TECHNICAL BULLETIN

Ubiquitinating Enzyme Systems

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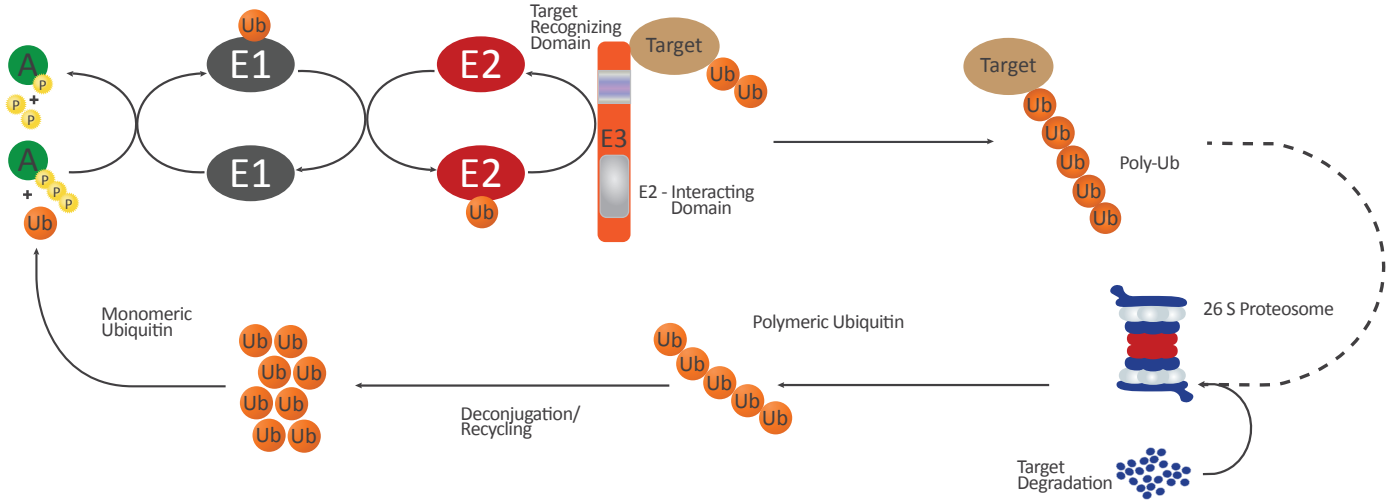

Protein modifications by ubiquitin (Ub) or ubiquitin-like proteins (UBLs) participate in many critical cellular processes such as cell-cycle regulation, DNA repair, oncogenesis, antiviral pathways and most notably, proteasomal degradation of target proteins. Ubiquitination and modification by UBLs share a similar catalytic cascade which requires the sequential action of three classes of enzymes: E1 activating enzymes, E2 conjugating enzymes and E3 ligases. Recent research has linked dysregulation of the Ub/UBLs modification system to numerous diseases including cancer, immunological disorders and neurodegeneration. Thus, the high substrate specificity provided by combinations of over 30 E2's and over 600 E3's makes these enzymes emerging drug targets.

In response to a growing market demand, SignalChem has developed an extensive array of products encompassing enzymes, Ub/UBL modifiers and substrates in the ubiquitination, SUMOylation, ISGylation and NEDDylation processes. Using Promega's AMP-Glo™ technology and an optimized assay protocol, we have identified and validated a variety of functional combinations of the enzyme components. With the established protocol, each enzyme in the catalytic cascade has been assessed for their activity towards generation of free AMP. In addition, inhibition profiles of the ubiquitinating enzymes have been obtained using the assay system, further demonstrating their potential to be used in high-throughput screening to identify lead compounds for drug discovery and development programs.

