# Determining E2/E3 cognate pairs with the E2 Profiling Kit

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## BACKGROUND

### Ubiquitin and Polyubiquitylation in Cellular Processes

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to the ε-amino group of lysine residues on target proteins in either monomer form or as polymeric chains. The most well characterized polyubiquitin chains are those formed via conjugation of lysine 48 or 63 of ubiquitin (K48 or K63 chain linkage) and the most prevalent consequence of polyubiquitylation is proteasome-mediated degradation of the target protein, indicative of a K48 linkage[1]. Polyubiquitin modification is a reversible process; *i.e.* the chains are degraded and/or removed by a class of proteases known as deubiquitylases (DUBs)[2]. The complex nature of this enzymatic pathway (Fig. 1) represents a major obstacle in understanding the physiological role that ubiquitin and associated enzymes play in the cell[3].

## E2 Profiling Kit: A method to help identify cellular E2/E3 cognate pairs

LifeSensors has recently licensed assays and reagents that characterize and quantitate ubiquitin E3 ligase activity which can aid in deciphering the complicated network of related E2/E3 cellular pairs. One of these assays is based on the ability of an ubiquitin binding domain to preferentially bind polyubiquitin relative to monoubiquitin[4,5]. This binding event directly reports on the activity of an E3 ligase that builds ubiquitin chains in a dose-dependent manner. The benefit of this approach is that it allows the reaction components to be native, untagged, and free in solution.

Using this approach, it was observed that some E3 ligases can work with several E2 enzymes, however with different efficiencies. Interpreting the differences in E2:E3 ubiquitylation efficiency may help decode which E2:E3 pairs are true partners, a network which is poorly understood in the cell. E2 Profiling Kit allows fast and efficient screening of a specific E3 with a number of different E2s distinguishing which E2 supports E3 activity (Fig. 2). To further characterize these interactions, the E3LITE Customizable Ubiquitin Ligase Kit can be used to determine $K_m$ values for E2s identified from the E2 Profiling Kit with a specific E3 conjugating enzyme (Fig. 3). The $K_m$ encompasses both the affinity of a substrate for
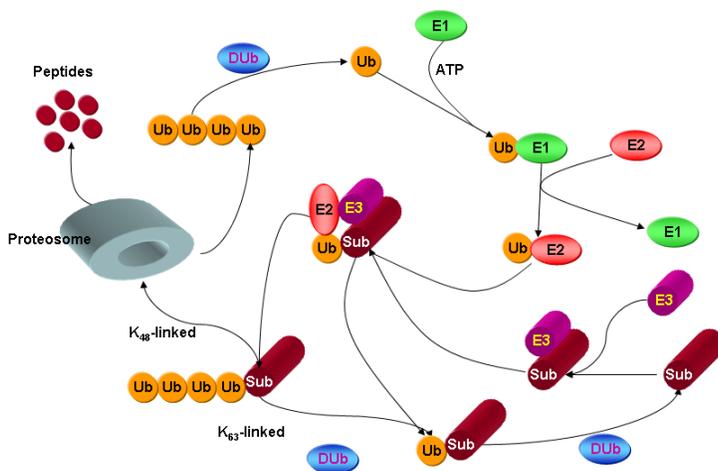


