Product Manual

HDAC Fluorometric Assay/Drug Discovery Kit

Catalog #: BML-AK500

A FLUOR DE LYS® Fluorescent Assay System

Designed to measure HDAC activity in cell or nuclear extracts, immunoprecipitates or purified enzymes.

For the latest product information, including support documentation, visit us online:
www.enzolifesciences.com

Technical Support (US): 800-942-0430
Technical Support (EU): +41 61 926 8989

USE FOR RESEARCH PURPOSES ONLY

Unless otherwise specified expressly on the packaging, all products sold hereunder are intended for and may be used for research use only. Not for use in diagnostic procedures. Purchase does not include any right or license to use, develop or otherwise exploit these products commercially. Any commercial use, development or exploitation of these products or development using these products without the express written authorization of Enzo Life Sciences, Inc. is strictly prohibited. Buyer assumes all risk and liability for the use and/or results obtained by the use of the products covered by this invoice whether used singularly or in combination with other products.

LIMITED WARRANTY; DISCLAIMER OF WARRANTIES

These products are offered under a limited warranty. The products are guaranteed to meet all appropriate specifications described in the package insert at the time of shipment. Enzo Life Sciences' sole obligation is to replace the product to the extent of the purchasing price. All claims must be made to Enzo Life Sciences, Inc., within five (5) days of receipt of order. THIS WARRANTY IS EXPRESSLY IN LIEU OF ANY OTHER WARRANTIES OR LIABILITIES, EXPRESS OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, AND NON- INFRINGEMENT OF THE PATENT OR OTHER INTELLECTUAL PROPERTY RIGHTS OF OTHERS, AND ALL SUCH WARRANTIES (AND ANY OTHER WARRANTIES IMPLIED BY LAW) ARE EXPRESSLY DISCLAIMED.

TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending. Enzo is a trademark of Enzo Life Sciences, Inc.

TABLE OF CONTENTS

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

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 transcription[1]. The best studied of these modifications, acetylation of the ε-amino groups of specific histone lysine residues, are catalyzed by histone acetyltransferases (HATs). Histone deacetylases (HDACs) are responsible for hydrolytic removal of these acetyl groups[2-4].

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 activity[1-4]. Conversely, HDACs are found to associate with transcriptional repression complexes such as NuRD or those including Sin3[1-7].

Thus far, eleven human HDACs have been identified, all trichostatin A-sensitive and all homologs of either RPD3 (Class I HDACs) or HDA1 (Class II HDACs), yeast histone deacetylases[8-16,20]. Interestingly, Sir2, the yeast mother cell longevity factor, and its mouse homolog, mSir2α, recently have been shown to be trichostatin A-insensitive, NAD^+-dependent histone deacetylases[17]. Human, archaeal and eubacterial Sir2 homologs also display NAD^+-dependent histone deacetylase activity[21]. These enzymes apparently function via a unique mechanism, which consumes NAD^+ and couples lysine deacetylation to formation of nicotinamide and *O*-acetyl-ADP-ribose[22-24]. The Sir2 family (sirtuins) thus constitutes a third class of HDACs, but its members have not been included in the HDAC (Class I/Class II) numbering scheme.

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 [^{3}H]acetyl-histone or [^{3}H]acetyl-histone peptides as substrates involves an acid/ethyl acetate extraction step prior to scintillation counting[8,18,19,25]. Unlabeled, acetylated histone peptides have been used as substrates, but reactions then require resolution by HPLC[17]. Enzo Life Science's *HDAC Fluorescent Activity 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). It has been used successfully with preparations of all class I HDACs-HDAC1, HDAC2, HDAC3 and HDAC8 (Figure 6, Figure 7, and References 26 and 27) - with

class II HDACs 6, 9 and 10[20,27] and with yeast Sir2 and its human homolog, SIRT1[28] (see also Figure 8).