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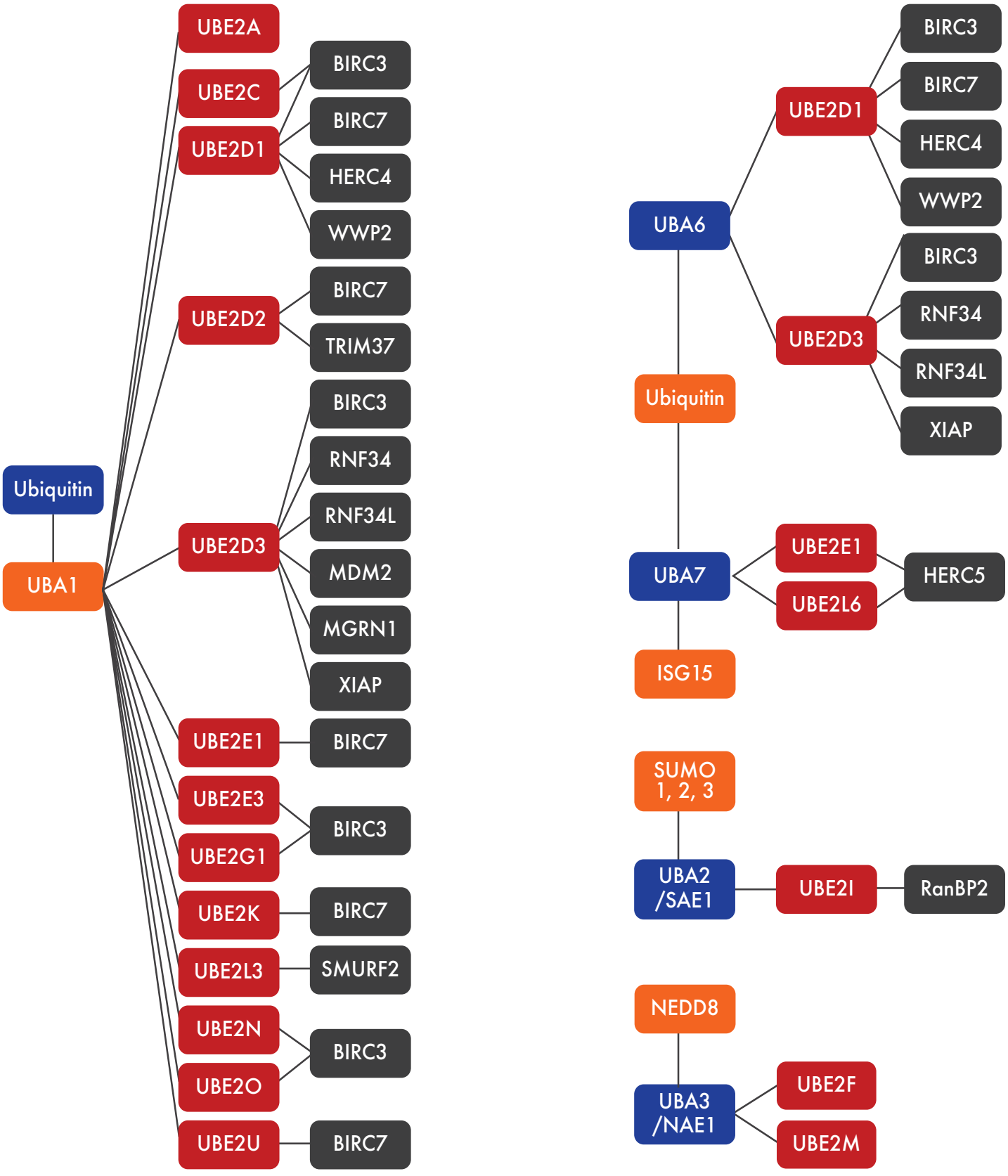

Figure 2. Combinations of ubiquitinating enzymes that are functionally validated at SignalChem.

Methods for Enzyme Assay

In order to obtain quantitative activity data for the different classes of enzymes catalyzing the ubiquitination cascade in a consistent setting, we have chosen to use the AMP-Glo™ Assay (Promega). By detecting the amount of the universal AMP generated on a bioluminescent assay platform, this method has the benefit of homogeneity, unparalleled sensitivity, high dynamic range and the potential to be adapted for high-throughput screening.

Because ubiquitin or ubiquitin-like protein (UBL) conjugation is proportional to AMP production, the presence of all components of the conjugation machinery (Ub or UBL, E1, E2 and E3) is required for maximal activity of the system. By progressively adding each of the three enzyme components, one can also determine their individual activities.