**Figure 1. The ubiquitin pathway.** A cartoon demonstrating the complex nature of the ubiquitin pathway. In the first step, ubiquitin is activated by an E1 activating enzyme, forming an ATP dependent, thioester with the activating enzyme. This enzyme:ubiquitin complex interacts with an E2 conjugating enzyme to transfer the ubiquitin moiety to the E2. The E2:ubiquitin complex then binds to a complex of an E3 conjugating enzyme and a target protein (Sub). The E3 facilitates the transfer of either monoubiquitin or a polyubiquitin chain to the substrate protein prior to release of the substrate from the complex. Depending on the nature of the polyubiquitin chain, the substrate is either targeted for degradation by the proteasome (*e.g.* K48 linkage) or for other fates (*e.g.* K63 linkage). In either case, deubiquitylases (DUB) can mediate these fates through removal of the conjugated ubiquitin. Polyubiquitin chains are also released from the proteasome and recycled by DUBs.
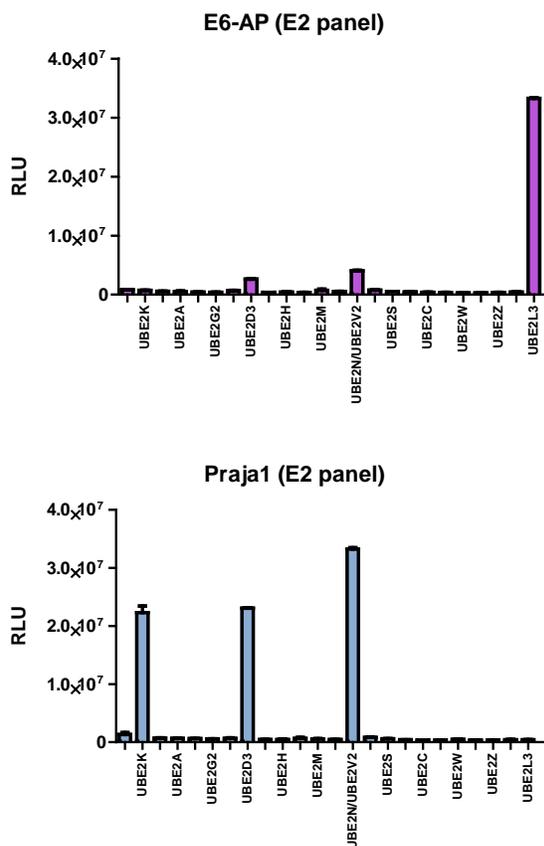
Under appropriate conditions, in which the reaction rate is limited by E3 concentration and at the time within the initial linear range of the overall time course, an apparent $K_m$ value can be generated for an E2:E3 interaction. For instance, the ubiquitin charged-E2 binds reversibly to the E3 ligase in the first step. In most cases this step cannot be quantified but the final product, autoubiquitylated E3 ligase created by the second reaction can be measured.

$$Ub\text{-}E2 + E3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Ub\text{-}E2::E3 \overset{k_2}{\longrightarrow} E2 + E3\text{-}Ub$$
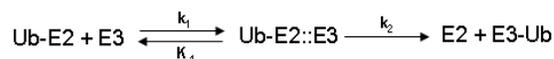
From this event, the rate of enzyme activity can be calculated (amount of autoubiquitylated E3 per time interval) as a function of charged-E2 concentration. This allows the calculation of an apparent $K_m$ value for the E2:E3 interaction.

## METHODOLOGY

**(For additional details, please refer to the E2 Profiling Kit manual[6] and the E3LITE Customizable Ubiquitin Ligase Kit manual[7])**

Using 12 E2s supplied in the E2 Profiling Kit, which encompass most E2 families from LifeSensors collection of more than 25 ligases, the E3 ligases E6AP and Praja1 were profiled (Fig.2). E2 conjugating enzyme (100 nM) was added to a cocktail mixture containing E1 activating enzyme (5 nM), the E3 ligases, E6AP (16 nM) or Praja1 (3 nM), and ubiquitin (1 μM). For controls, in addition to the E1+E2+E3+Ub reactions, a -E3 reaction was included adding E2 (100 nM) to a cocktail mixture of E1 (5 nM), and ubiquitin (1 μM). 50 μl of cocktail mixture with +/- E3 were dispensed into a 96-well E2 Profiling Kit plate in triplicates. To initiate the E3 ubiquitylation reaction, 50 μl of ATP (0.2mM final) was added to each well containing enzyme mixture. After one hour at room temperature, the reaction plate was washed 3x with PBS with 0.1% Tween (PBST). Subsequently, 100 μl of LifeSensors Detection reagent 1 in PBST containing 5% BSA was added to each well. After an additional hour of incubation, the plate was washed 3x with PBST before addition of 100 μl of streptavidin-HRP (Rockland; 1:10,000 dilution) in PBST containing 5% BSA for 1 hour. The plate was washed 4x with PBST before the addition of 100 μl ECL reagent (Millipore). E3 dependent ubiquitylation was detected by measuring the luminescent signal using a luminescence plate reader.



**Figure 2. E2 profiling of E6AP and Praja1 with the E2 Profiling Kit.** 12 E2s from different families were tested with the E3 ligases E6AP and Praja1. Activity with specific E2s is clearly identified. Once active E2:E3 combinations are identified, Km values can be determined to identify the favored E2 for a specific E3 (see Fig. 3).

the enzyme and the rate at which the substrate binds to the enzyme and is converted to a product. By comparing $K_m$ values for different E2 enzymes with a specific E3 ligase, the optimal E2 partner can be determined giving insight on ubiquitylation efficiency. For this reason, establishing $K_m$ values for many E2:E3 pairs is necessary to decode this complex network for better understanding of this critical cellular pathway. Although this assay platform is not formulated to determine accurate $V_{max}$ values, the $K_m$ values represent *apparent* $K_m$ values which have proven to be very accurate.

