Enabling Discovery in Life Science®

HDAC2 Fluorimetric Drug Discovery Kit

A complete system for screening and characterizing modulators of HDAC2 activity.

Instruction Manual
BML-AK512

For research use only

✦ *HDAC2 Fluorimetric Drug Discovery Kit** ✦
Fluor de Lys®-Green HDAC Assay - *BML-AK512*

BACKGROUND

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

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

HDAC2, like the highly homologous HDAC1, is a class I HDAC first identified as a human homolog of the yeast histone deacetylase Rpd3[9,19]. HDAC2 found, along with HDAC1, in the Sin3[20,21], NuRD[22] and CoREST[23] complexes, also can act independently to deacetylate non-histone proteins such as transcription factors[24,25].

In contrast to class II HDACs, which repress cardiac hypertrophy, HDAC2 activity is required for the cardiac hypertrophic response[26]. This would suggest inhibition of HDAC2 as a possible therapeutic approach for heart disease[26]. An opposite approach, i.e. increased HDAC2 activity or expression, might be indicated for chronic obstructive pulmonary disease (COPD), in which an HDAC2 deficit is implicated in insensitivity to glucocorticoids[25]. The ability of the natural products theophylline and curcumin to increase HDAC2 expression is being investigated as an approach to this problem (see review[27]). High HDAC2 expression is indicative of a poor prognosis in a number of cancers[28-30] and, along with other HDACs, HDAC2 is a potential target for anti-cancer drug discovery.

Histone deacetylase inhibitors have shown promise as anti-tumor agents and naturally this has for some time stimulated interest in the screening of compounds for HDAC inhibition. Unfortunately, the classic techniques for HDAC assay were cumbersome. Use of [^{3}H]acetyl-histone or [^{3}H]acetyl-histone peptides as substrates involved an acid/ethyl acetate extraction step prior to scintillation counting[8,18,32,33]. Unlabeled, acetylated histone peptides have been used as substrates, but reactions then required resolution by HPLC[17]. The original *Fluor de Lys®* HDAC assay addressed these problems by providing an assay that can be carried out in two simple mixing steps, all on the same 96-well plate. The *Fluor de Lys®-Green* assay has those same advantages, but also, due to its higher wavelength excitation and emission, avoids interference by quenching or fluorescence from compounds absorbing and/or emitting in the near UV and blue.

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. X. Zhou *et al. Proc. Natl. Acad. Sci. USA* 2001 **98** 10572
18. P.L. Jones and Y.B. Shi *Curr. Top. Microbiol. Immunol.* 2003 **274** 237
19. A.J. de Ruijter *et al. Biochem. J.* 2003 **370** 737
20. C.D. Laherty *et al. Cell* 1997 **89** 349
21. Y. Zhang *et al. Cell* 1997 **89** 357
22. Y. Zhang *et al. Genes Dev* 1999 **13** 1924
23. A. You *et al. Proc Natl Acad Sci U S A* 2001 **98** 1454
24. Y.L. Yao *et al. Mol Cell Biol* 2001 **21** 5979
25. K. Ito *et al. J Exp Med* 2006 **203** 7
26. C.M. Trivedi *et al. Nat Med* 2007 **13** 324
27. P.J. Barnes *Annu. Rev. Physiol.* 2009 **71** 451
28. A.V. Bieliauskas and M.K. Pflum *Chem. Soc. Rev.* 2008 **37** 1402
29. W. Weichert *Cancer Lett.* 2009 **280** 168
30. H.H. Chang *et al Oral Oncol.*. 2009 **45** 610
31. O.H. Krämer *Trends Pharmacol. Sci.* 2009 **30** 647
32. P.A. Wade *et al. Methods Enzymol.* 1999 **304** 715
33. C.M. Grozinger *et al. J. Biol. Chem.* 2001 **276** 38837

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

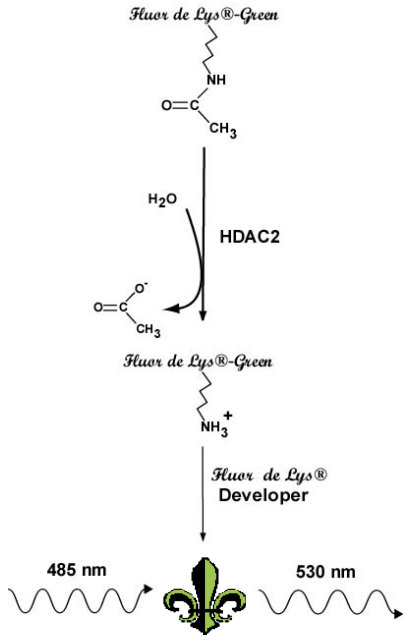

Figure 1. Reaction Scheme of the *Fluor de Lys®-Green HDAC2 Activity Assay.** Deacetylation of the substrate sensitizes it to the developer, which then generates a fluorophore (symbol). The fluorophor is excited with 485 nm light (470-500 nm) and the emitted light (~530 nm) is detected on a fluorometric plate reader.

