interfere with the Developer II, will display similarly rapid kinetics, although a lower final fluorescence. Trichostatin A (5 nM) 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 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 II. 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) HDAC3/NCOR1 reaction. Mix 40 µl of the diluted Standard with 10 µl inhibitor or 10 µl HDAC Assay Buffer II (see Table 2). Initiate development by adding 50 µl of 1x Developer II to each well. Follow fluorescence development by reading at 1 or 2 min. intervals for 30 min. If a test inhibitor sample reaches its final fluorescence more slowly than the control or if the final value is significantly below that of the control, then there is interference with the Developer II reaction.

5. Once it is determined that a particular substance does interfere with the Developer II 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 (e.g. 25 min. rather than 1 min.), 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) and diluting the Developer II Concentrate 2.5-fold, rather 5-fold. All three of these

approaches may be used separately or in combination.

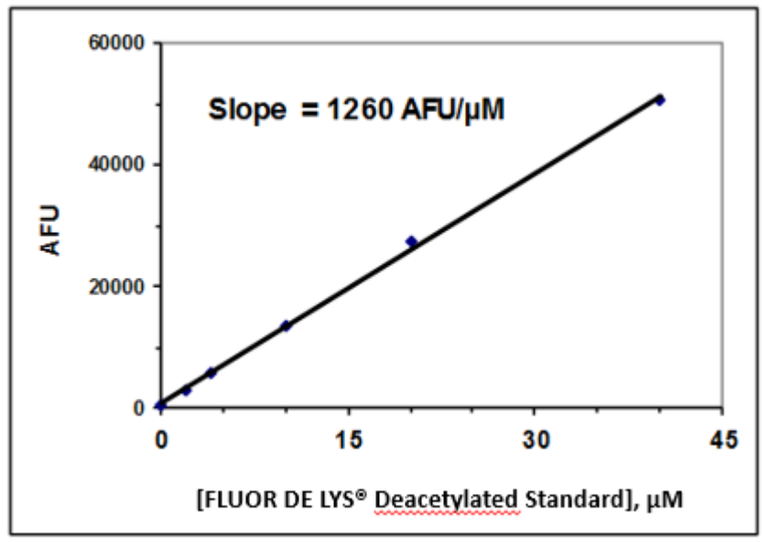

Figure 3. Fluorescence Standard Curve. 50 µl aliquots of FLUOR DE LYS® Deacetylated Standard, in Assay Buffer at the indicated concentrations, were mixed with 50 µl Developer II and incubated 10 min., 30°C. Fluorescence was then measured in the wells of the clear microplate with a CytoFluor™II fluorescence plate reader (PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=70).

Table 2. Assay Mixtures for Inhibitor Retesting with FLUOR DE LYS® Deacetylated Standard

Sample	HDAC Assay Buffer II	Inhibitor (5x)	Diluted* FLUOR DE LYS® deAc. Standard (1.25x)	DEVELOPER II (1x)
Control	10 µl	0	40 µl	50 µl
Tricho-statin A‡	0	10 µl	40 µl‡	50 µl
Test Inhibitor⌘	0	10 µl	40 µl⌘	50 µl

*The appropriate dilution of the FLUOR DE LYS® Deacetylated Standard, may be determined from the standard curve and should be the concentration producing a fluorescent signal equal to that produced by control (no inhibitor) samples in the HDAC3/NCOR1 assay. The dilution in HDAC Assay Buffer II is prepared at 1.25x this concentration to compensate for the 4/5 dilution due to addition of 10 µl of Assay Buffer or inhibitor.

‡Refers to dilution of Trichostatin A in HDAC Assay Buffer II, which will be 5x its final concentration in the 50 µl volume, prior to addition of Developer II. Example: As a model inhibitor that does not interfere with the Developer II, 25 nM Trichostatin A would produce a final 5 nM concentration.

⌘Refers to dilution of potential inhibitor in Assay Buffer, which will be 5x its final concentration in the 50 µl volume, prior to addition of Developer II.

APPLICATION EXAMPLES

The *HDAC3/NCOR1Fluorometric Drug Discovery Kit* has been used to investigate the kinetics of FLUOR DE LYS® SIRT1 (BML-KI177) deacetylation by HDAC3/NCOR1 (Figures 4 & 5) and the inhibition of HDAC3/NCOR1 by Trichostatin A (Figure 6).

