



Targeted Protein Degradation Discovery Using the E3scan™ Technology Platform and SPRINTer™ Cell Lines

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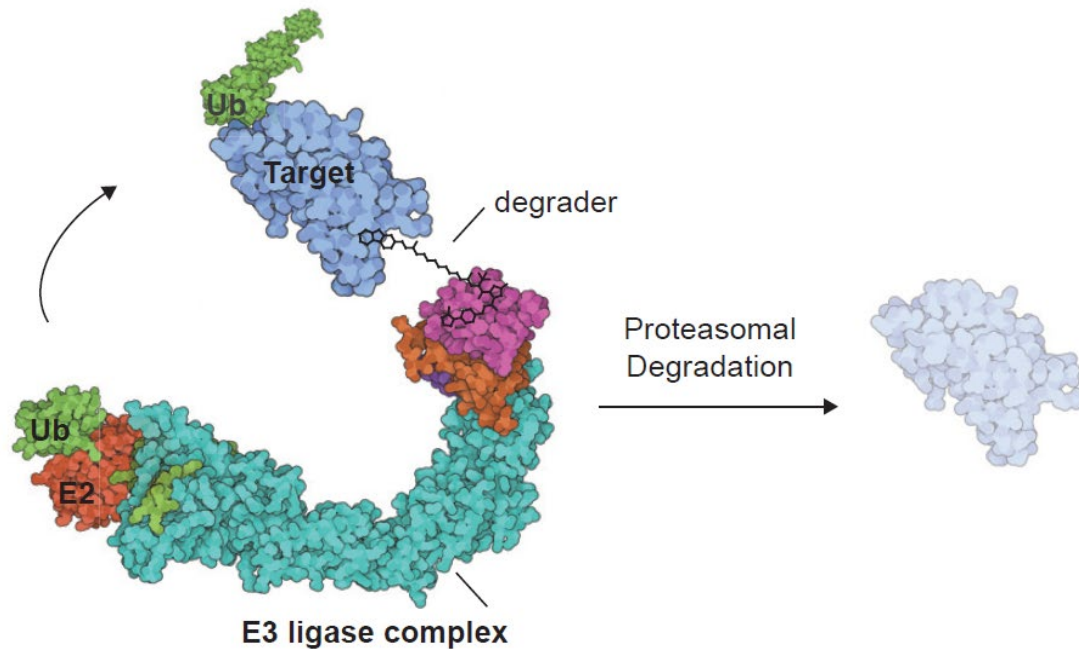
Discovery On Target September 28th, 2021

**A STRONG
FOUNDATION
FOR SUCCESSFUL
DRUG DISCOVERY**

- Targeted Protein Degradation (TPD) Introduction
- Overview of Eurofins Discovery TPD Portfolio
- E3scan™ Technology and Proof of Concept
- Other Biochemical Assays for Targeted Protein Degradation
- Eurofins Screening Capabilities
- SPRINTer™ Platform: A Cell-Based Functional Assay
- Q&A

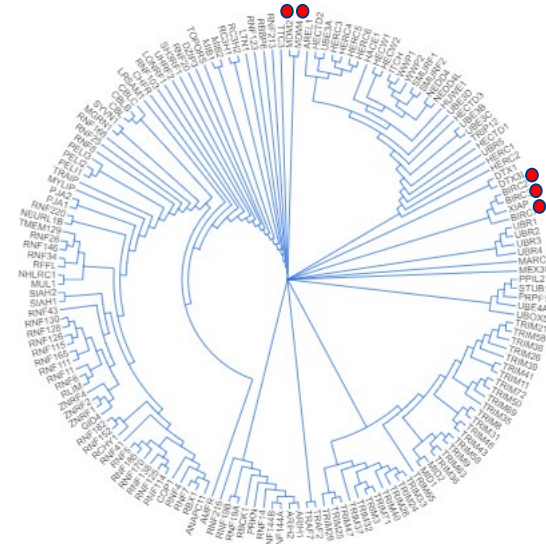
TPD Represents A Compelling New Drug Discovery Strategy