DESCRIPTION

The *HDAC2 Fluorimetric Drug Discovery Kit* is a complete system with which to screen for and characterize modulators of HDAC2 activity. Recombinant human HDAC2 is provided with the kit as are all the reagents necessary for fluorescent HDAC2 activity measurements and calibration of the assay. Both white and black 96-well microplates are also included.

The ***Fluor de Lys®-Green*** HDAC2 assay is based on the ***Fluor de Lys®-Green*** Substrate and ***Fluor de Lys®*** Developer combination. The assay procedure has two steps (Figs. 1 & 2). First, the ***Fluor de Lys®-Green*** Substrate, which comprises an acetylated lysine side chain, is incubated with HDAC2. Deacetylation of the substrate sensitizes the substrate so that, in the second step, treatment with the ***Fluor de Lys®*** Developer produces a fluorophore. ***Fluor de Lys®-Green*** is an especially sensitive substrate for HDAC2. Activity can readily be measured with enzyme amounts in the range of 1-10 ng/well (0.36 – 3.6 nM in 50 µl), thus enabling IC_{50} determinations for high affinity inhibitors (see Fig. 7). The assay is highly suitable for high-throughput screening, with a Z'-factor of 0.74 (Fig. 6).

COMPONENTS OF BML-AK512

BML-KI575-0030 HDAC2 (human, recombinant)
FORM: 30 ng/µl in 50 mM Tris, pH 8.0, 138 mM NaCl, 10% glycerol, 1 mg/ml BSA
STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 67 µl

BML-KI572-0050 ***Fluor de Lys®-Green*** Substrate
FORM: 50 mM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 50 µl

BML-KI105-0300 ***Fluor de Lys®*** Developer Concentrate (20x)
FORM: 20x Stock Solution; Dilute in Assay Buffer before use.
STORAGE: -70°C
QUANTITY: 300 µl

BML-GR309-9090 Trichostatin A (HDAC Inhibitor)
FORM: 0.2 mM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 100 µl

BML-KI605-0030 ***Fluor de Lys®-Green*** Standard
FORM: 1 mM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 30 µl

BML-KI422-0020 HDAC ASSAY BUFFER II
(50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/ml BSA)
STORAGE: -70°C
QUANTITY: 20 ml

80-2408 ½-VOLUME NBS MICROPLATES
1 black and 1 white, 96-well, non-binding
STORAGE: Room temperature

OTHER MATERIALS REQUIRED

Microplate reading fluorimeter capable of excitation at a wavelength in the range 470-500 nm and detection of emitted light in the range 520-550 nm.
Pipetman or multi-channel pipetman capable of pipetting 2-100 µl accurately
Ice bucket to keep reagents cold until use.
Microtiter plate warmer or other temperature control device (optional)

ASSAY PROCEDURES

Notes On Storage: *Store all components except the microtiter plate and instruction booklet at -70°C for the highest stability. HDAC2, BML-KI575, must be handled with particular care in order to retain maximum enzymatic activity. Defrost it quickly in a RT water bath or by rubbing between fingers, then immediately store on an ice bath. The remaining unused enzyme should be refrozen quickly, by placing at -70°C. If possible, snap freeze in liquid nitrogen or a dry ice/ethanol bath. To minimize the number of freeze/thaw cycles, aliquot the enzyme into separate tubes and store at -70°C. The* ***Fluor de Lys®-Green*** Substrate, BML-KI572, *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. Substrate deacetylation occurs first, in a total volume of 50 µl. The second stage, which is initiated by the addition of 50 µl of Developer, stops HDAC activity and produces the fluorescent signal. See "Preparing Reagents For Assay" (p. 3), Fig. 2, (p. 4) and Table I (p. 5). .

   Two types of ½-volume, 96-well microplates are provided. The signal obtained with the opaque, white plate will be greater than that obtained with the black plate, but the black plate may reduce background and well-well cross-talk.

   If needed, there is some leeway for change in the reaction volumes. The wells of the microplates provided (80-2408) can readily accommodate 150 µl. 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 µM concentration of Trichostatin in the final mix; 2) a minimum 2.5 µl/well amount of Developer Concentrate (BML-KI105). See "Preparing Reagents For Assay", Step #5, (p. 3).

2. Assays should include a "time-zero" (sample for which enzyme is added after addition of the Developer with TSA or other inhibitor) and/or a negative control (no-enzyme). The measure of a sample's deacetylation is the difference between its fluorescence and the fluorescence of a time-zero or no enzyme sample with the same substrate concentration.

