

MDM2 Self-Ubiquitylation: An Example of a High Throughput Assay for Inhibitors of E3 Ligase Activity

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Abstract

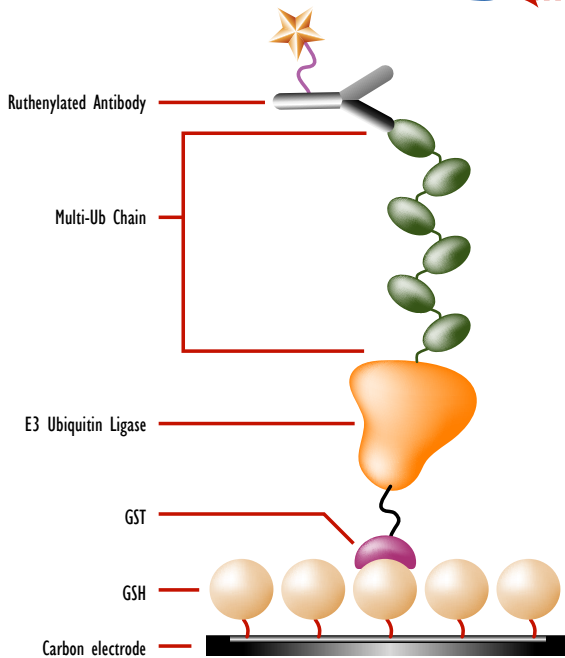
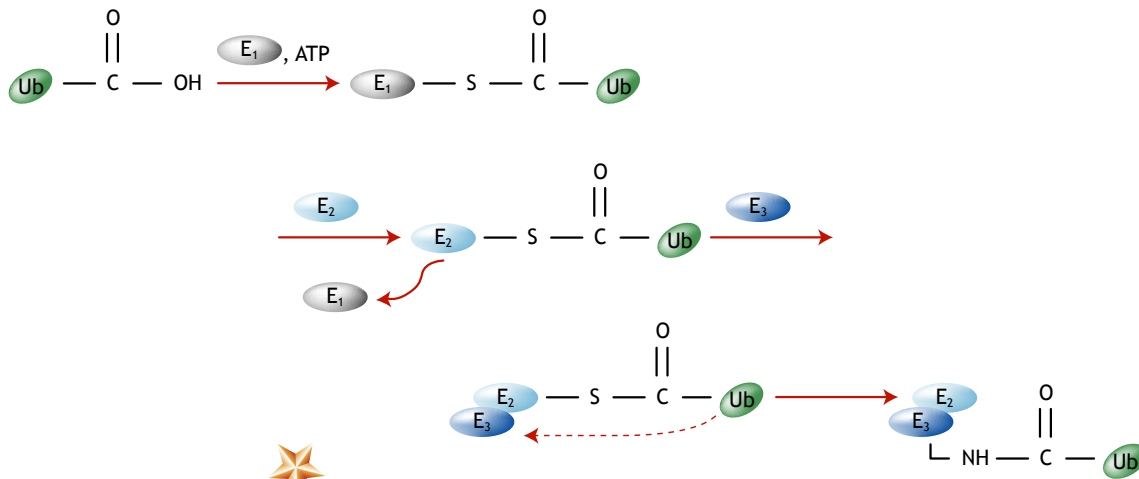
We developed a high-throughput assay for the measurement of self-ubiquitylation of MDM2 using Meso Scale Discovery's Multi-Array™ technology. Multi-Array technology combines arrays and electrochemiluminescence detection for sensitive, high-throughput assays. MDM2 is a mediator of p53 ubiquitylation and degradation, and its over-expression can lead to tumor growth by suppression of p53 function. We reconstituted the self-ubiquitylating activity of MDM2 using a well-characterized mixture of E1 and E2, the enzymes that activate and conjugate ubiquitylation. The simple, HTS-compatible assay has only reagent additions and may be conducted without washing. The assay parameters were optimized with a rate-limiting concentration of MDM2 to enhance the identification of E3-specific inhibitors. The MDM2 was expressed as a GST fusion, allowing its capture on a glutathione-coated 384-well Multi-Array Plate. The assay used a labeled anti-ubiquitin antibody to recognize ubiquitylated MDM2 and a Sector HTS™ Reader to quantitate the extent of ubiquitylation. We present the details of assay optimization and validation in preparation for screening. We employ secondary screens against other E3 ligases to stratify the hits according to their selectivity for a specific E3 or class of E3s (Ring or HECT). We also show results from a 10,000 compound screen performed with an automated workflow that allows at least one hundred 384-well plates per day. This assay format is broadly applicable to the high throughput measurement of homo- and hetero- ubiquitylating activities of many other E3 ligases.



