LifeSensors DUBs Screening & Profiling Platform

LifeSensors

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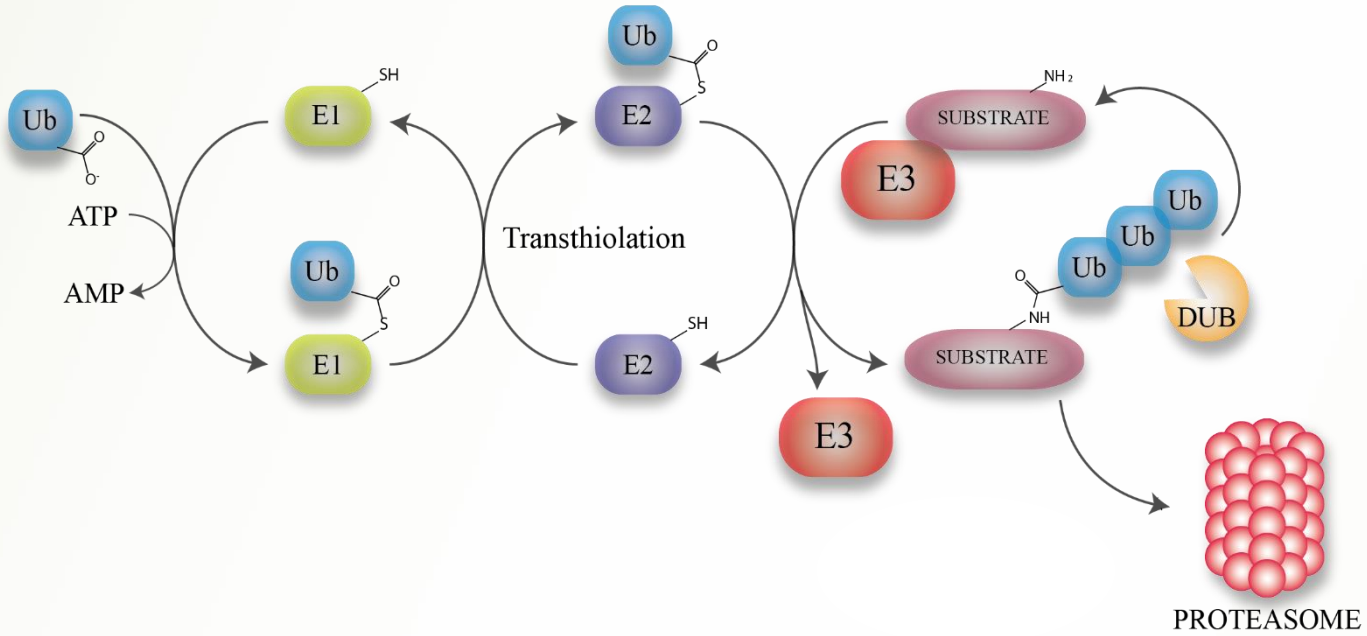
LifeSensors Inc. Mission

- Leadership in UPS, PROTAC, DUBTAC, and Molecular Glues
- Drug Discovery, UPS Enzymes, DUBs, PROTAC Screening Services
- Biomarker Development and Collaborative Research
- ~500 Products, DUBs, E3 ligases, Ubiquitin Affinity Matrices (TUBEs), Assay Kits and Proprietary Protein Expression Systems (SUMO)
- Profiling Compounds Against Ubiquitin Ligases and DUBs

Ubiquitin Proteasome System



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**

DUB-based Drug Discovery Capabilities

- ~40 biologically active DUBs for compound profiling and selectivity panel
- Developed ~25 DUB assays for a variety of HTS and validation strategies
- Ability to screen ~500,000 compounds
- Establish pre-clinical and clinical biomarkers for DUB, DUBTAC ligands
- Determine compound MOA, cellular and target tissue PD markers

DUB Families

DUB Family	Representative DUBs
USP	USP2, USP5, USP7, USP8, USP15, USP20, USP21, USP30, USP34, USP47, USP51
OTU	OTUB1, Cezanne,
UCH	UCHL1, UCHL3, UCHL5
Viral & Bacterial	PLPro, PLP2, Ssel
MJD	Ataxin 3, JosD1
JAMM	AMSH
DeSUMOylase	Ulp1, SENP2, SENP6
DeNEDDylase	Den1
MINDY	MINDY1

LifeSensors DUB Selectivity Panel

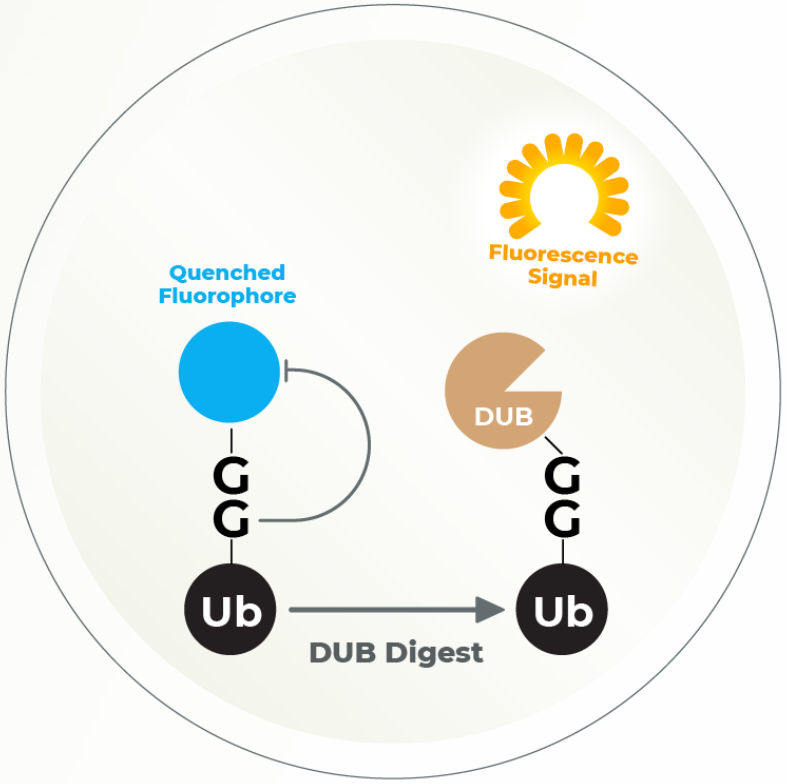
(Largest collection of functional DUBs in the industry and growing)

DUB Panel	Representative DUBs
Panel I (10 DUBs)	USP2c, USP4, USP5, USP7, USP8c, USP15, USP30, UCHL1, OTUB1, OTUD7B (Cezanne).
Panel II (22 DUBs, includes DUB from panel I as well)	USP2c, USP4, USP5, USP7, USP8c, USP10, USP15, USP21, USP28, USP30, USP33c, USP47, OTUB1, OTUB2, AMSHc, Cezanne, UCHL1, UCHL3, UCHL5, SENP1c, SENP6c, PLPro.

Each DUB assay has been validated. LifeSensors profiles inhibitory or activation properties of every compound in Panel I followed by Panel II.

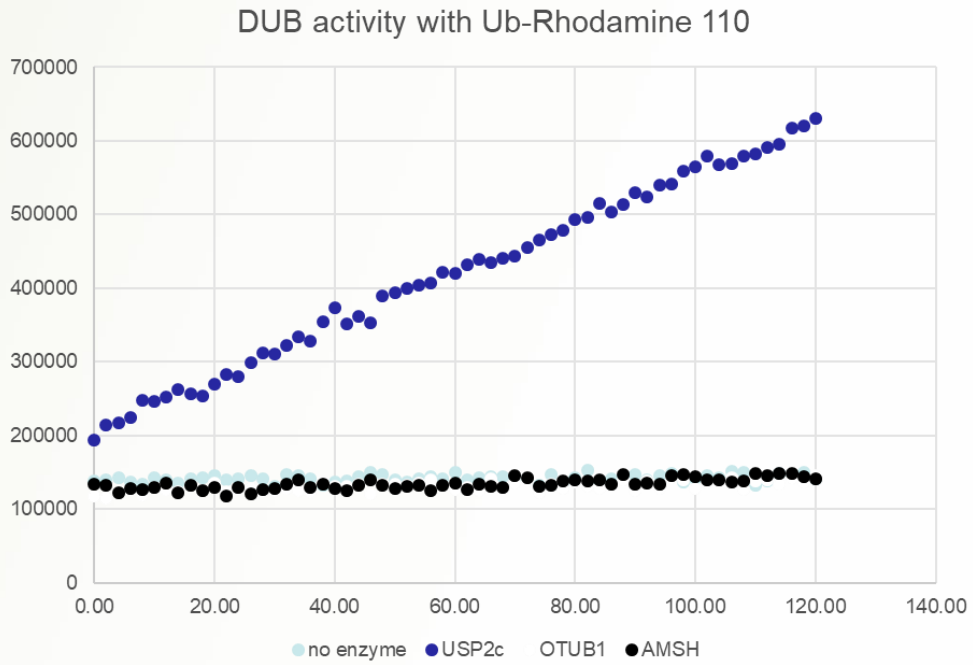
Step One: DUB Assay Development, Optimization and HTS