3. Reaction progress curves for a broad range of substrate concentrations (5-100 µM ***Fluor de Lys®-Green*** Substrate), remain linear for at least 60 min. at 37°C, 4 ng HDAC2/well and longer at lower temperatures (e.g. 25°C) or with less enzyme. A time course experiment will aid in the selection of an incubation time and/or enzyme amount, which yield a signal that is both sufficiently large and proportional to enzyme rate (Fig. 4).

4. The best-fit K_m for HDAC2 with the ***Fluor de Lys®-Green*** Substrate is ~48 µM (Fig. 5A) and $K_{(0.5)}$ from a fit to the Hill equation is ~28 µM (Fig. 5B). Use of a substrate concentration below these values (e.g. 10 µM) will help avoid substrate competition effects, which could mask the effectiveness of a potential inhibitor.

5. It is conceivable that some compounds being screened for inhibition of HDAC2 may interfere with the action of the *Fluor de Lys*® Developer. Assuming the concentration of the test compound(s) does not exceed 0.15 mM, any interference will in all likelihood result in fluorescence development slowing down rather than stopping. It is recommended that, after addition of Developer, fluorescence be read at regular intervals (e.g. every 5 min.) until fluorescence readings stabilize at a higher value. If a sample's fluorescence continues to increase significantly after 20 min. at 25°C, this may indicate interference. Nevertheless, a valid final reading may still be obtained by continuing to monitor the fluorescence until it does stabilize. A follow-up assay in which the amount of Developer Concentrate is increased (e.g. using 50 µl of a 10x Developer Concentrate dilution) should display faster kinetics of fluorescence development and may be useful for confirming the first reading.

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 HDAC2 (BML-KI575) diluted in Assay Buffer (BML-KI422) to provide for the assays to be performed (# of wells x 15 µl). Dilutions of HDAC2 ranging from 450-fold to 45-fold will provide 1 to 10 ng of HDAC2 per 15 µl (Table 1).
3. Prepare dilution(s) of Trichostatin A and/or Test Inhibitors in Assay Buffer (BML-KI143). Since 10 µl will be used per well (Table 1, Fig. 2), and since the final volume of the HDAC reaction is 50 µl, these inhibitor dilutions will be 5x their final concentration.
4. Prepare dilution(s) of the *Fluor de Lys®-Green* Substrate (BML-KI572; 50 mM) in Assay Buffer (BML-KI422) that will be 2x the desired final concentration(s). For inhibitor screening, substrate concentrations at or below the K_m are recommended. Twenty-five µl will be used per well (Table 1). Initial dilutions of 50-fold or greater in Assay Buffer (1.0 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®-Green* Substrate solutions in Assay Buffer may cause precipitation of the Substrate. Dilute only 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 (50 µl per well). First, dilute the *Fluor de Lys®* Developer Concentrate 20-fold (e.g. 50 µl plus 950 µl Assay Buffer) in cold Assay Buffer (BML-KI422). Second, dilute the 0.2 mM Trichostatin A (BML-GR309-9090) 100-fold in the 1x Developer just prepared (e.g. 10 µl in 1 ml; final Trichostatin A concentration in the 1x Developer = 2 µM; final concentration after addition to HDAC/Substrate reaction = 1 µM). 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. Fig. 2 depicts a schematic of the procedure.)
2. Add diluted HDAC2 to all wells except those that are to be "No-Enzyme Controls."
3. Allow diluted *Fluor de Lys®-Green* 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 (25 µl) to each well and mixing thoroughly.
5. 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. or transfer to fluorimeter to monitor signal development. Once fully developed, the signal is stable and can even be read hours later.
6. Read samples in a microplate-reading fluorimeter capable of excitation at a wavelength in the range 470-500 nm and detection of emitted light in the range 520-550 nm.

TABLE 1. ASSAY MIXTURE EXAMPLES[6]

Sample	Assay Buffer	HDAC2 (Dilution)	Inhibitor (5x)	*FdL.-Green* Substrate (2x)
Blank (No Enzyme)	25 µl	0	0	25 µl
Control	10 µl	15 µl	0	25 µl
Trichostatin A[‡]	0	15 µl	10 µl[‡]	25 µl
Test Sample[⌘]	0	15 µl	10 µl[⌘]	25 µl

[6] HDAC reaction mixtures, prior to addition of *Fluor de Lys®* Developer.

[‡] 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 1 µM concentration and essentially complete HDAC inhibition; 2) As a model inhibitor "hit", 2.5 nM would produce final 0.5 nM and ~50% inhibition at 10 µM substrate.