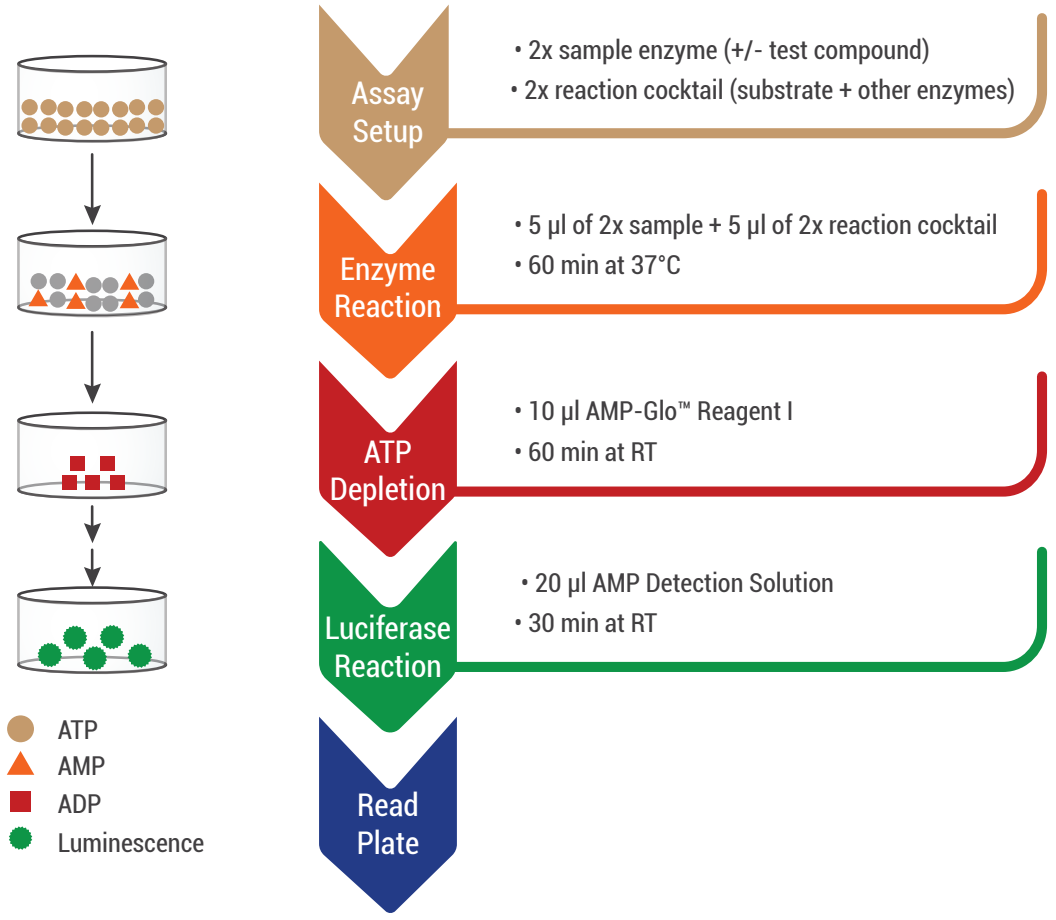

Figure 3. Assay principle and general procedure for detection of ubiquitinating enzyme activity (Ref. 1). Note: the assay volume described in figure is suitable for 384-well or half-area 96-well plate. For other plate formats please refer to AMP-Glo™ Assay Technical Manual. The reaction time and temperature can also be adapted to individual requirement.

Reagents and Materials

Ubiquitinating enzyme samples
Ubiquitin or ubiquitin-like protein (SUMOs, ISG15, NEDD8, etc.)
Protein substrate
Ubiquitination Buffer
1M DTT
Test compounds of interest (optional)
Assay plate
Plate sealer
Incubator with thermostat
Multi- or single-channel pipettes
Luminometer
AMP-Glo™ Assay (Promega, Catalog #: V5011)

- AMP, 10 mM
- Ultra Pure ATP, 10mM
- AMP-Glo™ Reagent I
- AMP-Glo™ Reagent II
- Kinase-Glo™ One Solution

Directions in Designing Enzyme Assays

SignalChem's wide range of ubiquitinating enzymes have been extensively tested for their in vitro activity using AMP-Glo Assays. While challenged by the inherent complexity of the ubiquitination system, in particular the concerted yet progressive action of multiple components, we have taken several approaches to validate function of these enzymes and explore their capacity for use in biochemical characterizations.

Combining Enzymes for Ubiquitin Conjugation

The specificity of the ubiquitination pathway is achieved at the level of the E2 and E3 enzymes. Although knowledge of active E2/E3 pairs is critical in guiding biological studies of the pathway as they remain largely undiscovered. The difficulty in identifying these functional pairs partly arises from the modest affinity and transient nature of E2/E3 complexes, as well as the lack of an effective tool to systematically predict E2-E3 interactions (Ref. 2).

By taking advantage of our access to an extensive array of ubiquitinating enzymes, we have been able to design quantitative assays to compare the ubiquitination activity of different E2/E3 combinations (Figure 4). These results have so far been consistent with the E2-E3 physical interactions reported earlier (Ref. 3).

