Fluorescent High-Throughput Conjugation and Deconjugation Assays for Ubiquitin-like Proteins SUMO and NEDD8

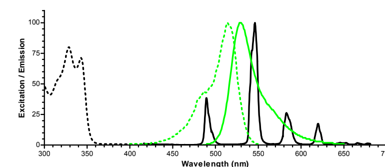
Robert Horton, Coby Carlson, Steve Riddle, and Kurt Vogel

Invitrogen Corporation • 1600 Faraday Avenue • Carlsbad, California 92008 • USA

Introduction

The broad involvement of ubiquitin and ubiquitin-like proteins (Ubls) in key regulatory processes, including proliferation of cancer cells, provides an attractive set of potential drug targets. Traditional methods for the analysis of ubiquitination include autoradiography, ELISA, or immunoblotting, which lack the necessary throughput to effectively identify modulators of these events in a high-throughput screening (HTS) environment. We have developed a set of reagents for monitoring conjugation and deconjugation of ubiquitin, the ubiquitin-like proteins SUMO-1/2/3, and Nedd8. By directly labeling ubiquitin and the ubiquitin-like proteins with a FRET donor (terbium) or a FRET acceptor (fluorescein), we have developed a flexible set of reagents for robust high throughput screening applications that can be read in either kinetic or endpoint mode. Additionally, the time-resolved and ratiometric format of the assays reduces interference from colored or fluorescent library compounds.

Figure 1 – Principle of LanthaScreen™ TR-FRET Technology



The excitation and emission spectra of the Tb chelate FRET donor (black) and Topaz GFP acceptor (green) are shown above. When the donor and acceptor are in proximity, FRET is detected. Because of the long fluorescent lifetime of the Tb chelate, FRET can be measured after interfering signals have completely decayed. This, in addition to the ratiometric readout, makes the LanthaScreen™ TR-FRET ideally suited for HTS, due to its resistance to common forms of assay interference.

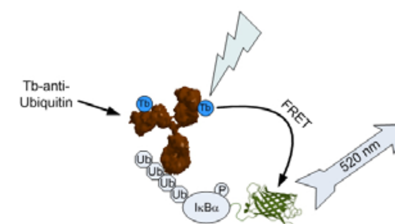
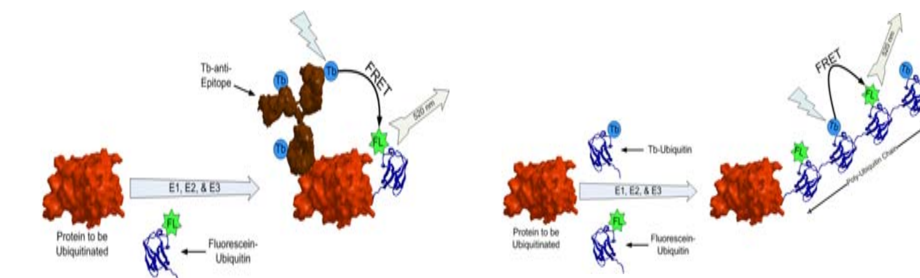


Figure 2 – LanthaScreen™ Conjugation Assays

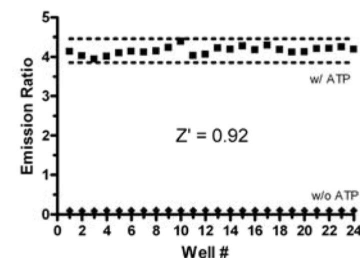


Detection of Ubiquitination of Epitope-tagged proteins

The anti-epitope ubiquitination assay utilizes fluorescein-labeled ubiquitin and a terbium labeled anti-epitope antibody to complete the TR-FRET pairing. The anti-epitope format can detect both mono- and polyubiquitination of the target protein.

Detection of Poly-Ubiquitin Chain Formation

When an epitope tag is not available, the intrachain ubiquitination assay format can be used. Since both the TR-FRET donor (Tb-ubiquitin) and acceptor (fluorescein-ubiquitin) are present in the polyubiquitin chain, no development step is required for the intrachain assay. This makes the intrachain assay especially useful when real-time kinetic information on ubiquitination is desired.

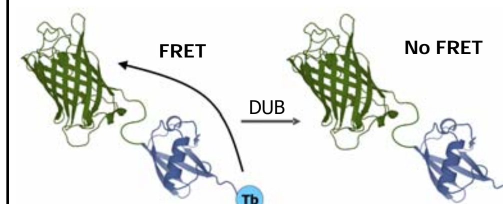


Assay Sensitivity

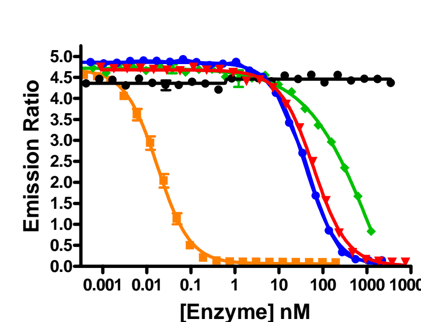
Z' values above 0.7 are commonly observed with the LanthaScreen™ ubiquitination assays.

Figure 4 – LanthaScreen™ Deubiquitination Assays

Deubiquitinating (DUB) enzymes proteolytically cleave ubiquitin from proteins. The functions of most DUB enzymes are not known, but it has long been speculated that DUBs play a regulatory role by "rescuing" target proteins from degradation by the proteasome. Recently, USP2 and UCH37 have been shown to deubiquitinate tumor-growth-promoting proteins, and other DUBs have been shown to be over expressed in cancer cells. Therefore inhibition of DUBs is of interest as a potential therapeutic strategy for treating cancer.