Goal: Expanding the TPD toolbox with more options for E3 ligase targets

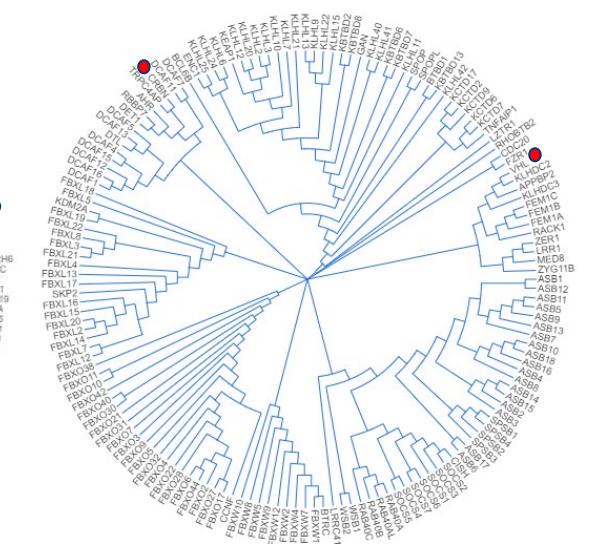


Donovan et al., 2020, Cell

Simple E3 ligases tree

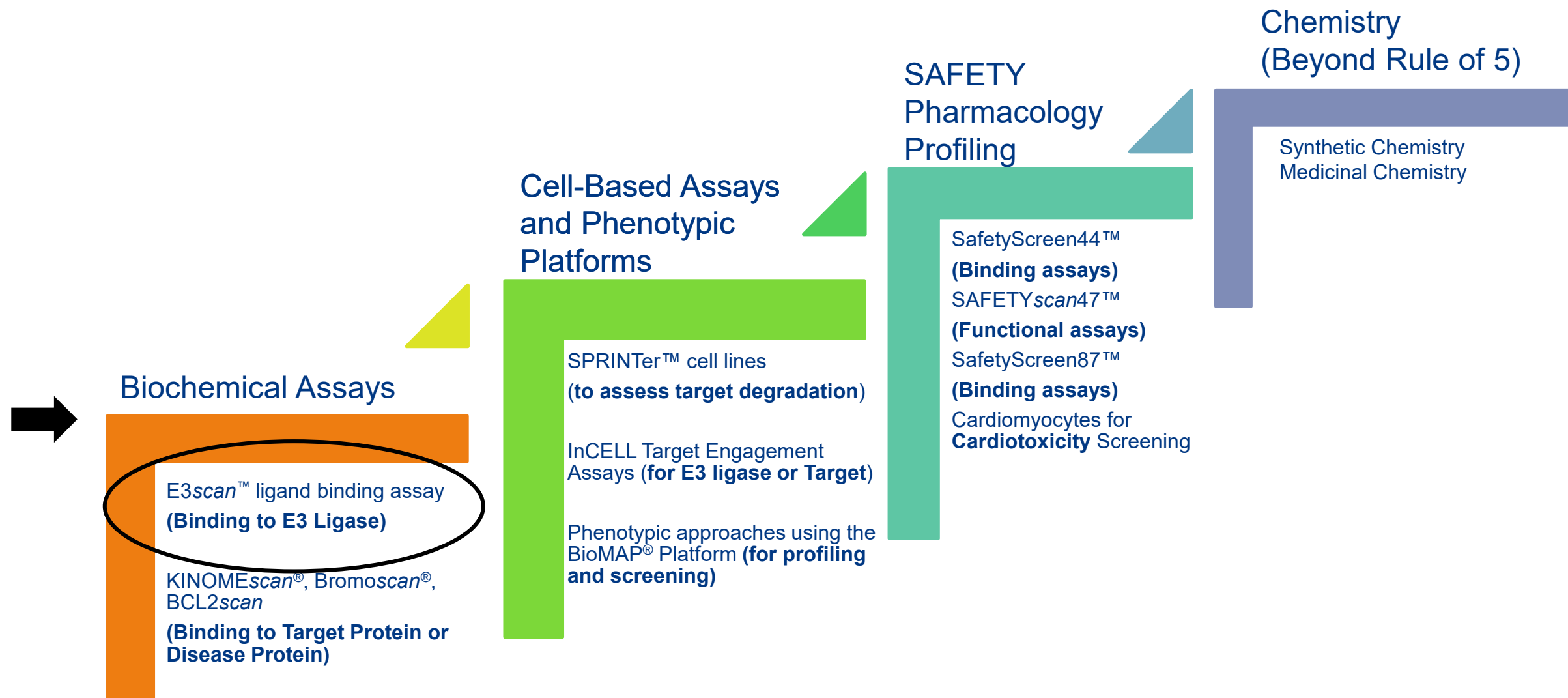


Complex E3 ligases tree



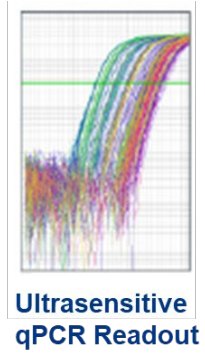
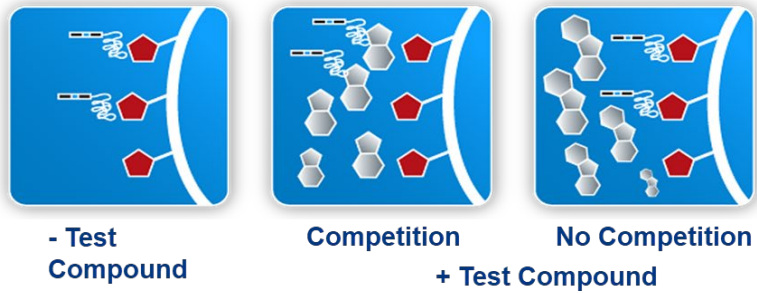
<https://ubihub.thesgc.org/static/UbiHub.html>

~1% of the entire 600 E3 ligase family has been explored in TPD



Expanding the Novel E3 Family-Wide Screening and Profiling Technology