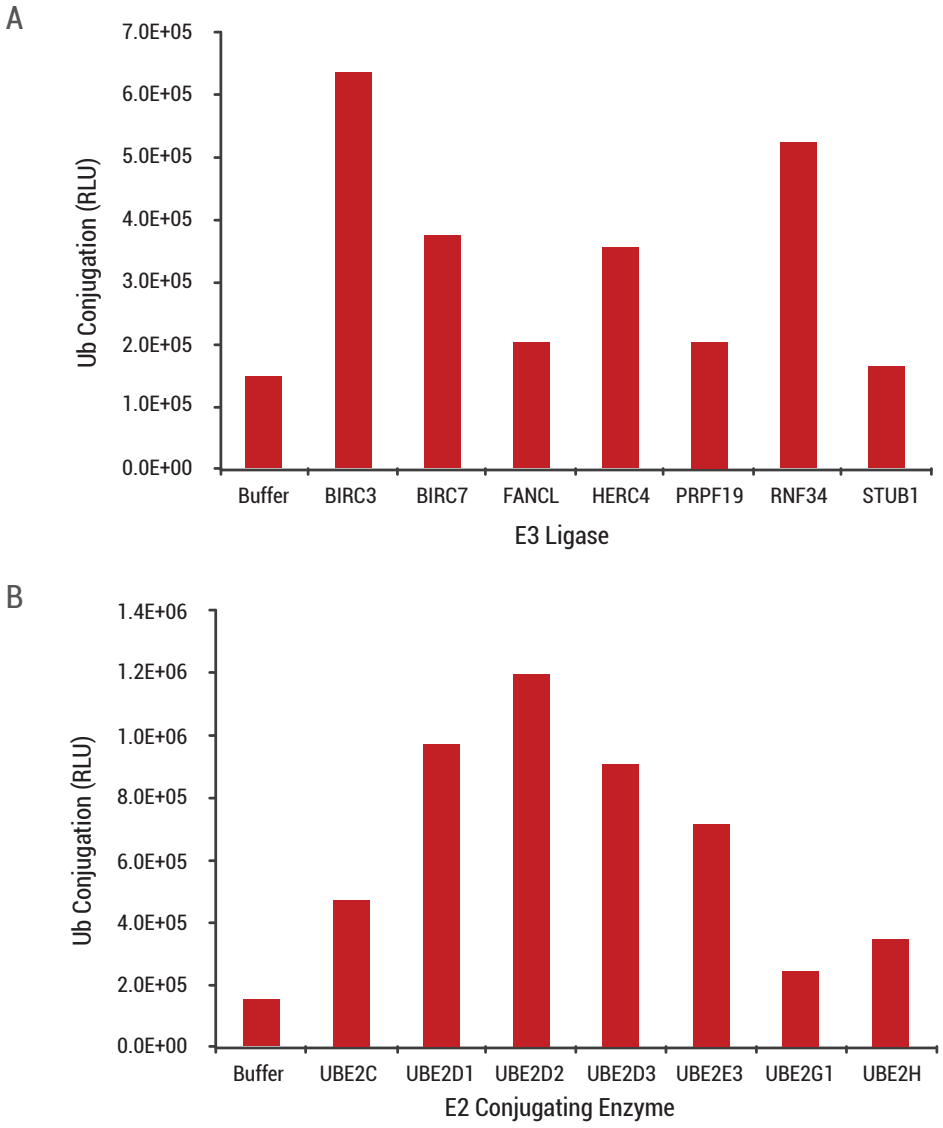

Figure 4. Ubiquitin conjugation catalyzed by various combinations of ubiquitinating enzymes. Ubiquitination reaction containing **A.** Ub (10 μM), UBA1 (25 nM), UBE2D3 (400 nM) and several different E3 ligases (100 nM), and **B.** Ub (10 mM), UBA1 (25 nM), RNF34 (100 nM) and several different E2 conjugating enzymes (400 nM), was initiated by the addition of 25 μM ATP. The reactions were incubated at 37°C for 60 minutes. The AMP-Glo assay was performed. Luminescence was recorded on a GloMax® Multi+ Detection System. Results shown are averages of duplicate wells. Ubiquitin conjugation is represented in relative light units (RLU).

Optimizing Enzyme Concentration

For many enzyme reactions, the velocity is often directly proportional to the enzyme concentration showing a linear dependence, in contrast to the hyperbolic dependence on substrates and cofactors. When planning an assay, the amount of the enzyme should be as low as possible- only catalytic amounts are necessary, whereas the concentration of all substrates and cofactors directly involved in the enzyme reaction should be saturating, so that they will not be rate limiting. As a prerequisite prior to performing most high-throughput assays, the concentration of enzymes must be optimized. Here we show example data of E1, E2 and E3 titrations, from which the linear range of different enzymes at given assay conditions can be observed.

