MANUAL

E1/E2 LITE - Ubiquitin Transfer Assay Kit

Catalog Number UC106

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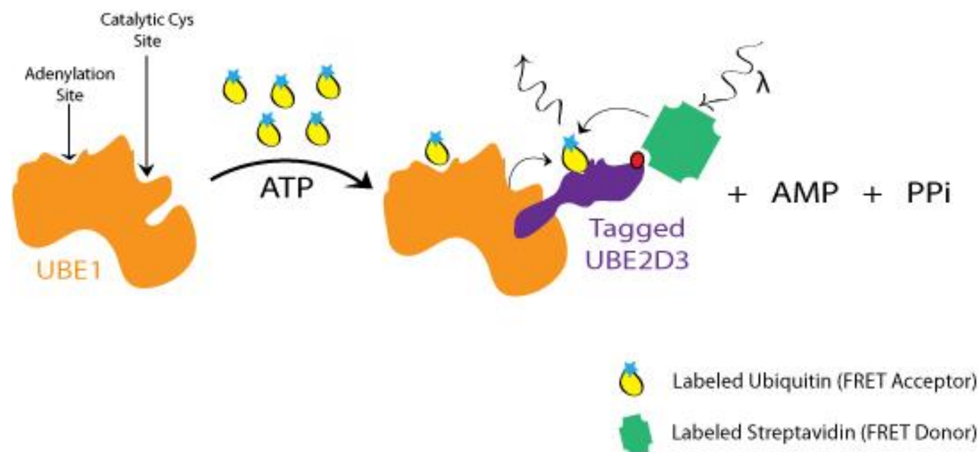
BACKGROUND

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. Since all E3 assays require the participation of an E1 and an E2, it is important to have facile assays for deconvoluting the actual target of compounds that are positive in an E3 screen, i.e., compounds that inhibit substrate ubiquitylation or auto-ubiquitylation through modulation of E1 or E2 activity thus indirectly affecting E3 activity. In addition, there could be pathological conditions characterized by excessive ubiquitylation in which down-regulation of the activity of the E1 or E2 enzymes might provide therapeutic relief.

ABOUT THE ASSAY

We have developed a high-throughput assay for measuring E2 thioester formation that can be used either as a secondary screen to deconvolute E3 assay “hits” or as a primary screen for inhibitors of E2 thioester formation. The assay is based on Förster resonance energy transfer (FRET), in which energy transfer between donor and acceptor fluorophores in close proximity results in an increased fluorescent signal. Ubiquitin thioester formation on the E2 conjugating enzyme brings a donor fluorophore on the ubiqituin in close proximity to the acceptor fluorophore associated with the E2 to allow FRET and an increased fluorescence signal in proportion to the amount of ubiquitin thioester. 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 is configured with the E1 activating enzyme UBE1 (UBA1) and the E2 conjugating enzyme UBE2D3 (UbcH5c). The assay can be run in a ½-volume 96-well or 384-well format and produces a reproducible signal with a Z’ $\geq$0.7.



E2 thioester formation is measured by TR-FRET between a streptavidin-Terbium donor associated with the E2 enzyme and fluorescein-labeled ubiquitin bound to the E2. 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 E2 thioester formation
2. Homogenous assay compatible with high-throughput screening
3. Robust assay with a S/B >10 and a Z’ >0.7
4. Compatible with 1/2 –volume 96-well and 384-well formats

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SUGGESTED USES

1. Counterscreening hits from E3 Ligase screens for activity against representative E1 and E2 enzymes (UBE1 and UBE2D3)
2. Screening for inhibitors of UBE1 and UBE2D3
3. Characterization of the kinetics of E2 thioester formation.
4. Characterization of the mechanism of E1 or E2 inhibition

COMPONENTS

Unless otherwise indicated, 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 (20X)** (store at 4°C or below)

   Size:1 x 1ml

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

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

   Size: 1 x 35µl (300nM)

3. **Fluorescein-labeled Ubiquitin (300X)**

   Size:1 x 35µl (15µM)

4. **Biotinylated UBE2D3 (UbcH5c) (300X)**

   Size:1 x 35µl (1.5µM)

5. **Mg-ATP Solution (3mM)**

   Size:35µl (3mM)

6. **Microtiter plate** (store at RT)

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

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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

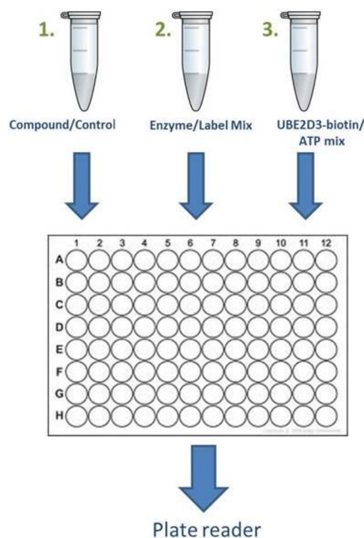
   b. Streptavidin-Tb – 5,000 tests (Cat. # 610SATLA) from CisBio.

