UbiQ
targeting the ubiquitin system

In vitro ubiquitination assays

version	V1, July-2016
applicable for	UbiQ Triple E Probes, Activity-based probes for E1-E2-E3 enzymes
reference	Mulder, M. P. C. et al. a cascading activity-based probe sequentially targets E1–E2–E3 ubiquitin enzymes. *Nature chemical biology.* **12,** 523–530 (2016). doi:10.1038/nchembio.2084.

protocol

stability

1. assess the stability of thioether-linked UBE2D3–UbDha adduct by incubating 5 µM purified thioether conjugate at 37°C in combination with 2 µM TRAF6 or 2 µM GST–NEDD4

The stability of the UBE2N–UbDha adduct was assessed similarly but with the accessory E2 variant UBE2V2 with or without TRAF6

1. samples were collected at 0-, 1-, 3-, and 24-h time points for visualization on Coomassie-stained SDS–PAGE gels
2. control reactions containing 0.5 µM UBE1, 5 µM E2, 20 µM Ub and the relevant activating factors demonstrate the Ub chain formation activity under normal *in vitro* conditions

competitive inhibition

Single-turnover ubiquitination assays were established for UBE2N with an initial activation stage containing

1. 10 µM UBE2N, 0.5 µM UBE1, 20 µM Ub, 2.5 mM ATP, and 5 mM $MgCl_2$ to generate native thioester-linked UBE2N~Ub after 30 min at 37 °C
2. this reaction was then quenched by addition of apyrase to prevent any further formation of UBE2N~Ub.
3. preactivated UBE2N~Ub was then diluted twofold into a reaction containing 5 µM UBE2V2 alone or in addition to 1µM GST–cIAP
4. samples were collected at 0-, 15-, and 30-min time points for visualization of diUb product formation on Coomassie-stained SDS–PAGE gels
5. to test for competitive inhibition using the thioether-linked UBE2N–UbDha adduct, increasing concentrations (1.25 µM, 2.5 µM, 5 µM, 10 µM, and 20 µM) of purified conjugate were added to reactions containing UBE2N~Ub, UBE2V2, and GST–cIAP as specified above
6. samples were collected at 30-min intervals to compare levels of diUb product formation with the control experiment

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