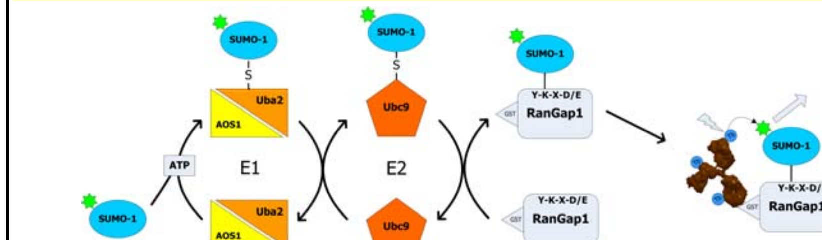
We have prepared an N-terminal GFP fusion of ubiquitin with a short C-terminal extension containing an engineered cysteine residue that has been labeled with a terbium chelate. The intact substrate shows a high degree of FRET, whereas DUB-dependant cleavage leads to a decrease in FRET.



Cleavage of LanthaScreen™ Deubiquitination Substrate

GFP-Ub-Tb was tested as a substrate (at 10 nM) against UCH-L3 (●), USP-2 (●), USP-15 (●), UCH-L1 (●), USP-5 (●) and USP-14 (●). USP-14 is not expected to show activity in the absence of association with components of the 26S proteasome. USP-2 and USP-15 are indistinguishable. The deubiquitination enzymes were purchased from BIOMOL.

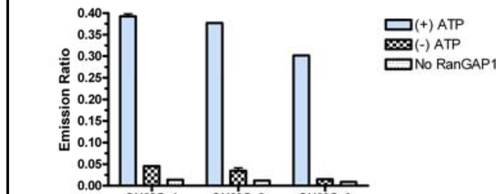
Figure 3 – LanthaScreen™ Ubiquitin-like Protein Conjugation Assays



Conjugation reactions with Ubiquitin-like proteins

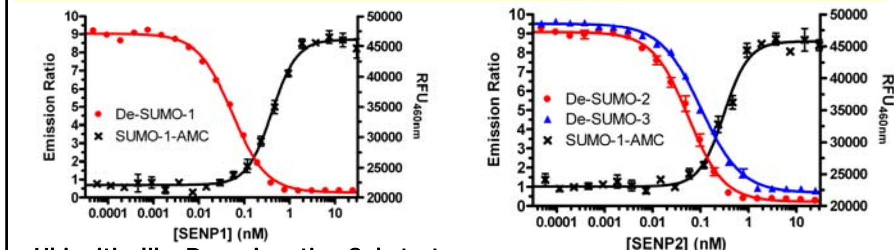
A similar principle has been applied to the ubiquitin-like proteins including SUMO-1/2/3 and NEDD8. In this particular example, fluorescein labeled SUMO-1 is conjugated to the Sumo activating enzyme (Aos1/Uba2) via a thioester bond in an ATP-dependent reaction. The charged SUMO activating enzyme then transfers the fluorescein-SUMO-1 to Ubc9. Ubc9 will attach the fluorescein-SUMO-1 to the target protein (GST-RanGAP1) via a isopeptide bond. At the completion of the SUMOylation reaction, a terbium-labeled anti-GST antibody is added to the reaction mixture to complete the FRET pairing.

The SUMOylation assay has been extended to other target proteins including SP100 and p53 (Data not shown).



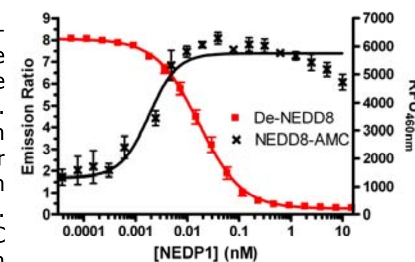
Representative data from the SUMOylation reaction outlined above is shown to the left. Similar reactions have been performed with related ubiquitin-like proteins including SUMO-2 or SUMO-3. On average, a ten-fold increase in the assay signal is observed. The E1, E2, and RanGAP1 were purchased from BIOMOL.

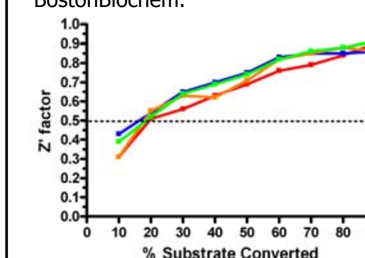
Figure 5 – LanthaScreen™ Ubl Deconjugation Substrates



Ubiquitin-like Deconjugation Substrates

Deconjugating substrates with the ubiquitin-like proteins (SUMO-1/2/3 and NEDD8) were developed in a manner analogous to the LanthaScreen™ deubiquitination substrate. These substrates were tested with a known deconjugation enzyme and compared to their respective AMC analogue ([LanthaScreen substrate] = 10 nM; [Ubl-AMC] = 100 nM). The deconjugation enzymes and the AMC analogues were purchased from BostonBiochem.





Assay Sensitivity

To evaluate assay sensitivity, Z' calculations were performed with the LanthaScreen™ De-SUMO-1 substrate (●), De-SUMO-2 (●), De-SUMO-3 (●), and De-NEDD8 (●). Z' values of ≥ 0.5 were obtained with as little as 20-30% conversion of the deconjugation substrates to product.

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