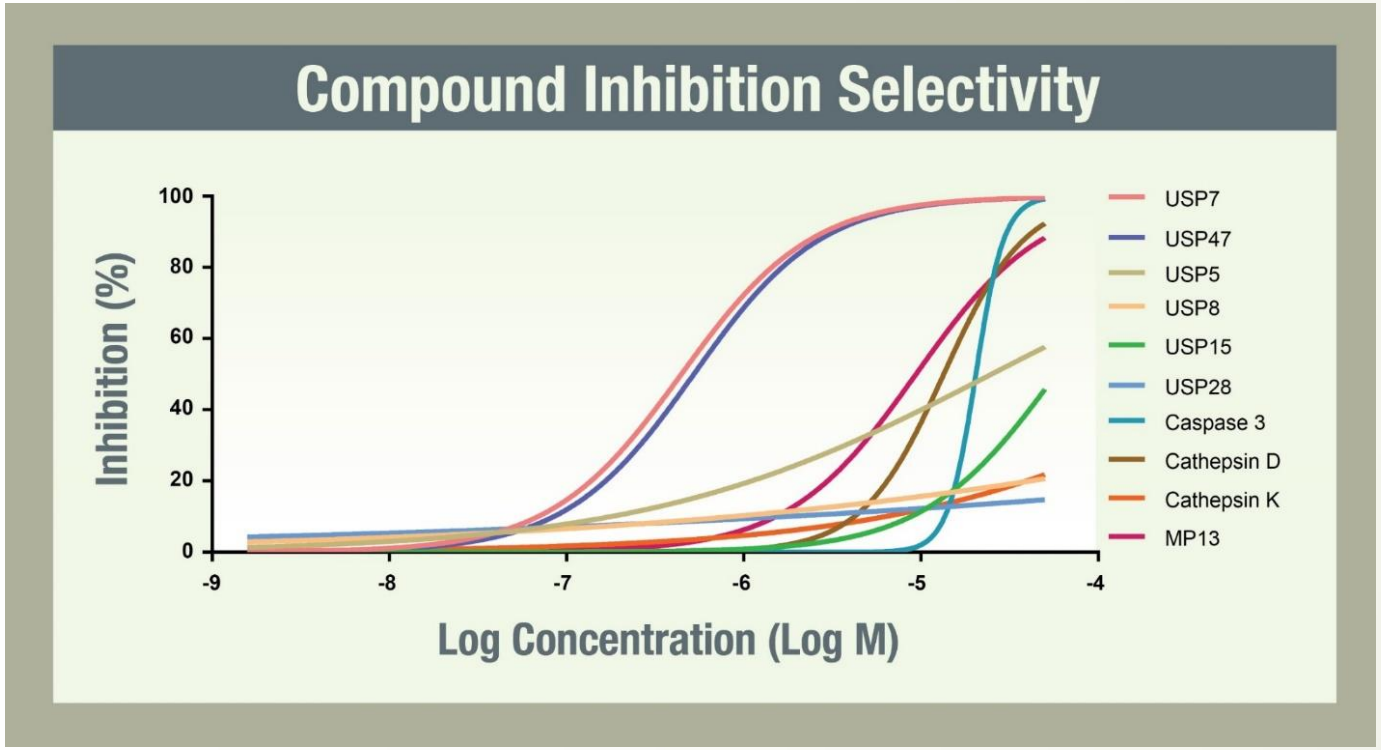
DUB HTS and Validation Assays



- The classic Ubiquitin Fluorophore Assay utilizes a ubiquitin molecule attached to a quenched fluorophore (e.g. Rhodamine 110).
- DUB activity releases the fluorophore resulting increased fluorescence.
- This simple and straight-forward assay is a great high-throughput screening option for most DUBs.

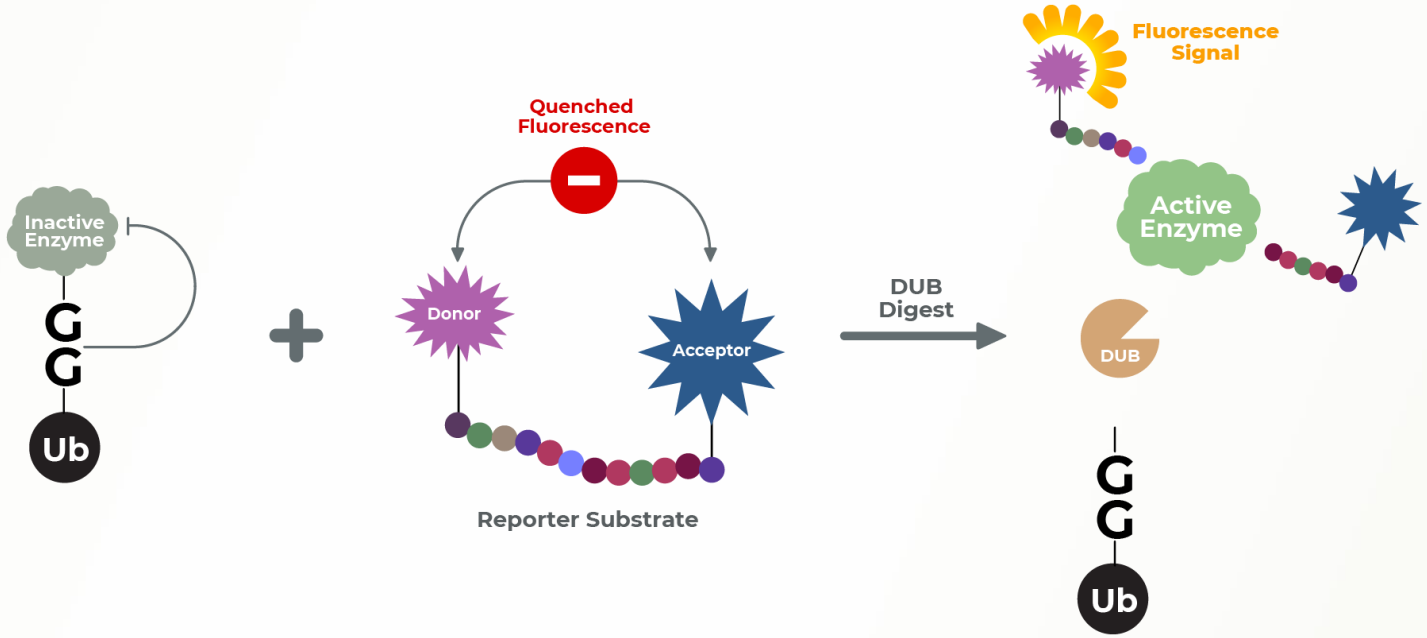
Example of DUB HTS and Validation Assays





DUB HTS and Validation Assays

Ub-CHOP-Reporter Deubiquitylation Assay

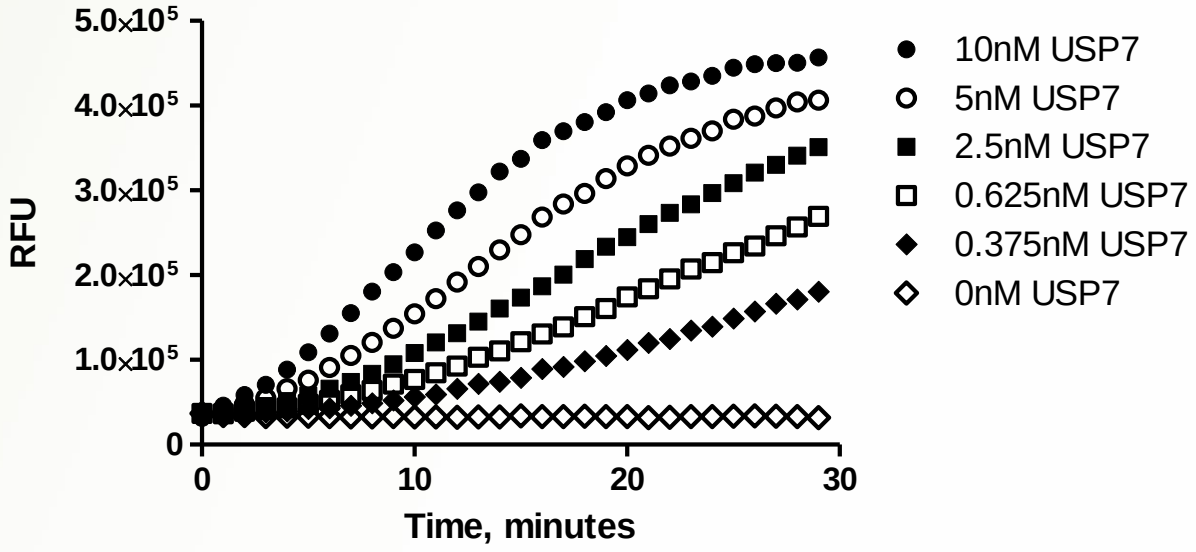


- The [Ub-CHOP-Reporter Deubiquitylation Assay Kit] consists of ubiquitin fused to a reporter enzyme.
- The fused reporter enzyme stays catalytically inactive.
- Cleavage of the ubiquitin by DUBs, releases the active reporter enzyme which acts on its fluorescence substrate.
- The fluorescence signal generated by the reporter enzyme is a quantitative measure of DUB activity.

