



HDAC Colorimetric Assay/Drug Discovery Kit

Catalog # BML-AK501

A COLOR DE LYS® Assay System

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Please read entire booklet before proceeding with the assay.



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



Please contact Enzo Life Sciences Technical Support if necessary.

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BACKGROUND

Histones form the protein core of nucleosomes, the DNA/protein complexes that are the subunits of eukaryotic chromatin. The 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 transcription¹. The best studied of these modifications, acetylation of the ϵ -aminos of specific histone lysine residues, are catalyzed by histone acetyltransferases (HATs). Histone deacetylases (HDACs) are responsible for hydrolytic removal of these acetyl groups²⁻⁴.

Histone hyperacetylation correlates with an open, decondensed chromatin structure and gene activation, while hypoacetylation correlates with chromatin condensation and transcriptional repression. Consistent with this, HATs have been shown to associate with several transcriptional activators and some transcriptional activators have been found to have intrinsic HAT activity¹⁻⁴. Conversely HDACs are found to associate with transcriptional repression complexes such as NuRD or those including Sin3¹⁻⁷. Thus far, eight human HDACs have been identified; all trichostatin A-sensitive and all homologs of either RPD3 or HDA1, yeast histone deacetylases⁸⁻¹⁶. Interestingly, Sir2, the yeast mother cell longevity factor, and its mouse homolog, mSir2 α , have recently been shown to be trichostatin A-insensitive, NAD⁺-dependent histone deacetylases¹⁷.

Histone deacetylase inhibitors have shown promise as anti-tumor agents and naturally this has stimulated interest in the screening of compounds for HDAC inhibition. Unfortunately, the standard techniques for HDAC assay are cumbersome. Use of [³H]acetyl-histone or [³H]acetyl-histone peptides as substrates involves an acid/ethyl acetate extraction step prior to scintillation counting^{8,18,19}. Unlabeled, acetylated histone peptides have been used as substrates, but reactions then require resolution by HPLC¹⁷. Enzo Life Sciences' *HDAC Colorimetric Assay/Drug Discovery Kit* addresses these problems by providing an assay that can be carried out in two simple mixing steps, all on the same 96-well plate (Figure 1).

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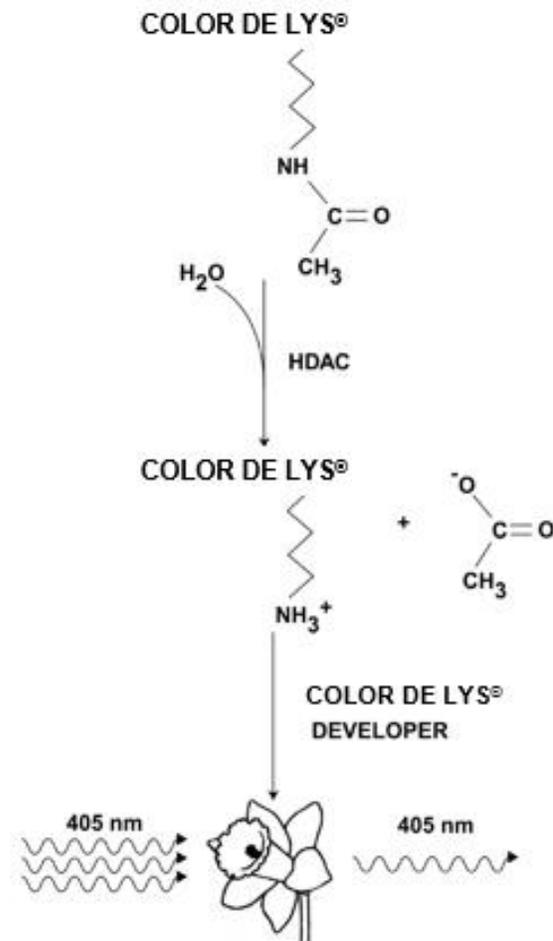


FIGURE 1. Reaction Scheme of the HDAC Colorimetric Activity Assay*. Deacetylation of the substrate sensitizes it to the developer, which causes an increase in yellow intensity and absorption at 405nm.

*Patent Pending.

