

UTILIZING E₃LITE (CUSTOMIZABLE UBIQUITIN LIGASE ASSAY) FOR HIGH- THROUGHPUT SCREENING (HTS)

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BACKGROUND

Ubiquitin and Polyubiquitylation

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to amine groups of lysine residues on targeted substrate proteins. This process utilizes a multi-step enzyme cascade involving three different enzymes (E1-E2-E3). Ubiquitin can be conjugated in either monomer form or as polymer chains, with polyubiquitylation at either lysine 48 or 63 of ubiquitin (K48 or K63 chain linkage) being the most characterized forms. The most prevalent consequence of polyubiquitylation is K48-mediated proteasomal degradation of the substrate protein. Polyubiquitin modification is a reversible process utilizing a class of proteases known as deubiquitylases (DUBs) that remove ubiquitin and ubiquitin chains from substrates. The dynamic and intricate nature of this signaling, therefore, represents a major obstacle to functional characterization of ubiquitylated proteins.

E₃ LITE: An improvement in screening small molecule modulators against E3 ligase activity

E3 ligase activity assays are notorious for being complex and not very robust. With multiple enzymes and a substrate in the reaction mixtures, deciphering E3 ligase inhibition can be complicated. Available assays generally try to overcome this obstacle by utilizing tagged or immobilized enzymes leading to assays that are expensive or complicated to set up and run.

LifeSensors' E₃ LITE kit can help characterize and quantify ubiquitin E3 ligase activity easily, overcoming many of the obstacles associated with HTS. The assay is based on the ability of an ubiquitin binding domain to preferentially bind polyubiquitin relative to monoubiquitin. This binding event directly reports on the activity of an E3 ligase that builds ubiquitin chains in a dose-dependent manner. In the absence of substrate, the building of ubiquitin chains is on the E3 ligase (autoubiquitylation). The benefit of this strategic approach allows the reaction components be native, non-tagged, and free in solution. The assay format is versatile allowing the detection of K48-linked or K63-linked polyubiquitin chains. In addition, the assay has been shown to detect activity of different E3 ligases from different classes including simple RING finger ligases, SCF ligases, and HECT domain ligases. For HTS the assay format is amenable to 96-well and 384-well plates (Figures 1A and 1B). The E₃ LITE Kit has been successfully utilized in a screening program finding inhibitors of the E3 ligase MuRF1 (Figure 2).

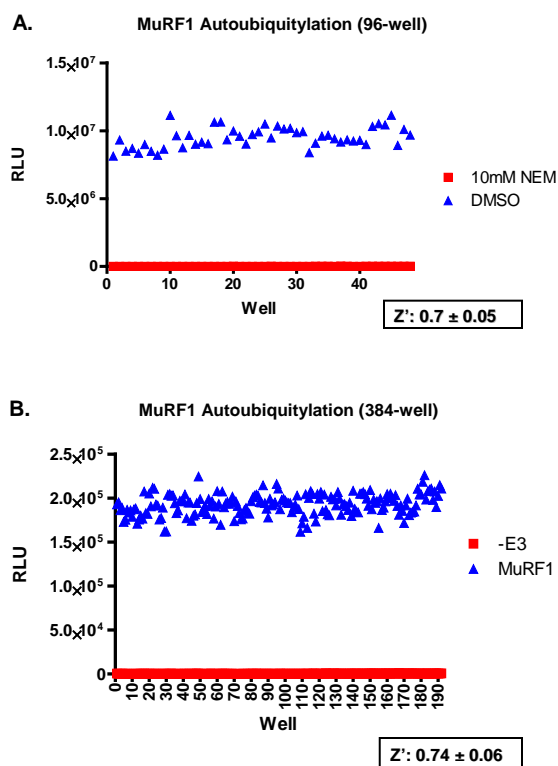


Figure 1: The Z' score for MuRF1 utilizing the E₃ LITE kit in 96-well plate format (A), and 384-well plate format (B). In both formats the Z' score is above 0.5 indicating that this assay is reproducible and robust. In the 96-well format the inhibitor sulfhydryl alkylating agent N-Ethylmaleimide (NEM) was used to inhibit the ubiquitylation reaction as a positive control. In the 384-well format the positive control used was minus MuRF1.

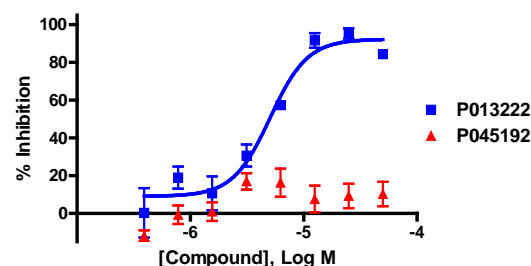


Figure 2: The inhibitor P013222 was identified from a primary screen of MuRF1 utilizing the E₃ LITE kit. P013222 (blue) inhibits MuRF1 with an EC₅₀ of 8.5 μM. Also shown is P045192 (red) which was identified as an inactive analog of P013222. EC₅₀ values for inhibitors can be quickly determined with the E₃ LITE kit.

SCREENING A SMALL MOLECULE COMPOUND LIBRARY FOR E3 INHIBITORS

Utilizing 96-well coated microtiter plates for 100 μ l reactions, 2 μ l of compound (50x concentration in 100% DMSO from chosen compound collection) is added to the wells with a liquid handler. Positive and negative controls of 2 μ l of NEM (250mM in 100% DMSO) or 2 μ l of DMSO (100%) are added to the control wells. An E1-E2-E3 enzyme cocktail (80 μ l, 1.25x concentration) is then added to all the wells and incubated for 30 minutes at room temperature. The reaction is initiated by the addition of 20 μ l (5x concentration) of an ATP-Ubiquitin cocktail. After a predetermined time (from initial Time/Dose experiments) the reaction is stopped by washing. Primary detection reagent 1 is added (100 μ l) to all the wells and incubated for one hour. After an additional wash, streptavidin-HRP is added (100 μ l) to all the wells and incubated for one hour. After the final wash, 100 μ l of chemiluminescence agent is added and the plates are monitored for loss of signal. Discovered inhibitors need to be further profiled against the other enzymes in the reaction (E1 and E2) to determine their selectivity and the mode of action.

After identification of a potential inhibitor, the potency of the compound can be ascertained by modifying the above procedure. Briefly, 2 μ L of various concentrations of compound are added to a 96-well plate in triplicate. For the control, 2 μ L of DMSO or 400mM NEM are added to wells in triplicate. 80 μ L of E1-E2-E3 enzyme cocktail is added and the plate is incubated at RT for 30 minutes. The reaction is initiated by the addition of 20 μ L of an ATP-ubiquitin mixture. The assay is completed as described above. The percent inhibition for each concentration of compound is calculated using DMSO and NEM values as controls. The data can be plotted as percent inhibition versus LOG compound concentration as shown in figure 2. Using standard analysis software such as GraphPad Prism an EC₅₀ can be calculated. Figure 2 shows the dose response for both an active (P013222) and an inactive (P045192) small molecules.

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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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