Example of DUB HTS and Validation Assays

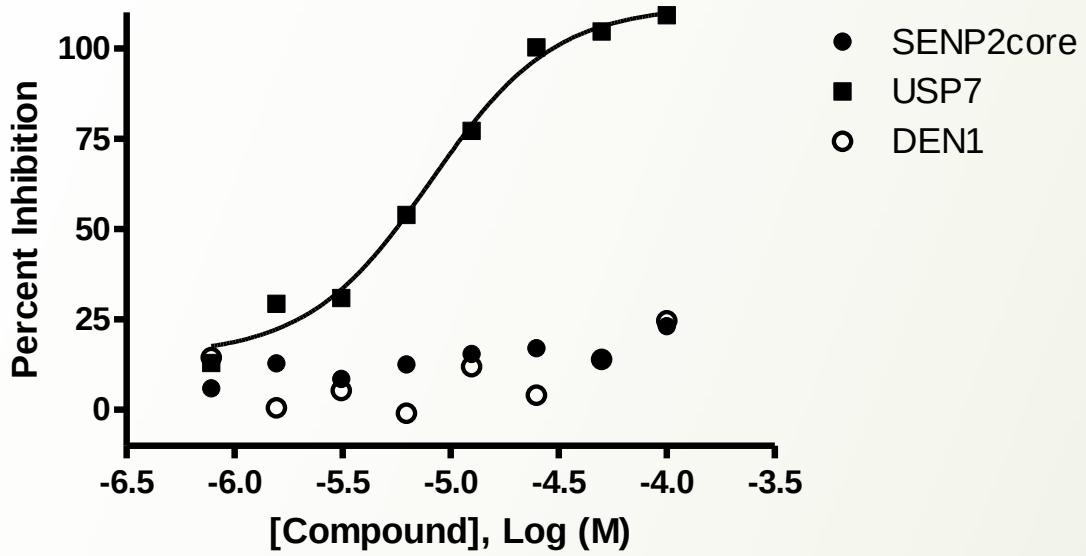
CHOP Assay

Dose response of USP7 in CHOP assay



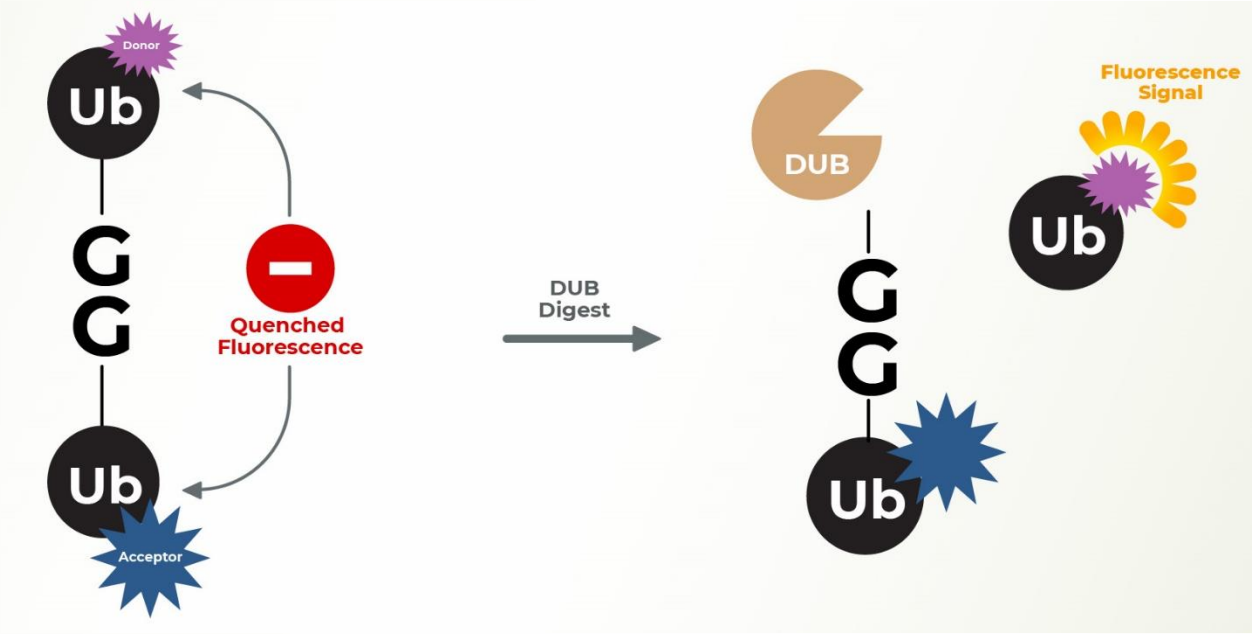
IC50 values of USP7 inhibitor compound X

SENP2core and DEN1 are negative controls



DUB HTS and Validation Assays

Internally Quenched Fluorescently Labeled DiUbiquitin assay



IQF DiUb panel

K48 panel:

K48-1, K48-2, K48-3, K48-4, K48-5, K48-6

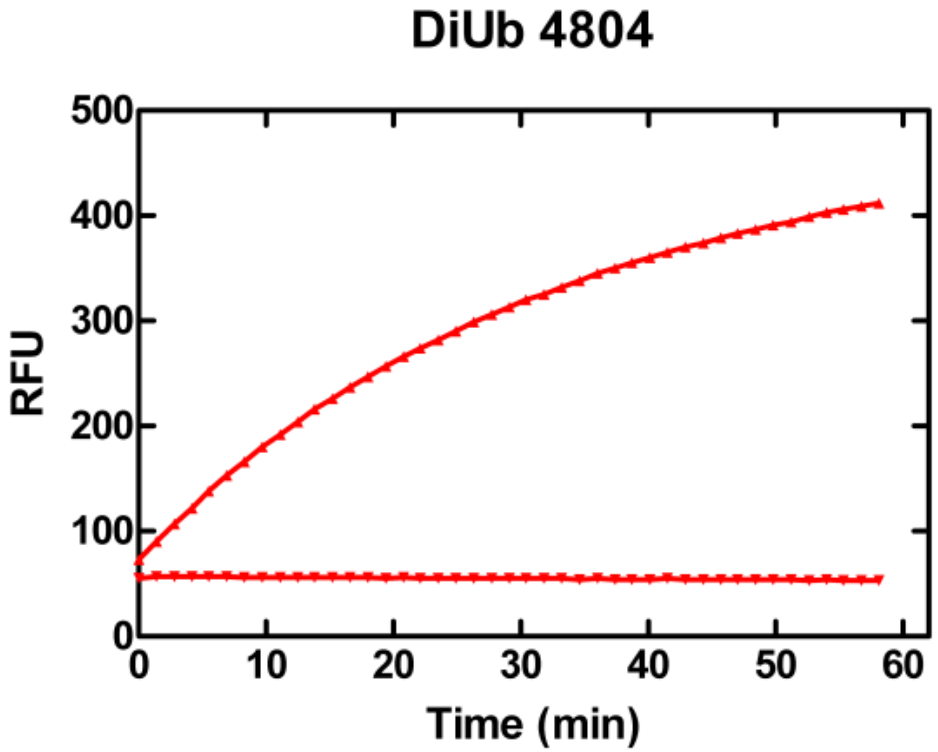
K63 panel:

K63-1, K63-2, K63-3, K63-4, K63-5, K63-6

- The **IQF Assay** utilizes a pair of conjugated ubiquitin proteins.
- One ubiquitin carries a fluorophore silenced by the nearby quencher.
- After DUB digestion the unquenched fluorophore can be detected by a plate reader.
- This assay is **physiologically relevant** since the DUB cleaves a **Ub-Ub isopeptide bond**.