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

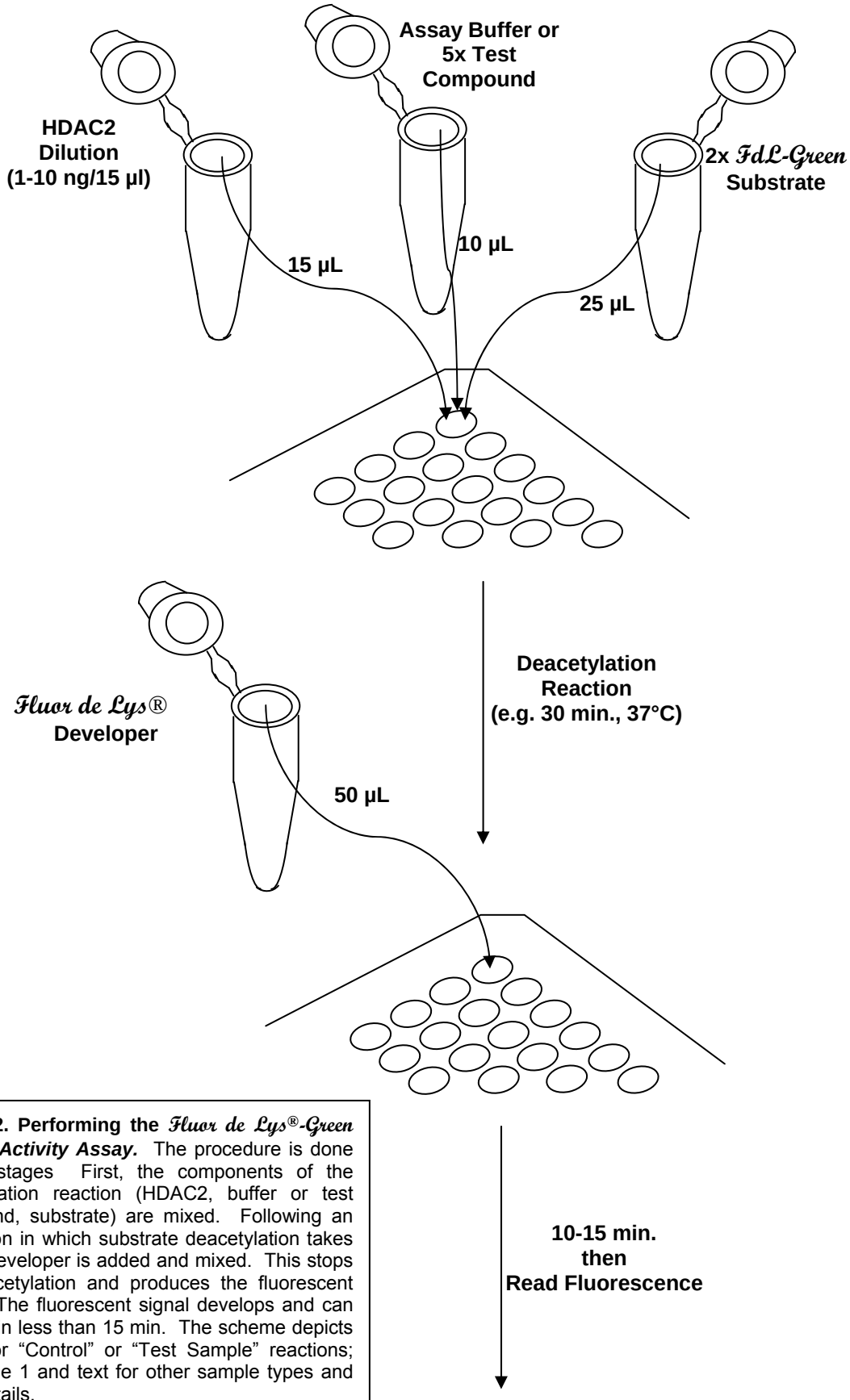

Figure 2. Performing the *Fluor de Lys®-Green HDAC2 Activity Assay.* The procedure is done in two stages First, the components of the deacetylation reaction (HDAC2, buffer or test compound, substrate) are mixed. Following an incubation in which substrate deacetylation takes place, Developer is added and mixed. This stops the deacetylation and produces the fluorescent signal. The fluorescent signal develops and can be read in less than 15 min. The scheme depicts mixes for "Control" or "Test Sample" reactions; see Table 1 and text for other sample types and more details.

