



HDAC Fluorometric Cellular Activity Assay

Catalog # BML-AK503

A FLUOR DE LYS® Assay System

NOTE: This version contains a change to reagent storage temperatures.

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Please read entire booklet before proceeding with the assay.



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

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BACKGROUND

Acetylation of lysine epsilon-amino groups extends well beyond histones to include, for example, α -tubulin and a wide variety of transcription factors. The importance of lysine acetylation as a regulatory post-translational modification may indeed rival that of phosphorylation¹. Nevertheless, the enzymes that catalyze lysine deacetylation still retain the name 'histone deacetylases' (HDACs).

There are two types of HDAC reaction mechanism. Class I enzymes (e.g. human HDACs 1-3, & 8) and class II enzymes (HDACs 4-7, 9 & 10) remove acetyl groups by hydrolysis and are sensitive to hydroxamate inhibitors such as trichostatin A. Class III HDACs, also known as sirtuins (e.g. yeast Sir2 or human SIRT1) employ a unique NAD⁺-dependent mechanism and are inhibited by the reaction product nicotinamide (NAM; see Figure 1).

Small-molecule modulators of HDAC activity are currently the focus of intense research and drug discovery efforts. Several inhibitors of class I and II (non-sirtuin) HDACs have shown promise as cancer therapeutics and are currently in clinical trials²⁻⁴. Other potential targets for class I/II HDAC inhibitors include neurodegenerative diseases such as amyotrophic lateral sclerosis, Huntington's disease and Alzheimer's disease⁵⁻⁸. Activation of SIRT1, a sirtuin (class III HDAC) may represent a strategy to treat various age-related diseases⁹, including neurodegenerative disorders¹⁰⁻¹³ and type II diabetes¹⁴.

The FLUOR DE LYS[®] Substrate (BML-KI104) is cell-permeable and thus is suitable for high-throughput cell-based deacetylase assays^{9,15,16}. It has been used in cellular assays to assess the effects of modulators of both class I/II HDACs¹⁶ and sirtuins^{9,15,17}. This kit provides the reagents needed and examples of protocols for determining rates of intracellular deacetylase activity with cultured cells.

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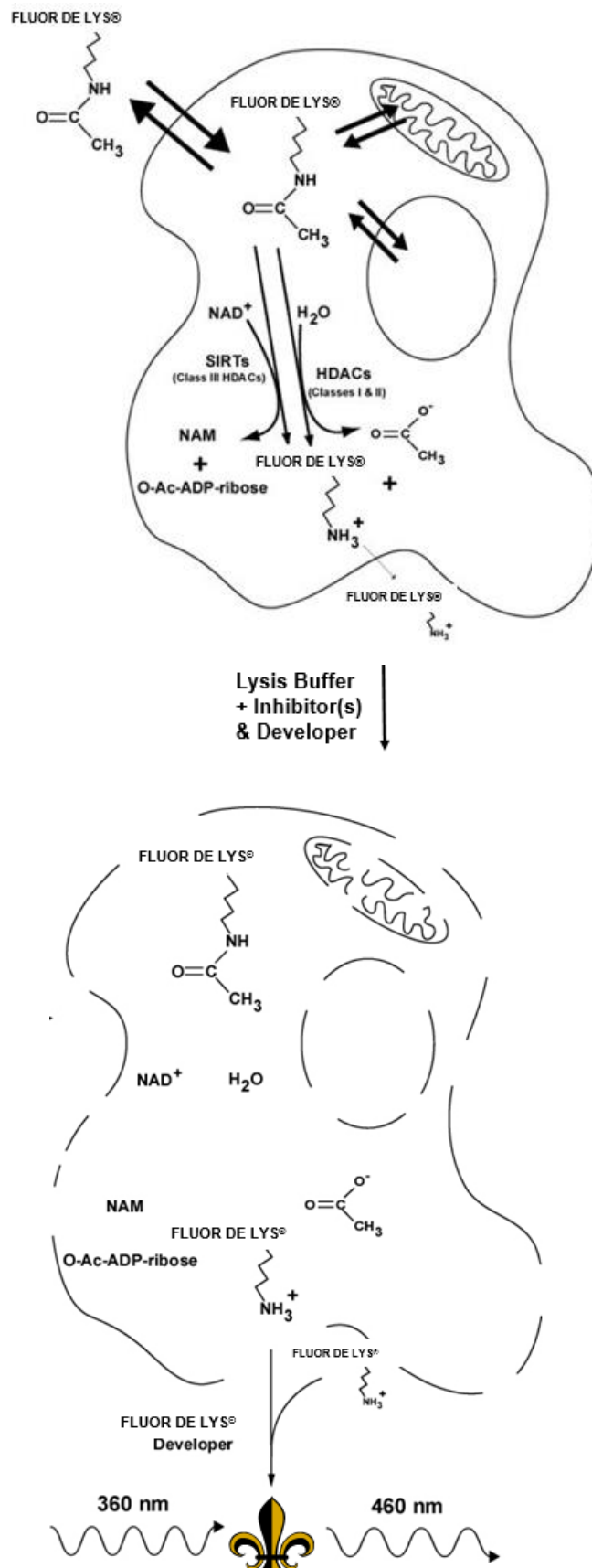


Figure 1. Reaction Scheme of the HDAC Colorimetric Activity Assay. Method for Assaying Intracellular HDAC Activity with the Fluorogenic, Cell-permeable Substrate, FLUOR DE LYS® (BML-K1104). FLUOR DE LYS® is added to cell growth medium and enters the cells. Deacetylation by class I/II HDACs or sirtuins yields the deacetylated form of FLUOR DE LYS® some of which may exit the cell. Lysis of the cells with detergent allows contact between the non-cell permeable Developer (BML-K1105) and both intra- and extracellular deacetylated substrate, thus producing a fluorescent signal (symbol). A strong class I/II HDAC inhibitor (e.g. trichostatin A) is added along with the lysis buffer containing detergent to insure that no deacetylation occurs after cell lysis.