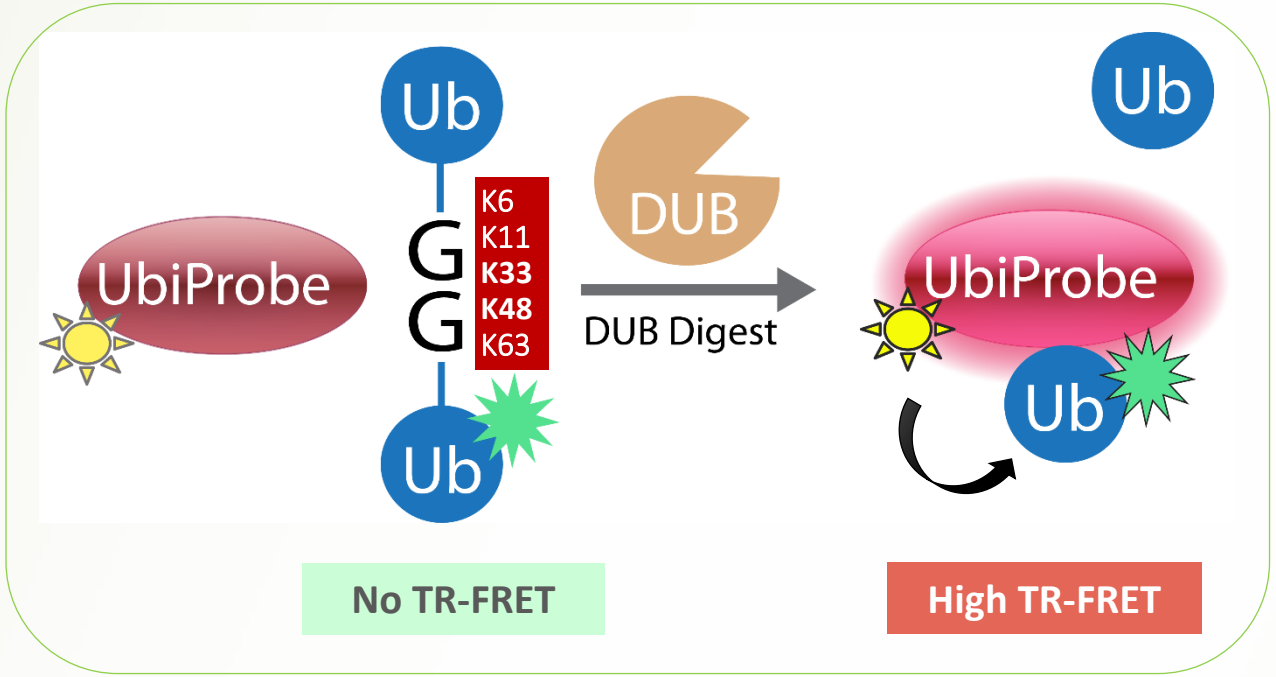
Example of DUB HTS and Validation Assays

Internally Quenched Fluorescently Labeled DiUbiquitin assay



- **Progression of DiUb cleavage by USP2core**
- DiUb K4804 (200 nM) was incubated with (curve) or without (flat line) 10nM USP2core.
- The increase in fluorescence was monitored.

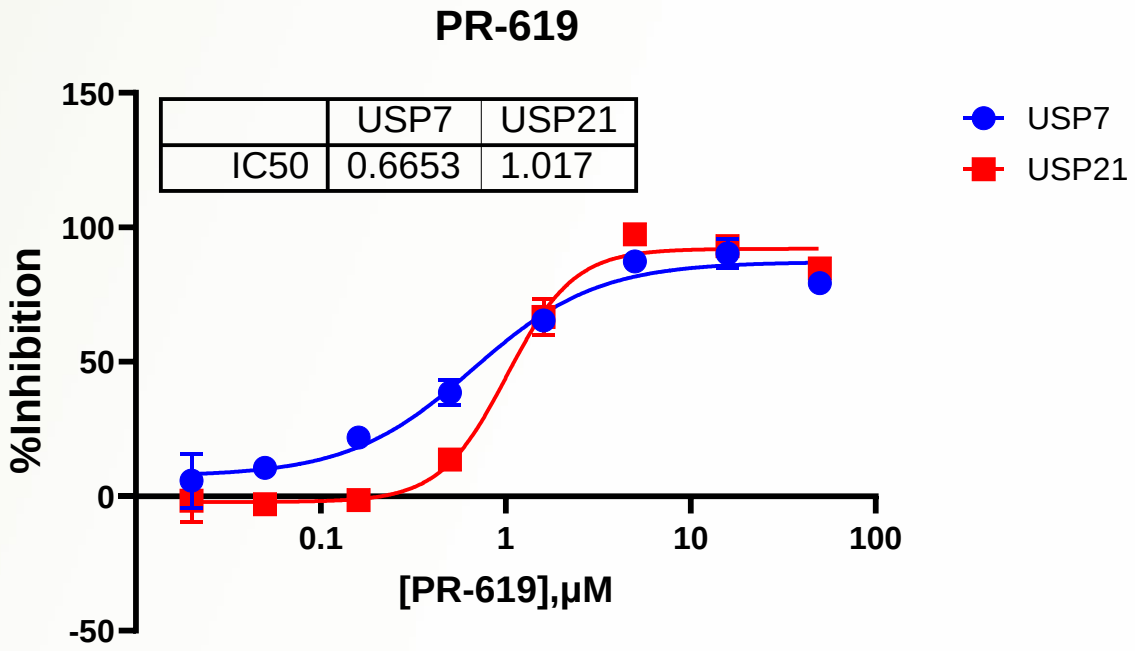
A novel TR-FRET DUB assay platform (UbiProbe)
(Highly sensitive, robust and HTS compatible DiUb assay)



Key Features

- Physiologically relevant DiUb substrates with native isopeptide linkages
- Highly sensitive (lower enzyme dose) and robust assay
- Far red shifted fluorophores ideal for HTS and compound screens
- Suitable for discovering/profiling DUB ligands, inhibitors and activators
- Better substitute for IQF DiUbiquitins

UbiProbe Assay Applications: DUB inhibitor profiling

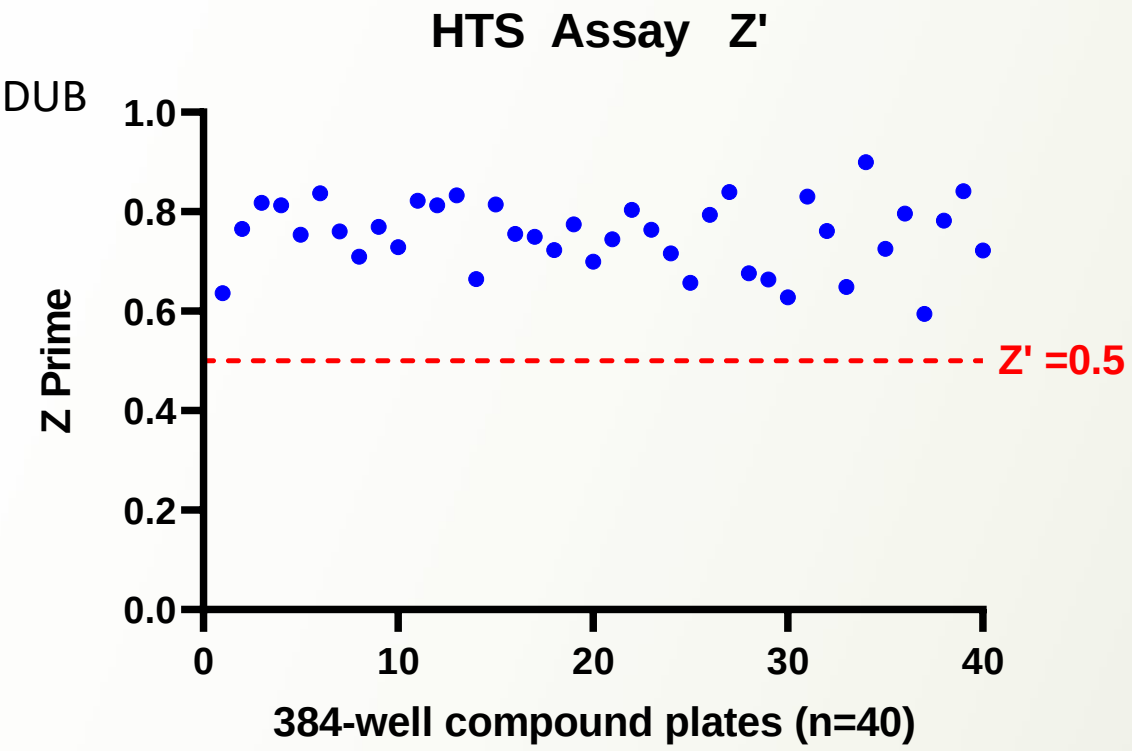


- PR-619 is a widely used pan DUB inhibitor
- PR-619 IC50 against USP7 and USP21 was determined using K48-DiUb in UbiProbe assay
- PR-619 inhibits USP7 with an IC50 of 0.66 µM
- PR-619 inhibits USP21 with an IC50 of 1.01 µM