USE OF THE *Fluor de Lys®-Green* STANDARDSTANDARD (BML-KI605)

Preparation of a Standard Curve:

1. The ***Fluor de Lys®-Green*** Standard (BML-KI605) will be used for preparing a standard curve. The best conditions will vary depending on the fluorimeter model, the gain setting, the plate chosen and the exact excitation and emission wavelengths used. We recommend making several dilutions of ***Fluor de Lys®-Green*** Standard (BML-KI605) in Assay Buffer (e.g. 1, 5 and 20 µM). The fluorescence signal should then be determined, as described below, after mixing 50 µl of the diluted standard with 50 µl of Developer. Readings taken with these samples may be used to adjust the gain, test excitation/emission wavelengths and cutoff filters and to plan an appropriate series of dilutions for a full standard curve. Under the conditions shown in Figure 3, fluorescence increases monotonically, although non-linearly, with concentration, up to about 20 µM. The relationship between Standard concentration and fluorescence remains linear to at least 0.4 µM. For most applications involving initial rate determinations, it should be possible to work with signals entirely within this linear range. Provided the same wavelength and gain settings are used each time, there should be no need to prepare a standard curve more than once.
2. After ascertaining an appropriate concentration range, prepare, in Assay Buffer, a series of ***Fluor de Lys®-Green*** Standard dilutions that span this range. Pipet 50 µl of each of these dilutions, and 50 µl of Assay Buffer as a 'zero', to a set of wells on the microtiter plate.
3. Prepare, as described in "Preparing Reagents For Assay", step #5. (p.3), sufficient ***Fluor de Lys®*** Developer for the standard wells (50 µl per well).
4. Mix 50 µl of the Developer with the 50 µl 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 470-500 nm and detection of emitted light in the range 520-550.
6. Plots of pmol. ***Fdl-Green*** DeAc. Std. (y-axis) vs. fluorescence are shown in Fig. 3, **A** and **B.** A plot covering a relatively narrow range of Standard quantities, such as 0-20 pmol/well (0-0.4 µM in 50 µL), will provide a good linear fit (**A**). Plotting a broader range of Standard quantities, such as 0-1000 pmol/well (0-20 µM in 50 µL), will yield a curve that fits well to a second-order polynomial (**B**). The accuracy of the fluorescence to pmol conversion will be best if the range chosen is the minimum necessary to cover the full range of fluorescence increases likely to be seen in your experimental samples. [NOTE: "Fluorescence Increase" = Sample – Background, where Background Fluorescence is the fluorescence of a time-zero or no-enzyme sample of the same substrate concentration.]

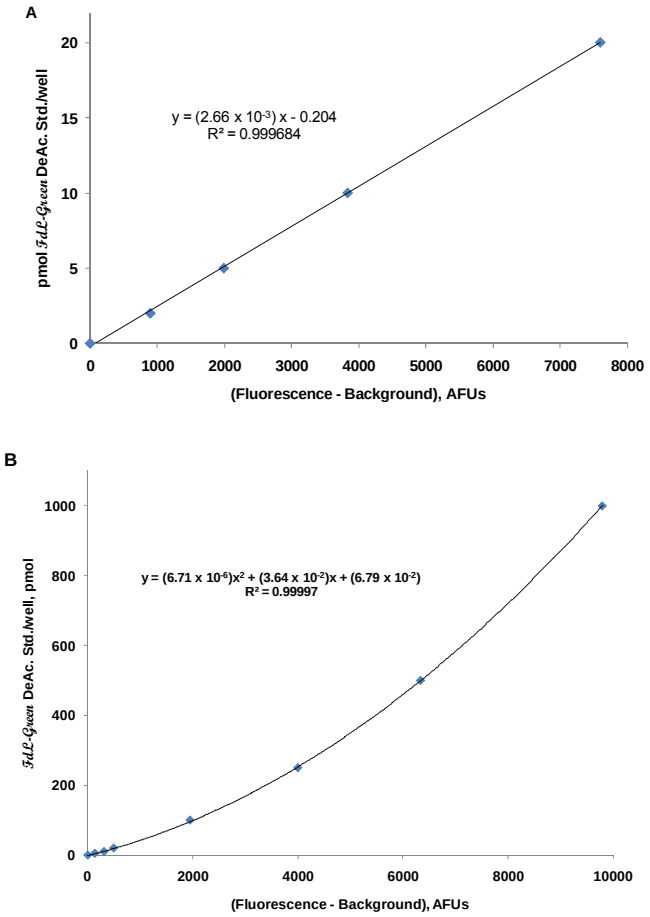

Figure 3. Fluorescence Standard Curves. ***Fluor de Lys®-Green*** Standard was diluted in Assay Buffer, to the concentrations of 0-0.4 µM (**A**) or 0-20 µM (**B**) and 50 µl aliquots mixed with 50 µl Developer. Fluorescence was measured in a black microplate (**A**) with a Synergy2 reader (BioTek, Ex. 485 nm, Em. 528 nm, gain=60) or a white microplate (**B**) with a CytoFluor™ II fluorescence plate reader (PerSeptive Biosystems, Ex. 485 nm, Em. 530 nm, gain=37). Linear or second-order best-fit curves were obtained with Microsoft XL (equations). Depending on the concentration range of substrate, an equation from one of these two plot types may be used to convert fluorescence increases due to deacetylation to the number of pmol of ***Fluor de Lys®-Green*** Substrate that have been deacetylated.