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2 Reaction Mechanism and Assay Format



Our biochemical assay for MDM2 self-ubiquitylation measures the final product of a multi-step cascade. The E1 component first binds ubiquitin through its enzymatic activity and subsequently transfers the ubiquitin to the E2. Subsequent interaction between the E3 and E2 components results in the transfer of multiple ubiquitin to the E3. In our case, we express the MDM2 E3 as a GST fusion protein. The GST-MDM2 is captured on a glutathione-coated Multi-Array Plate and the degree of ubiquitylation is measured by a ruthenylated antibody that recognizes the poly-ubiquitylated species.



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3 Assay Workflow and Automation

Assay Format

Dispense 15 μ l reaction mix
Dispense 1 μ l compound
Dispense 5 μ l ubiquitin
↓ Incubate 1 hr
Add Stop Reagent
↓ Incubate 1 hr
Read on Sector HTS Reader

Integrated Sector HTS Reader



The assay is formatted as a series of additions to 384-well Multi-Array Plates coated with glutathione. The reagent additions are followed by a room temperature incubation and subsequent addition of a stop solution containing ruthenylated antibody in MSD™ Read Buffer. We executed this workflow in two ways: 1) with a fully integrated Beckman Core system including an integrated Sector HTS Reader; and 2) in workstation mode with a Biomek FX and a standalone Sector HTS Reader. The integration was achieved using the Remote Instrument interface of the Sector HTS Reader software. The final protocol had no wash steps, however, some of the optimization experiments had a final wash step prior to analyzing the plates.