DESCRIPTION

The *HDAC Colorimetric Assay/Drug Discovery Kit* is a complete assay system designed to measure histone deacetylase (HDAC) activity in cell or nuclear extracts, immunoprecipitates or purified enzymes. It comes in a convenient 96-well format, with all reagents necessary for Colorimetric HDAC activity measurements and calibration of the assay. In addition, a HeLa nuclear extract, rich in HDAC activity, is included with the kit. The extract is useful as either a positive control or as the source of HDAC activity for inhibitor/drug screening. Also included is the potent HDAC inhibitor, Trichostatin A, which may be used as model inhibitor.

The *HDAC Colorimetric Assay/Drug Discovery Kit* is based on the unique COLOR DE LYS[®] Substrate and Developer combination. The COLOR DE LYS[®] system (Colorimetric 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 COLOR DE LYS[®] Substrate, which comprises an acetylated lysine side chain, is incubated with a sample containing HDAC activity (HeLa nuclear or other extract, purified enzyme, bead-bound immunocomplex, etc.). Deacetylation of the substrate sensitizes the substrate so that, in the second step, treatment with the COLOR DE LYS[®] Developer causes an increase in yellow intensity and absorption at 405nm. There is a linear correlation between the absorption and the amount of dye released within instrument limitations.

The COLOR DE LYS[®] Substrate is efficiently deacetylated by HDAC1 and HDAC2 (see Figure 6), the major contributors to HDAC activity in HeLa nuclear extracts. SIRT1, one of the human Sir2 homologs, also deacetylates the COLOR DE LYS[®] Substrate while SIRT2 does not (Enzo Life Sciences, unpublished results). It is also a poor substrate for HDAC3 (Figure 6) and recombinant human HDAC8 (Enzo Life Sciences, unpublished results). The activities of other HDAC isotypes with the COLOR DE LYS[®] Substrate have yet to be investigated. HDAC3, HDAC8 and SIRT2 do deacetylate the COLOR DE LYS[®] Substrate, which is the basis of the *HDAC Fluorometric Assay/Drug Discovery Kit* (Cat. # BML-AK500).

MATERIALS SUPPLIED

BML-KI137-0500 Nuclear Extract from HeLa Cells (human cervical cancer cell line)

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

QUANTITY: 500µl

BML-KI138-0050 COLOR DE LYS® Substrate

FORM: 50mM in DMSO

STORAGE: -80°C

QUANTITY: 50µl

BML-KI139-0300 COLOR DE LYS® Developer Concentrate (20x)

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

STORAGE: -80°C

QUANTITY: 300µl

BML-GR309-9090 Trichostatin A (HDAC Inhibitor)

FORM: 0.2mM in DMSO

STORAGE: -80°C

QUANTITY: 100µl

BML-KI141-0030 COLOR DE LYS® Deacetylated Standard

FORM: 10mM in DMSO

STORAGE: -80°C

QUANTITY: 30µl

BML-KI143-0020 HDAC ASSAY BUFFER

(50mM Tris/Cl, pH 8.0, 137mM NaCl, 2.7mM KCl, 1mM MgCl₂)

STORAGE: -20°C

QUANTITY: 20ml

80-2404 1/2 VOLUME MICROPLATE

1 clear, 96-well

STORAGE: Ambient

STORAGE

Store all components except the microtiter plate at -80°C for the highest stability. The HeLa Nuclear Extract 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 COLOR DE LYS[®] Substrate, 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

- Microtiter-plate reader capable of measuring A_{405} to ≥ 3 -decimal accuracy
- Pipette or multi-channel pipette capable of pipetting 2-100 μl accurately
- Ice bucket to keep reagents cold until use
- Microtiter plate warmer or other temperature control device (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 pipet by mouth.
3. Interpretation of the results is the sole responsibility of the user.

PROCEDURES

Some Things To Consider When Planning Assays:

1. The assay is performed in two stages. The first stage, during which the HDAC(s) acts on the Substrate, is done in a total volume of 50 μ l. The second stage, which is initiated by the addition of 50 μ l of Developer, stops HDAC activity and produces the Colorimetric signal. See "Preparing Reagents For Assay" and Table 1.

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 microtiter plate provided can readily accommodate 150 μ l. If planning a change to the volume of the Developer, it should be noted that it is important to keep two factors constant: 1) the 1 μ M concentration of Trichostatin in the final mix; 2) 2.5 μ l/well amount of Developer Concentrate. See "Preparing Reagents For Assay", Step #5.

