

High Throughput Assays for Inhibitors of Ubiquitin Carrier (E2) and Ubiquitin Ligase (E3) Proteins

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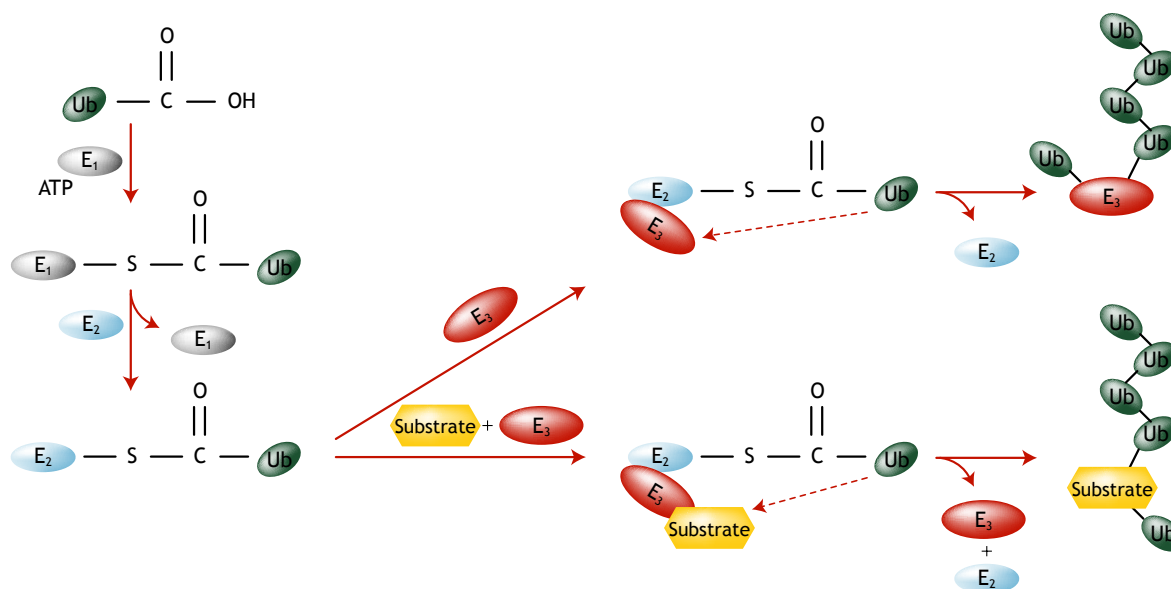
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1 Abstract

The emergence of specific information on ubiquitylation and the enzymes that affect these processes provides an increasing understanding of the biological phenotypes associated with the genetic or biochemical manipulation of various effectors of ubiquitylation. E3s constitute a family of proteins that select protein substrates for ubiquitylation and facilitate the transfer of ubiquitin from the carrier proteins (E2s) to substrate proteins. We present high-throughput *in vitro* assays for the measurement of E2 and E3 biochemical activities using Meso Scale Discovery's MULTI-ARRAY™ technology. Among others, we developed the E3 assays for MDM2, HRD1, A07/RNF25, MURF1/RNF28, RNF41/NRDP1, and EFP/TRIM25 (RING finger E3s), and Smurf1 and Nedd4 (HECT domain E3s). Four of the E3 assays were used in parallel screens for inhibitors from our library of ~150,000 compounds. Cross-correlation of the data allowed us to characterize the specificities of the inhibitors for each E3. The versatility of this screening approach demonstrates its general applicability.

2 Ubiquitylation

Ubiquitylation results in formation of *iso*-peptide bond between the C-terminal Gly-76 of Ub and an ϵ -amino group of one of internal Lys residues of substrate protein. Ubiquitylation of proteins occurs through a series of enzymatic steps involving E1, E2 and E3 proteins. Ub is first activated in an ATP-dependent step to form a thiol-ester with a specific Cys residue of E1 enzyme. Activated Ub is then transferred to one of a family of Ub-conjugating enzymes (E2). Finally, a Ub ligase (E3) selects protein substrates for ubiquitylation and transfers activated Ub from E2 to one of the lysines of a protein substrate. Most E3s can also self-ubiquitylate.