Unique competition binding assays with DNA-tagged protein targets and qPCR readouts eliminate the need for protein purification

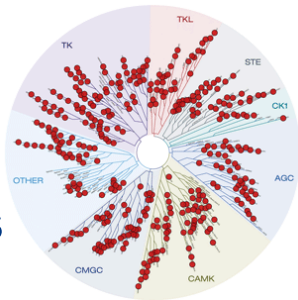


E3scan™ - NEW

12 family members are represented, and BIR domain selectivity available

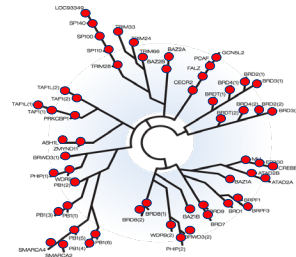
KINOMEscan®

489 assays



BROMOscan®

40 assays



BCL2scan

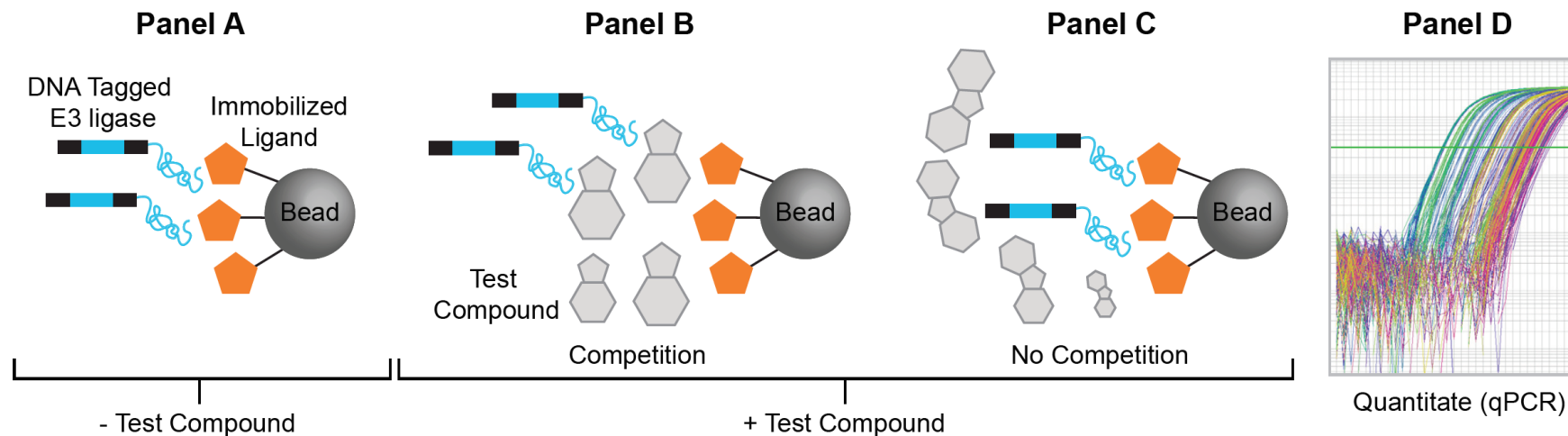
All 5 anti-apoptotic BCL2 family proteins represented

