MANUAL

E1 LITE - UBE1 Activity Assay Kit

Catalog Number UC105

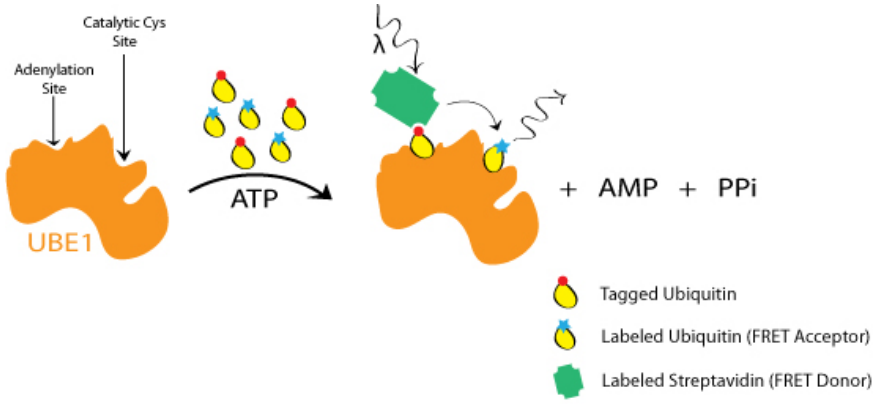
BACKGROUND

Conjugation of ubiquitin to a protein substrate involves the coordinated action of three separate proteins, an activating enzyme or E1, an intermediate carrier or E2, and the final mediator, the E3. The E3 may be actively involved or may serve primarily as a scaffold to bring the substrate and the charged E2 together. In either case, the general consensus is that the E3 is the principal source of substrate selectivity and, as such, is a primary target for the development of inhibitors or activators of substrate-specific ubiquitylation. In contrast, because UBE1 (UBA1) appears to be responsible for initiating the majority of ubiquitination events, inhibition of UBE1 would be expected to inhibit ubiquitination more broadly. Therefore, UBE1 may be an important target for anti-cancer therapeutics. This is supported by initial characterization of E1 inhibitors. The first in class NEDD8-activating enzyme (NAE) inhibitor, MLN-4924[1], which indirectly blocks ubiquitylation by cullin ring ligases, is in early stage clinical trials for hematologic and advanced non-hematologic malignancies. The first reported E1 inhibitor, PYR-41, increases p53 levels in cancer cells, resulting in p53-mediated apoptosis[2]. Similarly, the nitric oxide-producing prodrug JS-K inhibits ubiquitin E1, decreases total ubiquitination and elevates p53 levels in treated cells[3]. These examples suggest that ubiquitin E1 inhibitors are worthy of exploration as potential anti-cancer targets.

E1 enzymes catalyze ubiquitin activation through a multistep process that offers multiple opportunities for inhibition[4,5]. In a first step, E1 binds ubiquitin and Mg-ATP and catalyzes formation of a ubiquitin carboxyterminal acyl adenylate. The E1 catalytic cysteine then reacts with the ubiquitin-adenylate to form a ubiquitin thioester. Finally, another ubiquitin and Mg-ATP bind to form a fully-loaded E1 containing one ubiquitin-adenylate and one ubiquitin thioester, the latter activated for transfer to an E2. The E1 LITE - UBE1 Activity Assay Kit is based on FRET between donor and acceptor fluorophores associated with the bound ubiquitins to provide an HTS-compatible time-resolved fluorescent method for measuring E1 activity and screening for E1 inhibitors.

ABOUT THE ASSAY

Förster resonance energy transfer (FRET) is a mechanism of energy transfer between donor and acceptor fluorophores in close proximity that results in an increased fluorescent signal upon excitation of the donor. The E1 LITE - UBE1 Activity Assay Kit is based on FRET between donor and acceptor fluorophores associated with the two ubiquitin molecules bound to the activated UBE1 ternary complex (see figure below). The assay employs streptavidin terbium as the donor fluorophore, which is directed to the complex through binding to biotinylated ubiquitin, and fluorescein-labeled ubiquitin as the acceptor fluorophore. The long-lived fluorescence of the terbium donor allows a time-delayed reading of the FRET signal, or time-resolved FRET (TR-FRET), which reduces the contribution of short-lived background emissions resulting from buffers, proteins, and chemical compounds. The assay can be run in a 96-well or 384-well format and produces a reproducible signal with a Z' $\geq$ 0.7.