DESCRIPTION

The HDAC Fluorometric Cellular Activity Assay is a straightforward extension of the *in vitro* HDAC assay system (HDAC Fluorometric Assay/Drug Discovery Kit, Cat. # BML-AK500). Its design is based on two considerations, first that the FLUOR DE LYS[®] Substrate is cell-permeable and second that FLUOR DE LYS[®] Developer is not (see Figure 1). The simplest version of the assay therefore consists of adding substrate to cells, in culture medium, incubating for a desired length of time to allow substrate to be taken up and deacetylated and then adding a mixture which contains detergent to lyse the cells, Developer to generate a fluorescent signal from the deacetylated substrate and inhibitor(s) to stop deacetylase activity (see Figure 2 and Figure 3). When the fluorescence stops increasing, usually 15-30 min., the signal is read in a fluorometer (Ex. 350-380 nm; Em. 440-460 nm). Protocols are also provided for separate assay of deacetylated substrate in the cells and medium (see Figure 4 and Figure 5) and for use of the HeLa Nuclear Extract (BML-KI345) as positive control (see Figure 6 and Figure 7).

MATERIALS SUPPLIED

BML-KI345-0050 Nuclear Extract from HeLa Cells (human cervical cancer cell line)

FORM: 2 mg protein/ml in 0.1 M KCl, 20 mM HEPES/NaOH, pH 7.9, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF; Prepared according to a modification of J.D. Dignam *et al. Nuc. Acids Res.* 1983 **11** 1475 and S.M. Abmayr *et al. Genes Dev.* 1988 **2** 542.

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

QUANTITY: 50 µl

BML-KI104-0050 FLUOR DE LYS[®] Substrate

FORM: 50 mM in DMSO

STORAGE: -80°C

QUANTITY: 50 µl

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

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

STORAGE: -80°C

QUANTITY: 300 µl

BML-GR309-9090 Trichostatin A (HDAC Inhibitor)

FORM: 0.2 mM in DMSO

STORAGE: -80°C

QUANTITY: 100 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 MgCl₂

STORAGE: -80°C

QUANTITY: 500 µl

BML-KI142-0030 FLUOR DE LYS[®] Deacetylated Standard

FORM: 10 mM in DMSO

STORAGE: -80°C

QUANTITY: 30 µl

BML-KI143-0020 HDAC ASSAY BUFFER

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

STORAGE: -20°C

QUANTITY: 20 ml

BML-KI346-0020 Cell Lysis Buffer

FORM: 1.0 % NP-40 in HDAC Assay Buffer

STORAGE: -80°C

QUANTITY: 20 ml

80-2404 ½-VOLUME MICROPLATE

1 clear, 96-well

STORAGE: Ambient

80-2430 STERILE ½-VOLUME MICROPLATE, LID

One plate with lid

STORAGE: Ambient

STORAGE

Store all components except the microplate at -80°C for the highest stability. The HeLa Nuclear Extract, BML-KI345, 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 -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 extract into separate tubes and store at -80°C. The FLUOR DE LYS[®] Substrate, BML-KI104, when diluted in Assay Buffer, may precipitate after freezing and thawing. It is best, therefore, to dilute only the amount needed to perform the assays of that day.

ADDITIONAL MATERIALS NEEDED

- Microplate reading fluorometer capable of excitation at a wavelength in the range 350-380 nm and detection of emitted light in the range 440-460 nm.
- Pipette or multi-channel pipette capable of pipetting 2-100 μ l accurately
- Ice bucket to keep reagents cold until use.
- Equipment and materials for cell culture (media without phenol red is preferable).
- Microplate warmer or other temperature control device (optional)
- Additional microplates (optional)

SAFETY WARNINGS & PRECAUTIONS

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 pipette by mouth.
3. Interpretation of the results is the sole responsibility of the user.

PROCEDURES

Some Things To Consider When Planning Assays:

1. For convenience, a $\frac{1}{2}$ -volume sterile microplate is provided for performing assays (80-2430). Note, however, that other types of 96-well cell culture microplates may also be used. Alternatively, cell growth and assay procedures may be performed in a sterile microplate or other sterile vessel and samples transferred to non-sterile microplates for reading in a fluorometer. Be aware that use of opaque (black or white) plates requires a fluorometer configured so that excitation and fluorescence detection occurs from above the sample wells. Also be aware that calibrations or standard curves performed with the FLUOR DE LYS[®] Deacetylated Standard will vary with the type of plate used and should be done in the same type of plate in which the experimental samples are read.

2. Reasonable deacetylation signals can be obtained by incubating approximately 10^4 - 10^5 cultured mammalian cells, with 200 μ M FLUOR DE LYS[®] Substrate for one to several hours (see Figure 2 and Figure 3). In the examples provided below, this was achieved by growing adherent cells (HeLa) to confluence in the plate provided or with 100 μ l of a suspension of 10^6 cells/ml (Jurkat). The amount of activity may vary with other cell types or treatments and therefore, the optimal cell densities must be determined for your cell type and/or treatment.
3. A tube of HeLa Nuclear Extract (BML-KI345) is provided with the kit. This extract is rich in HDAC activity (especially class I HDACs, 1-3) and may be used as positive control to insure that Substrate and Developer are working properly. Assays may be performed in cell culture medium or HDAC Assay Buffer (BML-KI143). The non-sterile microplate provided (BML-KI101) may be used for assays with the HeLa Nuclear Extract. (See “Protocol Example III”, for details on assays with HeLa Nuclear Extract.)
4. The FLUOR DE LYS[®] Substrate itself has a low but measurable level of fluorescence at the assay wavelength. Cellular deacetylation rates should therefore be determined relative to a baseline control or ‘time zero’ sample. Such a sample may be prepared by adding the substrate simultaneously with, or immediately after, the Cell Lysis buffer/Developer/inhibitor addition.
5. After addition of FLUOR DE LYS[®] Developer to a deacetylation reaction, fluorescence increases and then plateaus, usually well within 15 to 30 min., at room temperature. Taking several fluorescence readings at 5 min. intervals can be used confirm that the signal is no longer increasing. After the signal reaches this plateau, it is stable for hours.
6. Some compounds may quench the fluorescence generated by substrate deacetylation and development. Phenol red, which is often present in cell culture media, is one such compound. It is recommended, if possible, that media without phenol red be used. In some cases, for example when testing potential HDAC inhibitors, the addition of quenching compounds may be unavoidable. In such cases, the addition of a defined spike of FLUOR DE LYS[®] Deacetylated Standard, after the cellular deacetylation has leveled off and been recorded, can be used to assess the degree of quenching and calibrate the assay (see “Uses Of The FLUOR DE LYS[®] Deacetylated Standard”).

