Unlocking the Impact of E3 Ligases in Drug Discovery

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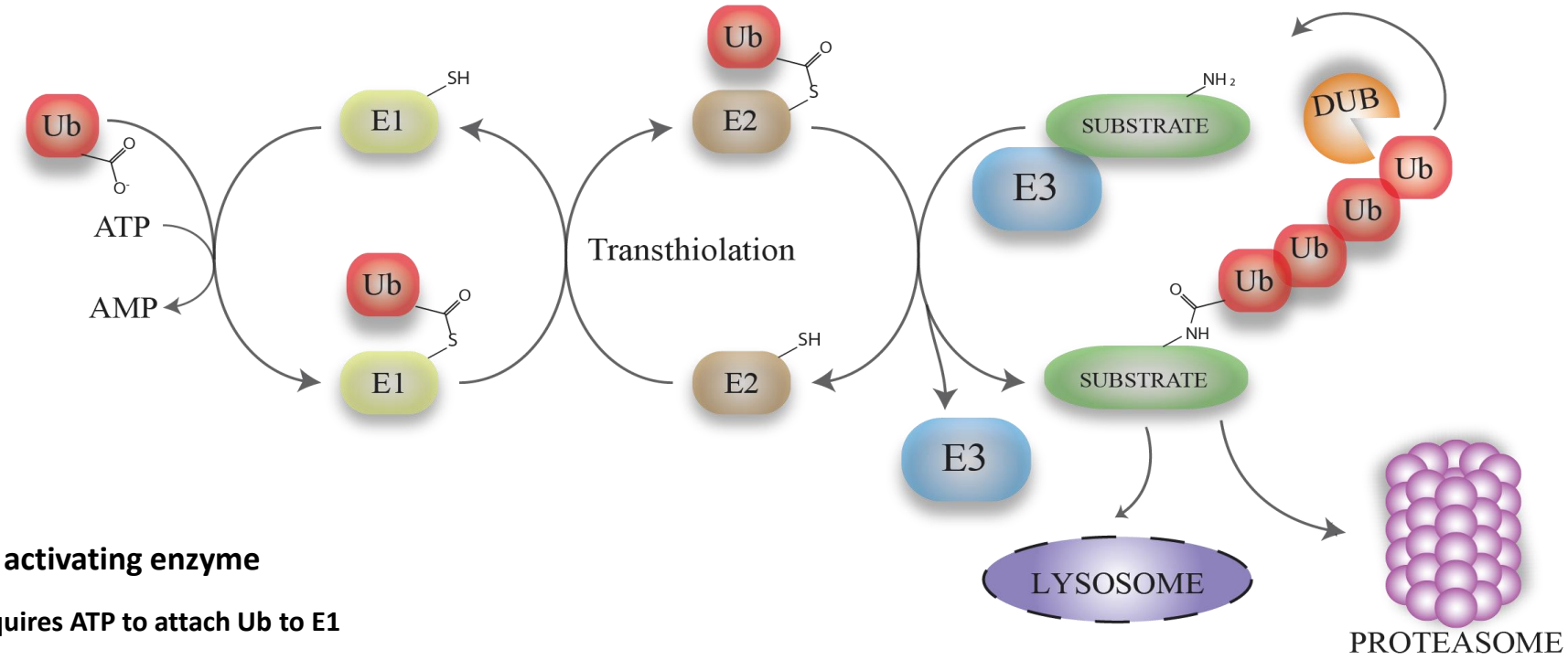
Understanding Ubiquitin Proteasome System in Drug Discovery