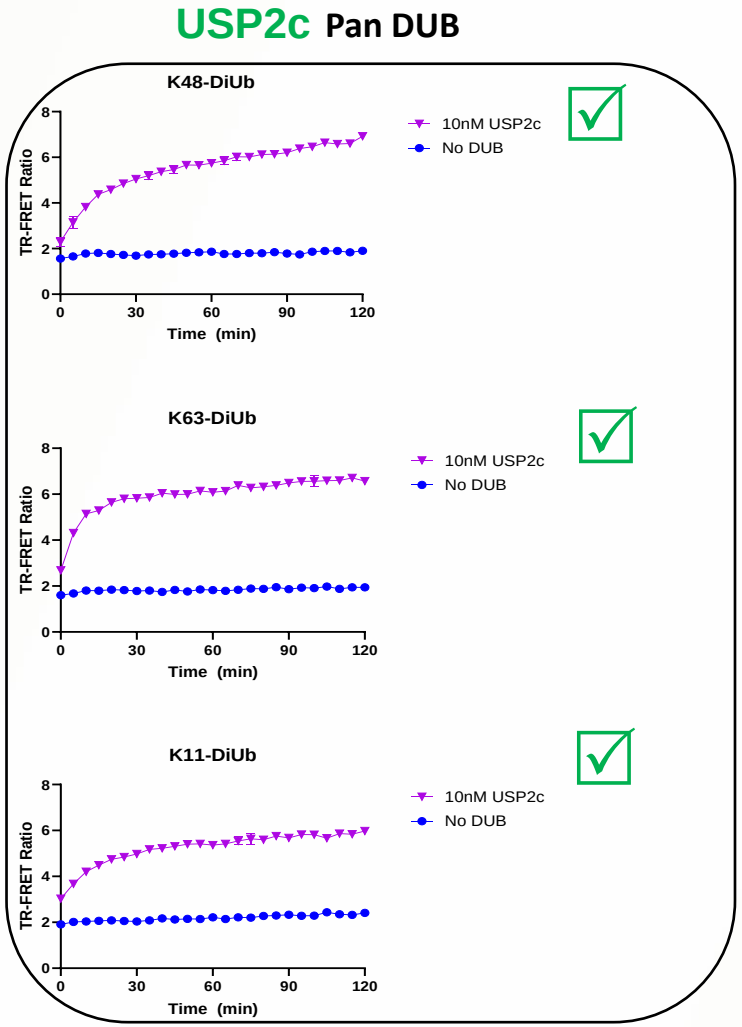
UbiProbe Assay Applications: HTS Assay Validation

(DUB with K63 DiUb)

- UbiProbe assay was validated for HTS using a K63-chain specific DUB
- ~13K compounds (40 plates) screened in 384-well format
- Z’ >0.6 across the 40 plates
- S/B =3.5
- CV= 7.44%



UbiProbe Assay Applications: DUB activity/specificity profiling

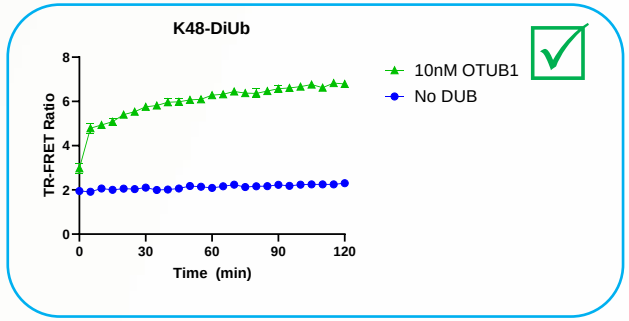


- USP2 is a pan Ubiquitin chain DUB
- USP2 DUB is active in Di-Ub linkages tested

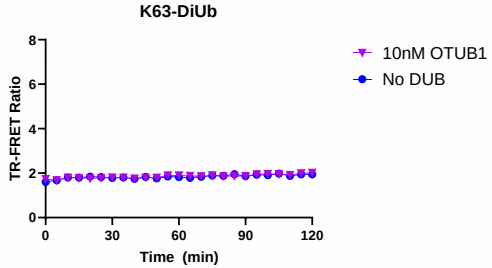
UbiProbe Assay Applications: DUB activity/specificity profiling

OTUB1 K48-Specific DUB

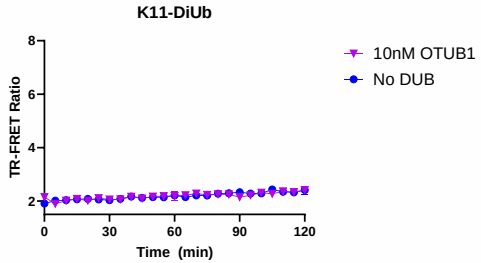
K48-DiUb



K63-DiUb



K11-DiUb

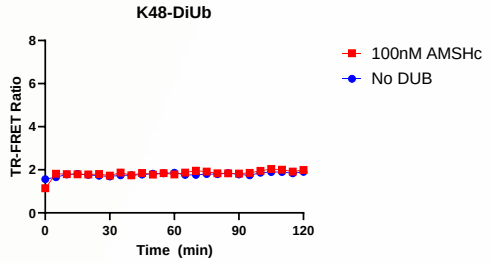


- **OTUB1 is a K48-Ubiquitin Chain specific DUB**
- **OTUB1 activity is only observed with K48-DiUb**

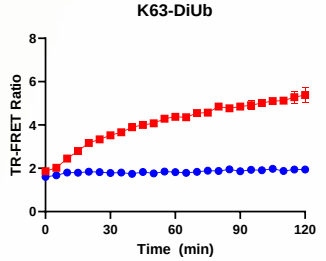
UbiProbe Assay Applications: DUB activity/specificity profiling

AMSHc K63-Specific DUB

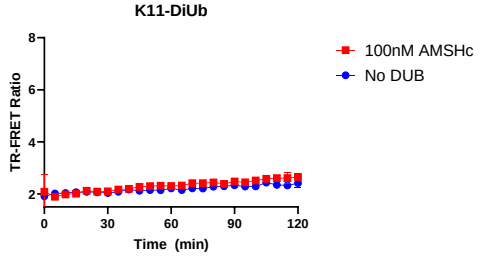
K48-DiUb



K63-DiUb



K11-DiUb

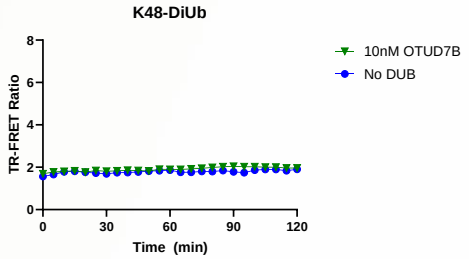


- **AMSH is a K63-Ubiquitin Chain specific DUB**
- **AMSH activity is only observed with K63-DiUb**

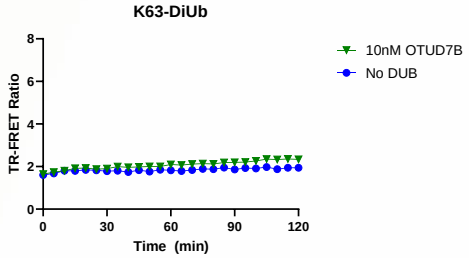
UbiProbe Assay Applications: DUB activity/specificity profiling

OTUD7B/Cezanne K11-Specific DUB

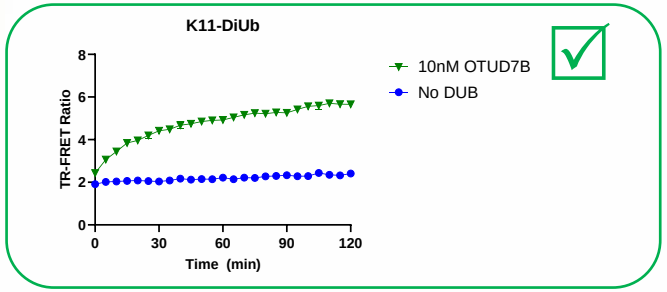
K48-DiUb



K63-DiUb



K11-DiUb



- **OTUD7B/Cezanne is a K11-Ubiquitin Chain specific DUB**
- **OTUDB7B/Cezanne is only active with K11-DiUb**

UbiProbe Assay SUMMARY

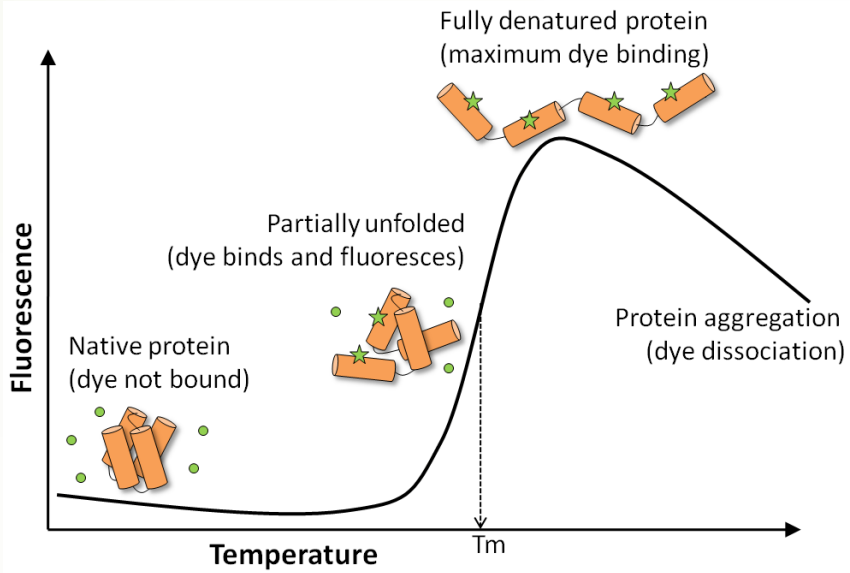
- **UbiProbe TR-FRET DUB assay:**
  - **Utilizes physiologically relevant DiUb substrates**
  - **Highly efficient and robust assay for HTS applications**
  - **Ideal for rapid compound profiling against DUBs**
  - **Suitable for studying DUB chain selectivity and mechanism of action**
  - **A better substitute for IQF DiUbiquitins**