7. It is conceivable that some compounds being screened for inhibition of HDAC activity or expression may interfere with the action of the FLUOR DE LYS[®] Developer. It is therefore important to confirm that apparent HDAC inhibitor “hits” are in fact acting only via HDAC inhibition. One approach to this involves retesting the candidate inhibitor in a reaction with the FLUOR DE LYS[®] Deacetylated Standard (BML-K1142) plus the FLUOR DE LYS[®] Developer. A detailed retesting procedure is described below, in the section “Uses Of The FLUOR DE LYS[®] Deacetylated Standard”. In some cases, it may be possible to avoid this retesting by means of measurements taken during the fluorescence development phase of the initial HDAC assay. This is also discussed in that section.
8. When using the cellular assay to test potential HDAC inhibitors, it should be considered that some test compounds may enter the cells more slowly than the FLUOR DE LYS[®] Substrate. Preincubation of the cells with the inhibitor (e.g. for 30 min.) may help avoid underestimation of inhibitor potency due to such permeability differences.
9. Note that sirtuins (Sir2 and Sir2-like NAD⁺-dependent HDACs) are insensitive to trichostatin A (TSA). Therefore, Developer prepared as described below with added TSA, will not necessarily block activity by these enzymes after cell lysis. However, since the lysis of the cells will produce a large dilution of the cellular contents, including the sirtuin co-substrate NAD⁺, sirtuin activity may nonetheless stop without the addition of an inhibitor. Under some circumstances, for example with cells that are expressing a sirtuin at a high level or when NAD⁺ has been added to the culture medium, addition of the sirtuin inhibitor nicotinamide (BML-K1283, 1-5 mM) to the Developer may be a worthwhile precaution.

10. Subsequent sections will describe three types of assay protocols. The first protocol (see “Protocol Example I”), which will be appropriate for most cellular studies, determines, with one measurement in a single well, the total amount of deacetylated substrate. This is done by simply adding Developer in Cell Lysis Buffer directly to cells in the medium in which they have been incubating with FLUOR DE LYS[®] Substrate. The second protocol separately determines the amount of intracellular deacetylated substrate and the amount that has translocated from the cells into the medium (see “Protocol Example II”). This protocol may be used to study transport of the FLUOR DE LYS[®] Substrate and/or its deacetylated form across the plasma membrane. It may also be useful in circumstances where the total deacetylation signal is low. Removal of the medium containing the FLUOR DE LYS[®] Substrate prior to cell lysis and development eliminates most of the fluorescence background due to acetylated substrate from the intracellular signal measurement. Since the intracellular part of the total signal can represent about half the total (see Figure 4 and Figure 5), this may improve the signal to noise ratio in the overall deacetylation measurement. Finally, the third protocol (see “Protocol Example III”) describes a non-cellular assay using the HeLa Nuclear Extract (BML-KI345). This assay is similar to those performed with the HDAC *Fluorometric Assay/Drug Discovery Kit* (Cat. # BML-AK500) and is intended for use as a positive control.

PREPARING REAGENTS FOR ASSAY

1. Determine which type of assay or assays your planned experiment will entail (see “Some Things To Consider When Planning Assays”, point 10., and Protocol Examples I-III).
2. Defrost the desired kit components. With the exception of the undiluted FLUOR DE LYS[®] Substrate (BML-KI104), keep these on ice until use. All undiluted kit components are stable for several hours on ice.
3. FLUOR DE LYS[®] Substrate (BML-KI104), the 0.2 mM TSA (GR-309-9090) and the FLUOR DE LYS[®] Deacetylated Standard (BML-KI142) are DMSO solutions that will freeze at ~18°C and so should be brought to room temperature before withdrawing aliquots for making dilutions in cell culture media, Cell Lysis Buffer (BML-KI346) or HDAC Assay Buffer (BML-KI143). In the case of the Substrate, these dilutions (e.g. to 200 µM) would typically be done in cell culture media warmed to 37°C or in HDAC Assay Buffer at the desired assay temperature.

4. Working dilutions of FLUOR DE LYS[®] Developer Concentrate (20x) (BML-KI105) in either Cell Lysis Buffer (BML-KI346) or HDAC Assay Buffer (BML-KI143) should be prepared shortly before use (<30 min.). Each assay well will require 50 μ l of 1x Developer. First, dilute the FLUOR DE LYS[®] Developer Concentrate 20-fold (e.g. 50 μ l plus 950 μ l buffer) in cold Cell Lysis Buffer or HDAC Assay Buffer. If desired for sirtuin inhibition (see “Some Things To Consider When Planning Assays”, point 9.), replace a portion of the diluting buffer with 50 mM nicotinamide (BML-KI283). (E.G., for 2 mM nicotinamide: 50 μ l 20X Developer plus 910 μ l Cell Lysis/HDAC Assay Buffer plus 40 μ l 50 mM nicotinamide.) Second, dilute the 0.2 mM Trichostatin A (TSA; GR309-9090) 100-fold in the 1x Developer just prepared (e.g. 10 μ l in 1 ml; final TSA concentration in the 1x Developer = 2 μ M; final concentration after addition to HDAC/Substrate reaction = 1 μ M). Addition of TSA to the Developer insures that HDAC activity stops when the Developer is added. Keep Developer on ice until use.