2. Experimental samples should be compared to a "time zero" (sample for which Developer is added immediately after mixing of the HDAC with substrate) and/or a negative control (no enzyme).
3. When 5 μ l/well of the HeLa Nuclear Extract is used as the source of HDAC activity, reaction progress curves, for a broad range of substrate concentrations (10-2500 μ M COLOR DE LYS[®] Substrate), remain linear for at least 30 min. (37°C). This will not necessarily be true if a different source of HDAC activity, a different amount of extract, or a different assay temperature is used. A time course experiment will aid in the selection of an incubation time, which yields a signal that is both sufficiently large and proportional to enzyme rate (Figure 3).

4. The apparent K_m of the HDAC activity in the HeLa Nuclear Extract for the COLOR DE LYS[®] Substrate is ~0.4mM (Figure 4). Use of a substrate concentration at or below the K_m will help avoid substrate competition effects, which could mask the effectiveness of a potential inhibitor. If a different source of HDAC activity is to be used, a rate vs. concentration experiment should be performed before selecting a substrate concentration for the screening experiments.
5. It is conceivable that some compounds being screened for inhibition of HDACs may interfere with the action of the COLOR 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 COLOR DE LYS[®] Deacetylated Standard (BML-KI141) plus the COLOR DE LYS[®] Developer. A detailed retesting procedure is described below, in the section “USES OF THE COLOR DE LYS[®] DEACETYLATED STANDARD”. In some cases, it may be possible to avoid this retesting by means of measurements taken during the color development phase of the initial HDAC assay. This is also discussed in that section.

PREPARING REAGENTS FOR ASSAY

1. Defrost all kit components and keep these, and all dilutions described below, on ice until use. All undiluted kit components are stable for several hours on ice.
2. Prepare a sufficient amount of HeLa Nuclear Extract or other HDAC source to provide for the assays to be performed (# of wells x 5 μ l). 5 μ l of undiluted Extract is needed for the standard assay (Table 1). If the undiluted HDAC source generates excessive signal, dilute it with HDAC Assay Buffer and re-assay.
3. Prepare dilution(s) of Trichostatin A and/or Test Inhibitors in Assay Buffer. Since 10 μ l will be used per well (Table 1), and since the final volume of the HDAC reaction is 50 μ l, these inhibitor dilutions will be 5x their final concentration.

4. Prepare dilution(s) of the COLOR DE LYS[®] Substrate (BML-KI138; 50mM) in Assay Buffer that will be 2x the desired final concentration(s). For inhibitor screening, substrate concentrations at or below the K_m are recommended. 25 μ l will be used per well (Table 1). Initial dilutions of the 50mM stock to 2.5mM or less in Assay Buffer yield stable solutions. Again, note that freezing/thawing of COLOR DE LYS[®] Substrate solutions in Assay Buffer may cause precipitation of the Substrate.
5. Shortly before use (<30 min.), prepare sufficient COLOR DE LYS[®] Developer for the assays to be performed (50 μ l per well). First, dilute the COLOR DE LYS[®] Developer Concentrate 20-fold (e.g. 50 μ l plus 950 μ l Assay Buffer) in cold Assay Buffer. Second, dilute the 0.2mM Trichostatin A 100-fold in the 1x Developer just prepared (e.g. 10 μ l in 1ml; final Trichostatin A concentration in the 1x Developer = 2 μ M; final concentration after addition to HDAC/Substrate reaction = 1 μ M). Addition of Trichostatin A to the Developer insures that HDAC activity stops when the Developer is added. Keep Developer on ice until use.

TABLE 1. ASSAY MIXTURE EXAMPLES⁶

Sample	Assay Buffer	HeLa Extract	Inhibitor (5x)	COLOR DE LYS [®] Substrate (2x)
Blank (No Enzyme)	25 μ l	0	0	25 μ l
Control	20 μ l	5 μ l	0	25 μ l
Trichostatin A [‡]	10 μ l	5 μ l	10 μ l [‡]	25 μ l
Test Sample ^{*†}	10 μ l	5 μ l	10 μ l ^{*†}	25 μ l

⁶ HDAC reaction mixtures, prior to addition of COLOR 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 μ M would produce final 1 μ M concentration and essentially complete HDAC inhibition; 2) As a model inhibitor “hit”, 250nM would produce final 50nM and ~50% inhibition.