Fluorescence Quenching and/or Fluorescence from Test Compounds

1. The ***Fluor de Lys®-Green*** assay avoids many instances of interference by compound quenching or fluorescence because it is unaffected by compounds absorbing and/or emitting in the near UV and blue parts of the spectrum. Given the excitation and emission spectra of the ***Fluor de Lys®-Green*** fluorophor (peak Ex. 496 nm, Em. 520 nm), test compounds that might quench assay fluorescence or fluoresce at the assay wavelength are likely to be highly colored.
2. If a compound is suspected of interfering with the assay by fluorescence or fluorescence quenching, prepare a standard curve with ***Fluor de Lys®-Green*** StandardStandard (BML-K605) in the presence of the working concentration of that compound and compare to curves done in its absence. (Fig. 3). If quenching is not too severe, it may be possible to correct for quenching by converting deacetylation-induced fluorescence increases to acetylation rates (e.g. in pmol/min) using the standard curve obtained in the compound's presence.
3. Interference from a compound that fluoresces, but does not quench the fluorescence of the ***Fluor de Lys®-Green*** Standard, can be corrected with a no-enzyme blank or time-zero sample that includes the compound. Note that in all cases, whether a fluorescent compound is present or not, deacetylation is a function of the increase in a sample's fluorescence above that of a corresponding no-enzyme blank or time-zero sample.

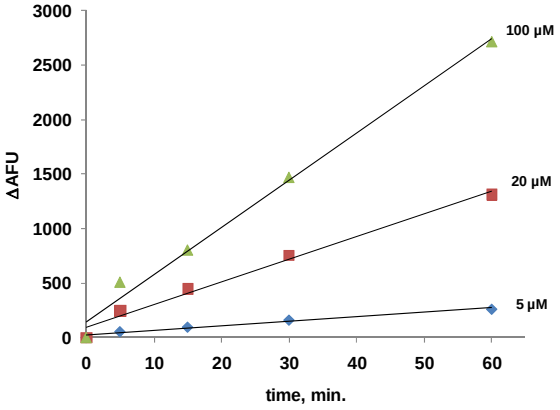

Figure 4. Time Courses of *Fluor de Lys®-Green* Substrate Deacetylation by HDAC2. HDAC2 (4 ng /well) was incubated (37°C) with indicated concentrations of substrate. Reactions were stopped at indicated times with ***Fluor de Lys®*** Developer and fluorescence measured (CytoFluor™ II, PerSeptive Biosystems, Ex. 485 nm, Em. 530 nm, gain=37), white plate. Each point represents a single determination with background (0 min.) fluorescence subtracted.

APPLICATION EXAMPLES

The ***Fluor de Lys®-Green*** HDAC Assay has been used to investigate the kinetics of ***Fluor de Lys®-Green*** Substrate deacetylation by HDAC2 (Figures 4 & 5). The dependence of HDAC2 initial rate on the concentration of ***Fluor de Lys®-Green*** Substrate exhibits a slightly sigmoidal character and fits of the rate data to both the Michaelis-Menten equation (Fig. 5A) and a form of the Hill equation (Fig. 5B) are shown.

In a black plate (Synergy2 reader, BioTek, gain = 60), under initial rate conditions, the maximum ratio of signal fluorescence to background fluorescence was achieved with a substrate concentration of ~10 µM. This concentration was used in determining the Z' factor for the assay (Fig. 6) and in obtaining the IC_{50} for the model inhibitor trichostatin A (Fig. 7)

