Application of the Transcreener™ AMP/GMP Assay for Interrogating Ubiquitinylation Cascades

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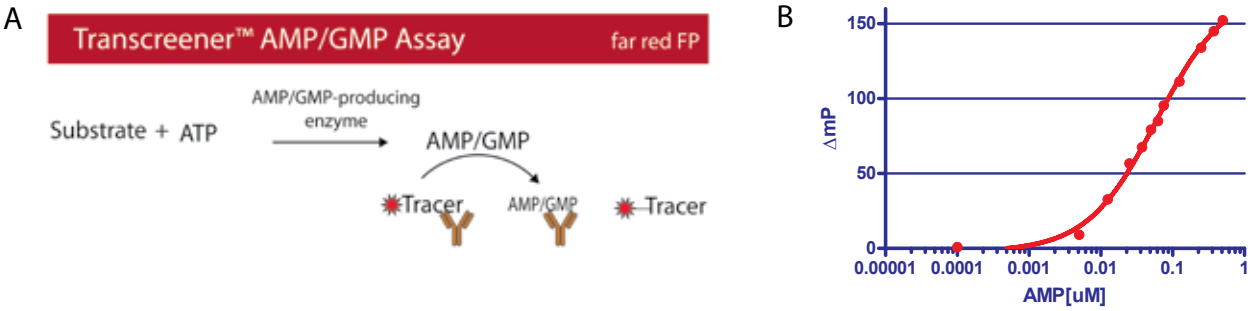
Introduction

Ubiquitin and ubiquitin-like molecules such as Ufm1, SUMO1, NEDD8 are important post-translational modifications that control the concentration of cellular proteins. Ubiquitins are ligated to their targets by related but distinct enzymatic cascades involving E1s (ubiquitin activating enzymes), E2s (ubiquitin conjugating enzymes), and E3s (ubiquitin ligases). Because of the complexity of the system and the large number of E3 enzymes present in the cell, development of robust biochemical HTS assays for ubiquitin ligases have been problematic. Previously developed assays are specific for a given E3/target protein pair. The E1 enzymes operate like many ligases, utilizing the phosphodiester bond of ATP to drive formation of a thioester linkage between ubiquitin and a catalytic cysteine and releasing AMP in the first step of the ubiquitin cascade pathway. Here we explore the use of the Transcreener AMP/GMP assay, which enables homogenous immunodetection of AMP, as a generic method for measuring protein ubiquitinylation. We examined an E3-dependent system comprised of the ubiquitin activation enzyme UBE1L2, the ubiquitin conjugating enzyme UbcH7, Parkin (an E3 ligase) and its target protein RanBP2. Low levels of AMP formation were detected using UBE1L2 alone, and AMP formation increased as each successive component of the enzyme cascade was added. Ongoing studies are focused on optimizing the levels of each enzyme in the cascade to maximize the target dependent assay signal. These studies demonstrate the concept of using AMP detection to measure the activity of protein ubiquitinylation enzyme cascades and suggest that the Transcreener AMP/GMP Assay could also be used as an *in vitro* assay method for similar modifications like sumoylation, isgylation, and neddylation.

Figure 1.

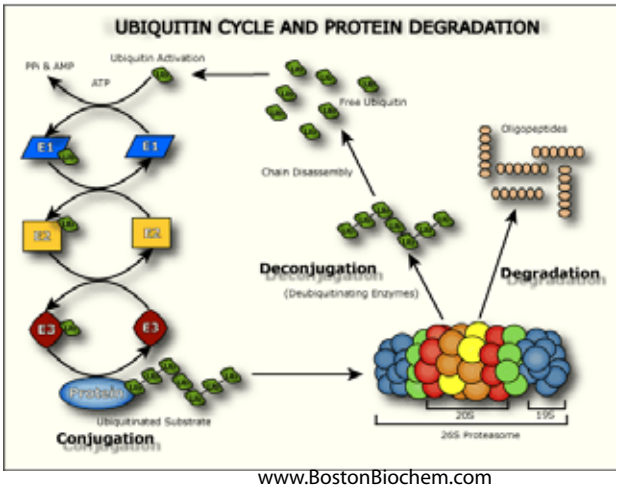
Assay Principle: Transcreener™ AMP-GMP Assay



The Transcreener assay platform developed at BellBrook Labs detects the reaction product of a group transfer reaction using homogenous fluorescent immunodetection. Ubiquitination is one such application of Transcreener AMP detection assay in which the AMP generated upon ubiquitin binding to the enzyme E1 can be detected and measured as millipolarization units. Figure 1A depicts the principle of Transcreener AMP-GMP assay. Figure 1B shows a typical standard curve for AMP. Error bars represent standard deviations of the mean (n=8).

Figure 2.