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 10 to 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 is suggested), the enzyme reaction is initiated by the addition of Biotinylated UBE2D3/ATP Mix. The reaction proceeds rapidly, approaching completion in 5-10 minutes. It is imperative to add Biotinylated UBE2D3/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: Running pilot assays to optimize conditions and plate reader settings prior to running a full plate of compounds is highly recommended.***



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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 Biotinylated UBE2D3/ATP Mix

We recommend 45µl/well total assay volume, i.e. 15µl/well of each solution, for 1/2 –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 handling into the plate (i.e. pipetting losses or reservoir requirements):

1/2-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-Terbium (SA-Tb)**

   Reconsititute the streptavidin-terbium according to the manufacturer’s instructions.

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

   Dilute **Fluorescein-labeled Ubiquitin (300X),** , and **UBE1 Enzyme (300x)** 100-fold each in assay buffer for 3X concentrations of 150nM Fluorescein-labeled ubiquitin, and 3nM UBE1. Add sufficient volume of **Streptavidin-Tb** to achieve 1.5nM SA-Tb (3X) For example, 25µl of each of **Fluorescein-labeled Ubiquitin** and **UBE1 Enzyme** stock solution and 14 ul 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 UBE2D3 (300x)** 100-fold in assay buffer for a 3X concentration of 15nM

5. **Biotinylated UBE2D3/ATP Mix (3X)**

   Dilute **Biotinylated UBE2D3 (300X)** and **Mg-ATP (**3mM**)** 100-fold each in **assay buffer** for a 3X concentrations of 15nM 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%. Concentrations of DMSO above 2% inhibit UBE1.

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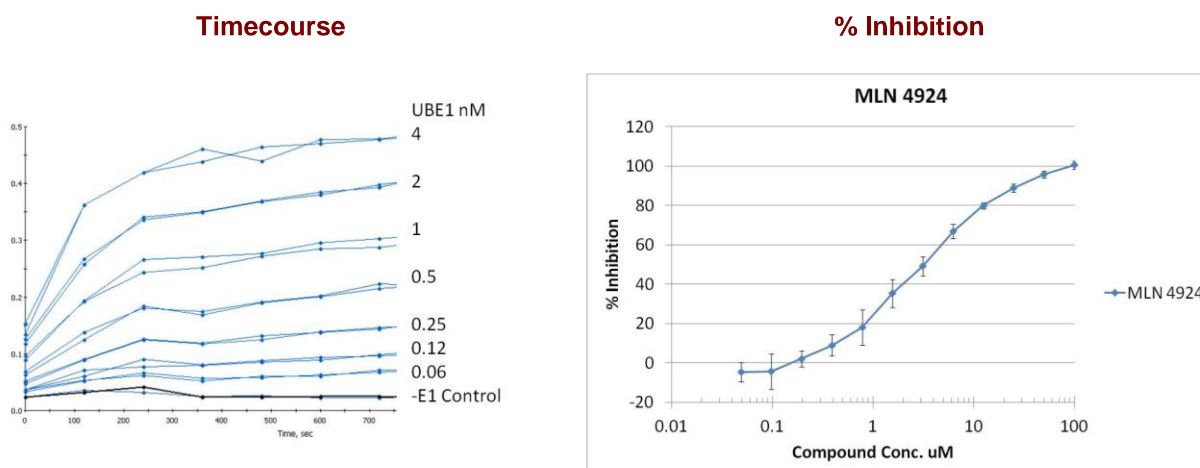
PROTOCOL

The following protocol is written for performing the assay in a 384-well plate with volumes recommended for 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 all 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 **Biotinylated UBE2D3/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 to 10 minute end-point.

SAMPLE DATA



Representative data for the E1/E2 LITE – Ubiquitin Transfer Assay. The left panel shows the progression of the reaction with increasing concentrations of UBE1. The right panel shows inhibition of the assay using 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].

REFERENCES

1. 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.

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