Step Two: Hit-to-lead optimization

Validation Assays

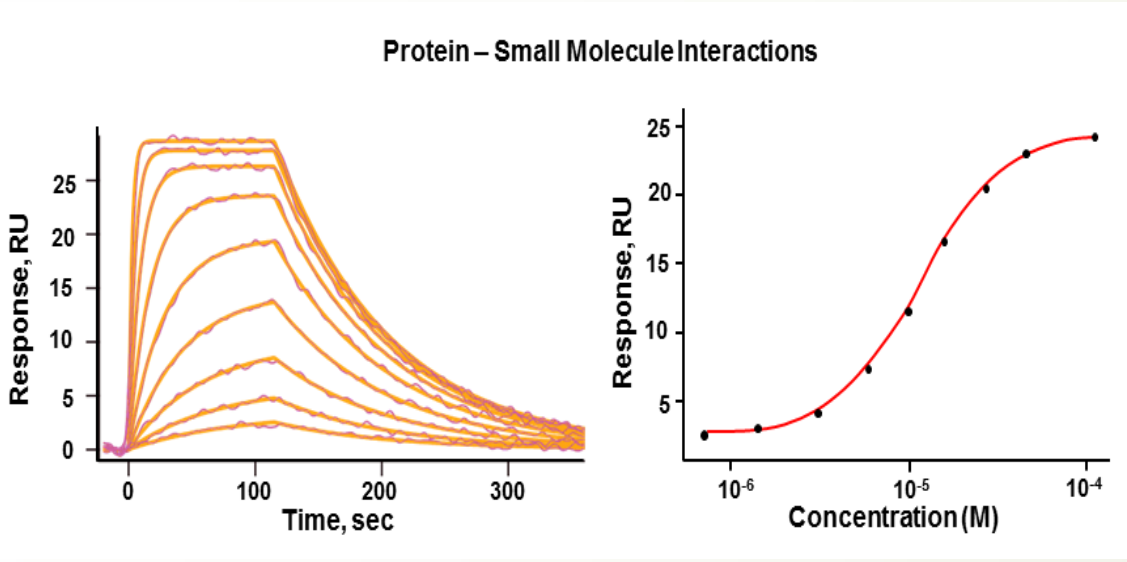
Thermal Shift Assay

HTS assay to measure compound binding/affinity to the target DUB

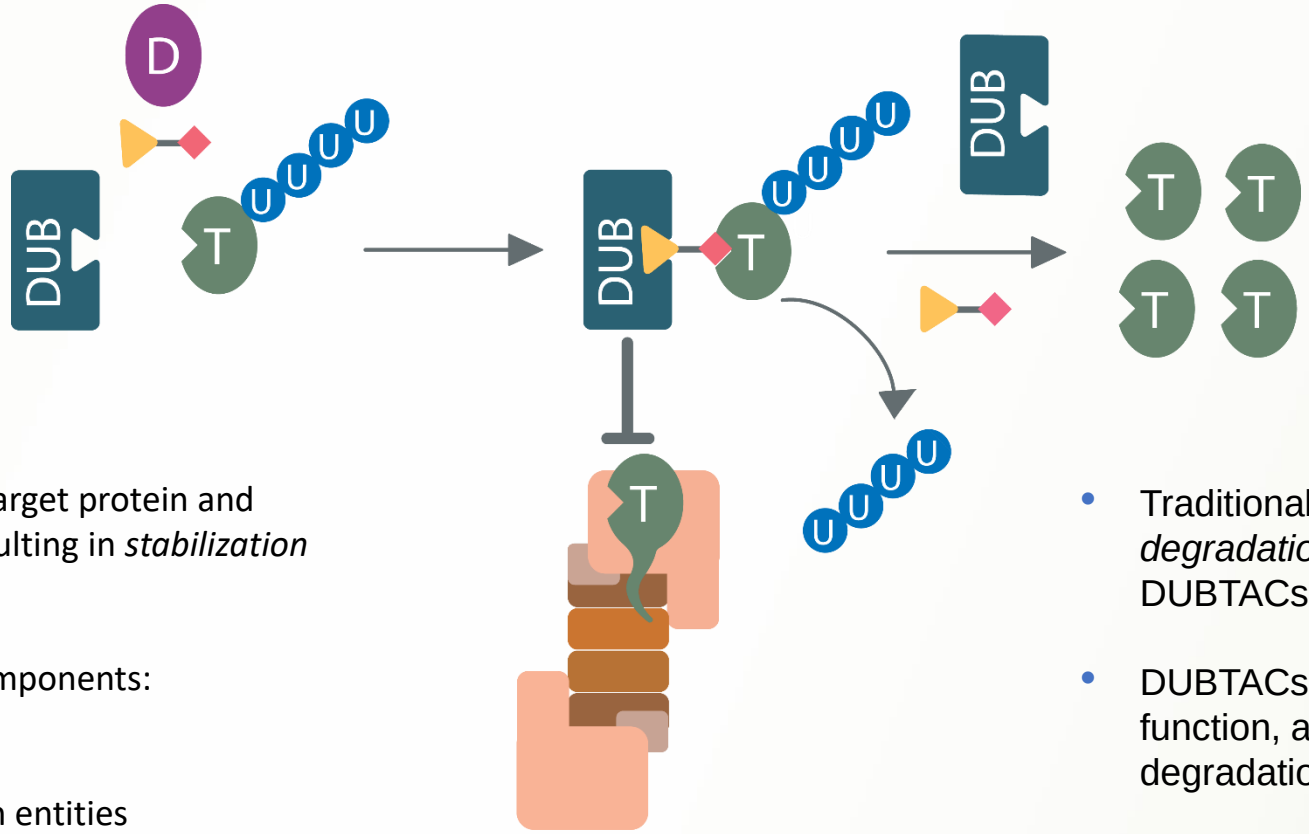


(SPR) Surface Plasma Resonance

Determines compound binding affinity to a target DUB



DUBTAC: An Emerging Therapeutic



- DUBTACs recruit DUBs to a target protein and remove ubiquitin chains, resulting in *stabilization of target proteins.*
- DUBTACs consist of three components:
  - DUB recruiter
  - Target protein binder
  - Linker connecting both entities

- Traditional PROTACs promote *degradation* of target proteins, whereas DUBTACs *stabilize* target proteins
- DUBTACs restore protein levels, function, and rescue target proteins from degradation via the proteasome

LifeSensors' Approach to DUBTAC Drug Discovery

1 **Validation of DUBTAC Ligands for Stabilizing Activity rather than Inhibition**

- Ub-Rh110/Ub-AMC assay
- CHOP reporter assay
- Diubiquitin internally quenched fluorescence (IQF) assay
- UbiSensor assay (TR-FRET)[New****]

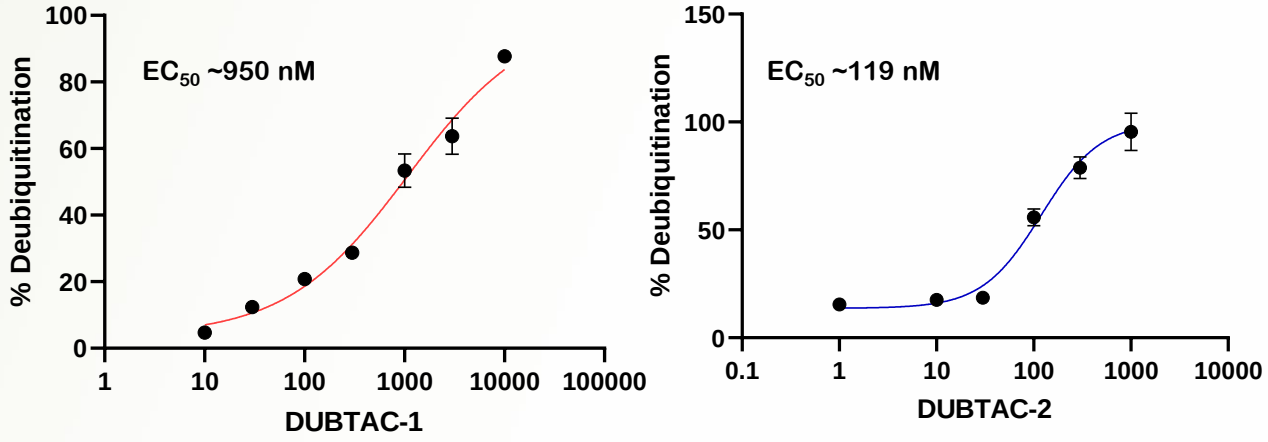
2 **Cellular Validation & DUBTAC Activity**