PROTOCOL EXAMPLE I: SINGLE WELL ASSAY OF TOTAL DEACETYLASE ACTIVITY IN HeLa CELLS

1. HeLa cells were seeded in 100 μ l media per well at $0.5 - 4 \times 10^4$ cells/well and grown to desired confluences (~30 or 80%; see Figure 2 and Figure 3) in a $\frac{1}{2}$ -volume sterile 96-well microplate (80-2430). Media used was MEM (e.g. Sigma M3024)/ 10%FBS/ 2.2g/L sodium bicarbonate/ 2mM glutamine (no phenol red).
2. Cells were grown to desired confluency and media was replaced with 50 μ l/well of media containing 200 μ M FLUOR DE LYS[®] Substrate (BML-KI104) and +/- 1 μ M TSA. (If test compounds were being screened, they would be included with the media/substrate added at this point. See “Some Things To Consider When Planning Assays”, point 8). Plates were incubated at 37°C for indicated times, with each condition represented in triplicate. To attain various incubation times, while developing and reading all wells simultaneously, incubations were begun in a reverse staggered fashion. For example, in the experiment of Figure 2, the media was replaced with media plus 200 μ M FLUOR DE LYS[®] Substrate first in the 4 hr. wells, then the 2 hr. wells etc.
3. Less than 30 minutes before termination of the assay, a 1x dilution of FLUOR DE LYS[®] Developer Concentrate (20x) (BML-KI105) was prepared in cold Cell Lysis Buffer (BML-KI346) and TSA was added to 2 μ M (see “Preparing Reagents for Assay”, point 4.).
4. To terminate HDAC activity and begin development of the fluorescence signal, 50 μ l per well of the 1x Developer + 2 μ M TSA was added and mixed by up and down pipetting. ‘Time 0’ samples were prepared by mixing equal volumes of media/200 μ M FLUOR DE LYS[®] Substrate and 1x Developer + 2 μ M TSA together in clean tube and then replacing the media in wells of untreated cells with 100 μ l of the mixture.
5. After Developer addition, plates were incubated for an additional 15 minutes at 37°C and fluorescence was read in a microplate-reading fluorometer (CytoFluor II, Perseptive Biosystems, Ex. 360 nm, Em. 460 nm). See “Some Things To Consider When Planning Assays”, point 5. for steps to take to insure that development is complete.)

(NOTE: If using this protocol with test compounds that might quench fluorescence, see “Some Things To Consider When Planning Assays”, point 6.)

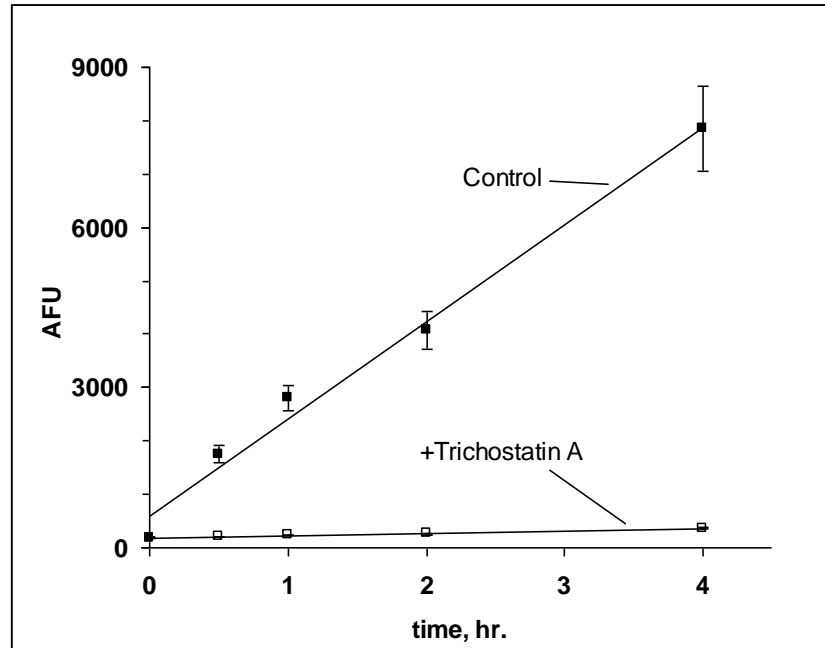


Figure 2. Time Course of FLUOR DE LYS® Substrate Deacetylation by HeLa Cells. HeLa cells were seeded at 4×10^4 cells per well in and grown overnight to 80% confluence. Cells were then incubated with 200 μ M FLUOR DE LYS® Substrate, +/- 1 μ M Trichostatin A, and fluorescence determined as described in Protocol Example I (AFU= Arbitrary Fluorescence units, CytoFluor II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain 70). Each point represents the mean of three determinations, with error bars representing +/- standard deviation.

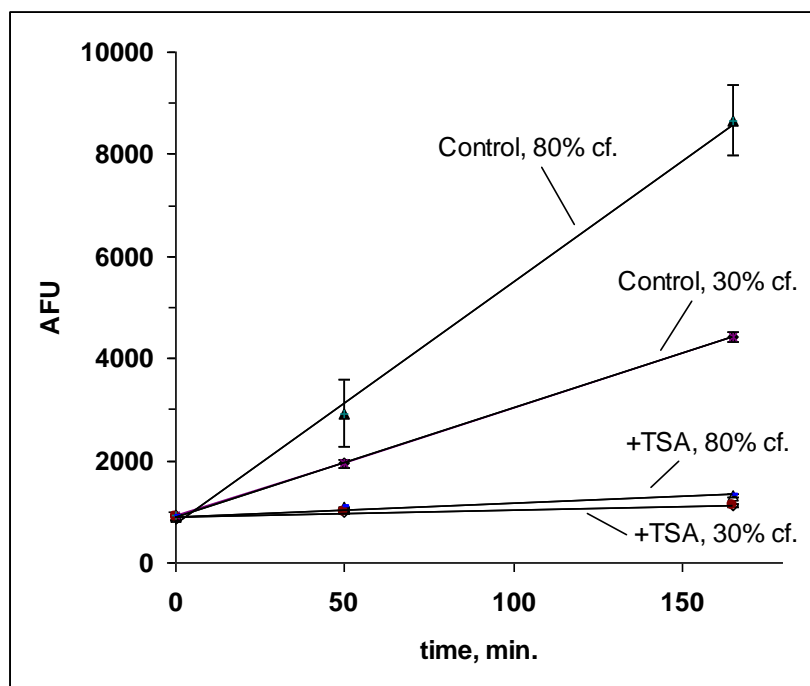


Figure 3. FLUOR DE LYS® Substrate Deacetylation at Two Cell Densities. HeLa cells were seeded at either 0.5×10^4 (30% confluence) or 2×10^4 (80% confluence) cells per well and grown two days to the indicated confluences. Cells were then incubated with 200 μ M FLUOR DE LYS® Substrate, +/- 1 μ M Trichostatin A, and fluorescence determined as described in Protocol Example I (AFU= Arbitrary Fluorescence units, CytoFluor II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain 85).