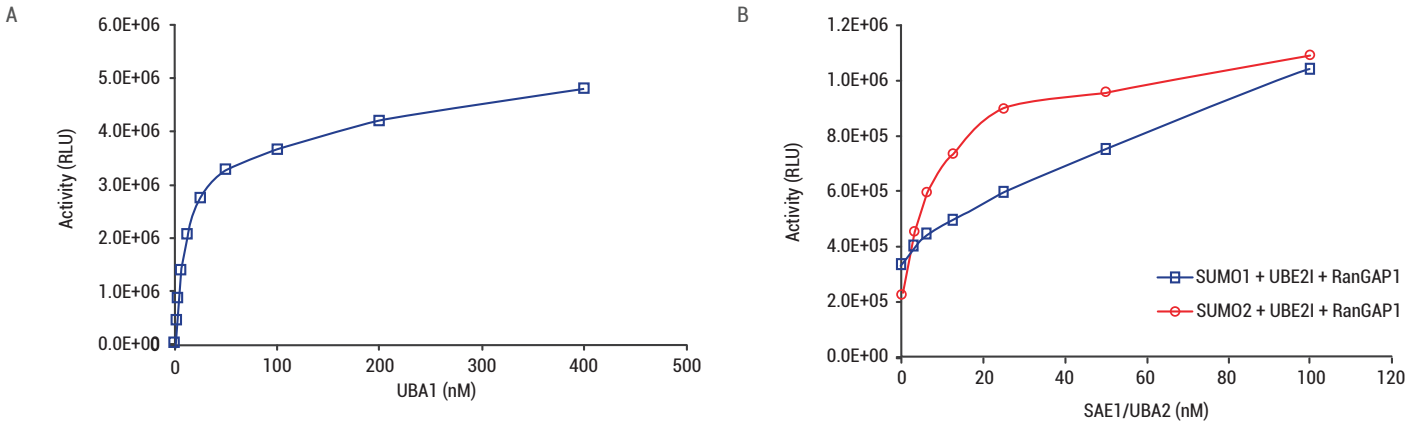

Figure 5. Titration of E1 activating enzymes assayed in 96-well plates using the AMP-Glo Assay. Panel A. Recombinant human UBA1 was serially diluted in Ubiquitination Buffer (40 mM Tris (pH 7.5), 20 μM $MgCl_2$, 0.1 mg/ml BSA, 0.5 mM DTT) and mixed with UBE2C (800 nM), BIRC3 (300 nM) and Ub (10 μM). Ubiquitination reaction was initiated by adding 25 μM ATP and incubated at 37°C for 45 minutes. **Panel B.** Co-expressed recombinant human SAE1 and UBA2 enzymes (1:1 molar ratio) were serially diluted in SUMOylation Buffer (50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM DTT) and mixed with UBE2I (400 nM), SUMO1 or SUMO2 (20 μM) and RanGAP1 (2 μM). SUMOylation reaction was initiated by adding 25 μM ATP and incubated at 30C for 120 minutes. Results shown are averages of duplicate wells. Activity of enzyme is represented as relative light units (RLU).