E1 – Ubiquitin activating enzyme

Requires ATP to attach Ub to E1

E2 – Ubiquitin conjugating enzyme

Transfers Ub from E1 to E3

E3 – Ubiquitin ligases

Transfers Ub to self or substrate

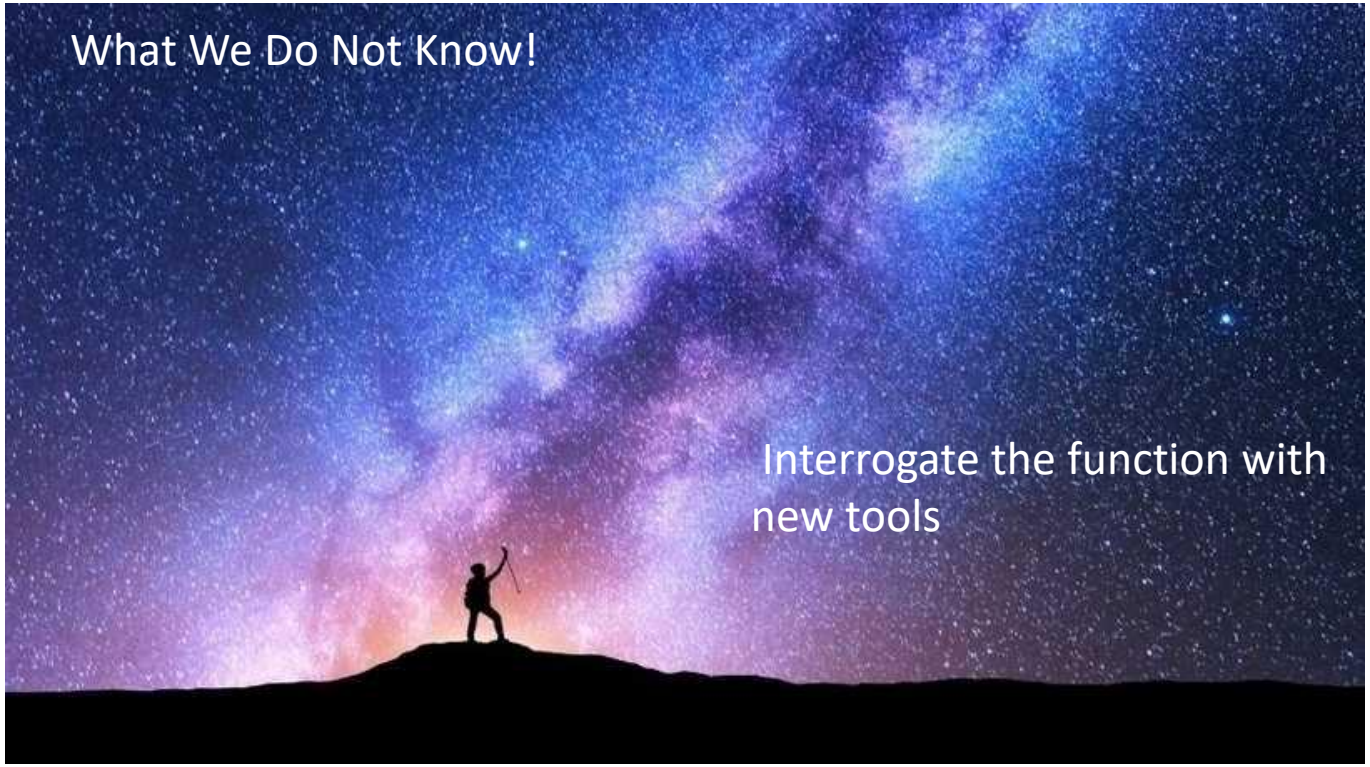
Forms mono-Ub or poly-Ub chains

DUB – Deubiquitinase

Removes mono-Ub or poly-Ub chains

Proteasome – Degrades ubiquitylated proteins

The Vast Universe (Functions) of E3 Ligases



Expression and Purification of Phsiologically Active E3 Ligases

- Expressed and purified ~40 biologically active E3 ligases
- Assays to monitor inhibition, activation, substrate ubiquitylation, PROTACs and Mol Glues
- TUBE embedded microtiter plate-based HTS screening and selectivity profiling
- Determine compound MOA, E3 substrates in cells and target tissue PD markers

Unlocking the Impact of E3 Ligases in Drug Discovery

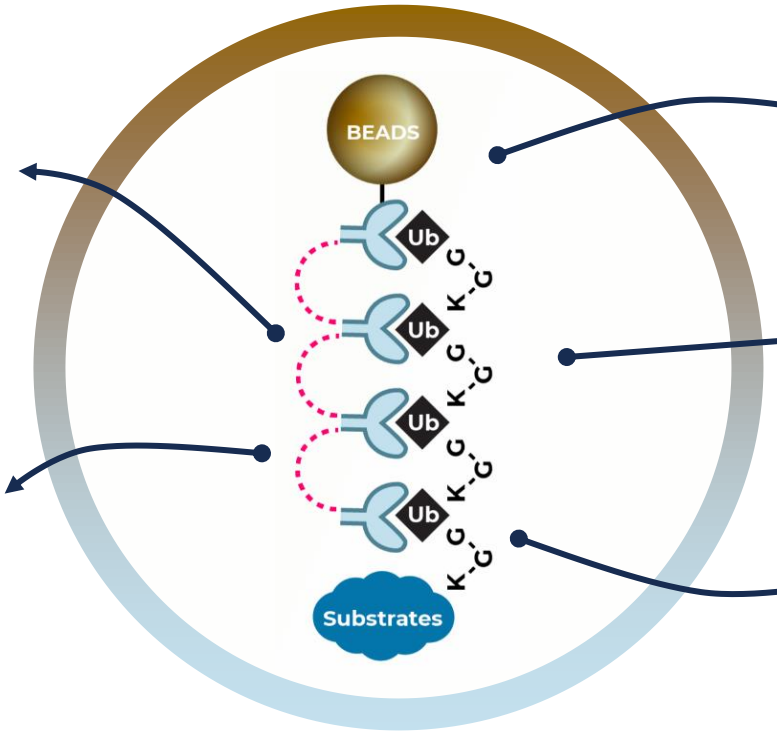
- **Step One: Assay Development, Optimization and HTS**
- TR-FRET E3 Assay
- E3 ELISA Assay
- **Step Two: E3 Ligase Selectivity Panel**
- Working with medicinal chemistry team
- Selectivity panel, compound profiling
- **Step Three: Validate Hits in Cellular Assays**
- UbiQuant S assay (ELISA)
- Cellular E3 Substrate Identification TUBE-Based Mass Spec Proteomics
- UbiTest (Immunoblot-based assay)