PROTOCOL EXAMPLE II: SEPARATE ASSAY OF DEACETYLATED SUBSTRATE IN CELLS AND MEDIUM

Introduction

When cells are incubated with the FLUOR DE LYS[®] Substrate, the media removed and Developer added separately to the media and cell lysate, a fluorescence signal is obtained in each of the two samples. This indicates that the FLUOR DE LYS[®] Substrate has entered the cells and some of the deacetylated substrate has left the cells and entered the media. In experiments with HeLa cells (Figure 4) and Jurkat cells (Figure 5) the amount of intracellular deacetylated substrate and the amount in the media were of similar magnitude. However, the much larger volume of the extracellular compartment (media), means that a large concentration gradient for the deacetylated substrate has developed between the cells and the media (Figure 4C and Figure 5C). In some cell types, for example *Drosophila* S2 cells^{1,2}, the deacetylated substrate does not accumulate in the cells and most will be found in the media. It has also been reported that the deacetylated form of the p53-based FLUOR DE LYS[®] SIRT1 Substrate (BML-KI177) is transported out of HT29 cells³.

The experiments in Figure 4 and Figure 5 were performed, respectively, with HeLa cells grown in full sized 96-well microplates and Jurkat cells grown in a flask in suspension culture. The following procedure describes an adaptation of the HeLa (adherent) cell version of the assay for ½-volume 96-well microplates.

REFERENCES

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2. K.T. Howitz *unpublished observations*.
3. V.C. de Boer *et al.* *Mech. Ageing Dev.* 2006 **127** 618

Performing the Assay

1. HeLa cells (or other adherent line in appropriate media) are grown in a ½-volume sterile 96-well microplate (80-2430) as described in 'Protocol Example I'.
2. Prepare 200 µM dilutions of FLUOR DE LYS[®] Substrate in media including TSA, other inhibitor or test compounds where desired. The 100 µl of media in each well will be replaced with 60 µl of media/substrate solution. After media replacement, allow the deacetylation to proceed for desired length of time (at 37°C in cell culture incubator).

3. Less than 30 minutes before termination of the assay, two different types of FLUOR DE LYS[®] Developer (BML-KI105) solutions should be prepared. The first, which is used for signal development in the media, is simply a twenty-fold dilution of the Developer Concentrate (20X) in cold HDAC Assay Buffer with 2 μ M TSA (e.g. 50 μ l FLUOR DE LYS[®] Developer Concentrate (BML-KI105) plus 950 μ l HDAC Assay Buffer (BML-KI143) plus 10 μ l 0.2 mM TSA (GR-309-9090)). **Each assay well will require 50 μ l of this 1x Developer in HDAC Assay Buffer, 2 μ M TSA.** The second type of Developer, solution, which is for developing the cell signal, is a 1:1 mixture of the solution just described (1x Developer in HDAC Assay Buffer, 2 μ M) with cold Cell Lysis Buffer (BML-KI346). **Each assay well will require 100 μ l of this mixture of 1x Developer in HDAC Assay Buffer, 2 μ M TSA with Cell Lysis Buffer (BML-KI346).**
4. As the end of the cellular deacetylation incubation approaches, have ready: 1) cold HDAC Assay Buffer, 2) the non-sterile 96-well plate (80-2404).
5. End the cellular deacetylation reaction and begin signal development as follows:
 - a) Withdraw 50 μ l of media from each well and transfer to an empty well in non-sterile plate (80-2404).
 - b) Rinse the cells in each well with 100 μ l cold HDAC Assay Buffer (BML-KI143) and then remove as much liquid as possible and discard.
 - c) Add 100 μ l of the 1x Developer/Cell Lysis Buffer mixture to the cells in the empty wells, pipetting up and down once.
 - d) Add 50 μ l of the 1x Developer to each of the 50 μ l aliquots of transferred media/Substrate in the non-sterile plate. Mix by pipetting up and down 2 or 3 times.
 - e) In order to prepare a 'Time 0' set of samples remove the growth media from an untreated well and pipet on 60 μ l of media plus 200 μ M Substrate plus 1 μ M TSA. Immediately remove 50 μ l to a well in the non-sterile plate and proceed to rinse the cells and add the Developer solutions as in steps b) through e).
 - f) Incubate both the cell (sterile) and media (non-sterile) plates at 37°C for 15 min.
6. Read and record the fluorescence in the wells of both plates (Ex. ca. 360 nm, Em. ca. 460 nm).

Use of the Deacetylated Standard to Combine or Compare Cell and Media Fluorescence Readings

1. In some cases, for example when a colored test compound has been added to the media/Substrate solution, the molar yields of fluorescence from deacetylated and developed substrate in the cellular and media parts of the assay may differ. Addition of a defined 'spike' of FLUOR DE LYS[®] Deacetylated Standard to each media and cellular well can be used to determine this yield and thus allow the addition or comparison of the cellular and media deacetylation data. (See also "USES OF THE FLUOR DE LYS[®] DEACETYLATED STANDARD")
2. Prepare enough of a 30 µM dilution of the FLUOR DE LYS[®] Deacetylated Standard (BML-KI142; 10 mM) in HDAC Assay Buffer (BML-KI143) such that 5 µl can be added to each cell and media assay well.
3. See "Some Things To Consider When Planning Assays", point 5. for steps to take to insure that development of the substrate deacetylation in the cell and media samples are complete and make sure these final readings are recorded.
4. Add 5 µl of the 30 µM Deacetylated Standard solution to each of the cell and media wells and mix. Do this as soon as possible after the completed development and recording of the substrate deacetylation reactions to insure that sufficient Developer activity remains to produce a complete signal from the spike of Deacetylated Standard.
5. Follow the development of the fluorescence from the Deacetylated Standard addition by taking periodic readings, e.g. every 5 min., until the signal stops increasing.
6. Record the new fluorescence readings and determine the increase in fluorescence by subtracting, for each cell and media well, the earlier fluorescence readings.
7. For each well calculate the fluorescence increase per pmol:
$$\frac{(2\text{nd Reading (AFU)} - 1\text{st Reading (AFU)})}{(5 \mu\text{l} \times 30 \mu\text{M})} =$$
$$\text{Fluorescence Increase (AFU)} / 150 \text{ pmol} =$$
$$\text{Conversion Factor (AFU/pmol)}$$
8. Using these Conversion Factors and the 1st Readings minus the appropriate 'Time 0' readings, calculate the number of pmol of Substrate deacetylated by HDAC activity represented in each cell or media well. NOTE: Since each media sample was 50 µl out of a total of 60 µl, the final number of pmol calculated for the media wells should be multiplied by 1.2.