Ligand binding site-directed competition binding assays

Three key components

- E3 ligase tagged with DNA (low pM E3 ligase concentration in assay)
 - Expression in mammalian cells or by using proprietary T7 phage display system
- Known E3 ligase ligand (small molecule or peptide) immobilized on solid support
- Test compound or solvent control

Measure amount of E3 ligase captured by solid support in the presence or absence of a test compound (ultrasensitive qPCR readout)



Advantages of E3scan™ Platform

- Broad sensitivity & dynamic range (pM to mM K_D values)
- All assays run in parallel on single platform
- The largest assay panel available on a single technology platform
- High throughput, run in 384-well format
- Rapid turnaround time of 5 days for weekly submissions
- 20 days turnaround time for library screens
- Custom assay development

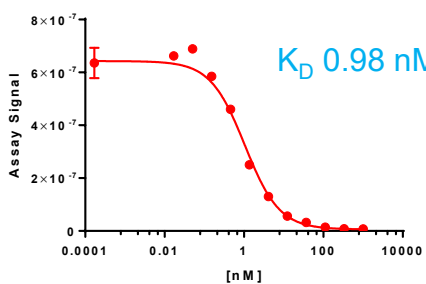
E3scan Assays currently available

cIAP1 BIR2	CRBN	VHL-ELO_B/C
cIAP1 BIR3	CRBN-DDB1	XIAP BIR3
cIAP1 FL	ITCH WW1-2	XIAP FL
cIAP2 BIR2	MDM2	WWP1
cIAP2 BIR3	MDMX	WWP2
cIAP2 FL	NEDD4 WW2-3	SMURF2