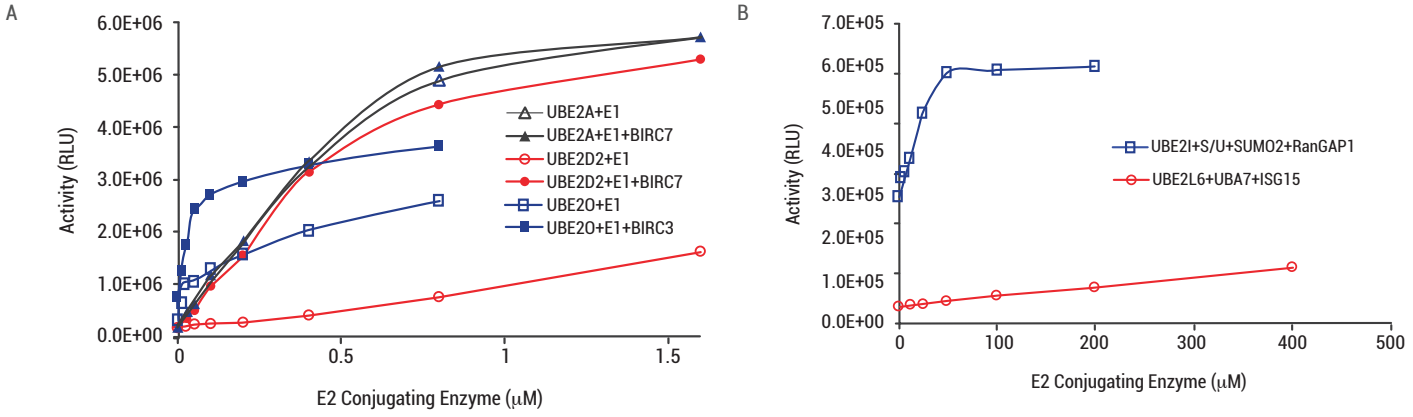

Figure 6. Titration of E2 conjugating enzymes assayed in 96-well plates using the AMP-Glo Assay. Panel A. Recombinant human UBE2A, UBE2D2 or UBE2O was serially diluted in Ubiquitination Buffer and mixed with UBA1 (50 nM), BIRC7 (400 nM) or BIRC3 (200 nM) and Ub (10 μM) as indicated. Ubiquitination reaction was initiated by adding 25 μM ATP and incubated at 37°C for 60 minutes. **Panel B.** Recombinant human UBE2I or UBE2L6 was serially diluted in SUMOylation Buffer and mixed with SAE1/UBA2 (10 nM), SUMO2 (20 μM) and RanGAP1 (2 μM), or UBA7 (25 nM) and ISG15 (10 μM), respectively as indicated. SUMO or ISG15 conjugation reaction was initiated by adding 25 μM ATP and incubated at 30°C for 120 minutes. Results shown are averages of duplicate wells. Activity of enzyme is represented as relative light units (RLU).

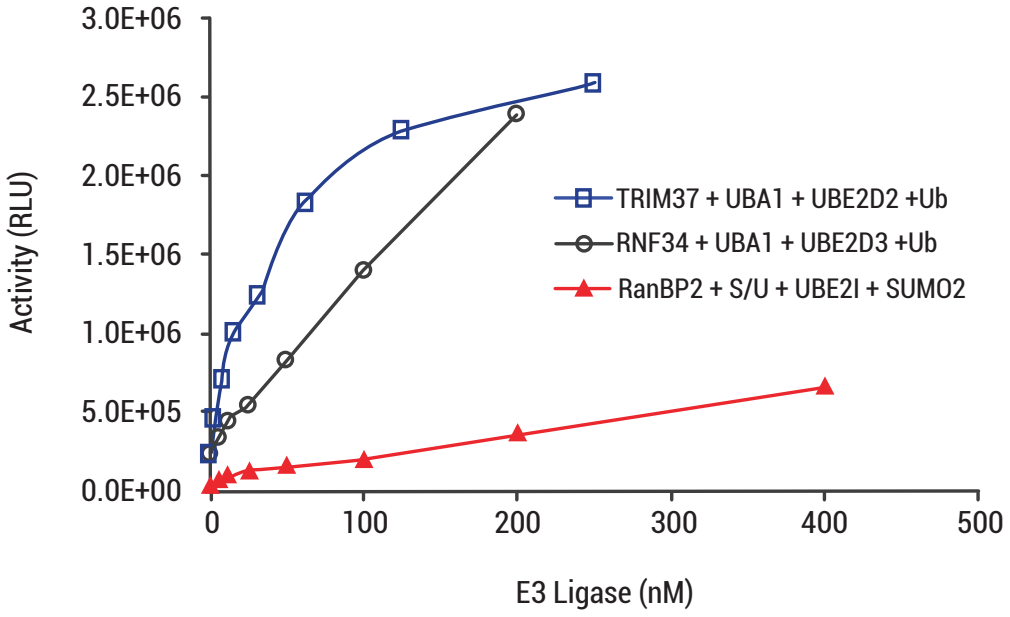

Figure 7. Titration of E3 ligases assayed in 96-well plates using the AMP-Glo Assay. Recombinant human TRIM37, RNF34 or RanBP2 was serially diluted in reaction buffer and mixed with UBA1 (50 nM), UBE2D2 (400 nM), Ub (10 μM), or UBA1 (50 nM), UBE2D3 (400 nM), Ub (10 M), or SAE1/UBA2 (10 nM), UBE2I (250 nM), SUMO2 (20 μM), respectively as indicated. Conjugation reaction was initiated by adding 25 μM ATP to all wells and incubated at 30°C for 120 minutes. Results shown are averages of duplicate wells. Activity of enzyme is represented as relative light units (RLU).