^{*†} Refers to dilution of potential inhibitor in Assay Buffer, which will be 5x its final concentration.

PERFORMING THE ASSAY

1. 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.

2. Add HeLa extract or other HDAC sample to all wells except those that are to be “No Enzyme Controls.”
3. Allow diluted COLOR DE LYS[®] Substrate and the samples in the microtiter plate to equilibrate to assay temperature (e.g. 37°C).
4. Initiate HDAC reactions by adding diluted substrate (25µl) to each well and mixing thoroughly.
5. Allow HDAC reactions to proceed for desired length of time and then stop them by addition of COLOR DE LYS[®] Developer (50µl). Incubate plate at 37°C for 10-15 min. Signal is stable for at least 30 min. beyond this time.
6. Read plate in a microtiter-plate reader at 405nm.

PREPARATION OF A STANDARD CURVE (USES OF THE COLOR DE LYS[®] DEACETYLATED STANDARD)

1. Standard curves prepared with the COLOR DE LYS[®] Deacetylated Standard (BML-KI141) can be used to correlate changes in OD_{405nm} with molar amounts of deacetylation (see Figure 2). Concentration ranges of 0-250µM or 0-500µM will both produce good results. A single standard curve will suffice; there is no need to do a standard curve each time assays are done. However, it can be useful to include a single well of Deacetylated Standard (e.g. at a concentration of 50µM) as a positive control, each time an assay is performed. (See also the next section on testing for interference with the Developer.)
2. After choosing a concentration range, prepare, in Assay Buffer, a series of COLOR DE LYS[®] Deacetylated Standard dilutions that span this range. Pipet 50µl of each of these dilutions, and 50µl 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 #5, sufficient COLOR DE LYS[®] Developer for the standard wells (50µl per well).
4. Mix 50µl of the Developer with the 50µl in each standard well and incubate 5-10 min. at 37°C.
5. Read samples in a microtiter-plate reader at 405nm.
6. Plot absorbance (y-axis) versus concentration of the COLOR DE LYS[®] Deacetylated Standard (x-axis). Determine slope as OD/µM. See example in Figure 2.

Testing of Potential HDAC Inhibitors for Interference with the COLOR DE LYS[®] Developer:

1. The COLOR DE LYS[®] Developer is formulated so that, under normal circumstances, the reaction goes to completion in less than 1 min. at 37°C. That, together with the recommended 10-15 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.

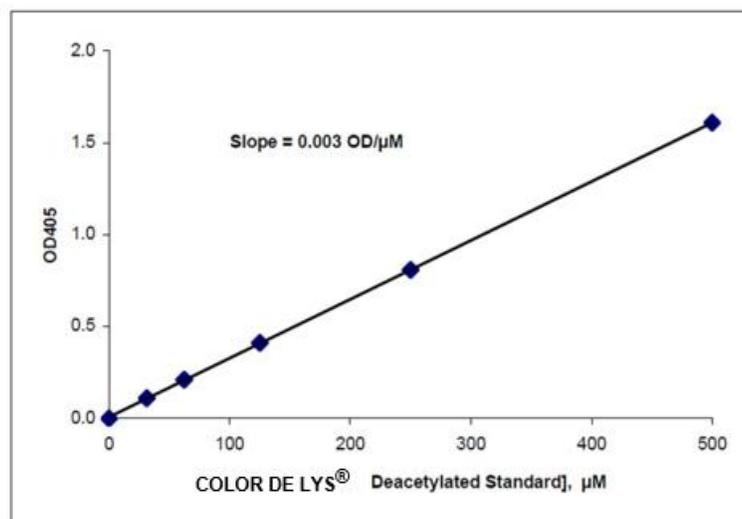


Figure 2. Deacetylated Standard Curve. 50 μl aliquots of COLOR DE LYS[®] Deacetylated Standard, in Assay Buffer at the indicated concentrations, were mixed with 50 μl Developer and incubated 10 min., 37°C. Absorbance at 405nm was then measured in the wells of the microtiter-plate.