TANDEM UBIQUITIN BINDING ENTITIES

TUBEs: A Versatile Tool in E3 Ligase Assays

Ubiquitin Binding Domains

Engineered for Chain Selectivity

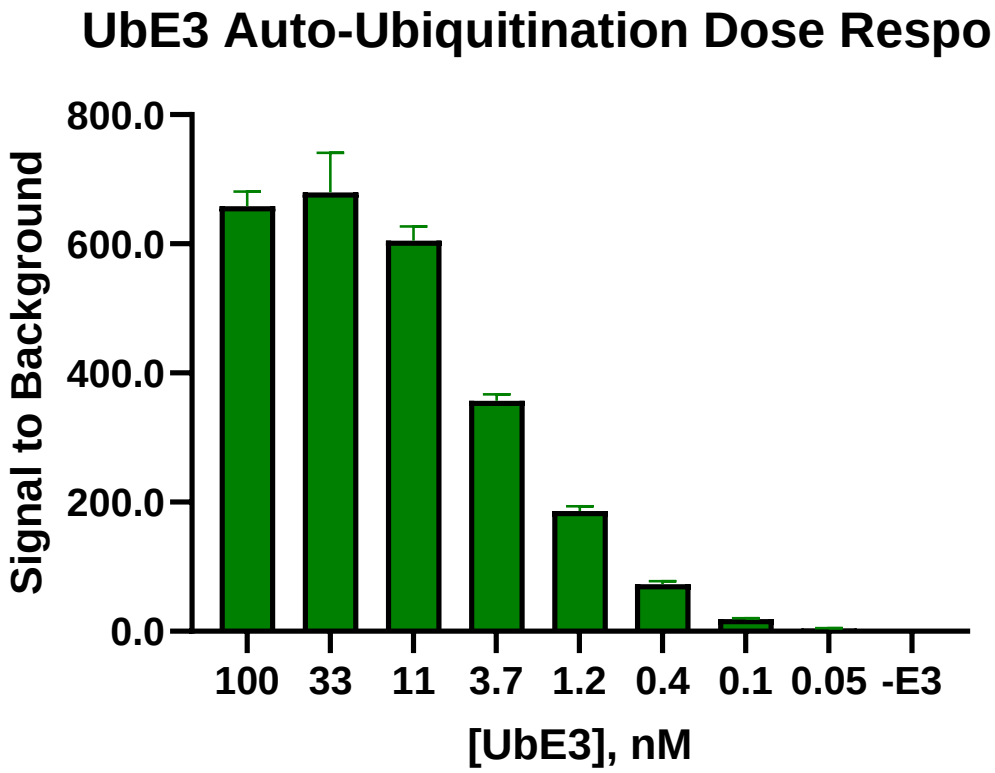


Versatile applications

Sub-nanomolar affinity binding

Protection against intrinsic DUBs

Highly Sensitive Assay to Capture E3 Ligase Activity

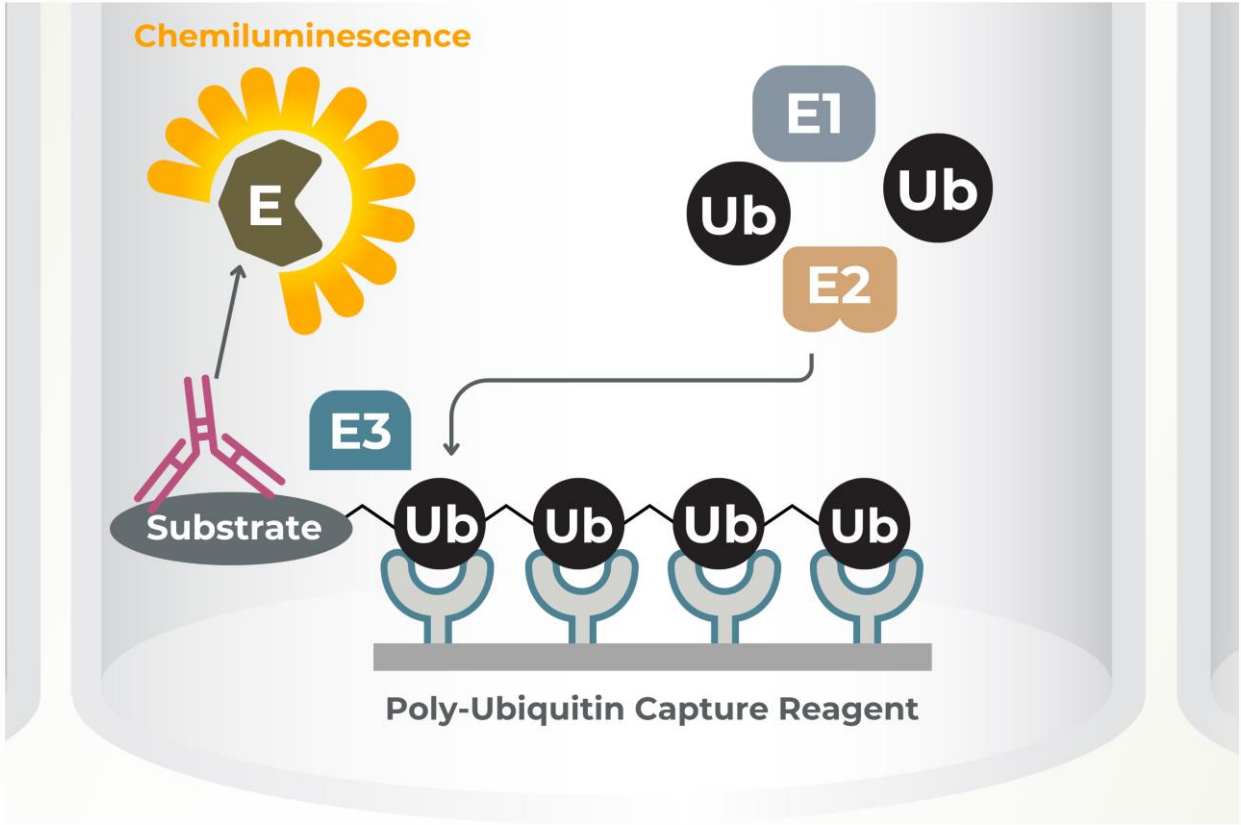


E3 auto-ubiquitination levels, detected using LifeSensor's UE905 plate with TUBE1 Biotin, showed a dose-dependent increase reflecting enzymatic activity and assay dynamic range.