Ubiquitinylation Pathway



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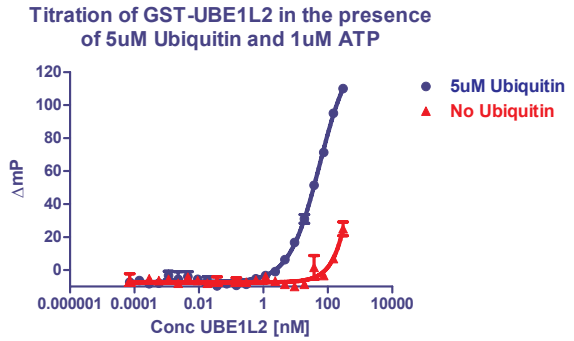
E1 Rxn: ATP + Ub + E1 → AMP + PPi + E1-Ub
E2 Rxn: E1-Ub + E2 → E1 + E2-Ub
E3 Rxn: E2-Ub + Target Protein → E2 + Target Protein-Ub

In some cases, E3 is also ubiquitinated as part of the cascade. Most target proteins are poly-ubiquitinated.

Figure 3.

AMP Formation by E1, UBE1L2, is Ubiquitin Dependent

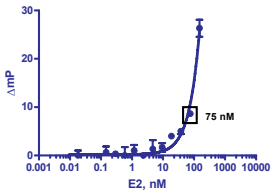


A proportional increase in ΔmP was observed with increasing concentrations of UBE1L2 in the presence of 5μM ubiquitin and 1μM ATP. Plates were read after 120 minutes essentially to completion at 30°C on a Tecan Safire^{2TM} reader. A small amount of AMP formation was seen at very high concentrations of GST-UBE1L2 in the absence of ubiquitin.
Error bars represent standard deviations of the mean, n=3.

Figure 4.

Optimization of UBE1L2 Concentration in the Presence of UbCH7

An optimal concentration of 2nM of UBE1L2 that gave a 30 mP shift was selected for further evaluation of E3. Pilot experiment was performed to determine maximal amount of E2 that could be tolerated without causing significant AMP formation (see inset).
Error bars represent standard deviations of the mean, n=3.



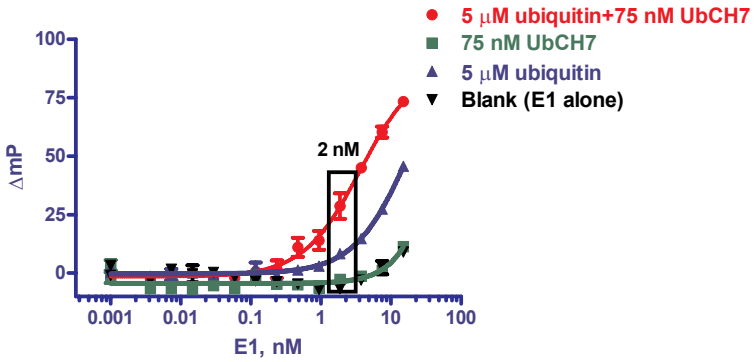
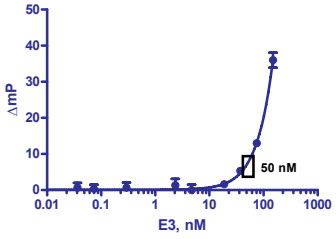


Figure 5.

Optimization of E2 (UbCH7) Concentration in the Presence of E3

An optimal concentration of 25 nM UbCH7 that gave a 20 mP shift was selected for further evaluation of E3. Pilot experiment was performed to determine optimal E3(see inset).
Error bars represent standard deviations of the mean, n=3.



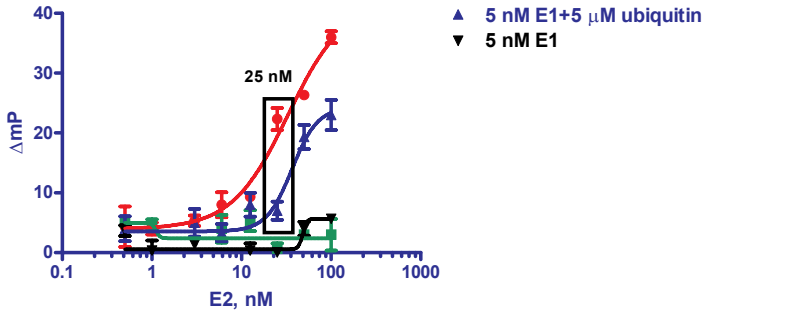


Figure 6.

Optimization of E3 (His Parkin) Concentration in the Presence of RanBP2, target protein

An optimal concentration of 15 nM E3 that gave a 40 mP shift was selected for further evalaution of target protein. Pilot experiment was performed to determine optimal concentration of RanBP2 (see inset). Error bars represent standard deviations of the mean, n=3.