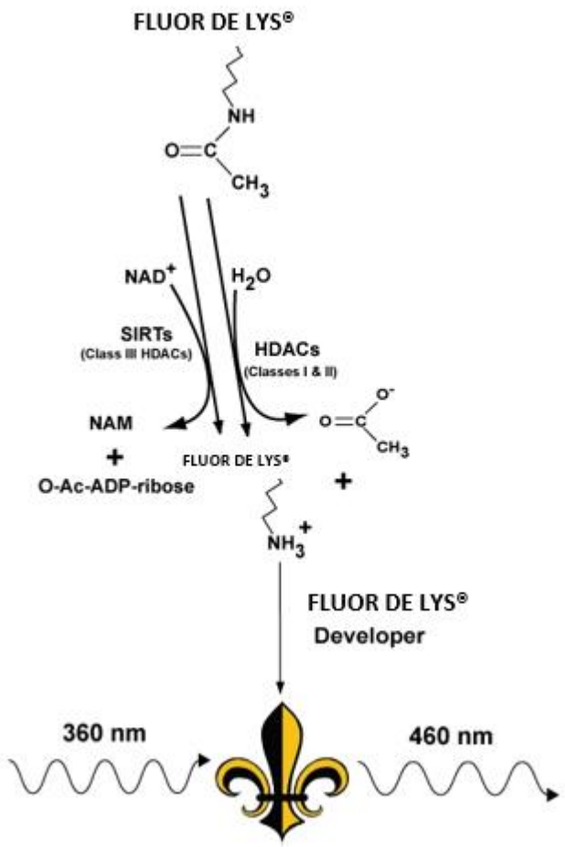


Figure 1. **Reaction Scheme of the *HDAC Fluorescent Activity Assay*.*** Deacetylation of the substrate sensitizes it to the developer, which then generates a fluorophore (symbol). The fluorophore is excited with 360nm light and the emitted light (460nm) is detected on a fluorometric plate reader.

INTRODUCTION

The *HDAC Fluorescent Activity 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 fluorescent 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 Fluorescent Activity Assay* is based on the unique FLUOR DE LYS® Substrate and Developer 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® 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 FLUOR DE LYS® Developer produces a fluorophore.

MATERIALS SUPPLIED

BML-KI140-0100 Nuclear Extract from HeLa Cells (human cervical cancer cell line)
FORM: 0.1 M KCl, 20 mM HEPES/NaOH, pH 7.9, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 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: 100µl

BML-KI104-0050 FLUOR DE LYS® Substrate
FORM: 50 mM in DMSO
STORAGE: -80°C
QUANTITY: 50µl

BML-KI105-0300 FLUOR DE LYS® Developer Concentrate (20x)
FORM: 20x Stock Solution; Dilute in Assay Buffer before use.
STORAGE: -80°C
QUANTITY: 300µl

BML-GR309-9090 Trichostatin A (HDAC Inhibitor)
FORM: 0.2 mM in DMSO
STORAGE: -80°C
QUANTITY: 100µl

BML-KI142-0030 FLUOR DE LYS® Deacetylated Standard
FORM: 10 mM in DMSO
STORAGE: -80°C
QUANTITY: 30µl

BML-KI143-0020 HDAC ASSAY BUFFER
FORM: (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 1/2 VOLUME MICROPLATES
STORAGE: Ambient
QUANTITY: 1 clear and 1 white, 96-well

ADDITIONAL MATERIALS NEEDED

- Microplate reading fluorometer capable of excitation at a wavelength in the range 350-380nm and detection of emitted light in the range 440-460nm
- Pipette or multi-channel pipette capable of pipetting 2-100µl accurately
- Ice bucket to keep reagents cold until use
- Microtiter plate warmer or other temperature control device (optional)

PROCEDURES

Notes On Storage: *Store all components (except the microtiter plate) at -80°C for the highest stability. The HeLa Nuclear Extract, BML-KI140, 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.*

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 50µl. The second stage, which is initiated by the addition of 50µl of Developer, stops HDAC activity and produces the fluorescent signal. See "Preparing Reagents For Assay" 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 (see Figure 7). As long as the fluorometer 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 150µl. 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 1µM concentration of Trichostatin in the final mix; 2) 2.5µl/well amount of Developer Concentrate (BML-KI105). 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 0.5µl/well of the HeLa Nuclear Extract is used as the source of HDAC activity, reaction progress curves, for a broad range of substrate concentrations (10-2000µM FLUOR DE LYS® Substrate), remain linear for at least 30 min. (25°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 (BML-KI140) for the FLUOR DE LYS® Substrate is ~50µM (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 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-KI142) plus the FLUOR DE LYS® Developer. A detailed retesting procedure is described 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.

6. Note that sirtuins (Sir2 and Sir2-like NAD^+-dependent HDACs) are insensitive to Trichostatin A. Therefore, Developer prepared as described below with added Trichostatin A, will not completely block further deacetylation by these enzymes. If the kit is to be used to assay a sirtuin, we recommend either reading the fluorescence at a consistent time shortly after addition of Developer (e.g. 2-5 min.) or adding a sirtuin inhibitor[24,25] to the Developer.