Step One: Assay Development, Optimization and HTS

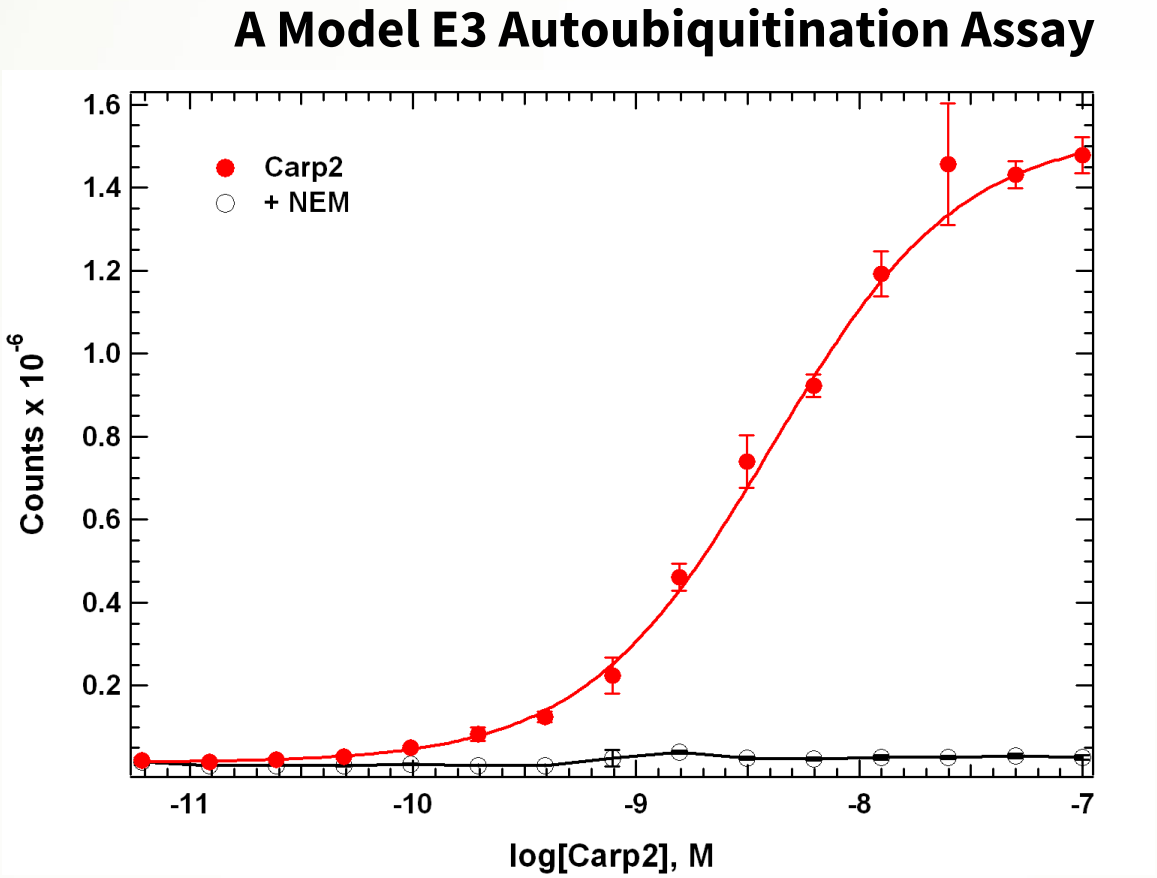
E3 Ligase ELISA Assays

Quantification of E3 ubiquitin ligase activity, employs a proprietary TUBE reagent to capture polyubiquitin chains formed in an E3 ligase dependent manner



- The polyubiquitylated E3 is detected using HRP-conjugated TUBEs.
- Polyubiquitinated substrate is detected using specific antibodies.
- The chemiluminescent signal can be followed over time in a homogenous format
- High-throughput format, ideal for small-molecule screening.