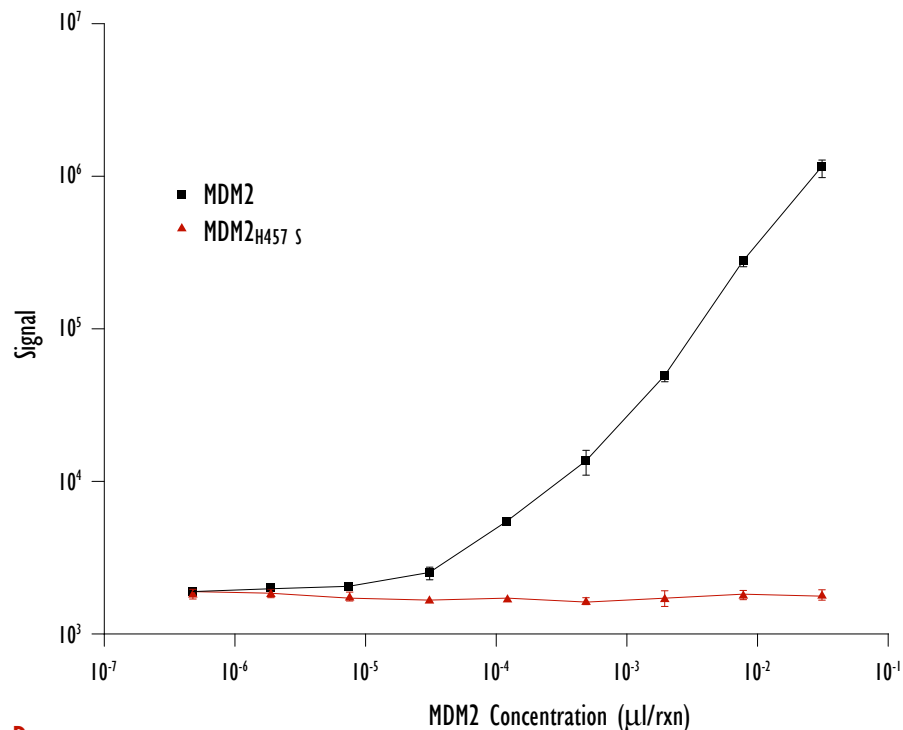


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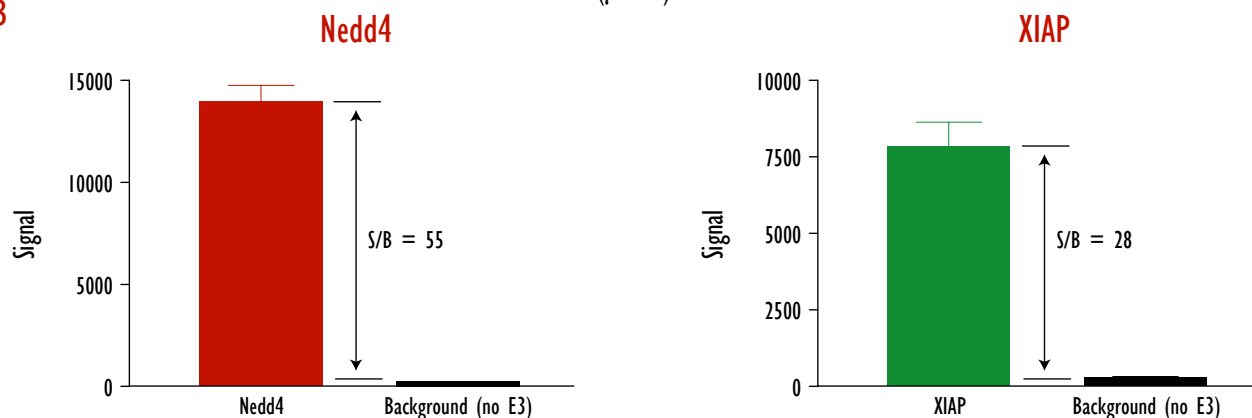
4 Assay Validation

A SIGNAL vs MDM2 CONCENTRATION



A. The assay signal increases with the concentration of GST-MDM2 but does not increase with the concentration of a mutant GST-MDM2 known to be defective as an E3 ligase. Total protein analysis was used to verify that both the functional and mutant GST-MDM2 were expressed at similar levels in E. Coli cells.

B



B. The assay conditions optimized for the MDM2 assay were adapted directly to demonstrate assays for two other E3 ligases - Nedd4 and XIAP. The signal to background ratios, observed in these two assays without further assay-specific optimization, are indicative of the system's flexibility to measure the activity of other Ring zinc finger or HECT-containing E3s.

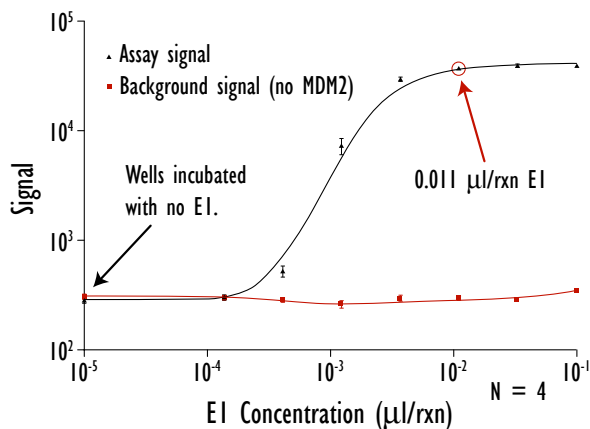


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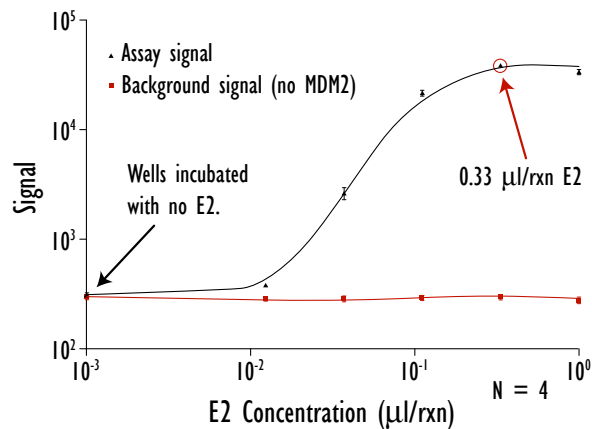
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5 Enzyme Optimizations

