P53-MDM2 System as a Target for High-Throughput Screening

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❶ Abstract

MDM2 regulates the activity of the tumor suppressor protein p53 by binding p53 and, further, by ubiquitylating it which can ultimately target p53 for proteasomal degradation. MDM2 also promotes self-ubiquitylation and thus regulates intracellular levels of both p53 and itself. This feature of MDM2, and the observation that overexpression of MDM2 is prevalent in many human cancers underscores its important role in cancer biology and its potential attractiveness as a target for therapeutic intervention in disease. Inhibition of the MDM2 ubiquitin ligase activity towards p53, for example, might lead to growth arrest and apoptosis in p53-positive cells resulting from metabolic stabilization of p53. We developed two types of high-throughput assays aimed at the discovery of inhibitors that might modulate the various activities of MDM2. The first was an HTS assay that measured the degree of self-ubiquitylation of MDM2 in a reconstituted system of enzymes that included the E1, an E2 (the enzymes that initiate ubiquitylation), MDM2, ubiquitin and ATP. This assay format proved straightforward, rapid and had a high signal-to-background ratio. We used secondary assays that incorporated other E3 ubiquitin ligases in the same format to sort potential inhibitors according to their specificity for MDM2; another secondary assay identified the inhibitors of the E1 or E2s. The second type of HTS assay measured the degree of interaction of full-length recombinant p53 and MDM2. Specificity of the assay was demonstrated by a dose-response inhibition with a known peptide inhibitor of p53-MDM2 binding. All our HTS assays utilize electrochemiluminescence-based detection on MSD's SECTOR™ Imager 6000 in combination with MULTI-ARRAY™ Plates.

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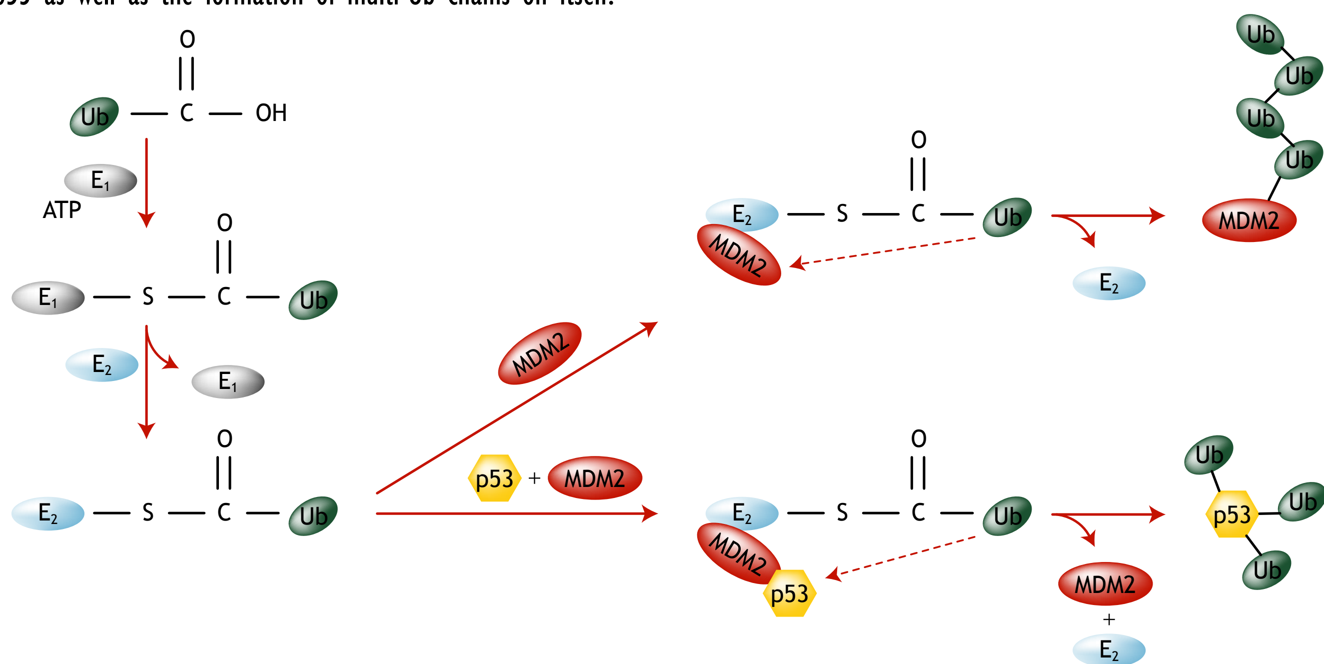
❷ Introduction