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3 E3s as Potential Drug Targets

E3s of the RING finger family:

E3	Substrate	Disease	Reference
MDM2	P53	Cancer	1,2
MURF1	?	Muscle atrophy	3
HRD1	?	Arthritis	4
A07/RNF25	p65?	Inflammation	5
NRDP1/RNF41	BRUCE, ErbB3		6,7
EFP/TRIM25	14-3-3 σ	Breast Cancer	8

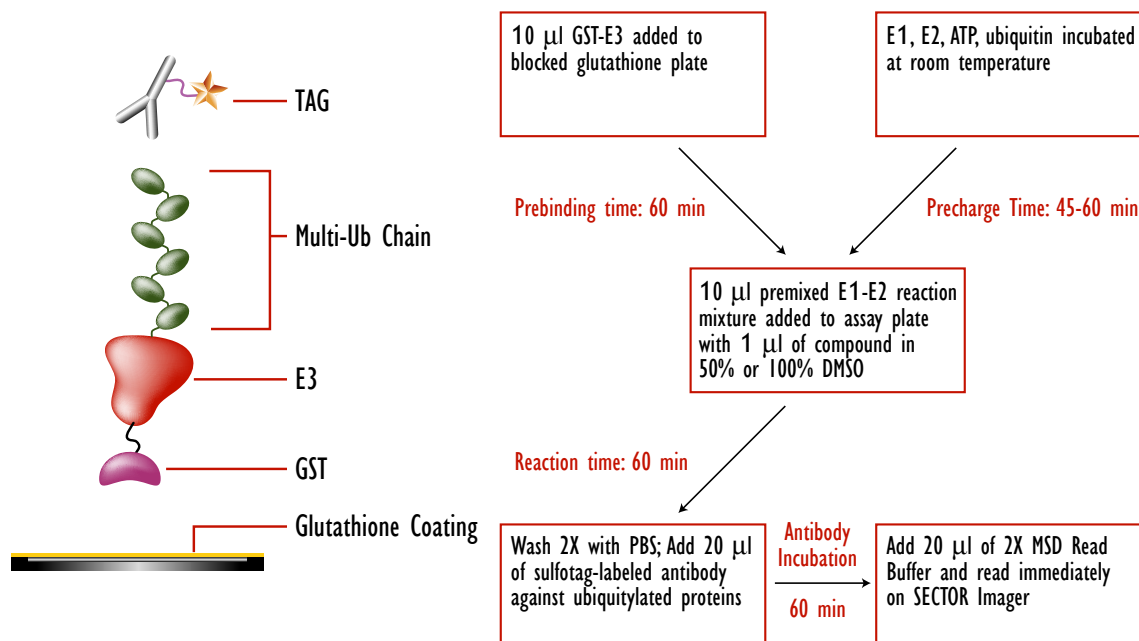
E3s of the HECT domain family:

E3	Substrate	Disease	Reference
SMURF1	SMAD1, SBFA1	Bone disorders	9
NEDD4	Gag	Retroviral infection	10

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4 E3 Self-Ubiquitylation Assay Format