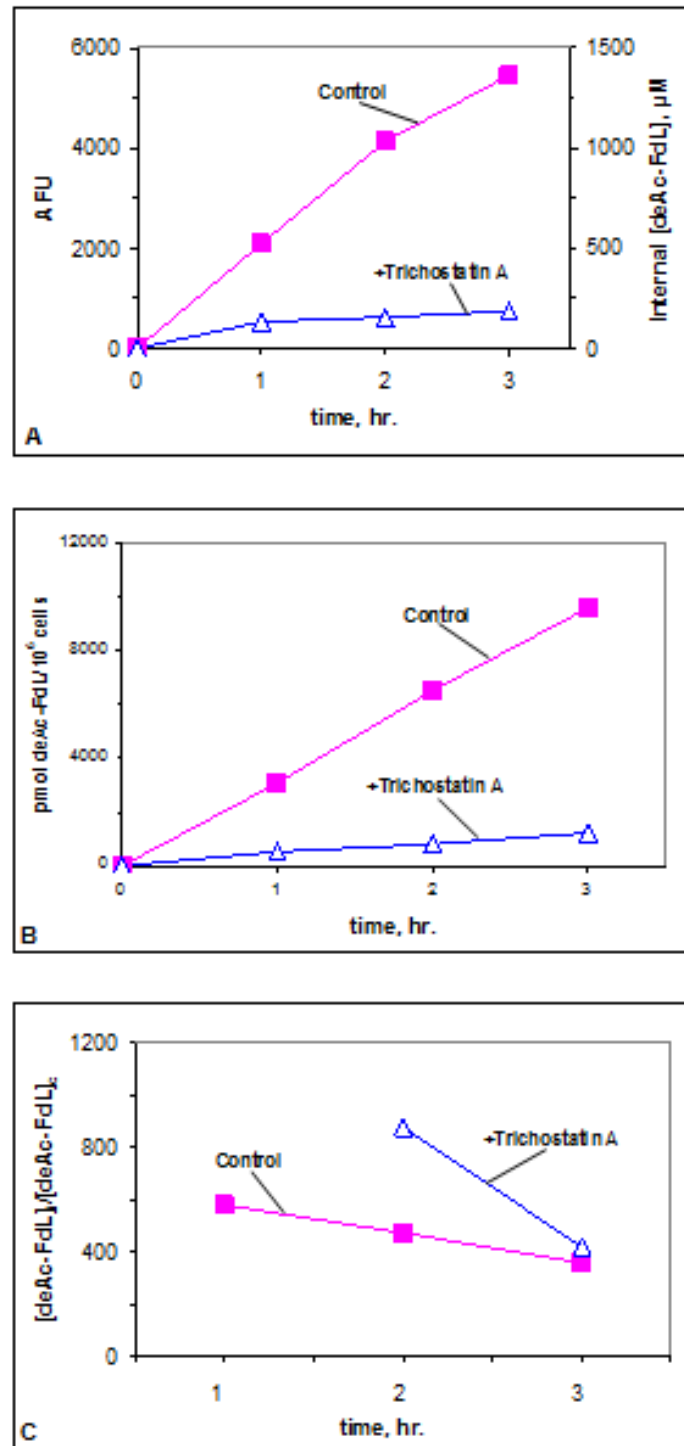


Figure 4. HDAC Activity of Adherent (HeLa) Cells Assayed with Separate Cell and Media Samples. HeLa cells were seeded at 6×10^4 per well (60% confluence; 96-well plate (Costar 3595); DMEM/10% FCS). When confluent, medium was replaced with 110 μl of 200 μM FLUOR DE LYS[®] Substrate in DMEM/10% FCS, +/- 1 μM trichostatin A, for indicated times. Medium was moved to separate wells for assay (100 μl plus 100 μl Developer) and cells rinsed 1x with PBS. FLUOR DE LYS[®] Developer (100 μl) was added to the cells and the plate incubated 5 min., 37°C. HDAC assay buffer plus 1% NP-40/1 μM trichostatin A (100 μl) was added to cell wells. After an additional 10 min. incubation, cell and medium fluorescence was measured (CytoFluor II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85). A. Cellular levels of deacetylated FLUOR DE LYS[®] Substrate (deAc-FdL. Calculated intracellular concentration of deAc-FdL (right axis) assumes cell volume of 4×10^{-9} ml. B. Time course of total deAc-FdL accumulation (cell pellet + medium) per million cells. C. Intracellular/extracellular ratio of deAc-FdL concentration as a function of treatment time with FLUOR DE LYS[®] Substrate.