MDM2 is a critical regulator of p53 function. MDM2 contains a COOH-terminal RING-finger domain having an E3 ubiquitin ligase activity towards p53 and itself. Ubiquitylation results in formation of an *iso*-peptide bond between the C-terminal Gly-76 of ubiquitin (Ub) and an ε-amino group on one of the internal lysine residues of the substrate protein. Ubiquitylation of proteins occurs through a series of enzymatic steps involving E1, E2 and E3 proteins. Ubiquitin (Ub) is first activated in an ATP-dependent step to form a thiol-ester with a specific cysteine residue on the E1 enzyme. Activated Ub is then transferred to one of a family of Ub-conjugating enzymes (E2). Finally, an Ub ligase (E3) binds protein targets for ubiquitylation and transfers activated Ub from E2 to one of the lysines on the protein substrate. MDM2 promotes multiple mono-ubiquitylation of p53 as well as the formation of multi-Ub chains on itself.



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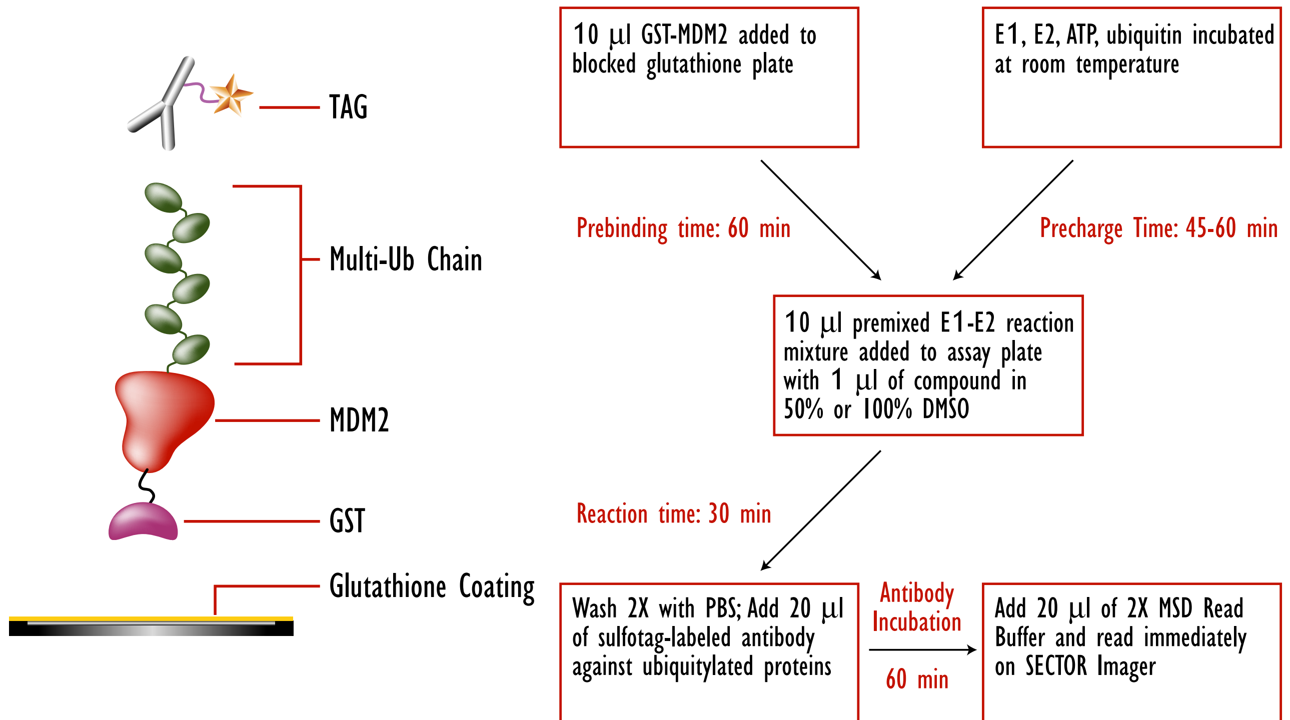
❸ MDM2 Self-Ubiquitylation Assay Format