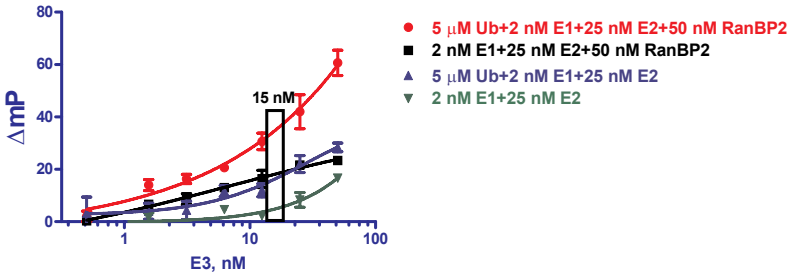
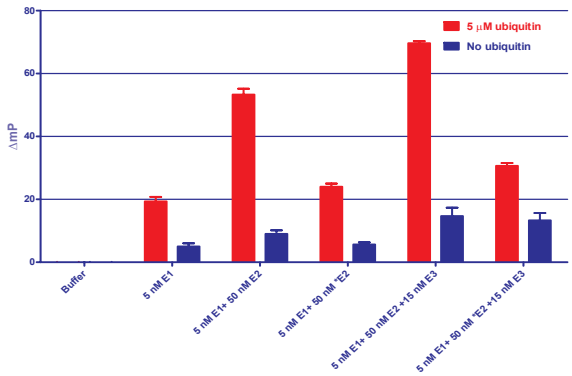


Figure 7

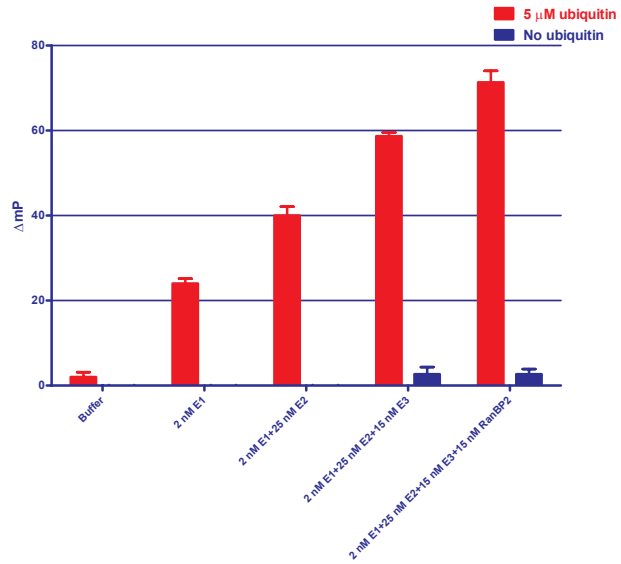
Disruption of the Cascade with an Inactive E2 Mutant Reduces AMP Formation



E2* with a point mutation in its active site makes it inactive and therefore incapable of forming a bond with ubiquitin. No AMP enhancement is seen in the presence of this inactive mutant. Error bars represent standard deviations of the mean, n=3.

Figure 8

Sequential AMP Enhancement in the Ubiquitin Cascade Pathway



Catalytic quantities of E1, E2 and E3 were sequentially combined with 5 μM ubiquitin. The target protein, Ran BP2, was used at 15 nM, a relatively low concentration, because it is reportedly poly-ubiquitinated. Error bars represent standard deviations of the mean, n=3.

Conclusions

- Ubiquitin dependent AMP formation by UBE1L2 can be detected using the Transcreener™ AMP/GMP Assay.
- An enhancement on AMP production was seen by titrating limiting amounts of ubiquitin ligase enzyme with the next sequential enzyme in the pathway.
- Use of an inactive E2 mutant reduced E2- and E3-dependent AMP formation.
- Ubiquitin dependent AMP production was observed upon sequential addition of E1, E2, E3 and target protein, the components of ubiquitination pathway.
- Further optimization of pathway components for target protein-dependant AMP formation is ongoing.

Materials and Methods

Ubiquitin Activating Enzyme (UBE1L2), Ubiquitin Conjugating Enzyme (UbCH7), Ubiquitin Ligase (His-Parkin), Ubiquitin and Inactive Ubiquitin Conjugating Enzyme (UbCH7*) were purchased from Boston Biochem. The target protein RanBP2-delta fragment was purchased from Biomol. The Transcreener AMP-GMP assay consisted of 15 μL reactions at 30° C, incubated for at least 2 hours. The 15 μL reaction was set in two parts-7.5 μL of 2X concentration of enzymes in master mix that consisted of 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM EGTA, 1% DMSO, and 0.02% Brij-35 in the presence or absence of 5 μM Ubiquitin. The ubiquitination reaction was thereafter started by adding additional 7.5 μL of 2X concentration of 1 μM ATP, 2 nM AMP/GMP AlexaFluor 633 tracer, 3.5 μg/mL of AMP/GMP antibody in 50 mM HEPES and 0.02% Brij-35. The reactions were initiated with the addition of ATP and were allowed to proceed until the reaction was completed. The assays were performed in black Corning 384-well, round bottom, low volume, polystyrene, non-binding surface plates (catalog # 3676). The plates were read using Tecan Safire^{2TM} at 635 emission and 670 excitation. Curve fitting and IC_{50} determinations were processed with Prism® software.

References

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