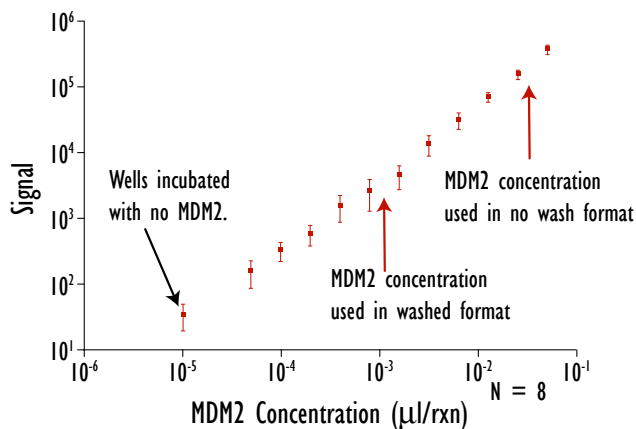
A SIGNAL vs EI CONCENTRATION



B SIGNAL vs E2 CONCENTRATION



C SIGNAL vs E3 CONCENTRATION



The relative concentrations of the E1, E2, and E3 components were optimized to improve the sensitivity of the assay to E3-specific inhibitors. A) The assay signal exhibits a plateau at high E1 concentration with fixed E2 and E3 concentrations. B) The assay signal exhibits a plateau at high E2 concentration with fixed E1 and E3 concentrations. The assay was performed at E1 and E2 concentrations where the signal is independent of changes in E1 and E2 concentrations. C) The assay was performed with MDM2 concentration in a regime where assay signal increased linearly with the concentration of MDM2 present in the reaction.

↑ Denotes concentration used in screen.