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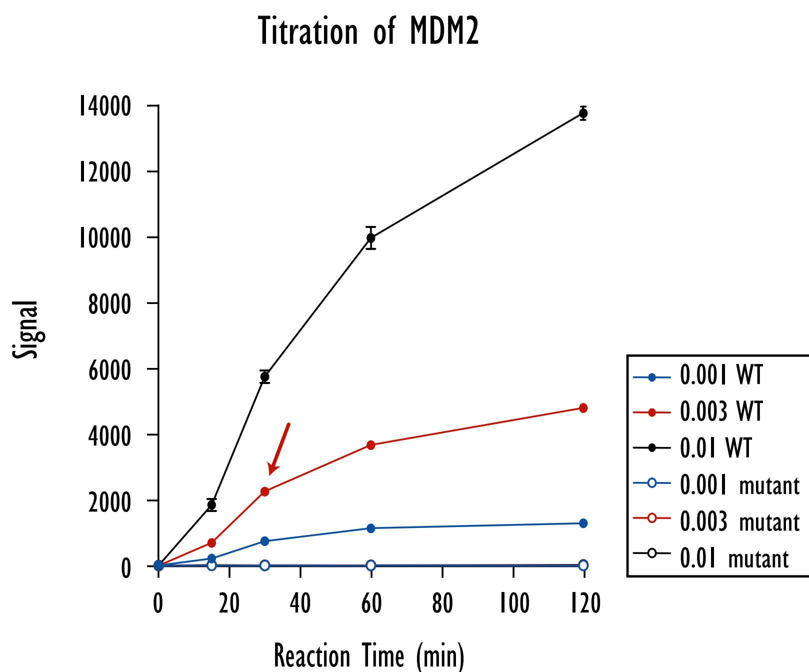
❹ Reaction Time Course – Titration of MDM2



Screening at the reaction conditions marked by the arrow maximized sensitivity of the system to inhibitors of E3s, had a high signal to background and was economic of reagents. The signal depended linearly on the MDM2 concentration, the reaction achieved the highest velocity, while showing substantially less dependence on the concentrations of E1 and E2s at these ratios of E1, E2 and E3 (data not shown).

The MDM2-H457S mutant, a mutation within the RING finger, showed no activity in the self-ubiquitylation assay.

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5 Self-Ubiquitinylation of Various E3s

A.

MDM2 NEDD4 XIAP RNF28

C U C U C U C U

-210
-134
-82

B.

MDM2 NEDD4 XIAP RNF28

C U C U C U C U

210-
134-
82-

MDM2-0.003

NEDD4-0.003

XIAP-0.01

RNF28-0.03

Signal

Time (min)

In vitro self-ubiquitylation of three different RING-finger type GST-E3s (MDM2, XIAP, and RNF28) and of a HECT-domain type GST-E3 (NEDD4). (A) The indicated GST-E3s prior to (control lanes "C") and after ubiquitylation reaction (lanes "U") were separated by SDS gel and stained by coomassie blue. Self-ubiquitylation of all the E3s is observed in lanes "U". (B) Western blot with antibodies against ubiquitylated proteins of the same samples as in panel (A). The signals in lanes "U" reflect self-ubiquitylation of the respective E3s. (C) Time courses of self-ubiquitylation of different E3s measured in electrochemiluminescence-based MSD assays. The data show that the self-ubiquitylation of various E3s can be measured in the same assay format and can be used in multiple parallel screens