Determining Compound IC_{50}

Although the dynamic rearrangement of multiple protein-protein interactions within the ubiquitination machinery has traditionally posed a deep challenge to disrupt with small molecules, the multi-layered system apparently offers several possibilities for therapeutic intervention (Ref. 4). Using optimized combinations of ubiquitinating enzymes, we have tested inhibition of E1, E2 and E3 with known inhibitors on the AMP-Glo assay platform. The IC_{50} values we obtained have been agreeable to those previously reported (Ref. 5, 6). Here we also report, for the first time, IC_{50} values of the anti-inflammatory drug Bay11-7082 (a.k.a. Bay11-7821) on the inhibition of UBE2N and UBE2L3 E2 conjugating enzymes.

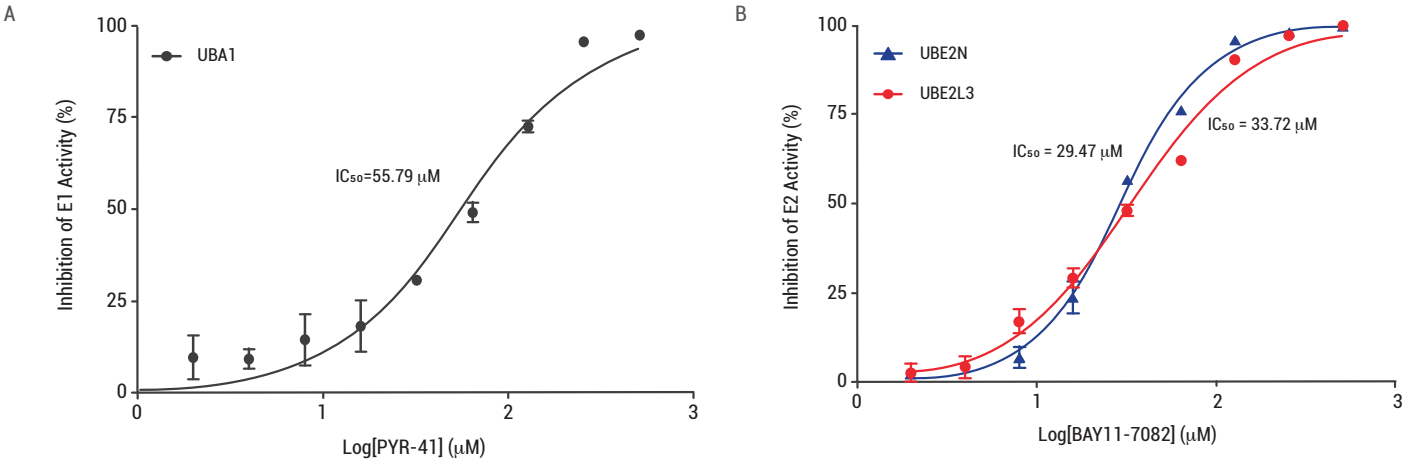

Figure 8. Inhibition of ubiquitination at different stages of the enzymatic cascade. The inhibitor concentrations that result in 50% inhibition (IC_{50}) of UBA1, UBE2N, UBE2L3 activity were determined for the specific inhibitors indicated. **Panel A.** Inhibition of UBA1 thioester bond formation by PYR-41. Inhibitor was serially diluted in Ubiquitination Buffer, combined with human recombinant UBA1 (100 nM), UBE2A (400 nM), Ub (10 μM) and pre-incubated at room temperature for 15 minutes. **Panel B.** Inhibition of UBE2N or UBE2L3 thioester bond formation by BAY11-7082. Inhibitor was serially diluted in Ubiquitination Buffer, combined with human recombinant UBA1 (3 nM), UBE2N (300 nM) or UBE2L3 (300 nM), Ub (5 μM) and pre-incubated at room temperature for 15 minutes. Reaction was initiated by adding 25 μM ATP to all wells and incubated at 30°C for 45 minutes. Background luminescence was subtracted from all data points. IC_{50} values were calculated from nonlinear regression fitting to a Variable Slope model using GraphPad Prism software.

Calculating Enzyme Specific Activity

Specific activity is a measure of enzyme processivity, at a specific (usually saturating) substrate concentration, and is usually constant for a pure enzyme. In protein isolation, the specific activity indicates the percentage of purification. It is the amount of product formed in an enzymatic reaction in a given amount of time under given conditions per milligram of total protein.

