E3 and DUB substrate identification using microarrays.

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LifeSensors
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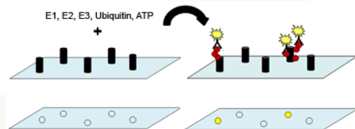


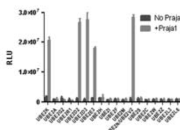
Ubiquitylation of Immobilized Substrates

INTRODUCTION

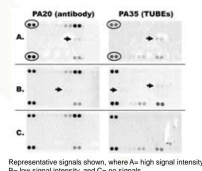
Ubiquitylation is a reversible modification to proteins necessary and essential for cellular homeostasis. First appreciated as the signal for proteasomal degradation, the consequences of protein ubiquitylation are now understood to be diverse and highly regulated. Conjugation of ubiquitin to target proteins is accomplished through the serial activity of enzymes called E1 (2 in human), E2 (~50), and E3's (~600). Target specificity is mostly determined by the E3's, which comprise the largest family of enzymes in the human proteome. Reversal of ubiquitin conjugation is the responsibility of the enzymes known as DUBs (deubiquitylases), of which there are ~90 in human. Both E3's and DUBs have currently attracted attention as diagnostic and therapeutic targets.

Although over 90% of cellular proteins will be ubiquitylated and deubiquitylated by these enzymes at some point in their lifecycle, few E3-substrate and even fewer DUB-substrate pairs have been confidently established. One problem is the transient nature of enzyme/substrate interaction; two-hybrid and mass spectrometric approaches are good at identifying cofactors, but not substrates.

Step 1. Optimize E2 pairing for the E3 of interest (Praja1 in this example). This is easily done using LifeSensors' E2 Profiling Kit (#UC102), which identified UBE2D3 as the optimal E2 for use with Praja1.

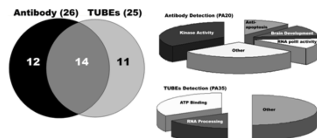


Step 2. Perform solution-based ubiquitylation reaction atop an array of immobilized proteins (Invitrogen ProtoArray, containing thousands of human proteins in this example). Putative E3 substrates are identified through detection of covalently bound ubiquitin. In this example, antibody (Sigma) recognizes mono- and polyubiquitin chains, while TUBEs (LifeSensors) are specific to polyubiquitin chains.



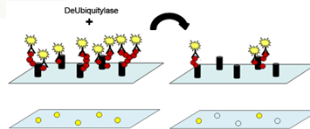
Representative signals shown, where A= high signal intensity, B= low signal intensity, and C= no signals

Step 3. Analysis
Extract array data, statistically determine putative hits, and use gene ontology (GO) clustering to help prioritize individual substrates for experimental validation.



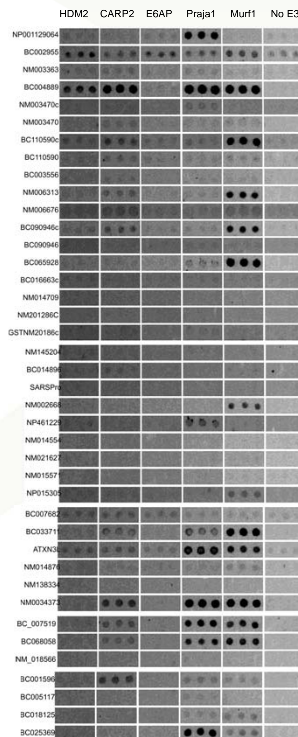
We (unpublished results) and others (Wada et al. 2006) have observed that certain substrates are specifically monoubiquitylated in vitro; differences between TUBEs and antibody identified substrates therefore likely reflect differences between polyubiquitylation and monoubiquitylation of different substrates. Because polyubiquitylation signals proteasomal degradation, a fate shared by the majority of proteins, one would predict that detection of polyubiquitylation events would result in a more general list of substrates compared with detection of monoubiquitylation (signaling) events. Consistent with this, GO clustering of our TUBEs-detected substrates resulted in fewer categories than analysis of antibody-detected substrates.

Step 4. You follow the leads we provide.
Praja1 is known to be highly expressed in brain, involved in memory formation, and implicated in X-linked mental retardation. The putative substrates representing the GO cluster "brain development" should therefore be prioritized for experimental follow up. MAGED1 is a protein involved in the apoptotic response, and has elsewhere been implicated as a cofactor of Praja1. The putative substrates representing "anti-apoptosis" might therefore be prioritized for independent experimental confirmation. Although MAGED1 was not present on the arrays, both array-detection methods in the current study identified another MAGE family protein, MAGEB4. Finally, although the GO clustering of array data qualitatively differed between the detection methods used (discussed above), involvement of Praja1 in protein translation might be suggested by the identification of putative substrates involved in "RNA Pol III activity" (antibody array) and RNA processing (TUBEs array).



De-ubiquitylation of Immobilized Substrates

E3 specificity for substrates is preserved in vitro on the microarray (exemplified by data to the right). This is good news if you want to identify E3 substrates, but poses a challenge to the array-based identification of DUB substrates…



Step 1. Drive ubiquitylation of immobilized substrates to the fullest extent possible, to maximize the ability to observe subsequent deubiquitylation events. Options include increasing E3 concentrations, temporally extending the ubiquitylation reaction (example to the right), or our prefered method (utilized for all following data): using a promiscuous E2 (UbcH5c) with a cocktail of E3's.
The absence of signals in reactions lacking either E1, E2, E3, or ubiquitin demonstrates that the signals observed under such conditions are still ubiquitylation dependent.

Duplicate, Identical subarrays tested under each experimental condition.

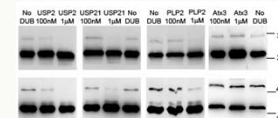
Complete
No E1
No E2
No E3
No Ubiquitin

Step 2. Apply DUB(s) to array and observe signal loss (compared with mock-treated array)

Because each of these arrays contained 16 identical subarrays containing triplicate printed features, we were able to fully screen the activity profile of 14 individual DUBs (plus two mock-DUB treated arrays; map to the left of image). Results indicated that the vast majority of these DUBs are unable to cleave ubiquitin from these substrates despite the fact that most were independently confirmed to be highly active enzymes (data not shown).

Step 3. Extract, analyze data and prioritize hits for experimental followup, exactly as with E3 substrate identification. *Note that the array used for this demonstration was insufficiently large (contained ~50 members of a single protein family) to necessitate such analysis.*

Step 4. Confirm array-identified substrates



Two substrates were selected from the array, ubiquitylated in a test tube, then treated with the indicated DUBs. In agreement with array data, USP2, USP21, and PLP2 were capable of reversing ubiquitylation from these substrates, but not Ataxin 3.

Conclusions

From identifying substrates of E3's or DUB's (and identifying the relevant E3 or DUB of any given substrate besides) LifeSensors' proteomics can successfully answer almost any question concerning the ubiquitin pathway. Additionally, LifeSesnors offers full spectrum, customizable proteomic services to help you answer your most important proteomic and ubiquitomic questions.

Arrays for the deubiquitylation study were manufactured from LifeSensors' reagents by GenTel BioSciences, Inc., 5500 Nobel Dr. Suite 230, Madison, WI 53711 **www.gentelbio.com**