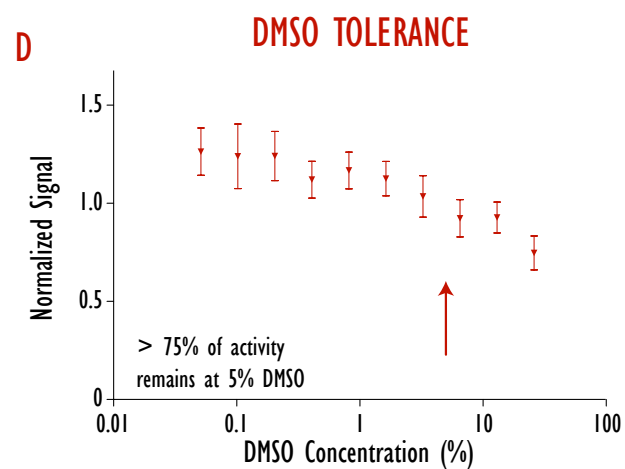
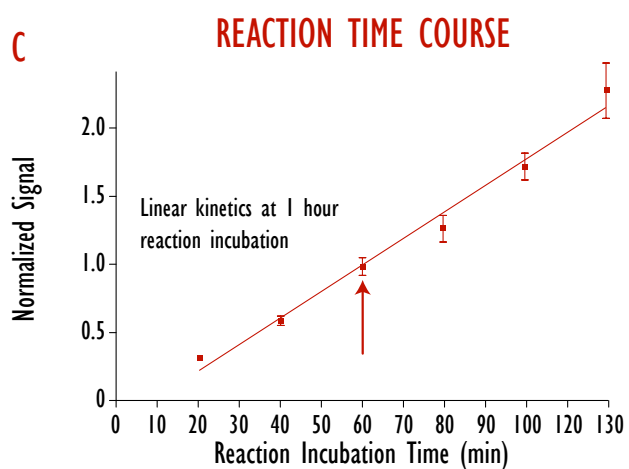
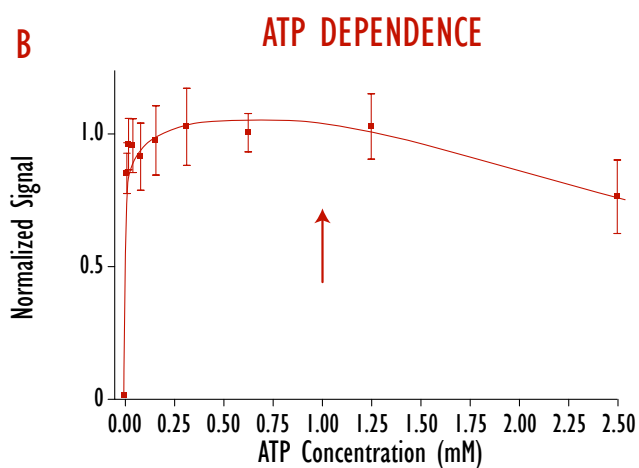
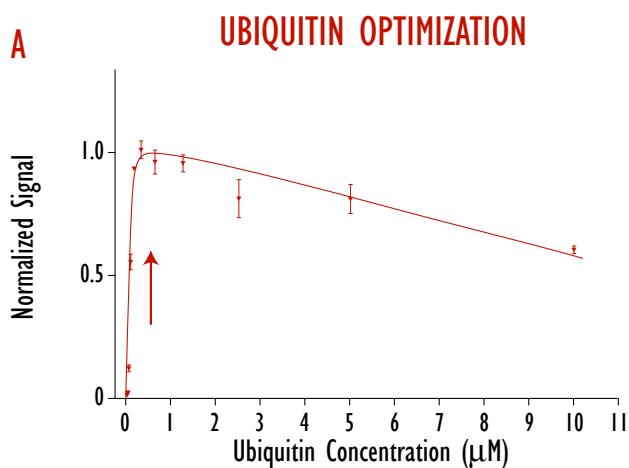
Unless noted otherwise, these experiments were conducted under the following conditions: [E1] = 0.011 $\mu\text{l}/\text{rxn}$, [E2] = 0.33 $\mu\text{l}/\text{rxn}$, [ubiquitin] = 0.5 μM , [ATP] = 1 mM, 5% DMSO



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6 Assay Characterization



↑ Denotes conditions used in screen.

Unless noted otherwise, these assay experiments were conducted without washing under the following conditions: [E1] = 0.011 µl/rxn, [E2] = 0.33 µl/rxn, [mdm2] = 0.032 µl/rxn, [ubiquitin] = 0.5 µM, [ATP] = 1 mM

The assay response was characterized extensively. A) The assay was optimized at a ubiquitin concentration that maximized signal and minimized the effect of variations in the ubiquitin concentration. B) No specific signal was observed without ATP. The assay exhibited a broad range of acceptable ATP concentration. C) The assay signal was linear in reaction time indicating that it was under kinetic control at 60 minutes. D) The assay tolerated the presence of DMSO during the reaction, maintaining significant activity even up to 5-10% DMSO concentration. In all cases, the signal was normalized relative to the conditions employed in the demonstration screen.