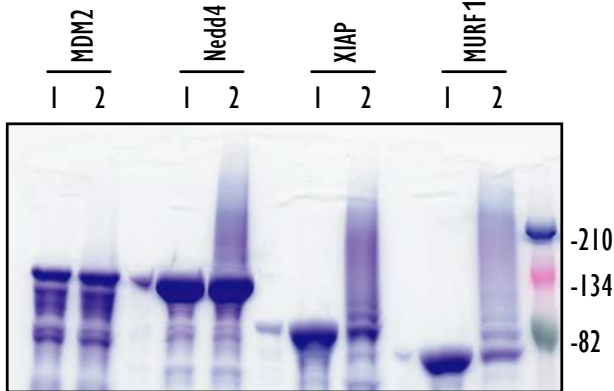


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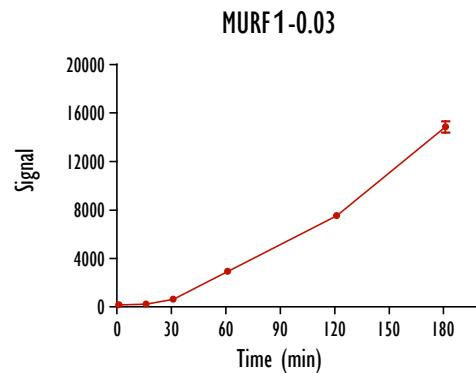
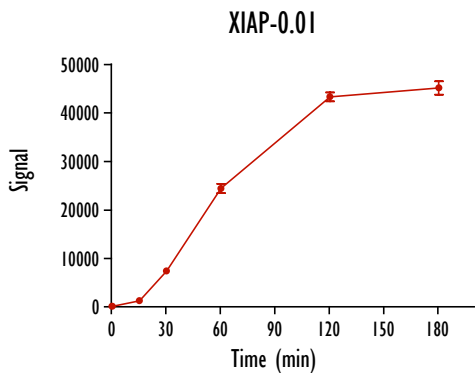
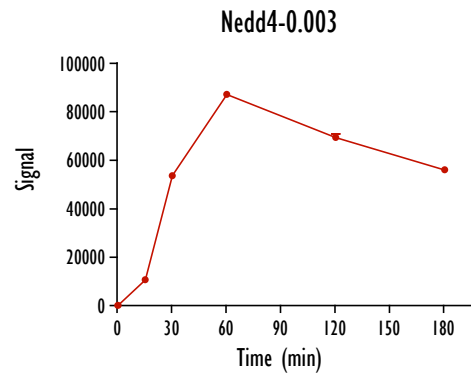
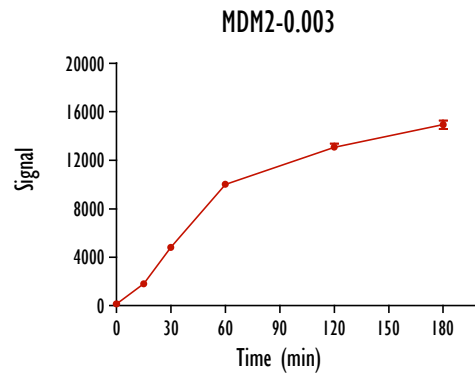
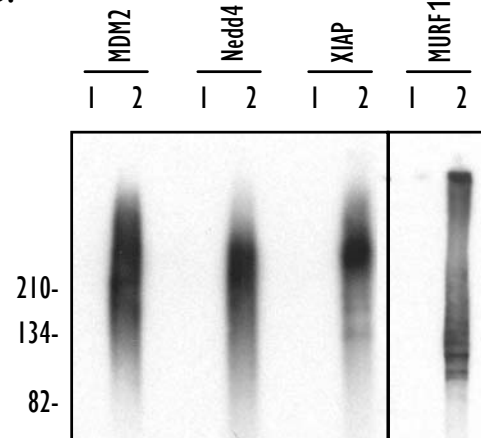
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5 Self-Ubiquitylation of Various E3s

A.



B.