2. It should be possible to identify many cases in which there is interference with the development reaction by taking a series of absorbance readings immediately following addition of the COLOR DE LYS[®] Developer (e.g. readings at 1 or 2 min. intervals for 30 min.). The absorbance 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 absorbance. Trichostatin A (5nM) provides a good model of this behavior. Any sample in which the approach to the final absorbance is substantially slower than in the above examples should be suspected of interference with the development reaction. For samples in which little or no color development has occurred, it may be impossible to assess the development kinetics.

3. 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 COLOR 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 40µl of the diluted Standard with 10µl inhibitor or 10µl Assay Buffer (see Table 2). Initiate development by adding 50µl of 1x Developer to each well. Follow color development by reading at 1 or 2 min. intervals for 30 min. If a test inhibitor sample reaches its final yellow color intensity 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.
4. 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 yellow color intensity 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 100µl) 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 COLOR DE LYS® DEACETYLATED STANDARD

Sample	Assay Buffer	Inhibitor (5x)	Diluted ⁶ COLOR DE LYS®	
			deAc. Standard (1.25x)	DEVELOPER (1x)
Control	10µl	0	40µl	50µl
Trichostatin A [‡]	0	10µl	40µl [‡]	50µl
Test Inhibitor [⊗]	0	10µl	40µl [⊗]	50µl

⁶ The appropriate dilution of the COLOR DE LYS® Deacetylated Standard, may be determined from the standard curve and should be the concentration producing a Colorimetric 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 10µl of Assay Buffer or inhibitor.

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

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

APPLICATION EXAMPLES

The *HDAC Colorimetric Activity Assay/Drug Discovery Kit* has been used to investigate the kinetics of COLOR DE LYS® Substrate deacetylation by HeLa nuclear extract (Figures 3 & 4). This activity is nearly totally sensitive to the HDAC inhibitor trichostatin A (Figure 5). HDAC1, immunoprecipitated from HeLa nuclear extract and bound to protein A agarose beads, deacetylates the COLOR DE LYS® Substrate (Figure 6).

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.

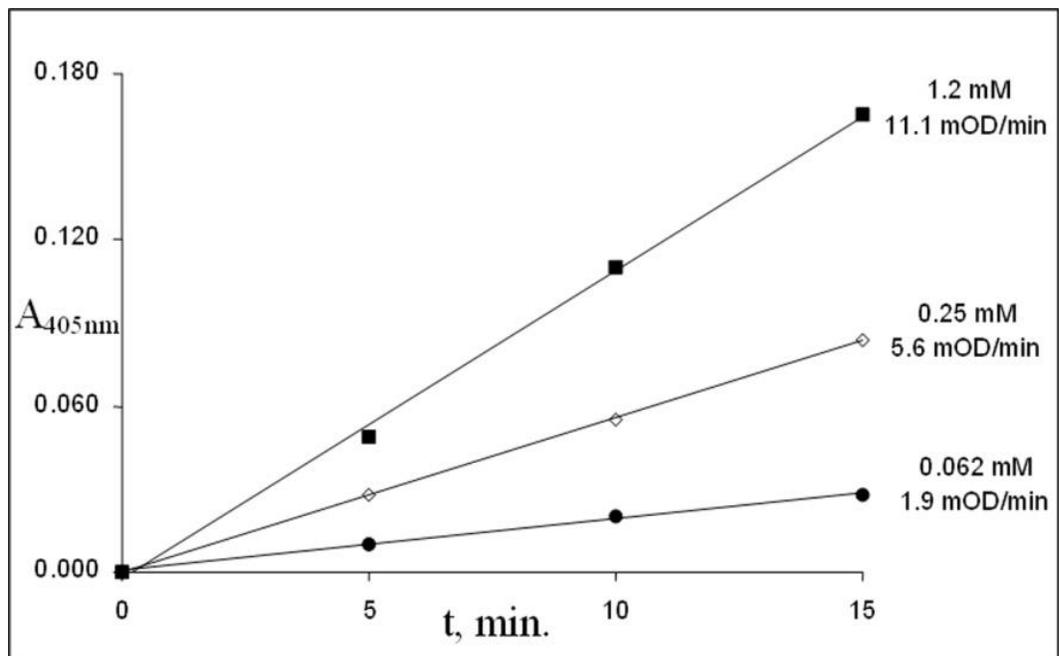


Figure 3. Time Courses of COLOR DE LYS® Substrate Deacetylation by HDAC. HeLa Nuclear extract (5 μ l /well) was incubated (37°C) with indicated concentrations of substrate. Reactions were stopped at indicated times with COLOR DE LYS® Developer and absorbance was measured at 405nm.