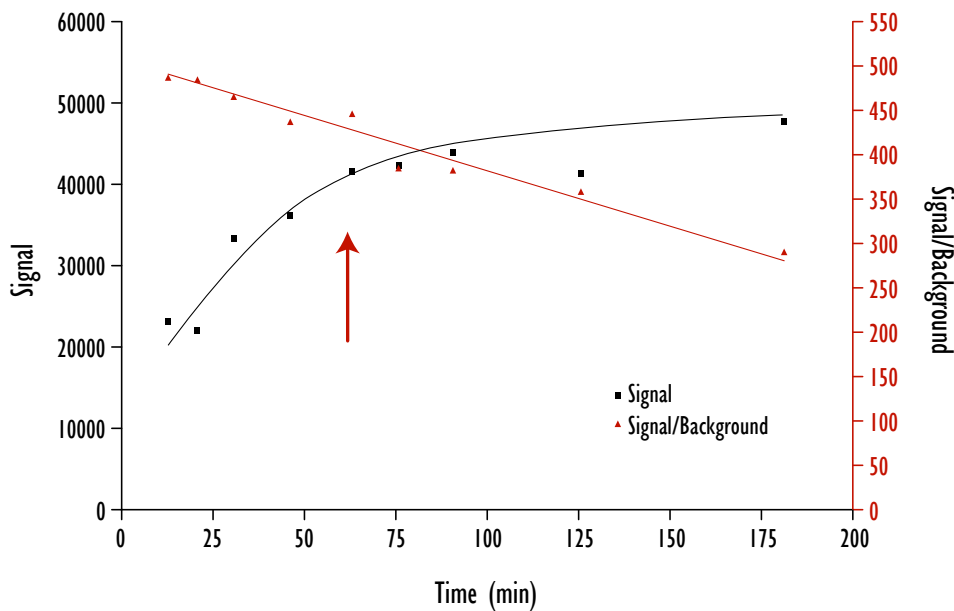


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7 Assay Flexibility

SIGNAL AND SIGNAL/BACKGROUND
VS
TIME AFTER ADDING STOP REAGENT



The no wash assay format is relatively insensitive to the time elapsed after addition of the stop reagent containing EDTA and labeled anti-ubiquitin antibody. This robustness makes the assay appropriate for a work-station mode of screening where the plates are run in batches. The graph contains data showing that both the absolute signal level and the signal to background ratio are stable. The assay is typically run with a 60 minute delay, as indicated by the red arrow on the graph.