Binding of biotinylated ubiquitin and fluorescein-labeled ubiquitin on E1 in the presence of streptavidin-terbium brings the terbium donor in close proximity with the fluorescein acceptor. Excitation at 340nm triggers energy transfer between the terbium and the fluorescein and increased fluorescence at 520nm.

BENEFITS

1. Eliminates the need for radioactive or Western blot-based detection of E1 - ubiquitin thioester formation
2. Homogenous mix and read assay compatible with high-throughput screening for UBE1 inhibitors
3. Time-resolved fluorescence reduces the number of false positives and false negatives due to autofluorescent or quenching compounds
4. Robust assay with a S/B of $\geq$8 and a Z' $\geq$0.7
5. Compatible with 96-well and 384-well formats

SUGGESTED USES

1. Screening for inhibitors of the ubiquitin E1 enzyme UBE1
2. Characterization of UBE1 kinetics
3. Characterization of the mechanism of E1 inhibitors
4. Secondary screening of E3 ligase, kinase or other target inhibitors for inhibition of UBE1

COMPONENTS

Unless indicated otherwise, store all materials at -80ºC, avoid cycles of freezing and thawing. All components are stable for at least 1 year from receipt

1. **Assay Buffer (store at 4ºC or below)**

   Size: 1 x 1ml (20X)

   Contents: 1M Tris-HCl, pH7.5, 100mM $MgCl_2$, 1% CHAPs

2. **UBE1 Enzyme (300X)**

   Size: 1 x 35µl (750nM)

3. Fluorescein-labeled Ubiquitin (150X)

   Size: 1 x 35µl (15µM)

4. **Biotinylated Ubiquitin (150X)**

   Size: 1 x 35µl (150nM)

5. **Mg-ATP Solution**

   Size: 35µl (3mM)

6. **Microtiter plate** (store at room temperature)

   Qty: 1 x ½-volume 96-well or 1 x 384-well polypropylene plate

ADDITIONAL ITEMS REQUIRED

1. **Streptavidin-terbium (streptavidin-Tb)**

   We recommend the following sources for streptavidin terbium

   a. LanthaScreen® Tb-Streptavidin (Cat. # PV3965) from LifeTechnologies. 50µg is sufficient for a vast excess of wells

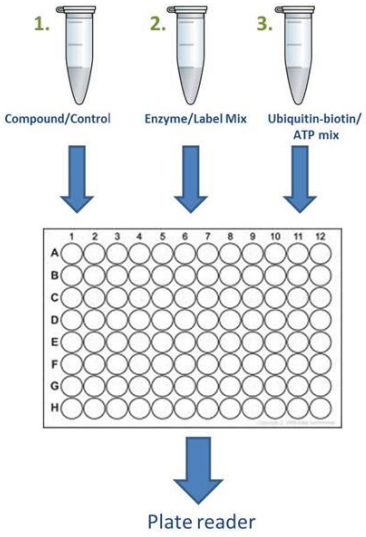
   b. Streptavidin-Tb – 5,000 tests (Cat. # 610SATLA) from CisBio.

2. **Fluorometric Plate Reader**

   A fluorescent plate reader capable of reading TR-FRET between a terbium donor and fluorescein acceptor is suitable for detection. Plate readers with excitation filters centered at 340nm with a 30nm bandpass, a primary emission filter centered at 520nm with a 20nm bandpass and a 2nd emission filter centered between 480-495nm with a 10nm bandpass is suitable. The fluorescein signal from the primary emission filter can be referenced to the terbium emission from the second emission filter to control for quenching or autofluorescence from the compounds or buffer components. Readers capable of a delay time of 100µs are recommended. The specifications of the emission filter are more critical than the excitation filter. Standard fluorescein filters are not recommended because they pass light associated with the terbium emission spectrum. Optimization of the plate reader optics (e.g. signal gain, plate height reads, etc.) is recommended.