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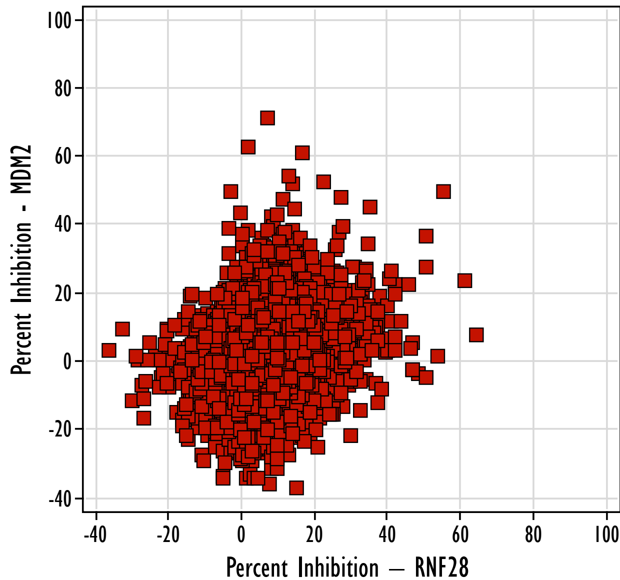
6 Compound Library Mini Screen



8000 compounds were screened using the MDM2 and RNF28 self-ubiquitylation assays in MULTI-ARRAY plates. Cross-correlation of the data allowed the identification of specific inhibitors of each E3. This mini-screen demonstrated the utility of using various E3s to characterize the selectivity of potential inhibitors.

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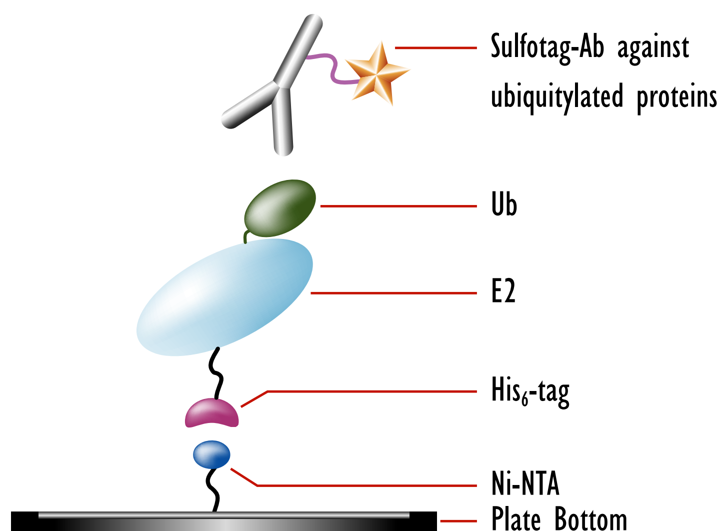
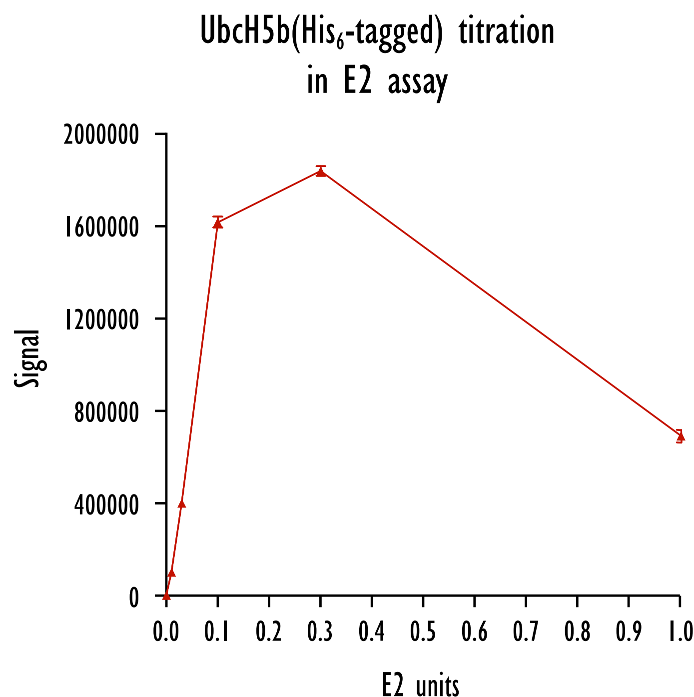
7 HTS Assay for E2 Activity