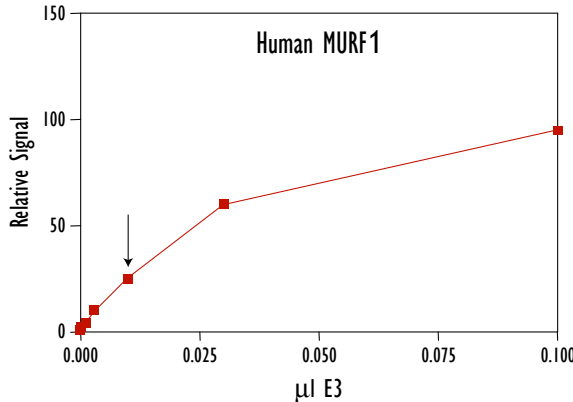
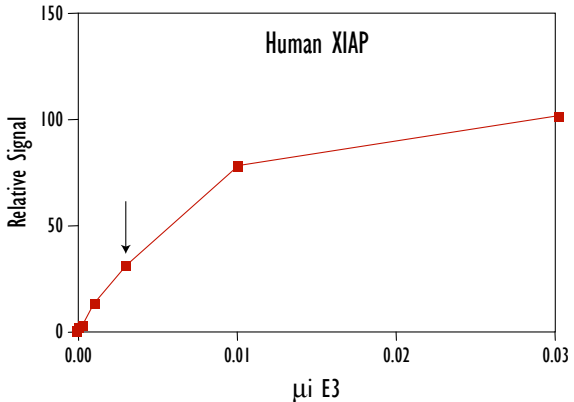
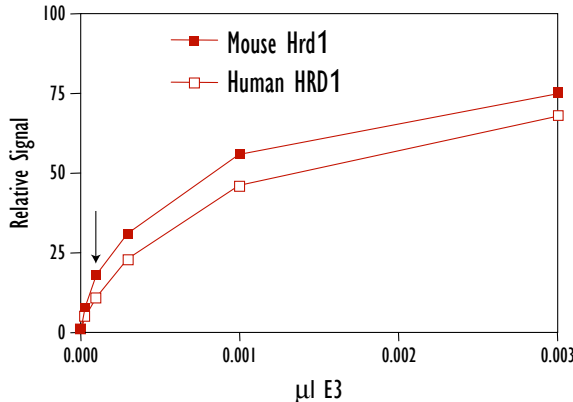
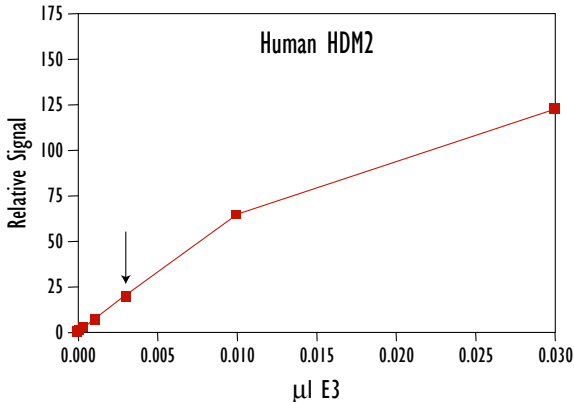
In vitro self-ubiquitylation of three different RING-finger type GST-E3s (MDM2, XIAP, and MURF1) as well as of a HECT-domain type GST-E3 (Nedd4) was studied by SDS gel (A), Western blot with antibodies against ubiquitylated proteins (B), or in electrochemiluminescence-based SECTOR Imager assay (C).