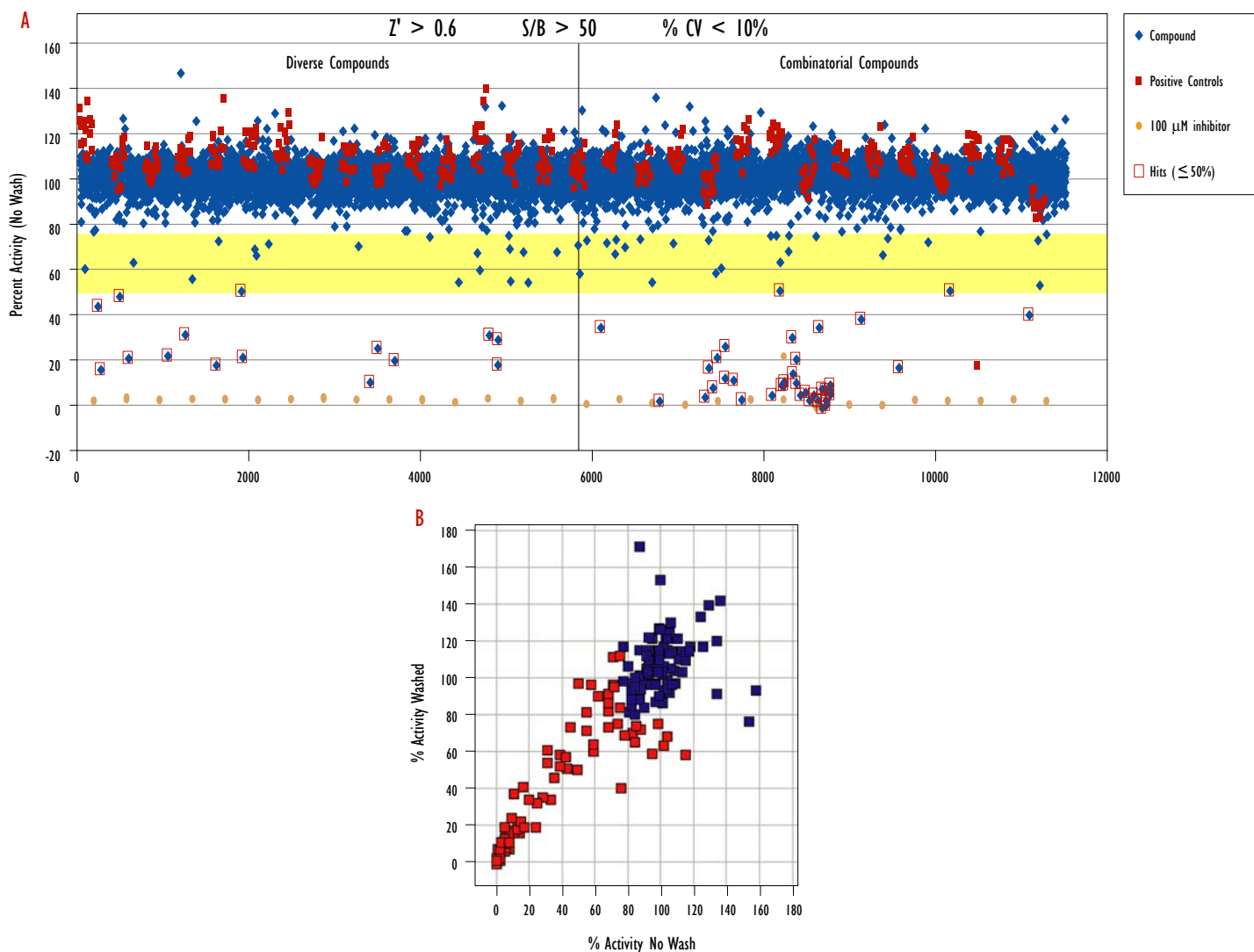


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8

Screen Against 10,000 Compound 'Robustness' Library



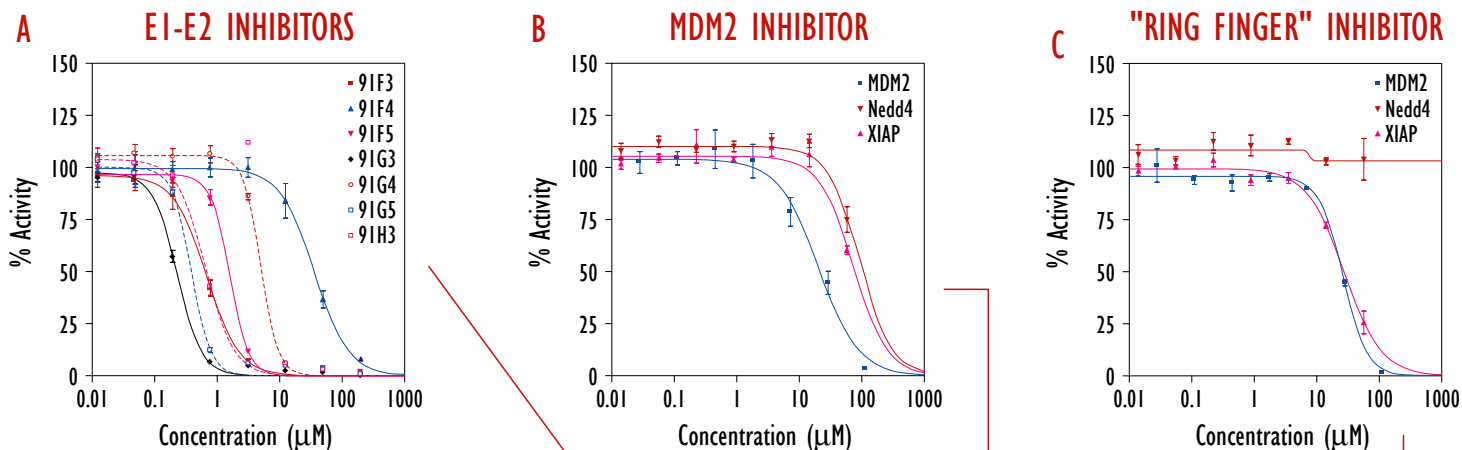
A) The assay was screened against 10,000 compounds from our library at 15 μ M in duplicate. The compound set was composed of a diverse set of 5,000 historical compounds and 5,000 "combichem" compounds. The low CVs ($< 10\%$) permitted selection of weak inhibitors for secondary screening (yellow region). 150 hits were selected where the percent activity was less than 75% on either screen. B) The hits were re-screened in both our standard no wash format and in an assay format including a final wash step to remove the compounds from the well during readout. Eighty-four compounds were selected where the activity was less than 75% (red). The correlation between the washed and unwashed formats indicated both a high rate of reconfirmation and little systematic effect between the two formats.