PROTOCOL OVERVIEW

The assay is a mix and read protocol that involves mixing equal volumes of three solutions and reading the ensuing reaction kinetically in a fluorometric plate reader. In a first step, compound or buffer control is mixed with an Enzyme/Label Mix. After the desired preincubation with compound (15 minutes), the enzyme reaction is initiated by the addition of Biotinylated Ubiquitin/ATP Mix. The reaction proceeds rapidly, approaching completion in 5-10 minutes. For kinetic reads, it is imperative to add Biotinylated Ubiquitin/ATP Mix and begin reading the signal as rapidly as possible. Negative control wells that lack ATP should be included. If testing for compound inhibition, we also recommend running appropriate vehicle controls. Each condition should be run in triplicate to assess the reproducibility and accuracy of your results. We recommend a total assay volume of 45µl and 15 µl for ½-volume 96-well and 384-well microtiter plate formats respectively. ***Note: Pilot assays are highly recommended prior to running a full plate of compounds to optimize conditions and plate reader settings.***



DETERMINATION OF STOCK SOLUTION VOLUMES

Each well receives an equal volume of the following solutions:

1) 1 volume compound or buffer control
2) 1 volume Enzyme/Label Mix
3) 1 volume Ubiquitin-biotin/ATP Mix

We recommend 45µl/well total assay volume, i.e. 15µl/well of each solution, for ½-volume 96-well microtiter plate format. For 384-well format, we recommend 15µl/well total assay volume, i.e. 5µl/well of each solution. Use the following formulas to calculate the total volume of each solution to prepare for your assay, taking into account appropriate controls and extra volume required to accommodate liquid handlers (i.e. pipetting losses or reservoir requirements):

½-volume 96-well format:	Solution volume required = # wells x 15µl x 1.2*
384-well format:	Solution volume required = # wells x 5µl x 1.2*

*Multiplying by 1.2 incorporates 20% extra volume to accommodate liquid handling into the plate

All solutions should be made immediately prior to use and kept on ice until ready for use.

PREPARATION OF SOLUTIONS

1. **Assay Buffer (1X)**

   Dilute the **20X Assay Buffer** 20-fold with water to make a sufficient volume of a 1X solution for the number of wells to be assayed (45µl/well for a ½-volume 96-well plate and 15µ/well for a 384-well plate) plus additional volume to accommodate pipetting losses and liquid handlers.

2. **Streptavidin-Tb (300X)**

   Reconstitute the streptavidin-Tb according to the manufacturer's instructions.

3. **Enzyme/Label Mix (3X)**

   Dilute **Fluorescein-labeled Ubiquitin (300X)** and **UBE1 Enzyme (150x)** 100-fold and 50-fold respectively in assay buffer for 3X concentrations of 150nM Ub-FAM, and 15nM UBE1. Add sufficient volume of **Streptavidin-Tb** to achieve 1.5nM Streptavidin-Tb (3X). For example, 25µl of each of **Fluorescein-labeled Ubiquitin** and **UBE1 Enzyme** stock solution and 14 µl of 267 nM Streptavidin-Tb stock solution in 2.436ml assay buffer for a total volume of 2.5ml

4. **Negative Control Mix**

   Dilute **Biotinylated Ubiquitin (300x)** 100-fold in assay buffer for a 3X concentration of 3nM

5. **Biotinylated Ubiquitin/ATP Mix (3X)**

   Dilute **Biotinylated Ubiquitin (300X)** and **Mg-ATP (**3mM**)** 100-fold each in **assay buffer** for a 3X concentrations of 3nM and 30µM respectively. For example, 25µl of each stock solution in 2.450ml assay buffer for a total volume of 2.5ml

6. **Compounds or buffer controls.**

   Dilute compounds to the desired concentration in assay buffer. Keep the DMSO concentration to <1%

PROTOCOL

The following protocol is written for performing the assay in a 384-well plate with volumes recommended for working with a ½-volume 96-well plate listed in parentheses.