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 (BML-KI140) or other HDAC source diluted in Assay Buffer (BML-KI143) to provide for the assays to be performed (# of wells x 15µl). A 30-fold dilution of the HeLa Extract means that 15µl contains 0.5µl of the undiluted Extract, an appropriate amount to use per well (Table 1).
3. Prepare dilution(s) of Trichostatin A and/or Test Inhibitors in Assay Buffer (BML-KI143). Since 10µl will be used per well (Table 1), and since the final volume of the HDAC reaction is 50µl, these inhibitor dilutions will be 5x their final concentration.
4. Prepare dilution(s) of the FLUOR DE LYS® Substrate (BML-KI104; 50 mM) in Assay Buffer (BML-KI143) that will be 2x the desired final concentration(s). For inhibitor screening, substrate concentrations at or below the K_m are recommended; 25µl will be used per well (Table 1). Initial dilutions of 25-fold or greater in Assay Buffer (2.0 mM or less) yield stable solutions (see NOTE on freezing and thawing below). Rapid mixing and dilution into room temperature buffer will help prevent precipitation at high substrate concentration. NOTE: Freezing/thawing of FLUOR DE LYS® Substrate solutions in Assay Buffer may cause precipitation of the Substrate. Dilute only the amount necessary for one day's experiment.
5. Shortly before use (<30 min.), prepare sufficient FLUOR DE LYS® Developer for the assays to be performed (50µl per well). First, dilute the FLUOR DE LYS® Developer Concentrate 20-fold (e.g. 50µl plus 950µl Assay Buffer) in cold Assay Buffer (BML-KI143). Second, dilute the 0.2 mM Trichostatin A (BML-GR309-9090) 100-fold in the 1x Developer just prepared (e.g. 10µl in 1 ml; final Trichostatin A concentration in the 1x Developer = 2µM; final concentration after addition to HDAC/Substrate reaction = 1µM). Addition of Trichostatin A to the Developer insures that HDAC activity stops when the Developer is added. Keep Developer on ice until use.

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 diluted HeLa extract or other HDAC sample to all wells except those that are to be “No Enzyme Controls.”
3. Allow diluted FLUOR DE LYS® Substrate and the samples in the microtiter plate to equilibrate to assay temperature (e.g. 25 or 37°C).
4. Initiate HDAC reactions by adding diluted substrate (25µl) to each well and mixing thoroughly.

TABLE 1. ASSAY MIXTURE EXAMPLES[6]

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, 5µM would produce final 1µM concentration and essentially complete HDAC inhibition; 2) As a model inhibitor “hit”, 25nm would produce final 5nm and ~50% inhibition.

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

5. Allow HDAC reactions to proceed for desired length of time and then stop them by addition of FLUOR DE LYS® Developer (50µl). Incubate plate at room temperature (25°C) for 10-15 min. Signal is stable for at least 30 min. beyond this time.
6. Read samples in a microtiter-plate reading fluorimeter capable of excitation at a wavelength in the range 350-380nm and detection of emitted light in the range 440-460nm.

PREPARATION OF A STANDARD CURVE (USES OF THE FLUOR DE LYS® DEACETYLATED 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 Assay Buffer (1 to 5µM). The fluorescence signal should then be determined, as described below, after mixing 50µl of the diluted standard with 50µl of Developer. The estimate of AFU(arbitrary fluorescence units)/µM obtained with this measurement, together with the observed range of values obtained in the enzyme assays can then be used to plan an appropriate series of dilutions for a standard curve. Provided the same wavelength and gain settings are used each time, there should be no need to prepare a standard curve more than once.
2. After ascertaining an appropriate concentration range, prepare, in Assay Buffer, a series of FLUOR DE LYS® 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. (p.10), sufficient FLUOR 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 room temperature (25°C).
5. Read samples in a microtiter-plate reading fluorimeter capable of excitation at a wavelength in the range 350-380nm and detection of emitted light in the range 440-460nm.
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 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 1 min. at 25°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.

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 5µM in the 50µl 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/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 HDAC reaction and that of the Deacetylated Standard 'spike'.

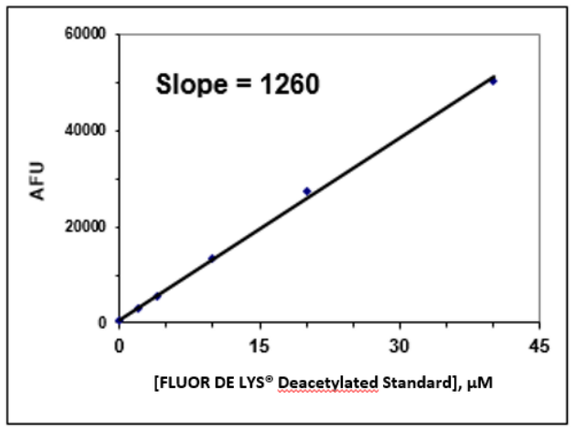


Figure 2. Fluorescence Standard Curve. 50µl aliquots of FLUOR DE LYS® Deacetylated Standard, in Assay Buffer at the indicated concentrations, were mixed with 50µl Developer and incubated 10 min., 25°C. Fluorescence was then measured in the wells of the clear microtiter-plate with a CytoFluor™ II fluorescence plate reader (PerSeptive Biosystems, Ex. 360nm, Em. 460nm, 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 (5nm) 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 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 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 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 FLUOR DE LYS® DEACETYLATED STANDARD