- UbiTest Assay
- UbiQuant ELISA

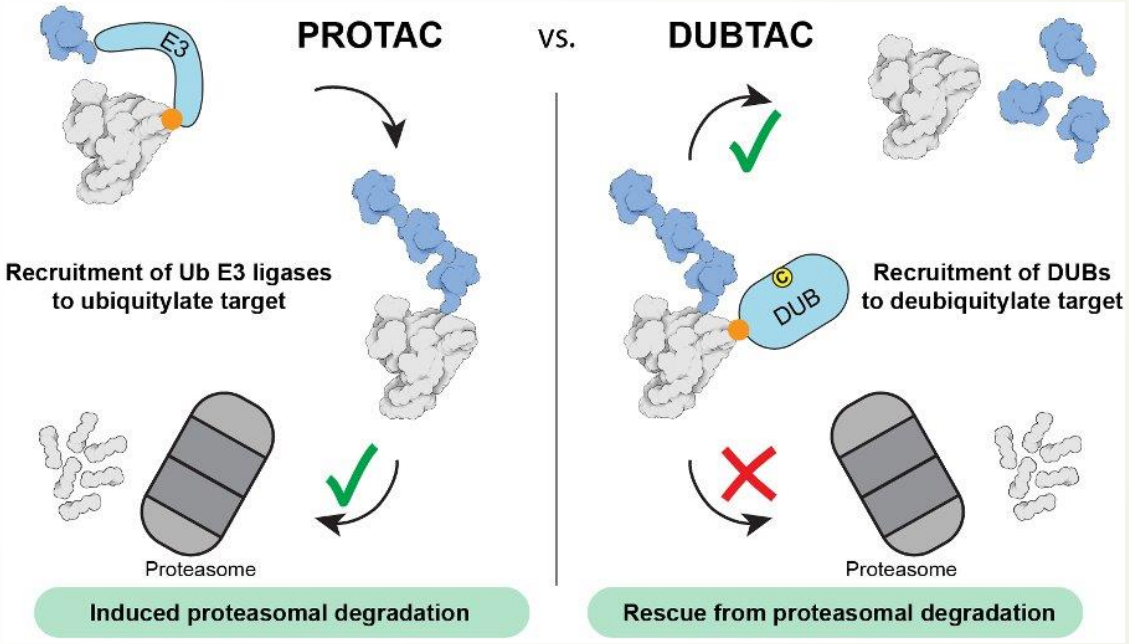
3 **Specificity Studies**

- TUBE-based mass-spectrometry studies

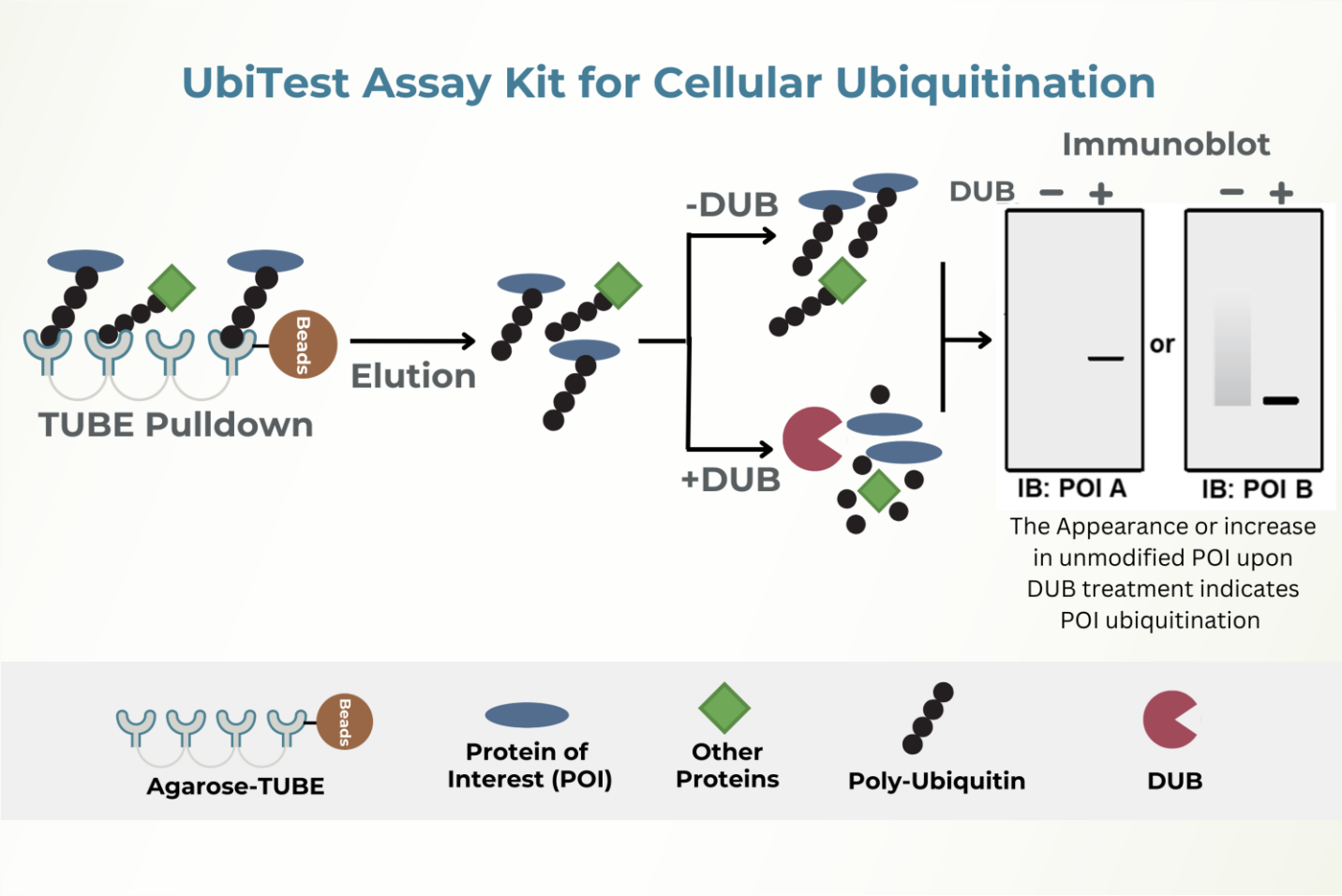
Rapid EC50 determination of DUBTACs using Lifesensors' assay platform



Monitoring deubiquitination – changes in compound mediated ubiquitination profiles mediated by DUBTAC to establish quantitative ubiquitination signals along with demonstrating EC_{50}'s of two test compounds



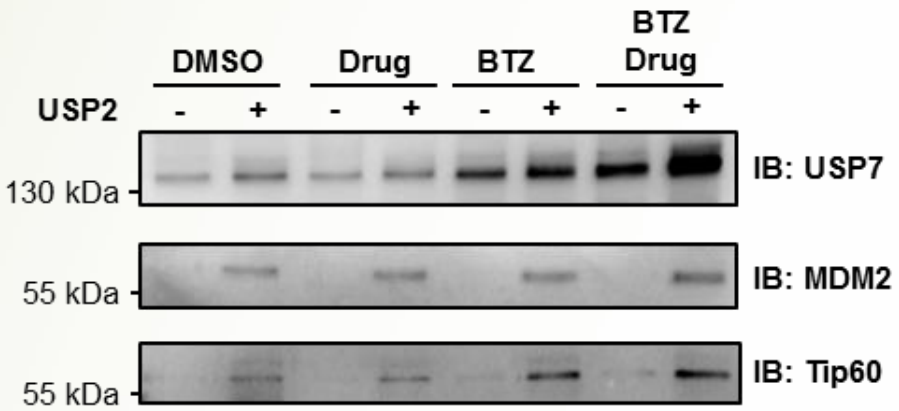
UbiTest: Cellular Assay to Measure Ubiquitylation of POI