Utilizing the E$_3$LITE Customizable Ubiquitin Ligase Kit[7], the determination of K$_m$ values for specific E2s with E6AP was performed. Serial dilutions of the E2 conjugating enzyme (800-12.5 nM) were added to a cocktail mixture containing E1 activating enzyme (5nM), E6AP (16 nM), and ubiquitin (1 $\mu$M). 50 µl of cocktail mixture with different concentrations of E2 were dispensed into a 96-well E2 Profiling Kit plate in triplicates. Subsequent steps were followed as above. Finally, raw data generated were divided by reaction time and fit to the Michaelis-Menten equation using Prism software (GraphPad, San Diego, CA) to determine K$_m$ values.

### Measuring K$_m$ values for E2 conjugating enzymes with the E3 ligase, E6AP

K$_m$ values were determined for three different E2s; UbcH7 (UBE2L3), UbcH5b (UBE2D2), and UbcH5c (UBE2D3) using E6AP dependent ubiquitylation, (Fig. 3). The plotted curve was hyperbolic, exhibiting typical saturation kinetics as a function of substrate concentration. In addition, the apparent K$_m$ values generated closely correlated with previously recorded K$_m$ values (personal communication, Dr. Arthur Haas, LSU Health Sciences Center School of Medicine). These data demonstrates that E6AP exhibits a greater affinity for UbcH7 than the UbcH5 isozymes and suggest possible physiological relevance to this particular pairing in the cell.
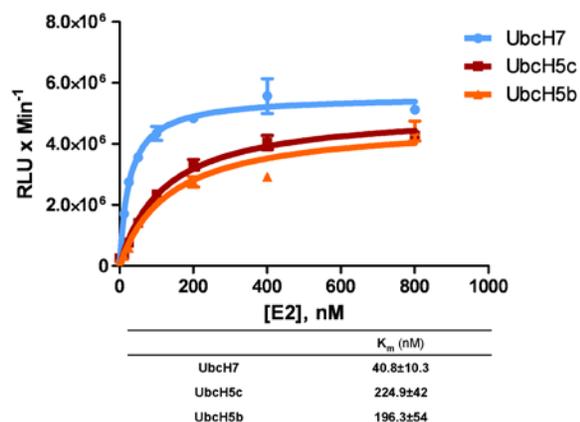


| | K$_m$ (nM) |
|---|---|
| UbcH7 | 40.8±10.3 |
| UbcH5c | 224.9±42 |
| UbcH5b | 196.3±54 |

**Figure 3. E2 kinetics with E6AP.** Serial dilutions of UbcH7, UbcH5b, and UbcH5c were added to E1 activating enzyme, E6AP, and ubiquitin. After the addition of ATP, E6AP dependent ubiquitylation was monitored and reported using the E3LITE Kit. The data were fit using Prism software to determine apparent Km values.

## ADDITIONAL EXPERIMENTS / PRODUCTS

### E3 substrate identification

Once the correct E2:E3 pair is identified, the discovery of E3 substrates can be pursued. LifeSensors has an E3 Substrate Identification Array Support Kit[8] which can be utilized to identify unique substrates for a specific E3. Using a highly sensitive proprietary detection reagent, a protein array can be profiled for substrates that get ubiquitylated from a specific E2:E3 pair.

## REFERENCES

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7. www.lifesensors.com/product-list.php?famid=42

8. www.lifesensors.com/product-list.php?famid=64

## About LifeSensors, Inc.

LifeSensors is a biotechnology company located 35 miles west of Philadelphia, Pennsylvania, USA. Founded in 1996, LifeSensors has developed a number of innovative protein expression technologies that enable efficient translation of the genome into proteome.

LifeSensors is well-known for its innovations in an important family of proteins consisting of ubiquitin and ubiquitin-like proteins (UBL) such as SUMO (Small Ubiquitin-like MOdifier).

LifeSensors has been granted several patents to cover the use of SUMO and other UBLs as gene fusion tags to improve the expression and purification of recombinant proteins. Additional patent applications are in various stages of review. Currently, LifeSensors is expanding its protein production capabilities and is developing protein micro array for drug discovery and diagnostics.

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