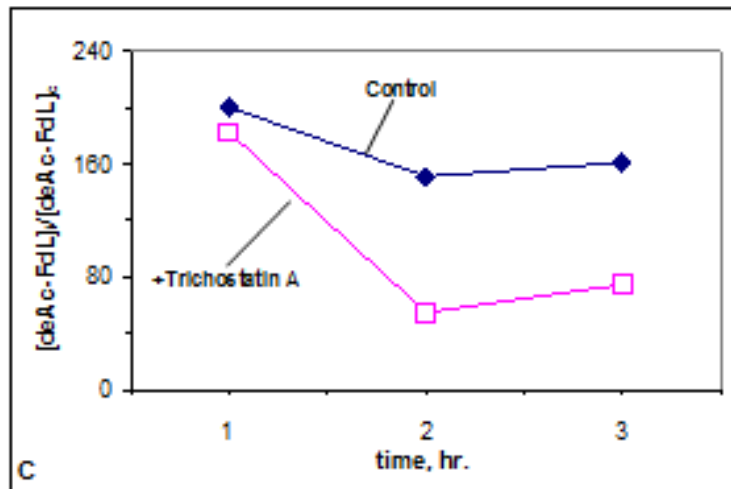


Figure 5. HDAC Activity of Suspension (Jurkat) Cells Assayed with Separate Cell and Media Samples. Jurkat cells (10^6 /ml) were incubated with 200 μ M FLUOR DE LYS[®] Substrate, +/- 1 μ M trichostatin A, in growth medium (RPMI 1640/10% FCS), for the indicated times and cells and medium separated by centrifugation. Cell pellets were extracted with 200 μ l HDAC assay buffer plus 0.5% NP-40 and 1 μ M trichostatin A. 50 μ l samples of media or cell extracts were mixed with 50 μ l FLUOR DE LYS[®] Developer, in a white plate, and fluorescence measured (CytoFluor II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85). A. Cellular levels of deacetylated FLUOR DE LYS[®] Substrate (deAc-FdL) in control and trichostatin A-treated cells. Calculated intracellular concentration of deAc-FdL (right axis) assumes a cell volume of 4×10^{-9} ml. B. Time course of total deAc-FdL accumulation (cell pellet + medium) per million cells. C. The intracellular/extracellular ratio of deAc-FdL concentration as a function of treatment time with FLUOR DE LYS[®] Substrate.

PROTOCOL EXAMPLE III: ASSAY OF HDAC ACTIVITY IN HeLa NUCLEAR EXTRACT

Introduction

HeLa nuclear extract (BML-KI345) has strong deacetylase activity with the FLUOR DE LYS[®] Substrate, contributed primarily by the TSA-sensitive, class I HDACs 1, 2 and 3 (see Figure 6 and Figure 7). An aliquot is provided with the kit (BML-KI345; 50 μ l, 100 μ g protein) as a positive control. Aside from being useful for confirming that the FLUOR DE LYS[®] Substrate and Developer are functioning properly, the extract may be useful for other types of troubleshooting. For example, if a known HDAC inhibitor has poor or no activity in a cell-based assay, an experiment comparing its effect on the HeLa extract activity when assayed in HDAC Assay Buffer versus that when assayed in cell culture media might be used to check for inactivation of the inhibitor by some component of the cell culture media.

Performing The Assay

1. Dilute HeLa Nuclear Extract (BML-KI345) in desired buffer for assay (e.g. HDAC Assay Buffer or cell culture media). HeLa Nuclear Extract amounts of 0.5-2 μl (1-4 μg protein) per well will provide useful signals in deacetylation reactions incubated at 37°C and running concurrently with cell-based assays (e.g. 1-4 hours). In the mixing scheme shown in TABLE 1, 15 μl of diluted HeLa extract is added per well.
2. Prepare a 2x FLUOR DE LYS[®] Substrate solution in desired buffer. (E.g., mix 2 μl 50 mM FLUOR DE LYS[®] Substrate plus 998 μl buffer for a 2x concentration of 100 μM and a final assay concentration of 50 μM .) The apparent K_m of the FLUOR DE LYS[®] Substrate for the HDAC activity in the HeLa extract is ~50 μM .
3. Add Assay buffer, diluted trichostatin A or test inhibitor to appropriate wells of the microtiter plate. TABLE 1 lists examples of various assay types and the additions required for each.
4. Add diluted HeLa extract to all wells except those that are to be “No Enzyme Controls.”
5. Allow diluted FLUOR DE LYS[®] Substrate and the samples in the microtiter plate to equilibrate to assay temperature (e.g., 25 or 37°C).
6. Initiate HDAC reactions by adding diluted substrate (25 μl) to each well and mixing thoroughly.
7. Allow HDAC reactions to proceed for desired length of time and then stop them by addition of FLUOR DE LYS[®] Developer (50 μl). Incubate plate at room temperature (25°C) for 10-15 min. Signal is stable for at least 30 min. beyond this time.
8. Read samples in a microtiter-plate reading fluorometer capable of excitation at a wavelength in the range 350-380 nm and detection of emitted light in the range 440-460 nm.

TABLE 1. ASSAY MIXTURE EXAMPLES⁶

Sample	Assay Buffer	HeLa Extract (Dilution)	Inhibitor (5x)	FLUOR DE LYS [®] Substrate (2x)
Blank (No Enzyme)	25µl	0	0	25µl
Control	10µl	15µl	0	25µl
Trichostatin A [‡]	0	15µl	10µl [‡]	25µl
Test Sample [⌘]	0	15µl	10µl [⌘]	25µl

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

[‡] Refers to dilution of trichostatin A in Assay Buffer, which will be 5x the final concentration. Examples: 1) As a measure of non-HDAC background, 5 µM would produce final 1µM concentration and essentially complete HDAC inhibition; 2) As a model inhibitor “hit”, 25 nM would produce final 5 nM and ~50% inhibition.

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

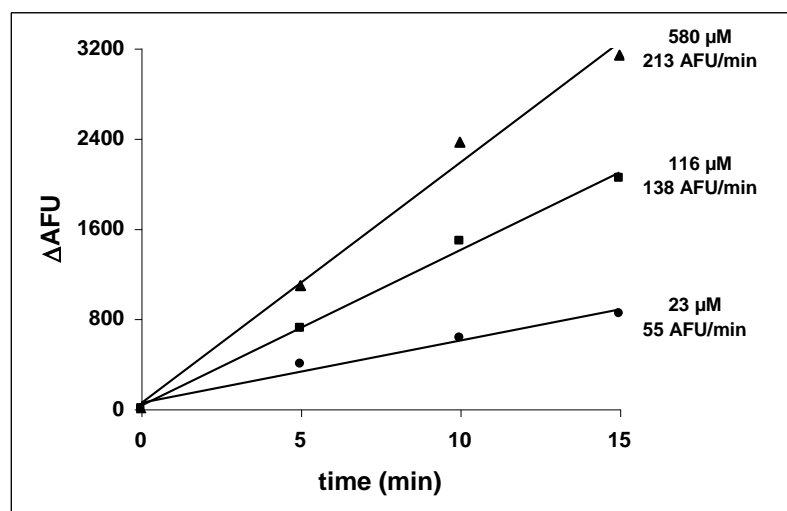


Figure 6. Time Courses of FLUOR DE LYS[®] Substrate Deacetylation by HDAC. HeLa Nuclear Extract (4 µg protein/well) was incubated (25°C) with indicated concentrations of substrate. Reactions were stopped at indicated times with FLUOR DE LYS[®] Developer and fluorescence measured (CytoFluor II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85).