Sample	Assay Buffer	Inhibitor (5x)	Diluted [6] FLUOR 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 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 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 Fluorescent Activity Assay/Drug Discovery Kit* has been used to investigate the kinetics of FLUOR DE LYS® Substrate deacetylation by HeLa nuclear extract (Figure 3 and Figure 4). This activity is nearly totally sensitive to the HDAC inhibitor Trichostatin A (Figure 5). HDAC1, HDAC2 and HDAC3, immunoprecipitated from HeLa nuclear extract and bound to protein A agarose beads, all deacetylate the FLUOR DE LYS® Substrate (Figure 6), as do recombinant human HDAC8 (Figure 7) and recombinant human Sirtuin 1 (Figure 8). The FLUOR DE LYS® Substrate is cell-permeable. Its deacetylation and the intracellular accumulation of its deacetylated form are trichostatin-sensitive (Figure 9 and Figure 10).

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.

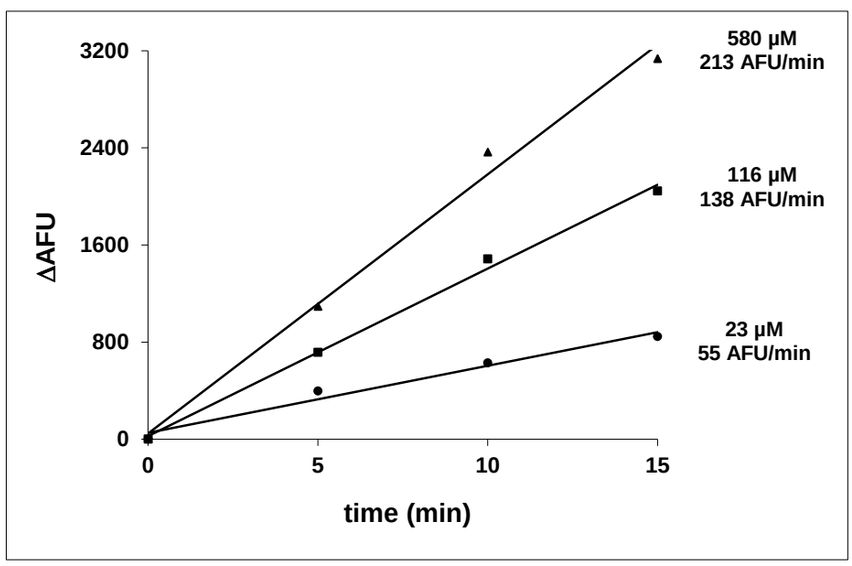


Figure 3. Time Courses of FLUOR DE LYS® Substrate Deacetylation by HDAC. HeLa Nuclear extract (0.5µl/well) was incubated (25°C) with indicated concentrations of substrate. Reactions were stopped at indicated times with FLUOR DE LYS® Developer and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360nm, Em. 460nm, gain=85).

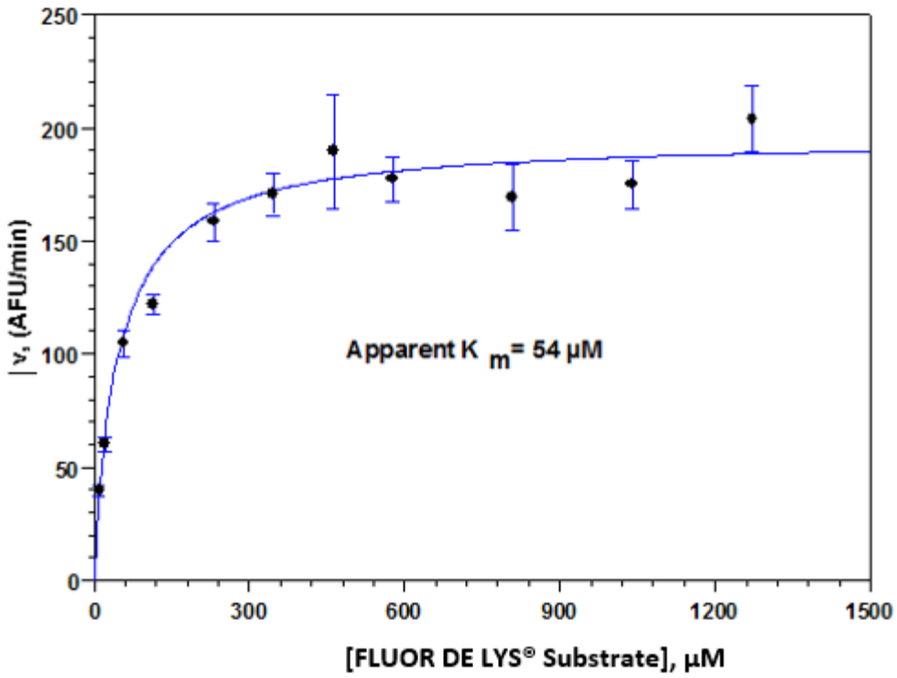


Figure 4. Kinetics of FLUOR DE LYS® Substrate Deacetylation by HeLa HDAC Activity. HeLa Nuclear extract (0.5µl /well) was incubated (25°C) with indicated concentrations of substrate. Reactions were stopped after 10 min. with FLUOR DE LYS® Developer and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360nm, Em. 460nm, gain=85). 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-Menten equation (Delta Graph 4.0, Deltapoint, Inc.).