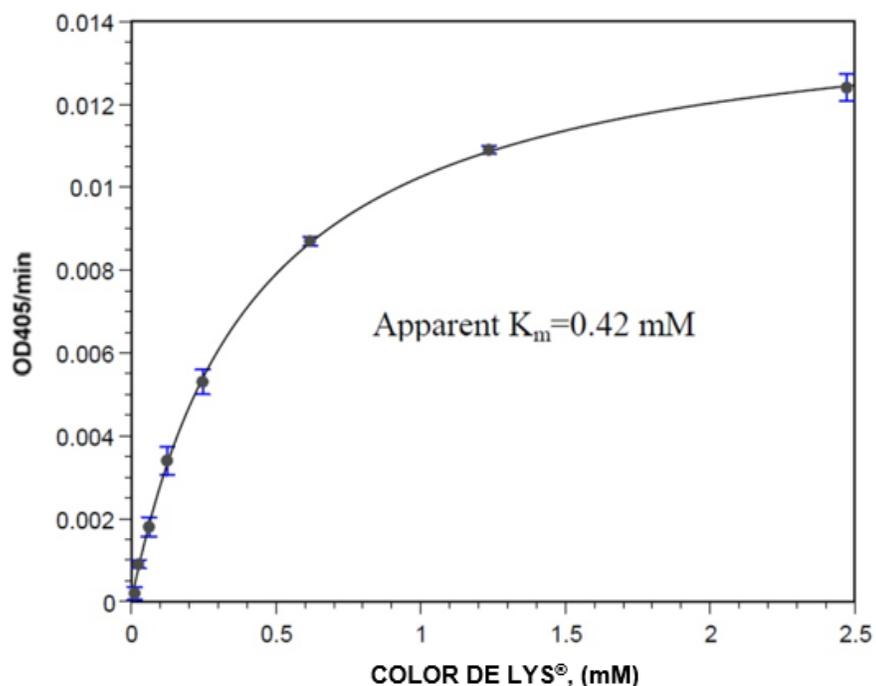


Figure 4. Kinetics of COLOR DE LYS® Substrate Deacetylation by HeLa HDAC Activity. HeLa Nuclear extract (5 μ l /well) was incubated (37°C) with indicated concentrations of substrate. Reactions were stopped after 10 min. with COLOR DE LYS® Developer and color development was monitored. Points are the 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 (Delta Graph 4.0, Deltapoint, Inc.).