The ubiquitylation of E2 was reconstituted *in vitro* from individual E1 and E2(His$_6$-tagged) enzymes as well as Ub and ATP. The E2 assay serves as a secondary screening strategy to separate compounds inhibiting E1 and E2 enzymes from those acting on E3.

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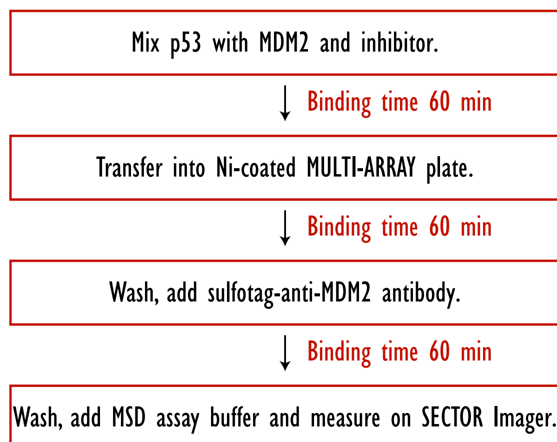
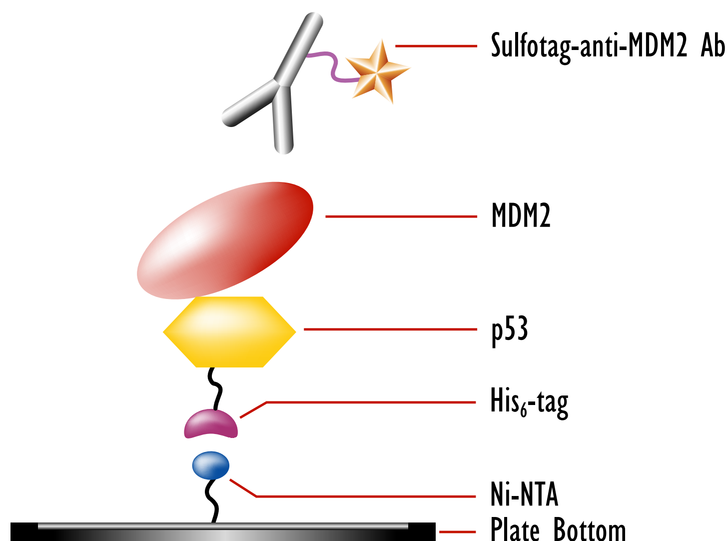
8 P53-MDM2 Binding Assay Format





Full-length baculovirus-expressed human p53 (His_6-tagged) and full-length bacterially expressed MDM2 (or GST-MDM2) were used for our p53-MDM2 binding assay.