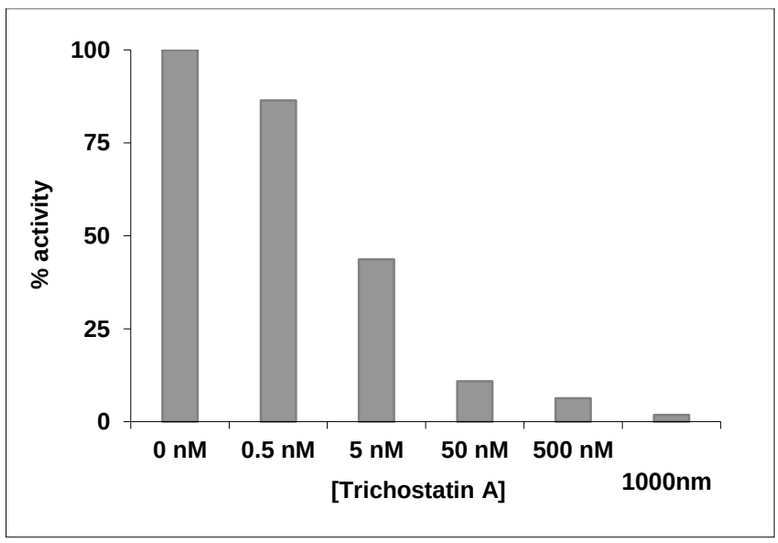


Figure 5. Trichostatin A Inhibition of FLUOR DE LYS® Substrate Deacetylation by HeLa Nuclear Extract. HeLa Nuclear Extract (0.5µl/well) were incubated (25°C) with 116µM substrate and indicated concentrations of Trichostatin A. Reactions were stopped after 10 min. with FLUOR DE LYS® Developer and fluorescence measured (CytoFluor™II, PerSeptive Biosystems, Ex. 360nm, Em. 460nm, gain=85).

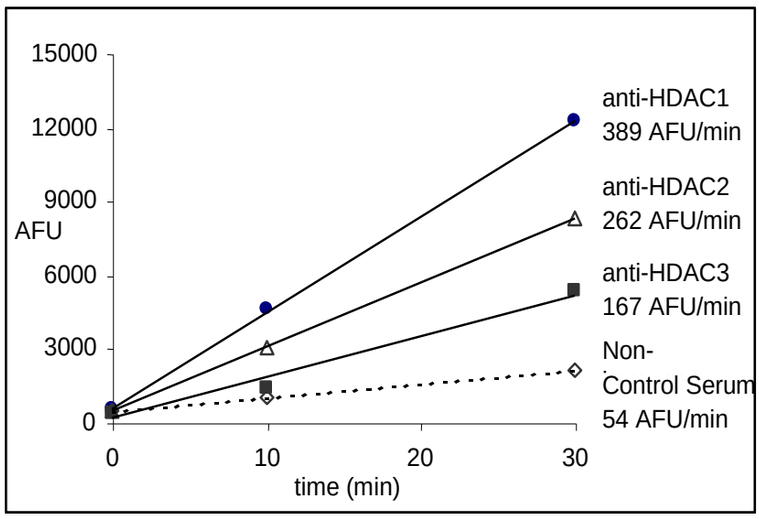


Figure 6. Immunoprecipitated HDAC1, HDAC2 and HDAC3 Deacetylate FLUOR DE LYS® Substrate. HDAC's were immunoprecipitated from HeLa Nuclear Extract (2.8 mg protein in 1.6 ml) using the Seize™ X Protein A Immunoprecipitation Kit (Pierce). Tenµg of each anti-HDAC antibody or 10µl of control serum were used (all rabbit polyclonals; Anti-HDAC's 1-3 are Cat. Nos. BML-SA401, BML-SA402, BML-SA403). The washed beads with bound HDAC/Anti-HDAC complexes were incubated with 150µl of 100µM FLUOR DE LYS® Substrate for indicated time, with rocking (25°C). Aliquots (30µl) were withdrawn, mixed with 20µl Assay buffer and 50µl FLUOR DE LYS® Developer, and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360nm, Em. 460nm, gain=85).