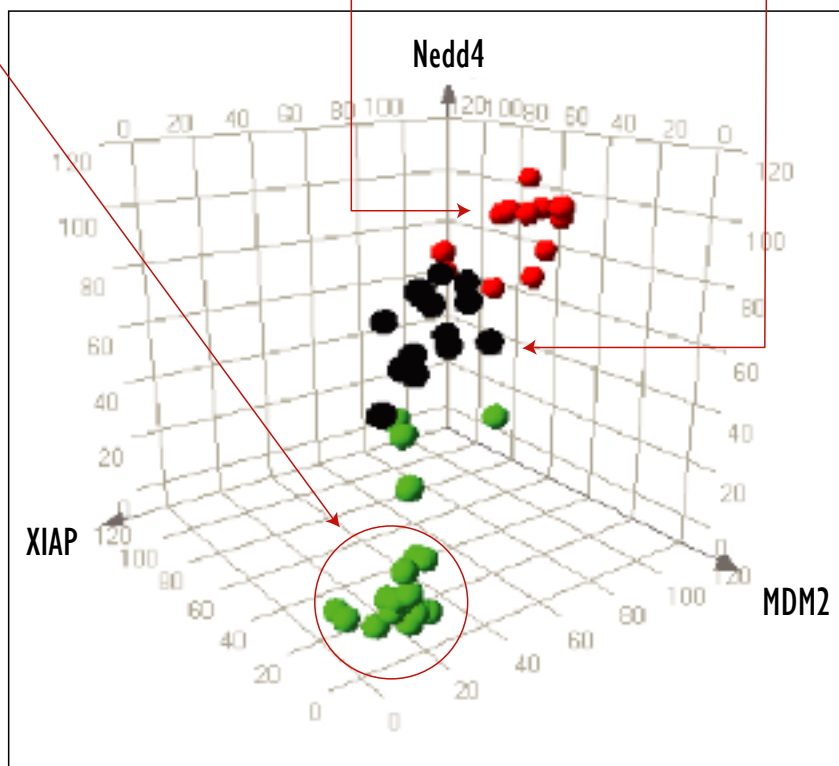
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9 Classification of Active Compounds by Type of Inhibition



The 84 hits confirmed in our MDM2 assay were titrated against two other E3 ligases (XIAP and Nedd4) in the same assay format. Fifty-four compounds could be classified according to the specificity of their activity. A cluster of compounds (green) showed strong inhibition of activity regardless of E3 and are thus likely to be E2 or E1 inhibitors. A) These compounds observed to inhibit all of the E3s investigated share a common chemical substructure. Changes in the side chains shift the IC50 from 200 nM to 30 μ M. A cluster of compounds (red) showed preferential specificity for MDM2. In B), a selected compound inhibits MDM2 activity at lower concentration than it inhibits Nedd4 and XIAP activity. A cluster of compounds (black) showed specificity for both ligases having a ring finger domain. In C), IC50 curves for a compound indicated specificity for RING zinc finger (MDM2 and XIAP) domain versus HECT domain (Nedd4) E3s. Such a stratification of hits in secondary screening results directly from the flexibility of the assay format to measure the activity of multiple E3 ligases.



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10 Conclusions

We have presented a simple-format, high-throughput assay for the measurement of self-ubiquitylation of MDM2 using Meso Scale Discovery's Multi-Array technology. The assay was characterized and optimized relative to the concentration of relevant assay components to produce a primary screen designed to be preferentially selective for MDM2-specific inhibitors. The compound screen was performed on a Beckman Core system with an integrated Sector HTS Reader. The assay format and workflow is capable of achieving an assay throughput of at least one hundred 384-well plates per day. The conditions used for the MDM2 screen were transferred to XIAP and Nedd4, providing secondary assays without additional assay development effort. These secondary assays were used to produce "high-content" information about the specificity of resulting hits. This type of multi-assay characterization contributes to an understanding of the structural activity relationship for the compounds that inhibit activity. This work demonstrates a broadly applicable, flexible and robust assay format suitable for high-throughput, automated compound screening against both hetero- and homo-ubiquitylating ligase activities.



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