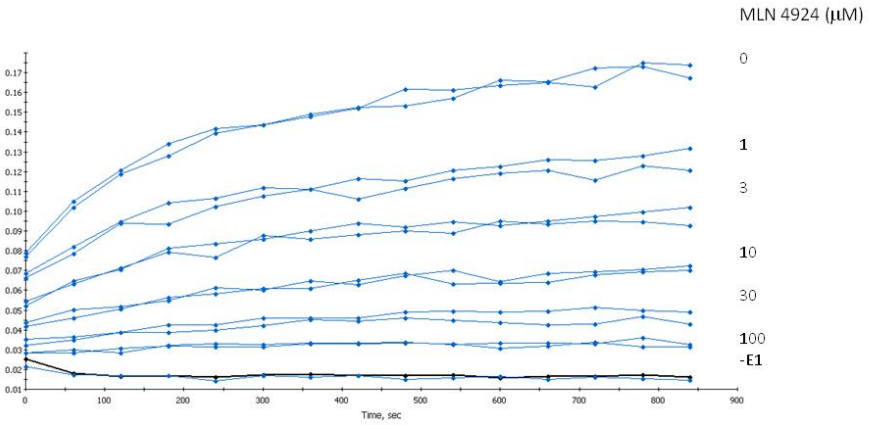
1. Dispense 5µl (15µl) of compound or buffer controls (prepared in step 5 above) into appropriate wells
2. Add 5µl (15µl) of **3X Enzyme/Label Mix** (prepared in step 2 above) to compound wells
3. Optional: Pre-incubate compounds with **3X Enzyme/Label Mix** for 15 minutes if desired
4. Add 5µl (15µl) of **Negative Control Mix** (prepared in step 3 in the previous section to appropriate negative control wells
5. Add 5µl (15µl) of **3X Ubiquitin-biotin/ATP mix** (prepared in step 4 in the previous section) to all wells except the negative control wells
6. Read the plate kinetically or at a 5 or 10 minute end-point

REFERENCES

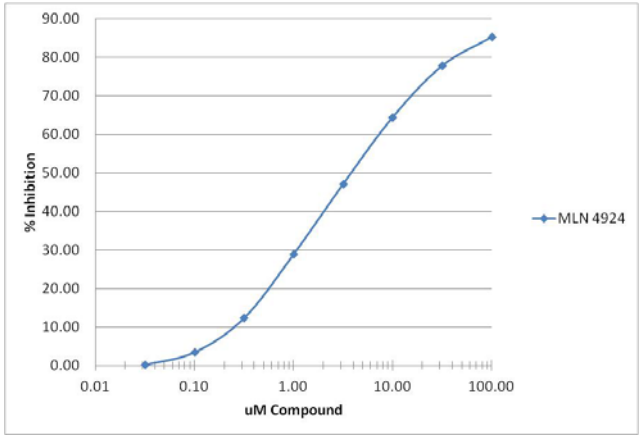
a. Soucy, T. A., P. G. Smith, et al. (2009). "An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer." Nature **458**(7239): 732-736.
b. Yang, Y., J. Kitagaki, et al. (2007). "Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics." Cancer Res **67**(19): 9472-9481.
c. Kitagaki, J., Y. Yang, et al. (2009). "Nitric oxide prodrug JS-K inhibits ubiquitin E1 and kills tumor cells retaining wild-type p53." Oncogene **28**(4): 619-624.
d. Haas, A. L., J. V. Warms, et al. (1982). "Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation." J Biol Chem **257**(5): 2543-2548.
e. Haas, A. L., J. V. Warms, et al. (1983). "Ubiquitin adenylate: structure and role in ubiquitin activation." Biochemistry **22**(19): 4388-4394.

SAMPLE DATA

Timecourse



% Inhibition



Inhibition of UBE1 with MLN-4924. The Nedd8-activating enzyme inhibitor, MLN-4924, has previously been shown to inhibit UBE1 activity with an IC_{50} of 1.5µM[1]. Here, we show inhibition of UBE1 using the E1 LITE - UBE1 Activity Assay Kit. Time courses of UBE1 activity in the presence of increasing concentrations of MLN-4924 are shown in the top panel and percent inhibition of UBE1 with increasing concentrations of MLN-4924 are shown in the bottom panel.