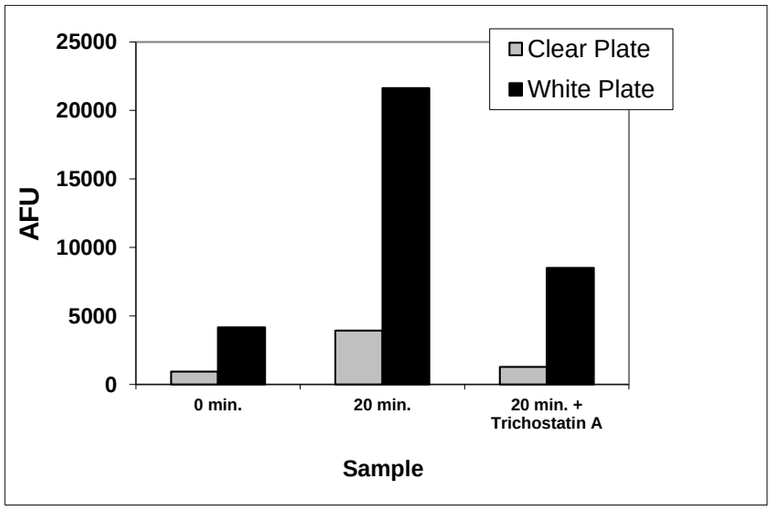


Figure 7. Recombinant Human HDAC8 Deacetylates FLUOR DE LYS® Substrate. Recombinant Human HDAC8 (Cat. # BML-SE145; 5 U/well) was incubated (37°C) with 250µM substrate for 0 or 20 min., with or without 5µM Trichostatin. Reactions were stopped with FLUOR DE LYS® Developer and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360nm, Em. 460nm, gain=85). Each bar represents the mean of two assays, performed and read in either a clear microplate or a white microplate.

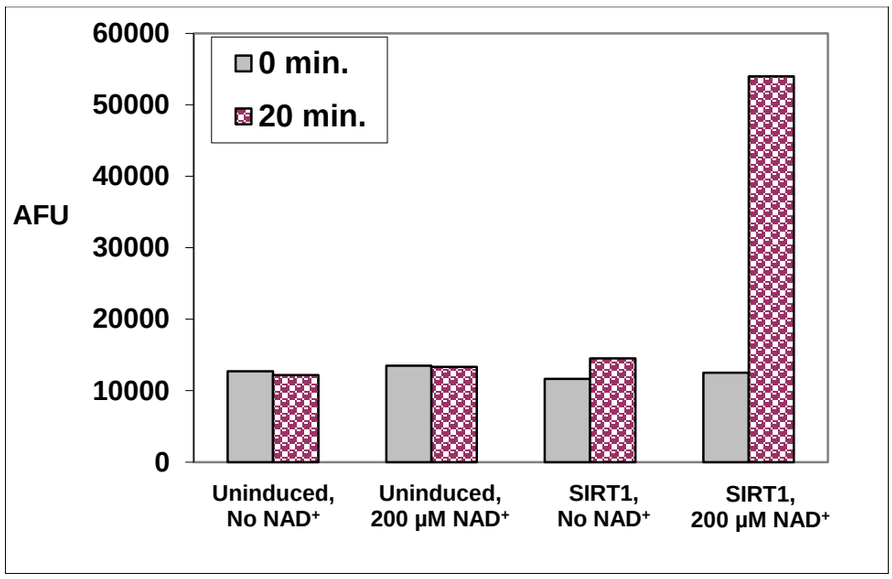


Figure 8. Human Sir2 Homolog, SIRT1 Performs NAD$^+$-Dependent Deacetylation of FLUOR DE LYS® Substrate. Soluble extracts were prepared from an *E. coli* strain either induced or not to express recombinant human SIRT1. Aliquots (20µl) of the extracts were mixed in a total volume of 50µl with 250µM FLUOR DE LYS® substrate, with or without 200µM added NAD$^+$. Parallel samples were either mixed immediately with 50µl of Developer or after a 20 min. incubation at 37°C. Fluorescence was read two min. after Developer addition, in a white microplate (SpectraFluor Plus, Tecan, Ex. 360nm, Em. 465nm, gain=52).