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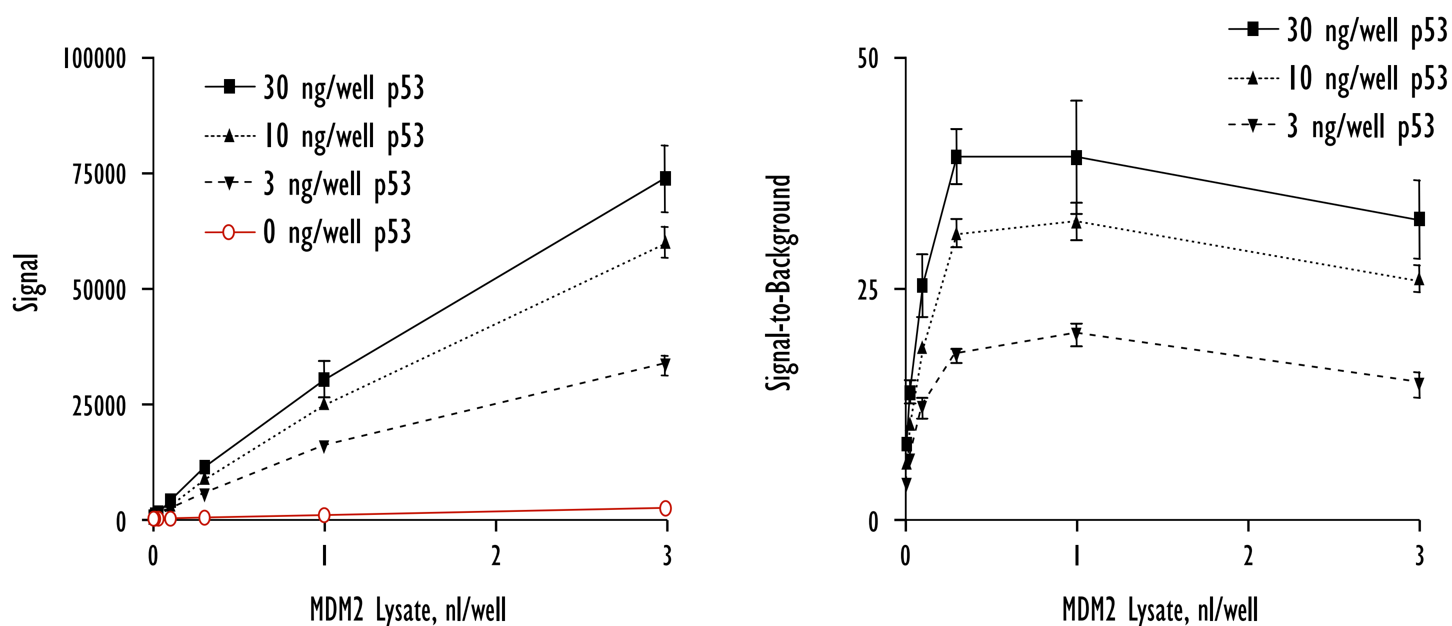
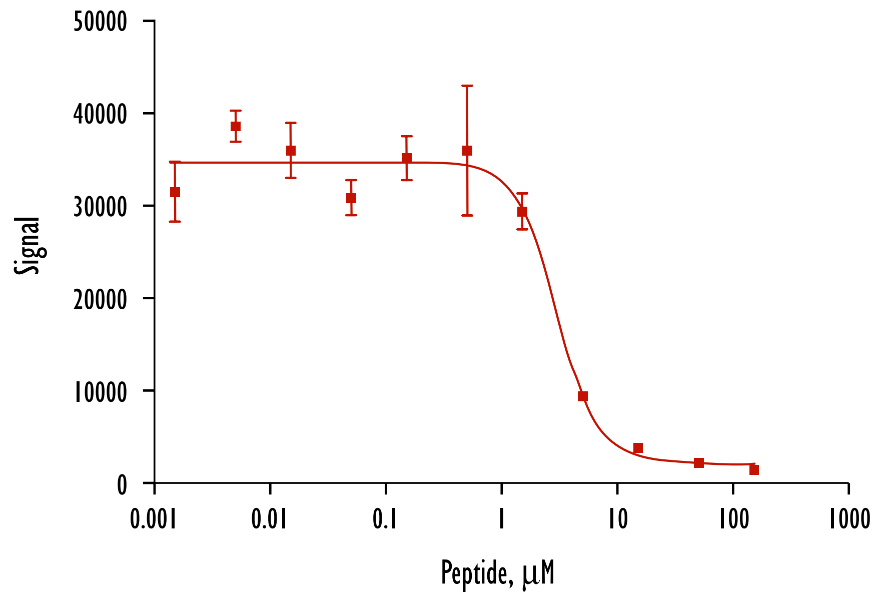
9 P53-MDM2 Binding Assay — Titration of the Components



The p53-MDM2 binding assay is a robust assay with maximum signal-to-background of ~35. The assay signals exhibit linear dependence on both p53 and MDM2 concentrations.