Example of E3 Ligase ELISA Assay

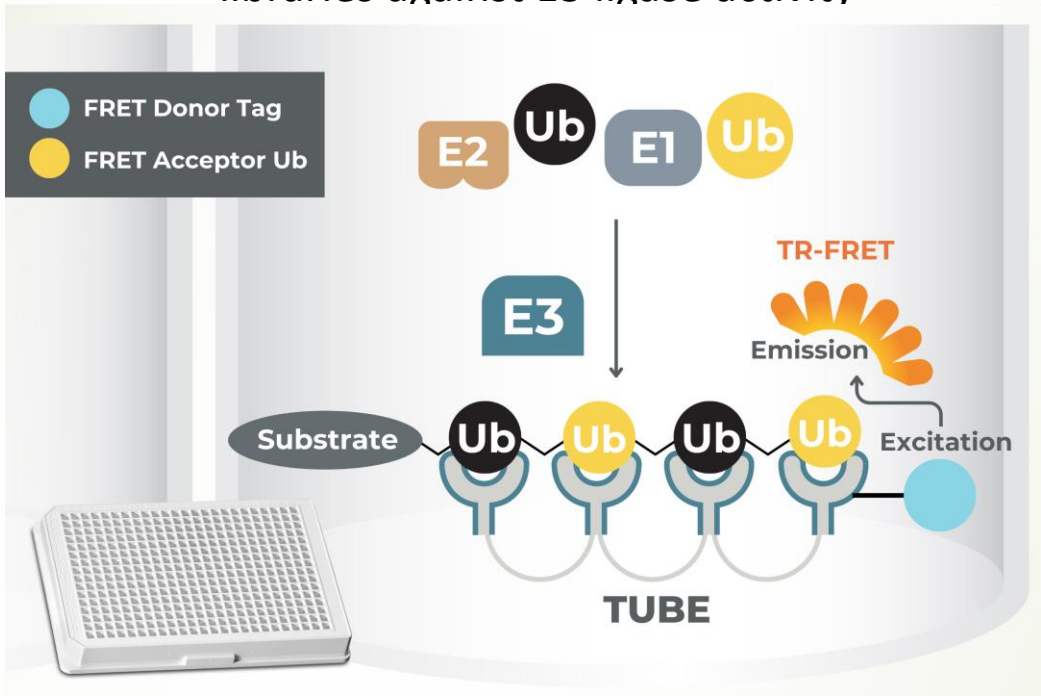


- E3 Dose dependent signal increase
- Robust Assay (Z' >0.8, S/B >15)
- E3 assay inhibited with NEM
- Assay also validated with TAK-243, an E1 inhibitor as positive control for inhibition

Step One: Assay Development, Optimization and HTS

TR-FRET E3 Ligase Assay

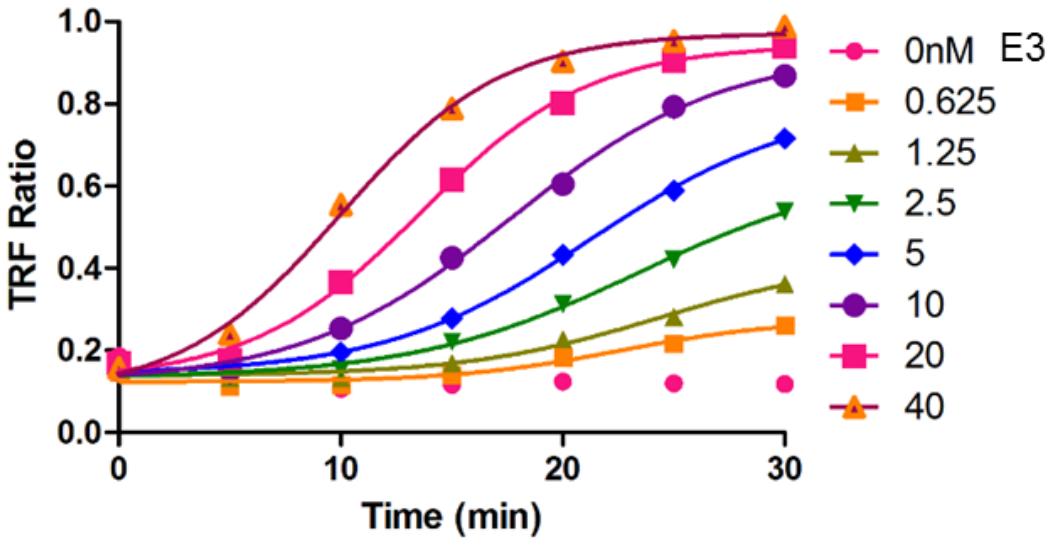
Fluorescence-based high-throughput assay system for screening compound libraries against E3 ligase activity