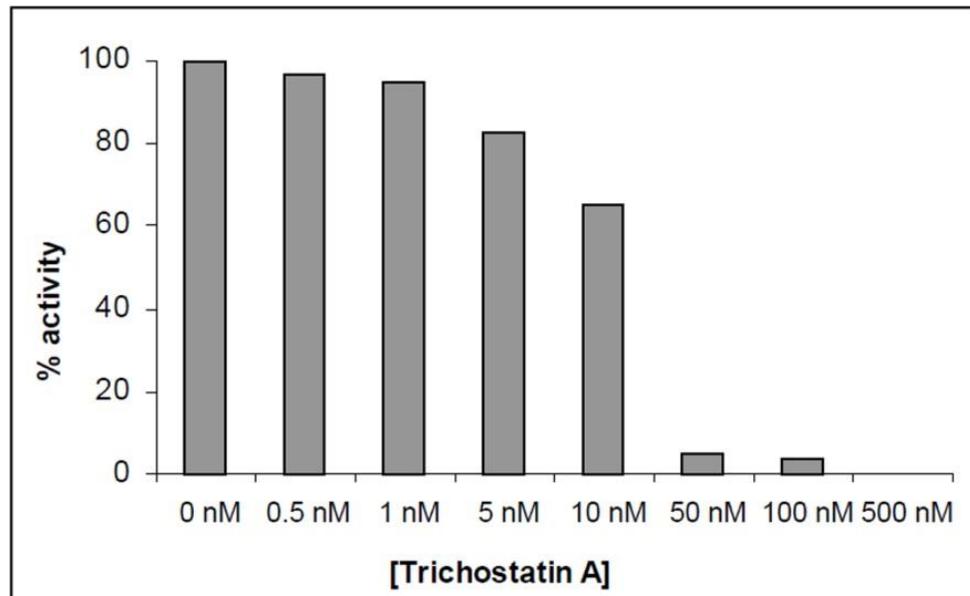


Figure 5. Trichostatin A Inhibition of COLOR DE LYS® Substrate Deacetylation by HeLa Nuclear Extract. HeLa Nuclear Extract (5µl/well) was incubated (37°C) with 0.2mM substrate and indicated concentrations of Trichostatin A. Reactions were stopped after 30 min. with COLOR DE LYS® Developer and the absorbance measured at 405nm.

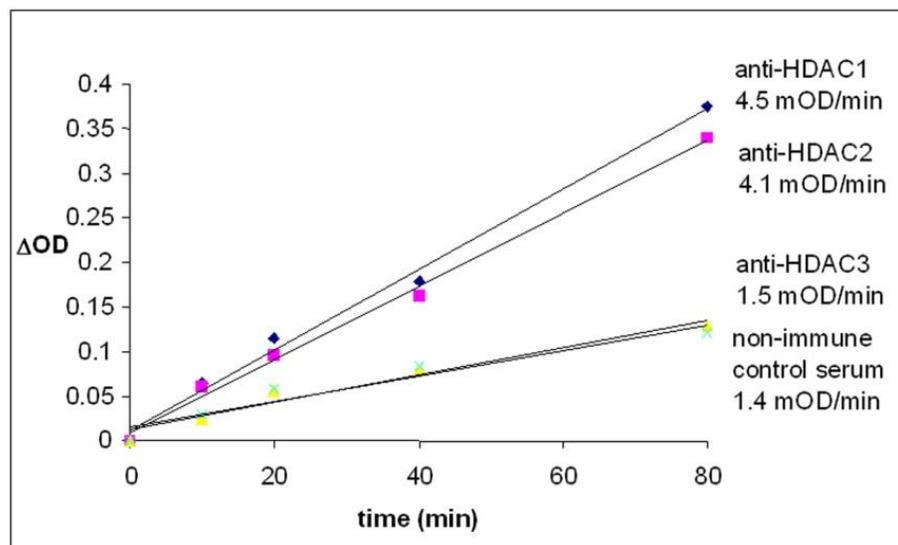


Figure 6. Immunoprecipitated HDAC1, HDAC2 Deacetylate COLOR DE LYS® Substrate. HDAC's were immunoprecipitated from HeLa Nuclear Extract (2.8mg protein in 1.6ml) using the Seize™ X Protein A Immunoprecipitation Kit (Pierce). 10µg of each anti-HDAC antibody or 10µl of control serum were used (all rabbit polyclonals; Anti-HDAC's 1-3 are Cat. Nos. BML-SA401, BML-SA402, BML-SA403). The washed beads with bound Anti-HDAC's were incubated with 150µl of 1mM COLOR DE LYS® substrate for indicated time, with rocking (37°C). Aliquots (30µl) were withdrawn, mixed with 20µl Assay buffer and 50µl COLOR DE LYS® Developer, and color development was monitored at 405nm.

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ALSO AVAILABLE SEPARATELY...

<u>PRODUCT</u>	<u>CATALOG #</u>
HeLa Nuclear Extract	BML-KI137
COLOR DE LYS® Substrate	BML-KI138
COLOR DE LYS® Developer	BML-KI139
HDAC8 (recombinant, human)	BML-SE145
Trichostatin A (HDAC Inhibitor)	BML-GR309
Suberoyl bis-hydroxamic acid (HDAC Inhibitor)	BML-GR323
Scriptaid (HDAC Inhibitor)	BML-GR326
HC Toxin (HDAC Inhibitor)	BML-GR320
Anti-HDAC1 (polyclonal Ab)	BML-SA401
Anti-HDAC2 (polyclonal Ab)	BML-SA402
Anti-HDAC3 (polyclonal Ab)	BML-SA403



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