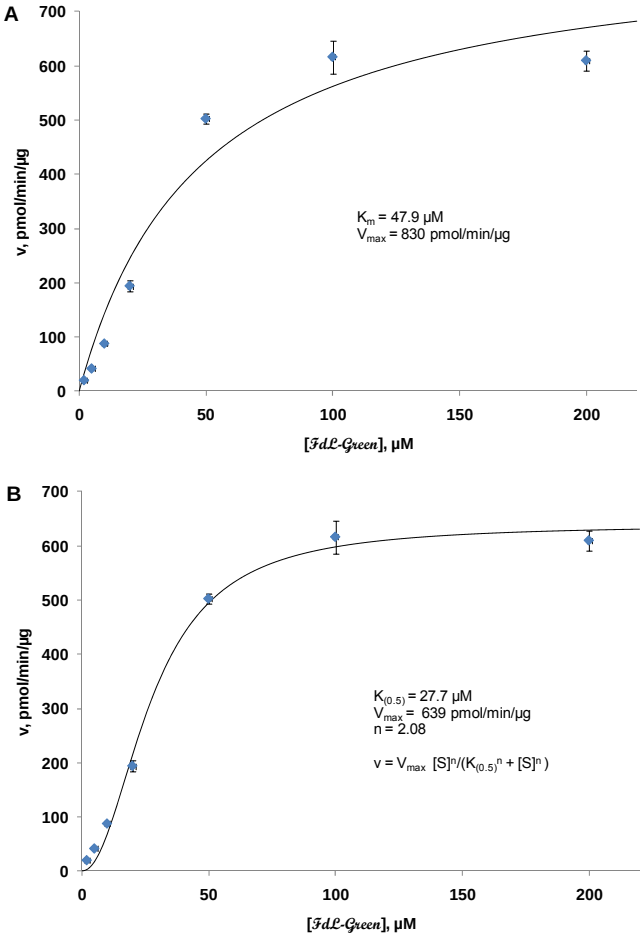

Figure 5. Kinetics of *Fluor de Lys®-Green* Substrate Deacetylation by HDAC2. HDAC2 (1.0 ng per well) was incubated (37°C) with indicated concentrations of substrate. Reactions were stopped after 20 min. with ***Fluor de Lys®*** Developer and fluorescence measured (Synergy2, (BioTek, Ex. 485 nm, Em. 528 nm, gain=60). Fluorescence increases were converted to pmol of deacetylated ***Fluor de Lys®-Green***.Substrate by means of a best fit equation from a fluorescence standard curve (see Fig. 1A). A fit of the data to the Michaelis-Menten equation is shown in **A**. The plot of rate vs. [***Fluor de Lys®-Green***] has a somewhat sigmoidal appearance and consequently is also shown with a fit to a form of the Hill equation:
$v = (Vmax \times [S]^n)/(K_{(0.5)}{}^n + [S]^n)$, where $K_{(0.5)}$ is the substrate concentration at half the maximal rate, V_{max}, and n is the Hill coefficient (**B**). Both of the non-linear least squares fits and the parameters shown were obtained with the Microsoft XL Solver tool.

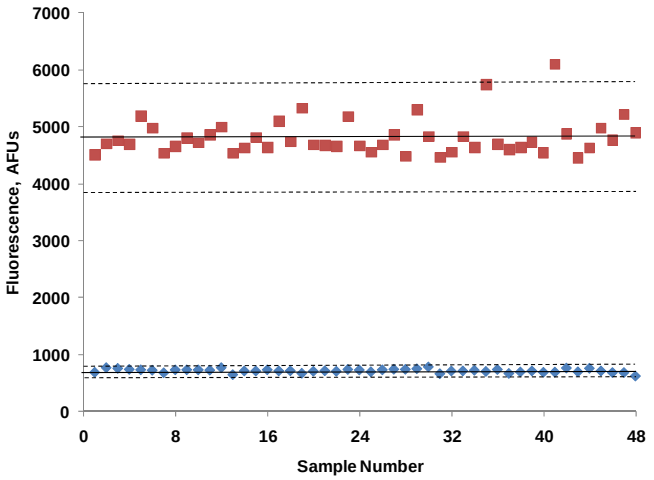

Figure 6. Determination of a Z'-Factor for the *Fluor de Lys*®-*Green* HDAC2 Assay. Half of a black microplate was devoted to positive control samples (1.0 ng per well, 60 min., 37°C, 10 µM substrate) and half to negative controls (no enzyme, same conditions). Reactions were stopped after 60 min. with ***Fluor de Lys***® Developer and fluorescence measured (Synergy2, BioTek, Ex. 485 nm, Em. 528 nm, gain=60). For the positive controls, the amount of substrate deacetylated was determined by reference to a standard curve (Fig. 3A) and determined to be 10 pmol (0.2 µM out of 10 µM in 50 µl) or 2% of the total substrate. Solid lines represent the mean fluorescence for each of the two sample groups and the dashed lines are placed three standard deviations above and below the means. The Z'-factor from the data was 0.74 (Z' = 1-(3 pos. cont. SD + 3 neg. cont. SD)/(mean pos. cont. – mean neg. cont.))