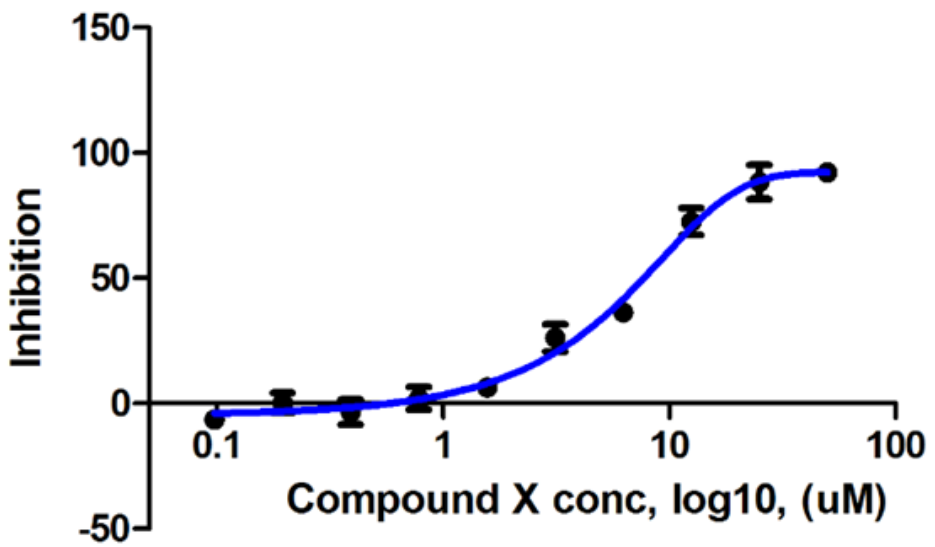
The **TR-FRET E3 Assay** involves Terbium-labeled TUBEs that bind to Fluorescein labelled polyubiquitin chains synthesized by the target E3 ligase. Terbium and Fluorescein are a FRET pair, so polyubiquitin chains containing Fluorescein-labeled ubiquitin yield a FRET signal when bound by a terbium-TUBE. This signal can be monitored over time in a homogenous, high-throughput format, making it ideal for small-molecule screening.

E3 Ligase Substrate Ubiquitination TR-FRET E3 Assay

E3 Titration
Substrate Protein X Ubiquitination



Protein X Ubiquitination
Inhibition



E3 TR-FRET assay and inhibitor dose response curve

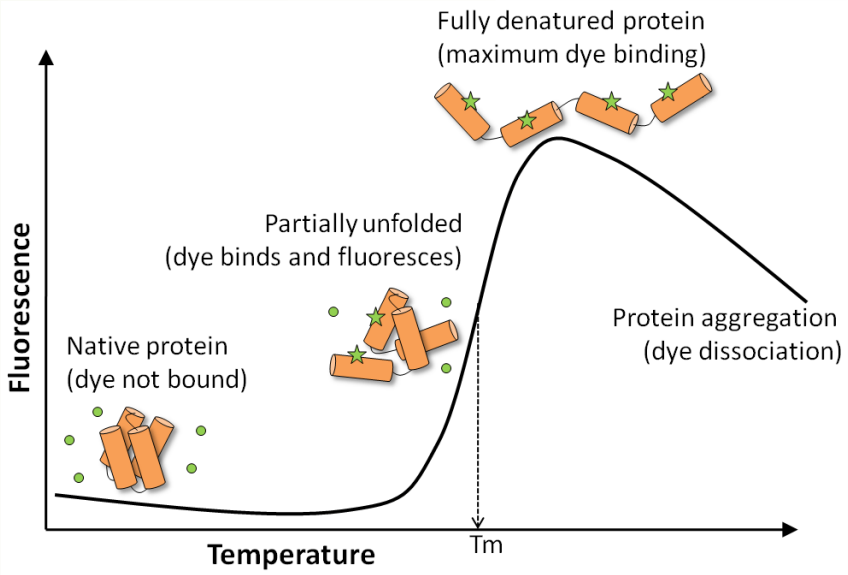
Protein X was used as a substrate for this E3 ligase. After initial TR-FRET high-throughput screening, selected compounds were used to determine IC_{50} by titration assay.