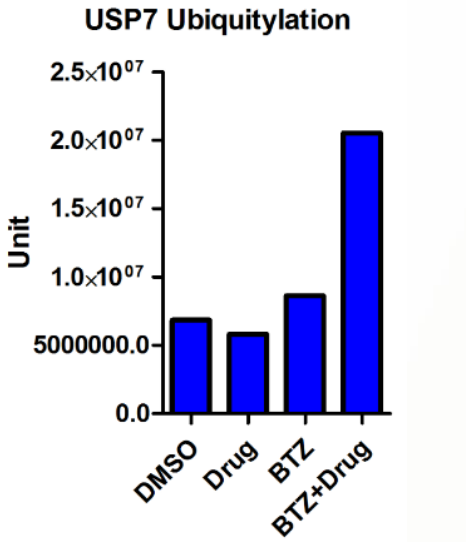


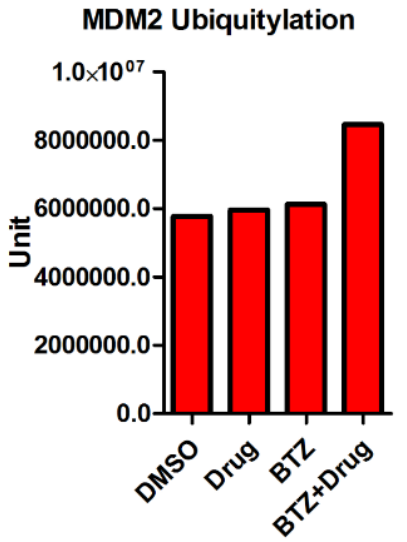
UbiTest –

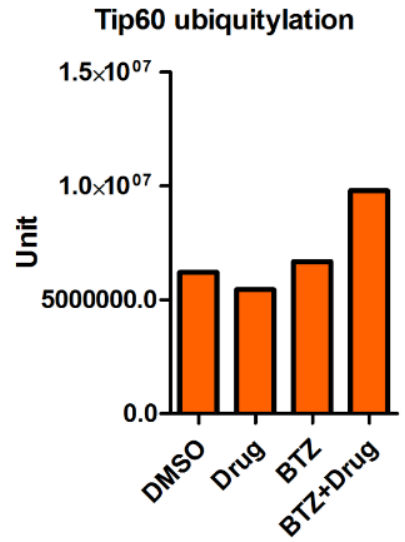
- A TUBE-based pull-down method that isolates total cellular ubiquitylated proteins.
- Subsequently, samples are treated with pan-selective DUBs to remove polyubiquitin chains.
- The target protein is identified by its native molecular weight and analyzed and quantified by immunoblotting.
- UbiTest is one of the most sensitive methods available to quantify ubiquitylation levels of proteins in vivo.

Example of UbiTest Assay









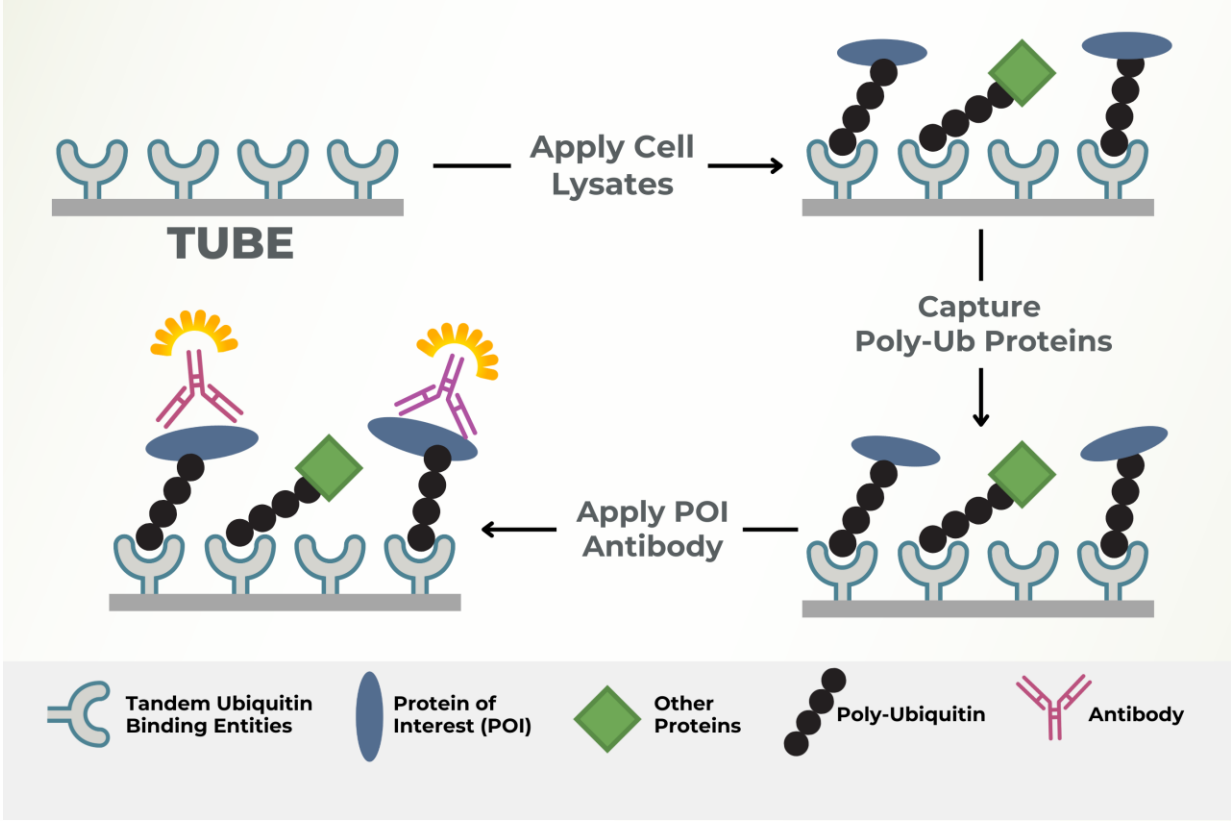
Determine endogenous target protein ubiquitylation using UbiTest

- Jurkat cells were treated with indicated compounds and lysed in RIPA buffer.
- Polyubiquitylated proteins were pulled down using TUBE1 agarose resin.
- Eluted proteins were incubated with DUB.
- Immunoblot (left) of the assay and quantitation (right) of the bands showed increased signal of USP7, MDM2, Tip60 after DUB treatment indicating they are polyubiquitylated in cells.

Determining *In Vivo* Drug Activity by Cellular Biomarker Assays

- UbiQuant S – ELISA

  Medium-throughput relative quantification of substrate ubiquitylation levels in a cellular context.

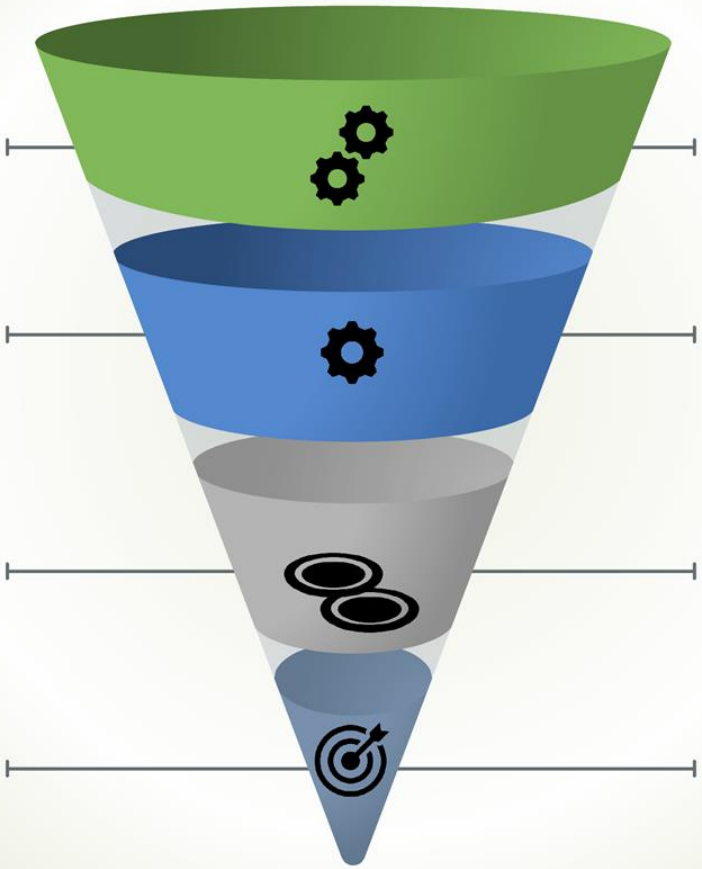


DUB Screening & Profiling Service

- Discover novel DUB ligands by HTS assays
- Confirmation and counter screen to eliminate off-target compounds
- Biophysical and biochemical assay development for DUBs
- Cell-based assays to establish target engagement by compounds
- Fee for service model, defined milestone-based agreement

DUB Screening & Profiling Service

Pathway to DUB Modulator Drug Discovery



IN VITRO ASSAY DEVELOPMENT
Ub-Rh110, CHOP, IQF, UbiProbe DUB assays

IC50 & RANK ORDER POTENCY
Identify most potent compounds for cellular validation

CELL-BASED ASSAY DEVELOPMENT
Substrate ubiquitylation and degradation

MASS SPEC & PROTAC SELECTIVITY
TUBE-based to establish total ubiquitome changes

ASSAY VALIDATION & COMPOUND SCREENING
HTS using validated DUB assay

COUNTER SCREENS & VALIDATION
Establish specificity and selectivity

VALIDATION & ESTABLISH IC50/EC50
Validate ubiquitylation via WBs and UbiTest

METHOD TRANSFER
Transfer technology for further validation studies

Preclinical/Clinical candidate(s)

Contact Us!

We are your partner for DUB drug discovery

Contact Information

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Custom Service & Assays	**BD**	**bd@lifesensors.com**	**610-644-8845 (ext 310)**