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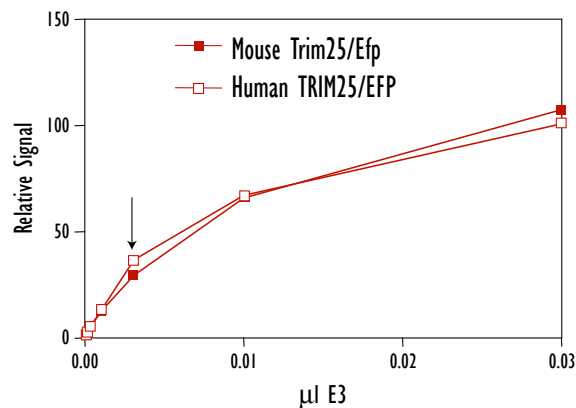
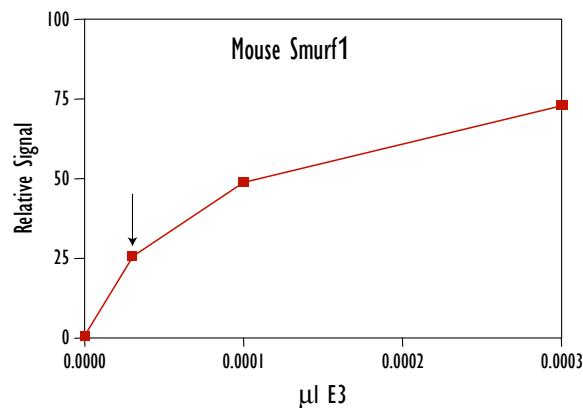
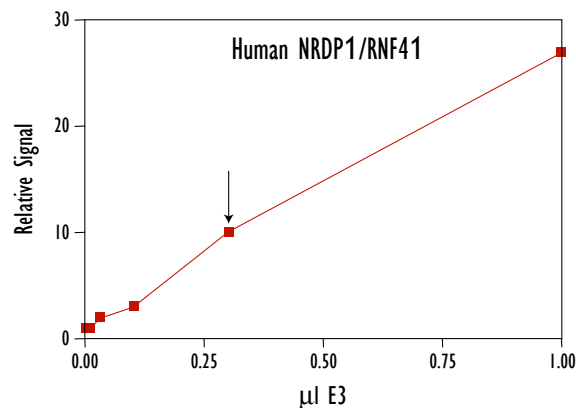
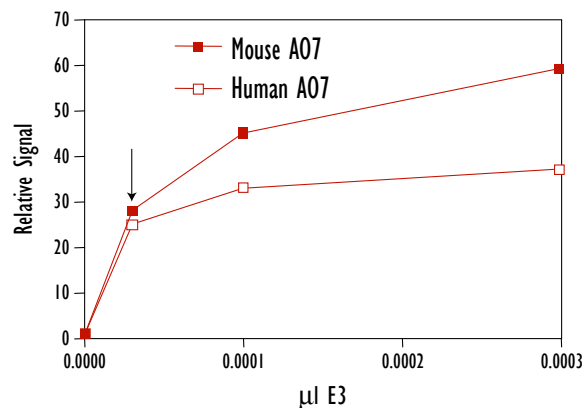
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6 Titration of Various E3s



High Throughput Assays for Inhibitors of Ubiquitin Carrier (E2) and Ubiquitin Ligase (E3) Proteins