Step Two: Hit-to-lead optimization

Validation Assays

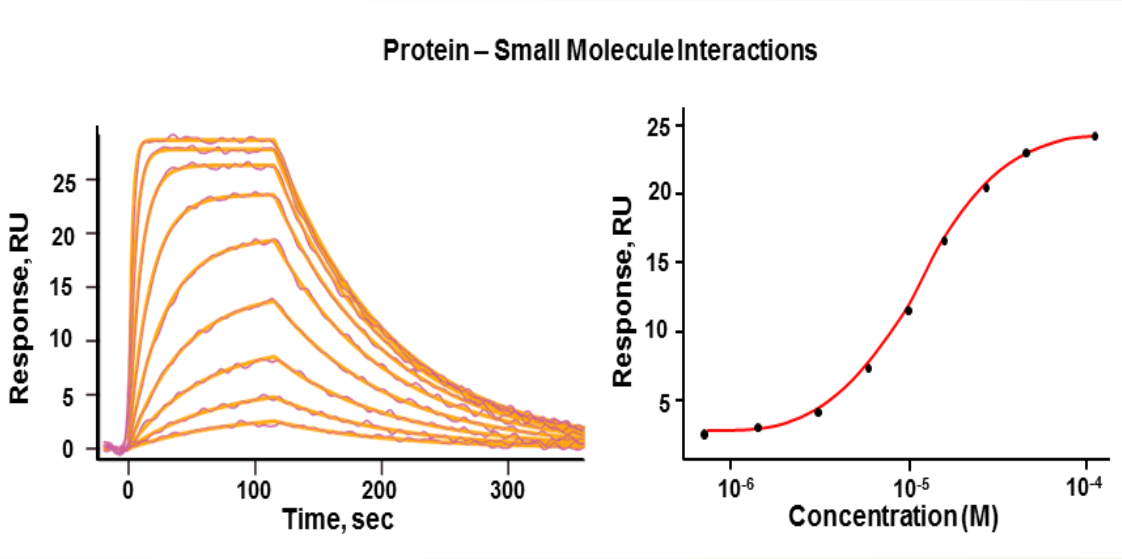
Thermal Shift Assay

HTS assay to detect compound binding to a target



Surface Plasma Resonance

Determination of a small molecule affinity to a target



Step Two: E3 Ligase Selectivity Panel

Selectivity Assays

E3 Ligase Panel	Representative E3s
Panel I (5 E3 ligases)	CRBN, CARP2, gp78, CHIP, Nedd4L
Panel II (10 E3 ligases)	CRBN, VHL, HDM2, cIAP2, CARP2, gp78, CHIP, Nedd4L, Praja1, Cbl-b
Panel III (29 E3 ligases, includes E3 from panel I as well)	CRBN, VHL, Hdm2, RNF4, CARP2, TRIM32, TRIM47, Cbl-b, c-Cbl, cIAP2, IDOL, SIAH, MURF1, MURF2, MURF3, Praja1, TRAF6, Parkin, E6-AP, Itch, Nedd4L, WWP1, WWP2, MARCH5, Hrd1, gp78, CHIP, RNF114, Nedd4, WWP1, WWP2.

Each ligase assay has been validated in TR-FRET assays regarding E2 pairing.
LifeSensors profiles inhibitory or activation properties of every compound in Panel I followed by Panel II.