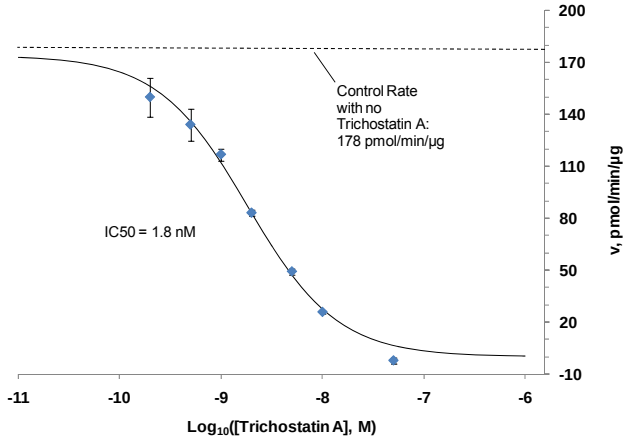

Figure 7. Trichostatin A Inhibition of *Fluor de Lys*®-*Green* Substrate Deacetylation by HDAC2. HDAC2 (1.0 ng/well) was incubated (37°C) with 10 µM substrate and indicated concentrations of Trichostatin A (TSA). Reactions were stopped after 60 min. with ***Fluor de Lys***® Developer and fluorescence measured (Synergy2, BioTek, Ex. 485 nm, Em. 528 nm, gain=60). The dose-response curve was derived from a least squares fit to a three parameter Hill-Slope model (bottom fixed at 0 pmol/min/µg.), $y = top/(1 + (x/IC_{50})^{slope})$ The fitted parameters were *top* = 174 pmol/min/µg, IC_{50} = 1.8 nM and slope = 0.98 ('Solver' tool, Microsoft XL). Error bars represent standard deviations of three determinations.

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.

LITERATURE CITATIONS OF *Fluor de Lys*® PRODUCTS

X. Zhou *et al. Proc. Natl. Acad. Sci. USA* 2001 **98** 10572
B. Heltweg and M. Jung *Anal. Biochem.* 2002 **302** 175
S. Milutinovic *et al. J. Biol. Chem.* 2002 **277** 20974
K. Ito *et al. Proc. Natl. Acad. Sci. USA* 2002 **99** 8921
K.J. Bitterman *et al. J. Biol. Chem.* 2002 **277** 45099
I.C. Jang *et al. Plant J* 2003 **33** 531
G.V. Kapustin *et al. Org. Lett.* 2003 **5** 3053
K.T. Howitz *et al. Nature* 2003 **425** 191
K. Zhao *et al. Nat. Struct. Biol.* 2003 **10** 864
D.-K. Kim *et al. J. Med. Chem.* 2003 **46** 5745
R.M. Anderson *et al. Science* 2003 **302** 2124
T. Suzuki *et al. Bioorg. Med. Chem. Lett.* 2003 **13** 4321
L.H. Wang *et al. Nature Medicine* 2004 **10** 40
C.M. Gallo *et al. Mol. Cell. Biol.* 2004 **24** 1301
N. Gurvich *et al. Cancer Res.* 2004 **64** 1079
F. Yeung *et al. EMBO J.* 2004 **23** 2369
J.G. Wood *et al. Nature* 2004 **430** 686
B.G. Cosío *et al. Am. J. Respir. Crit. Care Med.* 2004 **170** 141
A.J.M. de Ruijter *et al. Biochem. Pharmacol.* 2004 **68** 1279
J.L. Avalos *et al. Mol. Cell* 2005 **17** 855
T. Suzuki *et al. J. Med. Chem.* 2005 **48** 1019
K. Ito *et al. N. Engl. J. Med.* 2005 **352** 1967
E. Michishita *et al. Mol. Biol. Cell* 2005 **16** 4623
A. Mai *et al. J. Med. Chem.* 2005 **48** 7789
A. D. Napper *et al. J. Med. Chem.* 2005 **48** 8045
V.C. de Boer *et al. Mech. Ageing Dev.* 2006 **127** 618
S.L. Gantt *et al. Biochemistry* 2006 **45** 6170
W. Gu *et al. Bioorg. Med. Chem.* 2006 **14** 3320
D. Herman *et al. Nature Chem. Biol.* 2006 **10** 551
X. Li *et al. Cancer Res.* 2006 **66** 9323
P. Aksoy *et al. Biochem. Biophys. Res. Commun.* 2006 **349** 353
J.M. Solomon *et al. Mol. Cell. Biol.* 2006 **26** 28
V.M. Nayagam *et al. J. Biomol. Screen.* 2006 doi:10.1177/1087057106294710
P.H. Kiviranta *et al. Bioorg. Med. Chem. Lett.* 2007 **17** 2448
D.H. Kim *et al. Biochem. Biophys. Res. Commun.* 2007 **356** 233
T.F. Outeiro *et al. Science* 2007 **317** 516
S. Lain *et al. Cancer Cell* 2008 **13** 454
X. Hou *et al. J. Biol. Chem.* 2008 **283** 20015
Y. Nakahata *et al. Cell* 2008 **134** 329
S. Rashid *et al. J. Biol. Chem.* 2009 **284** 18115
Y. Chung *et al. Carcinogenesis* 2009 **30** 1387
H. Nian *et al. Carcinogenesis* 2009 **30** 1416
S. Agbor-Enoh *et al. Antimicrob. Agents Chemother.* 2009 **53** 1727
B.G. Cosio *et al. Thorax* 2009 **64** 424
P.D. N'Guessan *et al. Arterioscler. Thromb. Vasc. Biol.* 2009 **29** 380
J. Chen *et al. Blood* 2009 **113** 4038

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