10 Peptide Competitor of p53-MDM2 Binding



The peptide MPRFMDYWEGLN (*Boettger et al.*, 1996, Oncogene, Vol. 13, p. 2141) inhibited p53-MDM2 binding with an IC_{50} of ~3 µM. The observed IC_{50} was higher than the one previously reported for this peptide possibly because full-length (489-residue) MDM2 was used in the current experiment, whereas an N-terminal 1-188 fragment of MDM2 was used by *Boettger et al.*

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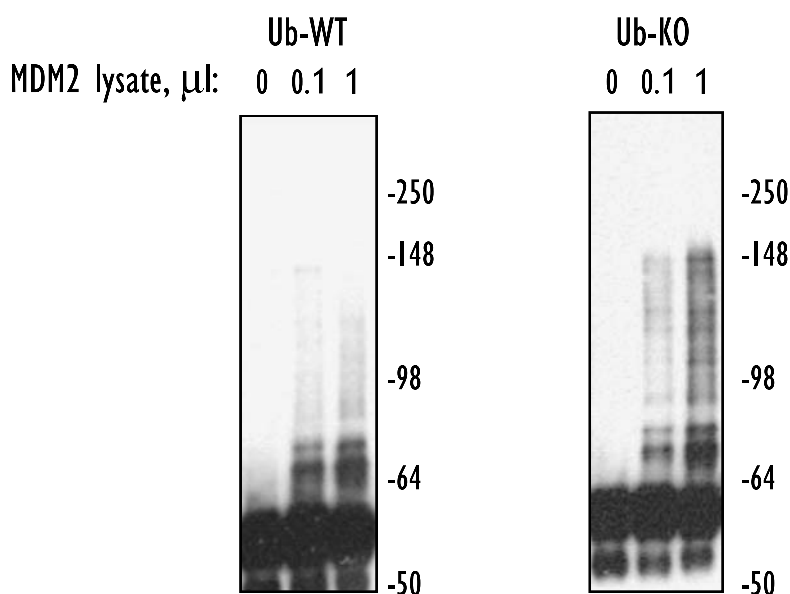
⓫ Ubiquitylation of p53 by MDM2

Western blot analysis with anti-p53 antibodies showed the ubiquitylation of p53 by MDM2. No significant difference in the pattern of p53 ubiquitylation occurred when an Ub mutant having all seven lysine residues replaced by arginines (Ub-KO) was used in the reaction instead of the wild type Ub (Ub-WT). This result suggested that p53 was largely modified by multiple mono-ubiquitylation (as first reported by *Lai et al.*, 2001, J. Biol. Chem., v. 276, p. 31357). The intensity of the ladder of ubiquitylated p53 was reproducibly stronger with Ub-KO than with Ub-WT, while the ladder observed for ubiquitylated MDM2 (self-ubiquitylation of MDM2 occurring in the same reaction in parallel with ubiquitylation of p53) was weaker with Ub-KO than with Ub-WT (data not shown).



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⓬ Conclusions

We presented a high-throughput assay that targets an E3 self-ubiquitylation activity of MDM2.

We developed secondary assays that measured self-ubiquitylation of two additional RING-finger type E3s (XIAP and RNF28) as well as a HECT-domain type E3 (NEDD4) that allowed further characterization of specificity of the inhibitors from the primary screen.

Other secondary assays measured E2 enzymatic activity and allowed segregation of inhibitors that acted on the E1 or E2 part of the pathway.

We developed a p53-MDM2 binding assay to screen for inhibitors that could disrupt the p53-MDM2 interaction. This assay utilized full-length, human recombinant p53 and MDM2 proteins. Specificity of the binding assay was confirmed by competition with a known peptide inhibitor of the p53-MDM2 interaction.