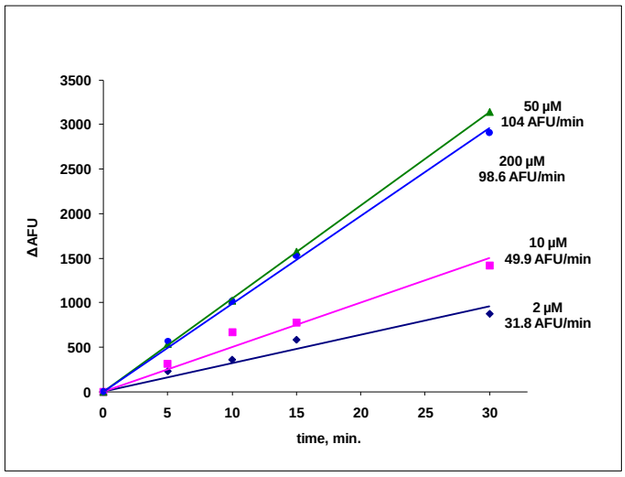

Figure 4. Time Courses of FLUOR DE LYS® - SIRT1 Substrate (BML-KI177) Deacetylation by HDAC3/NCOR1. HDAC3/NCOR1 Complex (10 ng/well) was incubated (37°C) with indicated concentrations of substrate. Reactions were stopped at indicated times with FLUOR DE LYS® Developer II and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=70). Data labels include deacetylation rates (AFU/min.) determined from linear best-fits to the data for each concentration of BML-KI177.

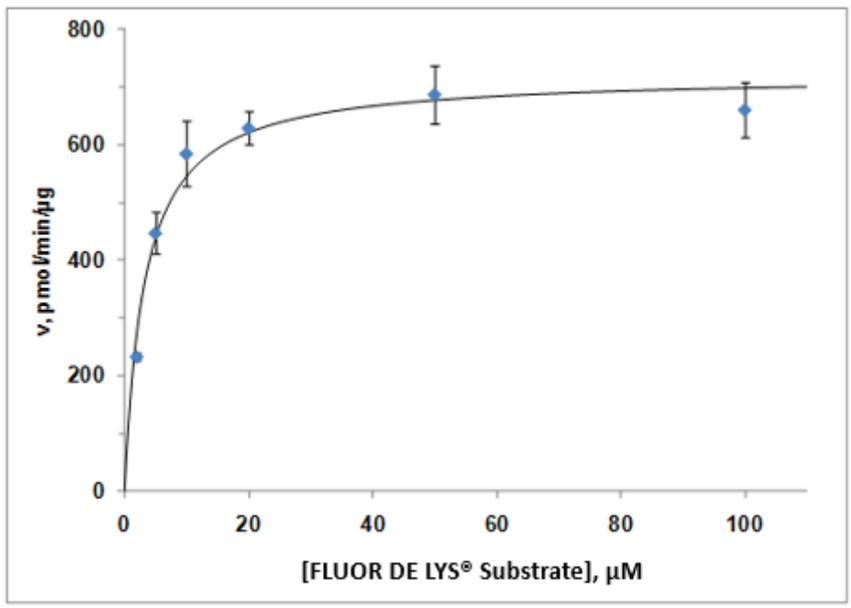

Figure 5. Kinetics of FLUOR DE LYS® SIRT1 (BML-KI177) Substrate Deacetylation by HDAC3/NCOR1. HDAC3/NCOR1 Complex (15 ng/well) was incubated (37 °C) with indicated concentrations of substrate. Reactions were stopped after 15 min. with FLUOR DE LYS® Developer II and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=70). Fluorescence increases (ΔAFU) above 'time zero' control samples were converted to pmol substrate deacetylated by a conversion factor determined with the Deacetylated Standard 'spiking' procedure described in section "PREPARATION OF A STANDARD CURVE (USES OF THE FLUOR DE LYS® DEACTYLATED STANDARD (BML-KI242)". Points are the mean of three determinations and error bars are standard deviations from the mean. Line is a non-linear least squares fit of the data to the Michaelis-Menton equation (Microsoft XL, Solver Tool). The best-fit K_m for BML-KI177 was 3.2 µM and the V_{max} was 721 pmol/min/µg.

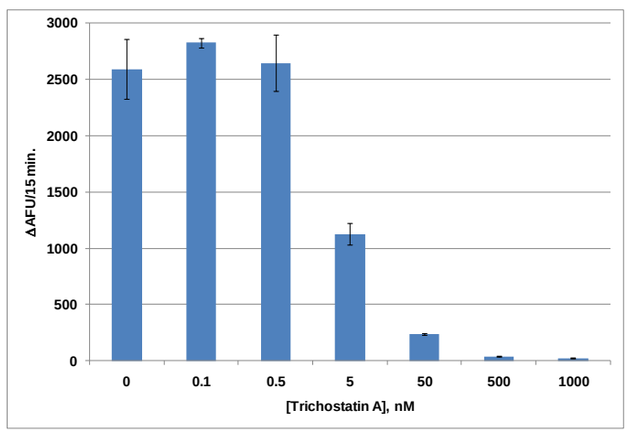

Figure 6. Trichostatin A Inhibition of HDAC3/NCOR1 Determined by FLUOR DE LYS® - SIRT1 (BML-KI177) Substrate Deacetylation. HDAC3/NCOR1 Complex (18 ng/well) was incubated (37 °C) with 5 µM substrate at indicated concentrations of Trichostatin. Reactions were stopped after 15 min. with FLUOR DE LYS® Developer II and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=70).

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