Step Two: Hit-to-lead optimization

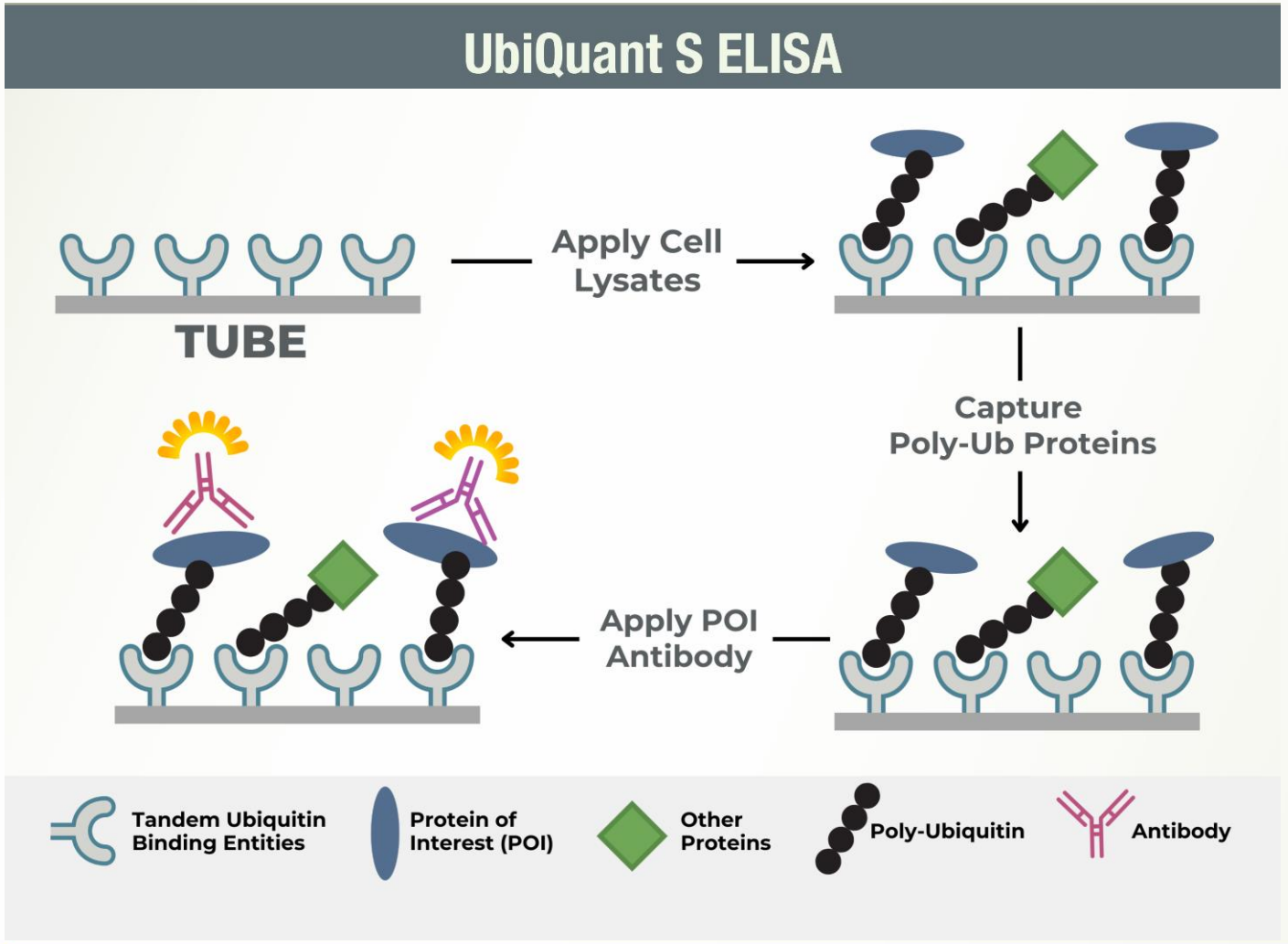
Mechanistic Validation Assays

Secondary screens to deconvolute hits from E3 screening (eliminating compounds that affect E1-E2 conjugation)

- **E3 Lite -** Measures E3 activity
- **E1 Lite -** Measures E1 activity
- **E1/E2 transfer -** Measures transfer between E1 to E2
- **E2 Profiling and Selection -** Finds the best E2 for your E3

Step Three: Validate E3 Ligase Hits in Cellular Ubiquitination Assays

Enables accurate determination of cellular substrate (POI) ubiquitination for monitoring the effects of various treatments



Step Three: Validate hits in cellular assays

TUBE-based Mass Spec Ubiquitin Proteomics

- TUBE-based proteomics to identify ubiquitination patterns specific to drug treatment
  - Optimized for cell and tissue lysates
  - Customer provides cell pellets, we do the rest
- Superior to Di-Gly proteomics method
- Assess specificity of E3 ligands, inhibitors, PROTACs and Molecular Glues
- Identify poly-ubiquitylation site(s) (number & position) on the protein sequence
- Fee for service model, defined milestone-based agreement

Identification E3 ligase Modulators for Clients:

Example #1

- **E3 ligase X: Assay development, validation and HTS**
- 50K small molecule library screen using TR-FRET E3 Assay
- 1600: number of primary hits, Z' >0.5
- 64: number of confirmed hits with selectivity
- 10: number of compounds with IC50s sub micromolar to nM
- **Step Two: Hit-to-lead optimization**
- Hit expansion (with medicinal chemistry team)
- Extended selectivity panel, compound profiling
- **Step Three: Confirm hits in cellular assays**
- 10 hits transferred to client for cellular validation

Identification E3 ligase modulators for Clients:

Example #2

- **E3 ligase X: Assay development, validation and HTS**
- Client's compounds screened using TR-FRET E3 Assay
- 10: number of confirmed compounds with selectivity
- 10: number of compounds with IC50s sub micromolar to nM
- **Step Two: Hit-to-lead optimization**
- Hit expansion (with medicinal chemistry team)
- Extended selectivity panel, compound profiling
- **Step Three: Confirm hits in cellular assays**
- Confirmation through Ubiquitin Mass Spec Proteomics

Identification of E3 ligase target for Molecular Glue degraders:

Example #3

- **E3 ligase X degrading target Y: Assay development, validation**
- Validation of target degradation and ubiquitination in cells (kinetics)
- Rescue of degradation using Proteasome/Lysosome inhibitors
- Determine the optimal dose and time needed to robustly ubiquitinate target
- **Step Two: Mass Spec Proteomics for E3 identification**
- Pull down target protein ubiquitination complex from cells treated with degrader
- Perform proteomics to identify interacting E3 ligases
- **Step Three: Validate hits in in vitro and cellular assays**
- Use recombinant E3s to confirm molecular glue mediated ubiquitination of target in vitro
- Validate the role of E3 in cells using CRISPR/Cas knock-out system

E3 Ligase Screening & Profiling Services

- We help customer discover E3 ligase ligands, inhibitors and activators
  - Express & purify biologically active E3 Ligases and substrates
  - Develop and optimize HTS assay for E3 ligase
  - Screen in house libraries or customer libraries at LifeSensors
  - Biophysical and biochemical assay development for target engagement
- Cell-based assays to determine target engagement by compound
- All IP and data belong to the customer
- Work performed under CDA and Master Service Agreement
- Fee for service model, defined milestone-based agreement

Contact Us!

We are your partner for E3 Ligase drug discovery

Contact Information

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