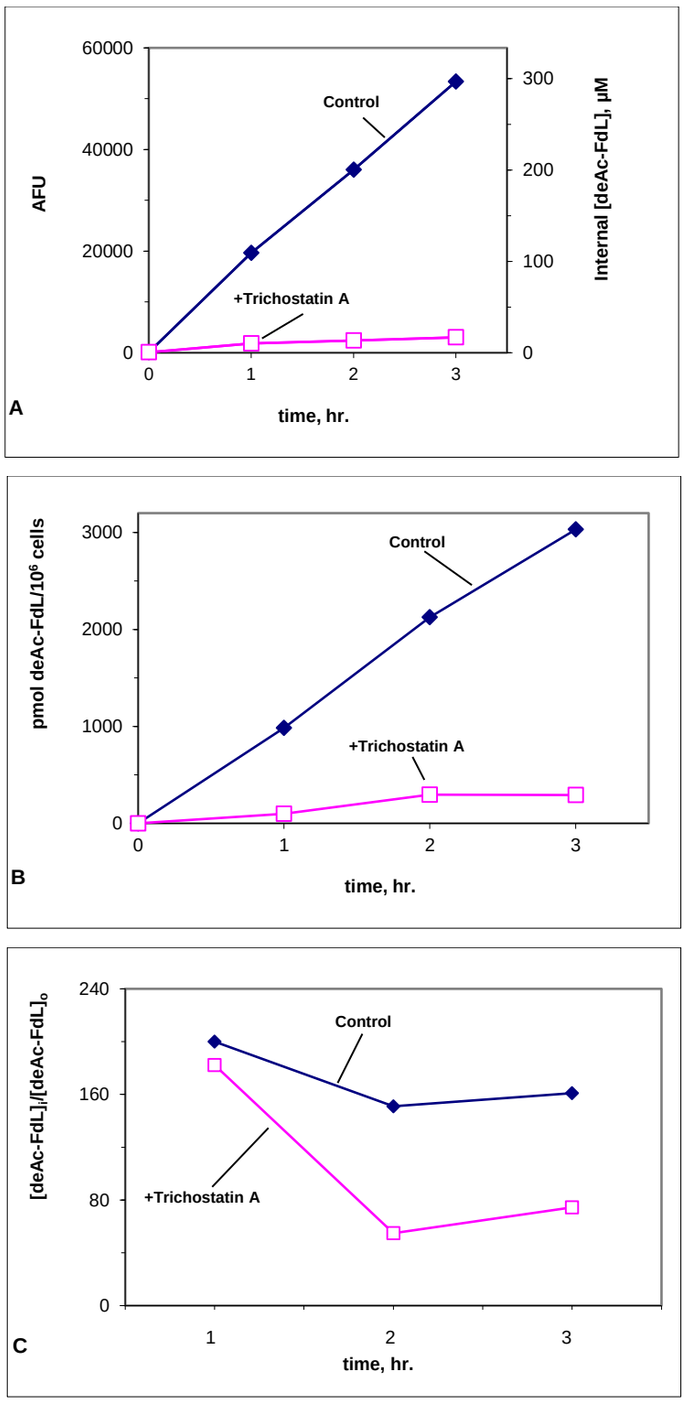
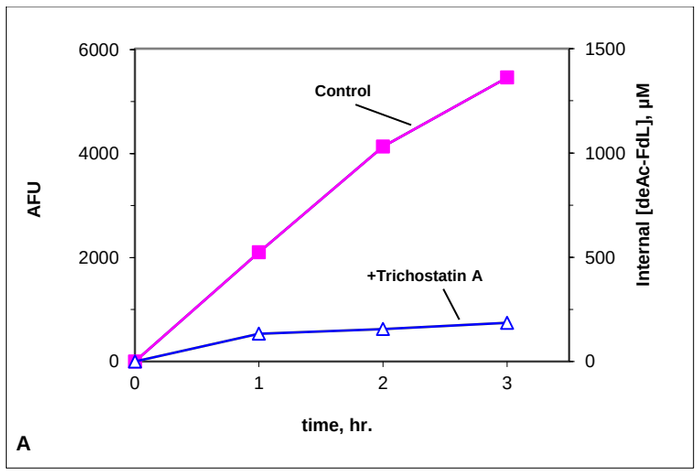
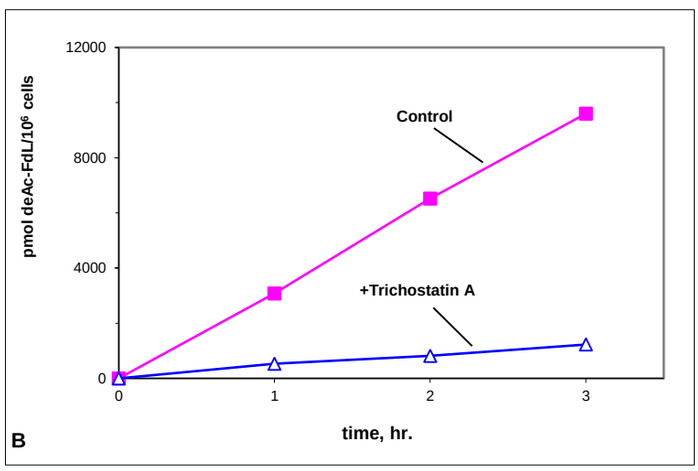


Figure 9. FLUOR DE LYS® Substrate is Cell-Permeable and is Deacetylated by Cellular HDACs. Deacetylated Substrate Accumulates Inside Cells. Jurkat cells (10^6/ml) were incubated with 200µM FLUOR DE LYS® Substrate, +/- 1µM Trichostatin A, in growth medium (RPMI 1640/10% FCS), for the indicated times and cells and medium separated by centrifugation. Cell pellets were extracted with 200µl HDAC assay buffer plus 0.5% NP-40 and 1µM trichostatin A. 50µl samples of media or cell extracts were mixed with 50µl FLUOR DE LYS® Developer, in a white plate, and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360nm, Em. 460nm, 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 x 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.