6 Titration of Various E3s (cont.)



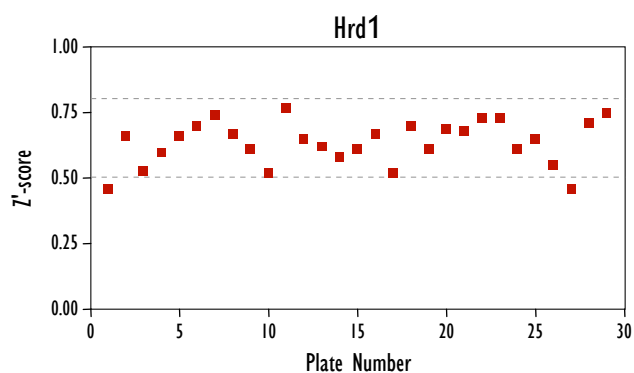
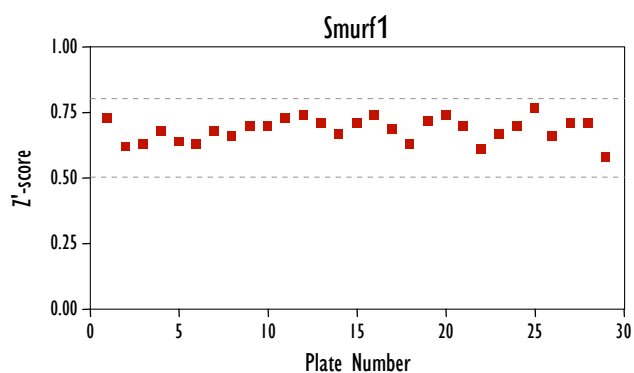
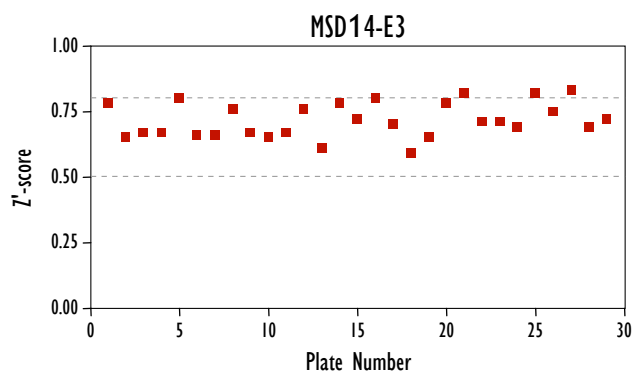
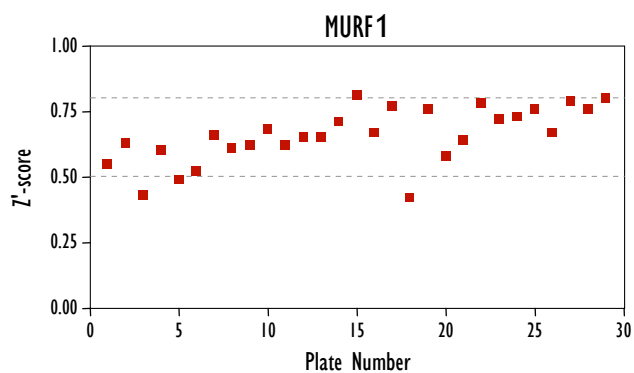
Various GST-E3s were diluted and their activities measured in the electrochemiluminescent assay. Each E3 was titrated individually to select their optimal concentrations in the reactions. Ideally, the reaction has limiting amounts of E3 to maximize the sensitivity to the detection of its inhibition in response to putative effectors during screening. Similarly, the assay signal should respond linearly to the reduction of E3 concentration. Values that met these criteria are marked by arrows for each E3. "Relative signal" represents assay signals divided by their background in the absence of E3s. Signal to background values in all the assays were greater than 10.



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7 Compound Library Screening