As with many other active enzymes marketed by SignalChem, the specific activity of ubiquitinating enzymes has been determined. The following equation was used to calculate the specific activity:

$$\text{Specific Activity} = \frac{[\text{AMP}](\mu\text{M}) \times \text{Reaction Volume}(\mu\text{l})}{\text{Reaction Time (min)} \times \text{Enzyme Amount (mg)}} \times 10^{-3} \text{ (nmol/min/mg)}$$

To obtain the concentration of AMP, a product of the enzyme reaction, a standard curve must be created at the same time the test enzyme is assayed. One can then calculate ΔRLU for the sample with and without the test enzyme, and use the ΔRLU to calculate AMP concentration from the standard curve (Figure 9).

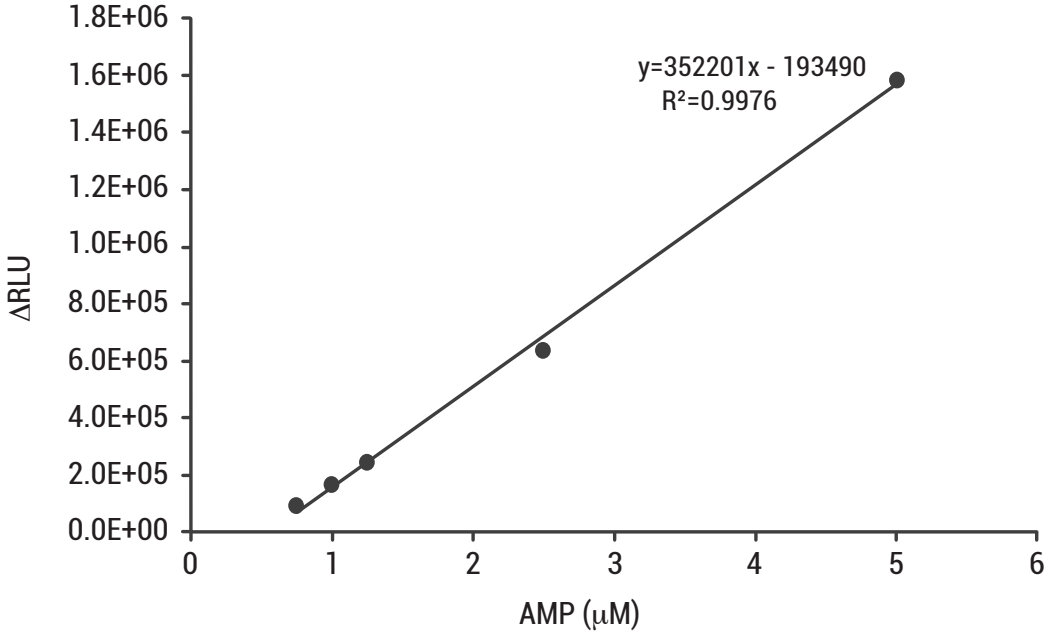

Figure 9. AMP standard curve. Each AMP standard solution was prepared in Ubiquitination Buffer, by mixing various amounts of AMP and ATP with a combined total nucleotide concentration of 25μM. Luminescence was recorded following AMP-Glo assay as described in Section 2. The difference in relative luminescence unit (ΔRLU) between the RLU at each AMP concentration and theRLU of the no-AMP (ATP only) control was plotted against each corresponding AMP concentration. The standard curve shown here covers the range of signals obtained from AMP-Glo assays using SignalChem's ubiquitinating enzymes. Each data point represents the average of duplicate wells.

References

1. Mondal S, Hsiao K, Goueli SA. A bioluminescent assay for monitoring conjugation of ubiquitin and ubiquitin-like proteins. Anal Biochem. 2016;510:41-51.

2. Wenzel DM, Stoll KE, Klevit RE. E2s: structurally economical and functionally replete. Biochem J. 2011;433(1):31-42.

3. Van wijk SJ, De vries SJ, Kemmeren P, et al. A comprehensive framework of E2-RING E3 interactions of the human ubiquitin-proteasome system. Mol Syst Biol. 2009;5:295.

4. Hoeller D, Dikic I. Targeting the ubiquitin system in cancer therapy. Nature. 2009;458(7237):438-44.

5. Yang Y, Kitagaki J, Dai RM, et al. Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. Cancer Res. 2007;67(19):9472-81.

6. Strickson S, Campbell DG, Emmerich CH, et al. The anti-inflammatory drug BAY 11-7082 suppresses the MyD88-dependent signalling network by targeting the ubiquitin system. Biochem J. 2013;451(3):427-37.

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