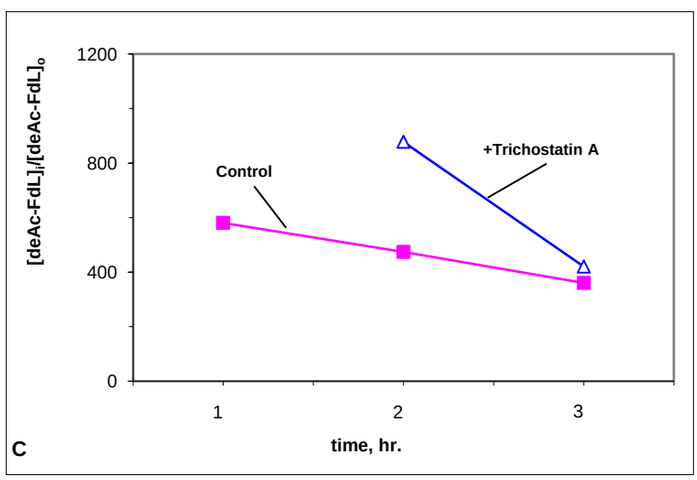


Figure 10. Adherent Cells Can Be Grown and Assayed for HDAC Activity in the Same Wells. HeLa cells were seeded at 6 x 10^4 per well (60% confluence; 96-well plate (Costar 3595); DMEM/10% FCS). When confluent, medium was replaced with 110µl of 200µM FLUOR DE LYS® Substrate in DMEM/10% FCS, +/- 1µM Trichostatin A, for indicated times. Medium was moved to separate wells for assay (100µl plus 100µl Developer) and cells rinsed 1x with PBS. FLUOR DE LYS® Developer (100µl) was added to the cells and the plate incubated 5 min., 37°C. HDAC assay buffer plus 1% NP-40/1µM Trichostatin A (100µl) was added to cell wells. After an additional 10 min. incubation, cell and medium fluorescence was measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 360nm, Em. 460nm, 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 x 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.

REFERENCES

1. B.D. Strahl and C.D. Allis *Nature.* 2000 **403** 41
2. M. Grunstein *Nature.* 1997 **389** 349
3. H. H. Ng and A. Bird *Trends Biochem. Sci.* 2000 **25** 121
4. W. L. Cheung *et al. Curr. Opin. Cell Biol.* 2000 **12** 326
5. D. Kadosh and K. Struhl *Mol. Cell. Biol.* 1998 **18** 5121
6. S.E.C. Rundlett *et al. Nature* 1998 **392** 831
7. Y. Zhang *et al. Mol. Cell* 1998 **1** 1021
8. J. Taunton *et al. Science* 1996 **272** 408
9. W. M. Yang *et al. Proc. Natl. Acad. Sci. USA* 1996 **93** 12845
10. W. M. Yang *et al. J. Biol. Chem.* 1997 **272** 28001
11. C.M. Grozinger *et al. Proc. Natl. Acad. Sci. USA* 1999 **96** 4868
12. W. Fischle *et al. J. Biol. Chem.* 1999 **274** 11713
13. A. Verdel and S. Khochbin *J. Biol. Chem.* 1999 **274** 24440
14. A.H. Wang *et al. Mol. Cell. Biol.* 1999 **19** 7816
15. H.-Y. Kao *et al. Genes Dev.* 2000 **14** 55
16. E. Hu *et al. J. Biol. Chem.* 2000 **275** 15254
17. S. Imai *et al. Nature* 2000 **403** 795
18. A. Inoue and D. Fujimoto *Biochem. Biophys. Res. Commun.* 1969 **36** 146
19. P.A. Wade *et al. Methods Enzymol.* 1999 **304** 715
20. X. Zhou *et al. Proc. Natl. Acad. Sci. USA* 2001 **98** 10572
21. J.S. Smith *et al. Proc. Natl. Acad. Sci. USA* 2000 **97** 6658
22. K.G. Tanner *et al. Proc. Natl. Acad. Sci. USA* 2000 **97** 14178
23. J.C. Tanny and D. Moazed *Proc. Natl. Acad. Sci. USA* 2000 **98** 415
24. J. Landry *et al. Biochem. Biophys. Res. Commun.* 2000 **278** 685
25. C.M. Grozinger *et al. J. Biol. Chem.* 2001 **276** 38837
26. G.V. Kapustin *et al. Org. Lett.* 2003 **5** 3053
27. N. Gurvich *et al. Cancer Res.* 2004 **64** 1079
28. K.J. Bitterman *et al. J. Biol. Chem.* 2002 **277** 45099

NOTES

NOTES

GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone: 631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE/ASIA
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
www.enzolifesciences.com