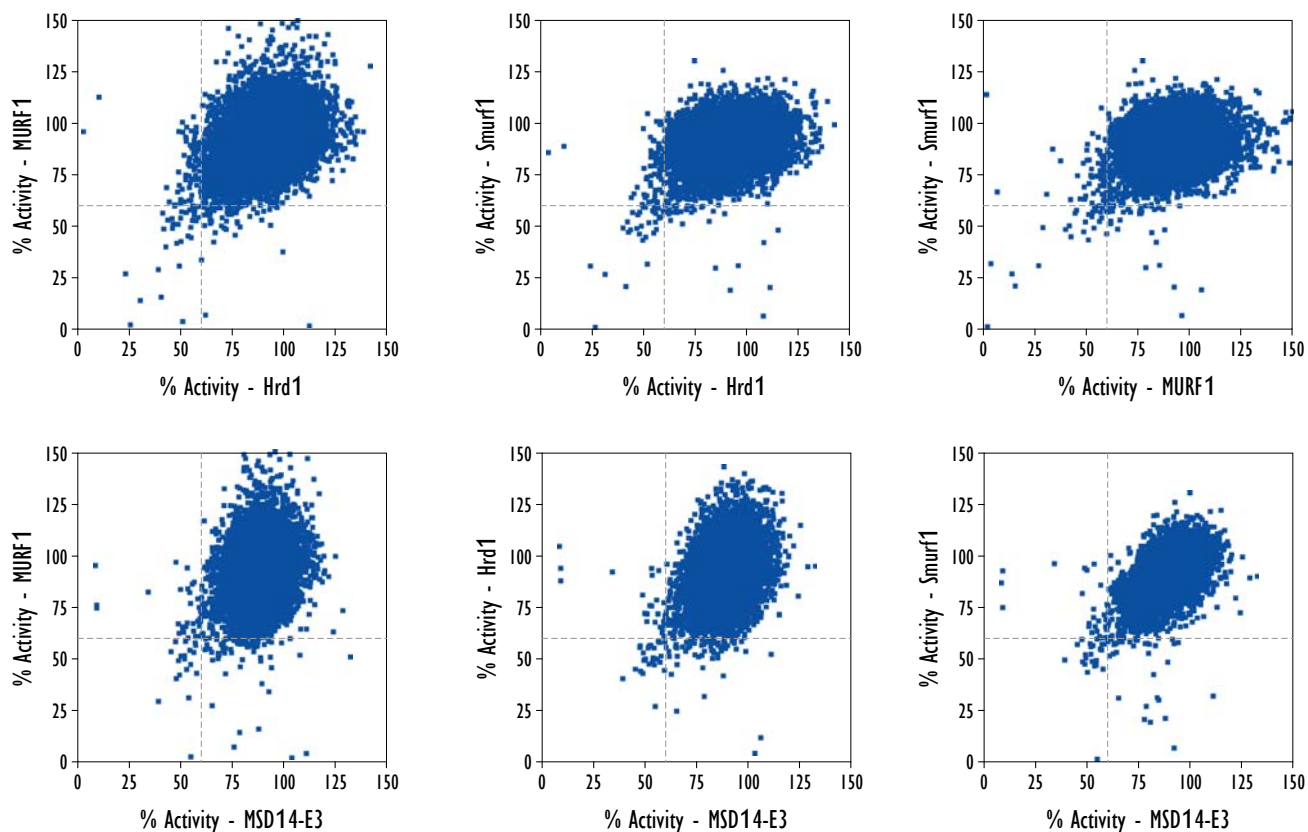
9600 compounds from MSD library were screened in 30 384-well plates against three different RING finger E3s (MURF1, MSD14-E3, and Hrd1) and one HECT domain E3 (Smurf1). Column 1 in each plate contained “No E3” controls. Column 2 contained DMSO controls. The figure shows the average Z' score over the plate where $Z' = 1 - 3 \times (SD_{DMSO} + SD_{NoE3}) / (\text{Mean}_{DMSO} - \text{Mean}_{NoE3})$.



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8 Cross-Correlation of the Screening Data