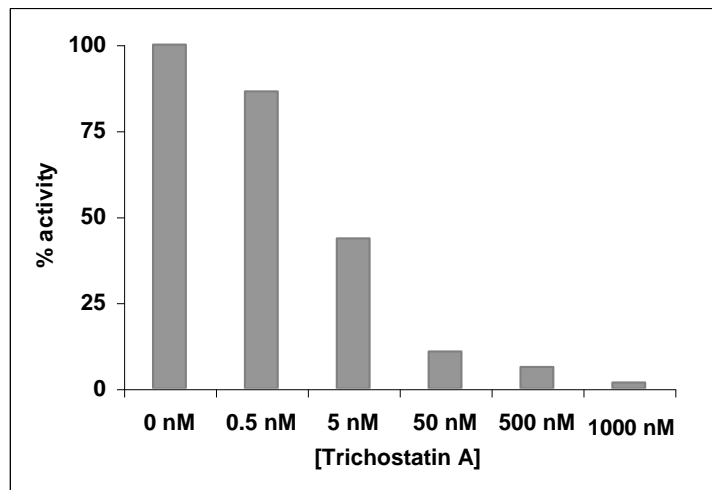


Figure 7. Trichostatin A Inhibition of FLUOR DE LYS® Substrate Deacetylation by HeLa Nuclear Extract. HeLa Nuclear Extract (4 μ g protein/well) was incubated (25°C) with 116 μ M substrate and indicated concentrations of Trichostatin A. Reactions were stopped after 10 min. with FLUOR DE LYS® Developer and fluorescence measured (CytoFluor II, PerSeptive Biosystems, Ex. 360 nm, Em. 460 nm, gain=85).

USES OF THE FLUOR DE LYS® DEACETYLATED STANDARD (BML-KI142)

Preparation of a Standard Curve:

1. The exact concentration range of the FLUOR DE LYS® Deacetylated Standard (BML-KI142) that will be useful for preparing a standard curve will vary depending on the fluorometer model, the gain setting and the exact excitation and emission wavelengths used. We recommend diluting some of the standard to a relatively low concentration with Assay Buffer (1 to 5 μ M). The fluorescence signal should then be determined, as described below, after mixing 50 μ l of the diluted standard with 50 μ l of Developer. The estimate of AFU (arbitrary fluorescence units)/ μ M or AFU/pmol 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 μ l of each of these dilutions, and 50 μ l of Assay Buffer as a 'zero', to a set of wells on the microtiter plate.

3. Prepare, as described in “Preparing Reagents For Assay”, step 4, sufficient FLUOR DE LYS[®] Developer for the standard wells (50 μ l per well).
4. Mix 50 μ l of the Developer with the 50 μ l in each standard well and incubate 5-10 min. at room temperature (25°C).
5. Read samples in a microtiter-plate reading fluorometer 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/ μ M. See example in Figure 8.

Testing of Potential HDAC 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, should help ensure 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 HDAC reaction to fully develop and stabilize (usually less than 5 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 μ M in the 50 μ l HDAC 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/ μ M or AFU/pmol, 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 HDAC reaction and that of the Deacetylated Standard ‘spike’.

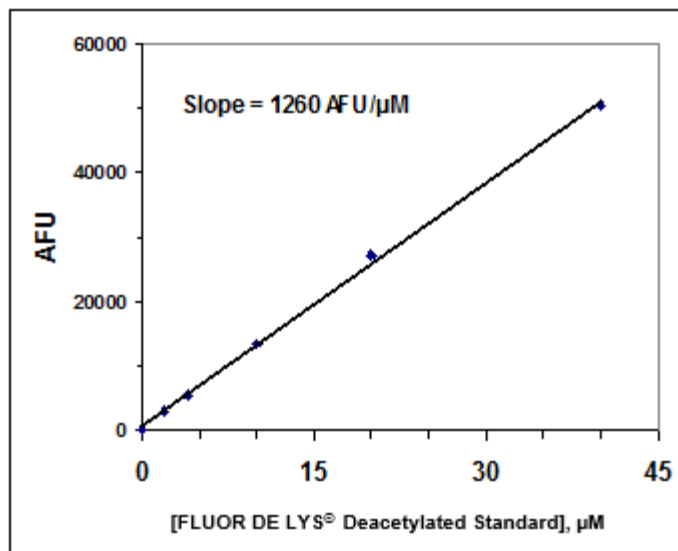


Figure 8. Fluorescence Standard Curve. 50µl aliquots of FLUOR DE LYS® Deacetylated Standard, in Assay Buffer at the indicated concentrations, were mixed with 50 µl Developer and incubated 10 min., 25°C. Fluorescence was then measured in the wells of the clear microtiter-plate (BML-K1101) with a CytoFluor 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 1 or 2 min. intervals for 30 min.). The fluorescence of control samples (no inhibitor) will change very little after the first or second reading. Samples containing compounds which inhibit HDACs, but which do not interfere with the Developer, 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 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) HDAC reaction. Mix 40 μ l of the diluted Standard with 10 μ l inhibitor or 10 μ l Assay Buffer (see Table 2). Initiate development by adding 50 μ l 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 more slowly than the control or if the final value is significantly below that of the control, then there is interference with the Developer reaction.
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 (e.g. 25 min. rather than 1 min.), 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 μ l) and diluting the Developer Concentrate 10-fold, rather 20-fold. All three of these approaches may be used separately or in combination.

TABLE 2. ASSAY MIXTURES FOR INHIBITOR RETESTING WITH FLUOR DE LYS[®] DEACETYLATED STANDARD

Sample	Assay Buffer	Inhibitor (5x)	Diluted⁶ FLUOR DE LYS[®] deAc. Standard (1.25x)	DEVELOPER (1x)
Control	10 µl	0	40 µl	50 µl
Trichostatin A [‡]	0	10 µl	40 µl [‡]	50 µl
Test Sample [⌘]	0	10 µl	40 µl [⌘]	50 µl

⁶ 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 HDAC 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 inhibitor.

[‡] Refers to dilution of trichostatin A in Assay Buffer, which will be 5x its final concentration in the 50 µl volume, prior to addition of Developer. Example: As a model inhibitor that does not interfere with the Developer, 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.

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NOTES



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