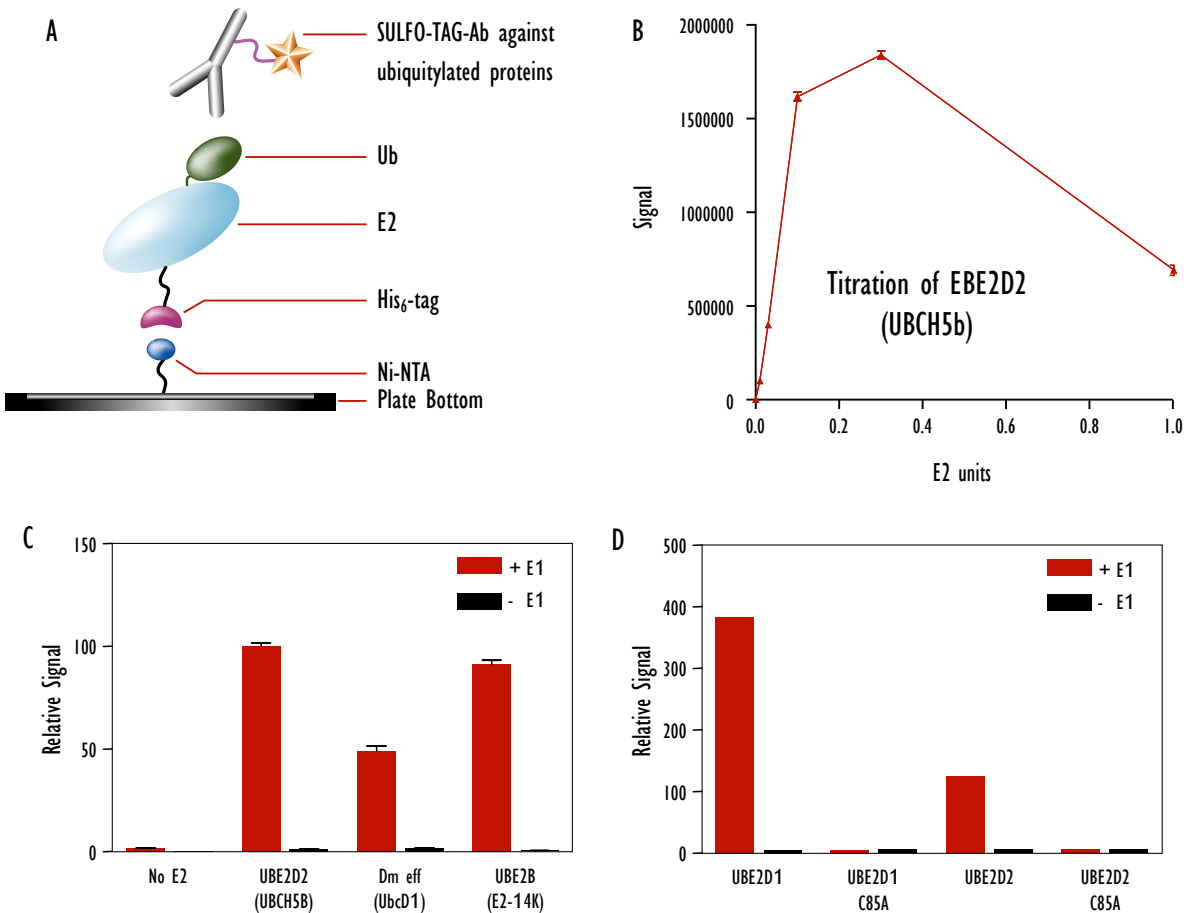
Cross-correlation of the screening data for different E3s allowed selection of compounds with specific activity against one E3. It also identified compounds that inhibited all the four E3s.



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9 HTS Assay for E2 Activity



(A) The principle behind an electrochemiluminescent E2 assay. Formation of the E2~Ub conjugate was reconstituted in vitro from individual E1 and E2(His₆-tagged) enzymes along with ubiquitin and ATP. Detection used Ni-NTA-coated MULTI-ARRAY plates with SULFO-TAG™ labeled antibodies against ubiquitin conjugates. (B) Titration of E2 enzyme in the assay. (C) Activity of various E2 enzymes was efficiently measured in the electrochemiluminescent assay. (D) Activity of E2 enzymes in the assay is dependent on the intact active site Cys residue.



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10 Conclusions

We present a versatile strategy for high-throughput *in vitro* screening of inhibitors of various E3 ubiquitin ligases both from RING finger and HECT domain families using electrochemiluminescence-based detection of their self-ubiquitylation.

Assays for several E3s, including MDM2, HRD1, MURF1/RNF28, XIAP, A07/RNF25, RNF41/NRDP1, EFP/TRIM25, and MSD14-E3 (RING finger E3s), and Smurf1 and Nedd4 (HECT domain E3s) were developed and validated.

Four E3 assays (Hrd1, MURF1, MSD14-E3 and Smurf1) were used to screen a subset of our compound library. The primary screening data was cross-correlated to identify compounds with preferential activity toward each of the four individual E3s.

We also present an electrochemiluminescence-based strategy to measure activities of various ubiquitin-conjugation (E2) enzymes. The assay was validated using several human (UBE2D1, UBE2D2, UBE2B) and *Drosophila* (eff) E2s.



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