Previously Launched E3scan™ Assays

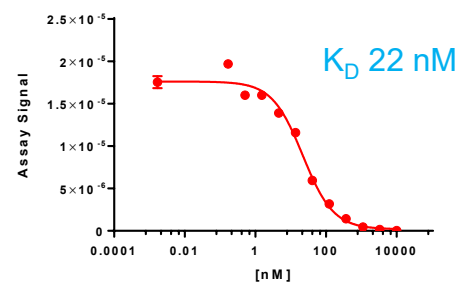
MDM2

Idasanutlin



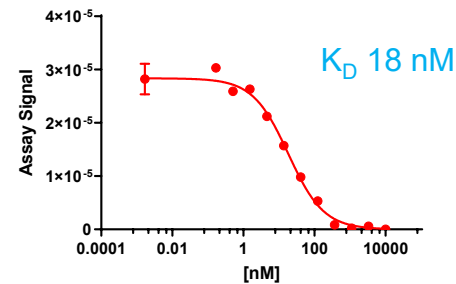
MDMX

pDI



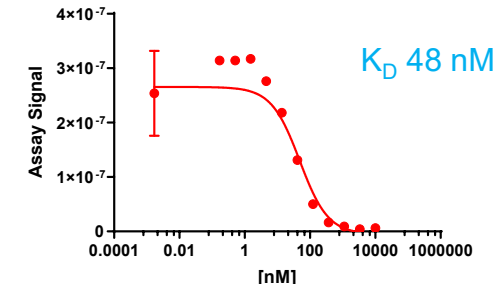
VHL-ELOB-ELOC

VH298



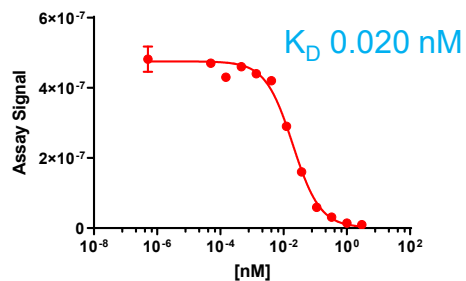
CRBN-DDB1

Lenalidomide



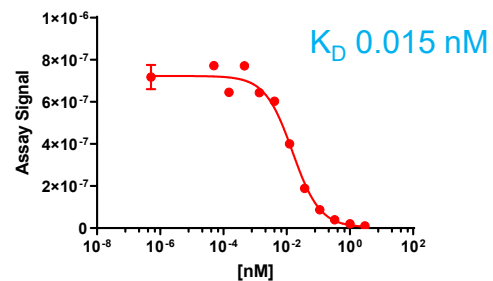
cIAP1

AZD5582



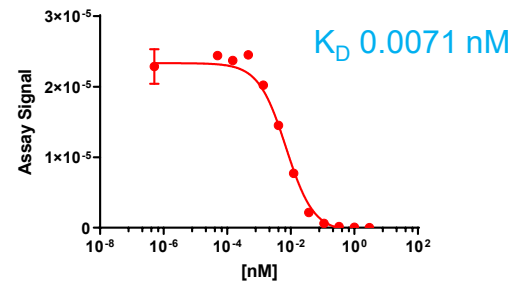
cIAP2

AZD5582



XIAP

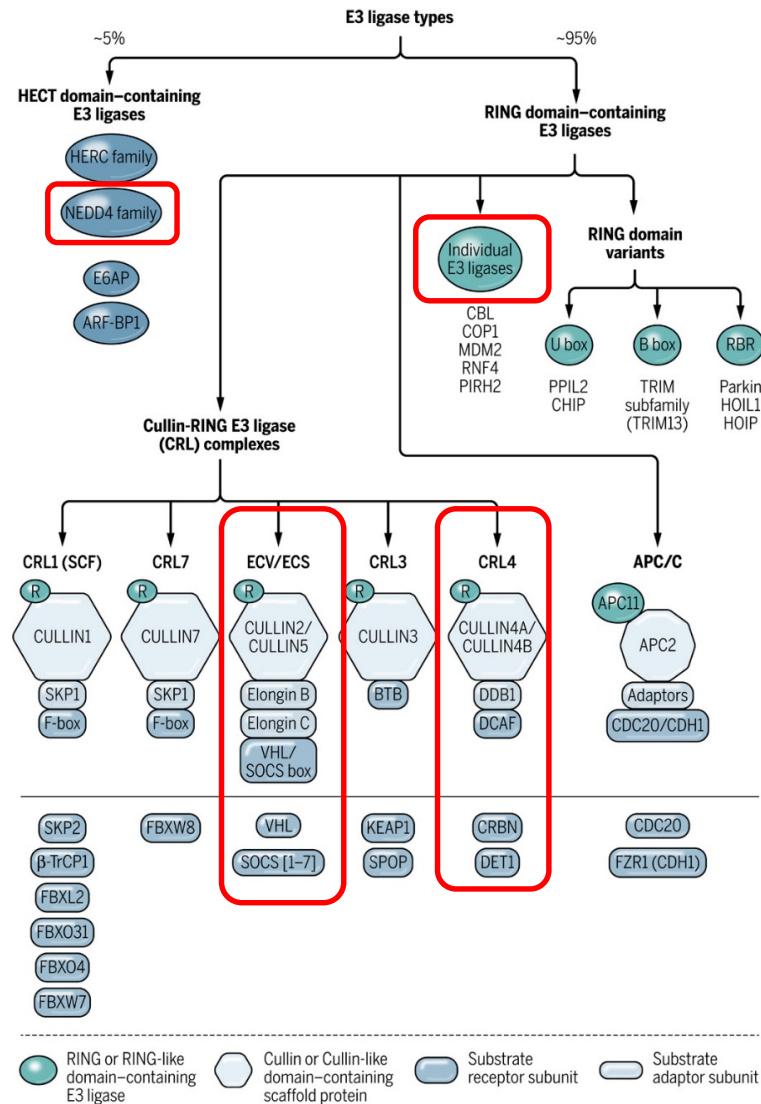
AZD5582



Individual BIR domain assays are now available, data presented in our poster

- We have previously launched E3scan assays for 7 commonly used E3 ligase targets in TPD
- Correct potency and rank order for the control inhibitors tested
- Assays do not approach the tight binding limit – even for pM compounds and stapled peptides

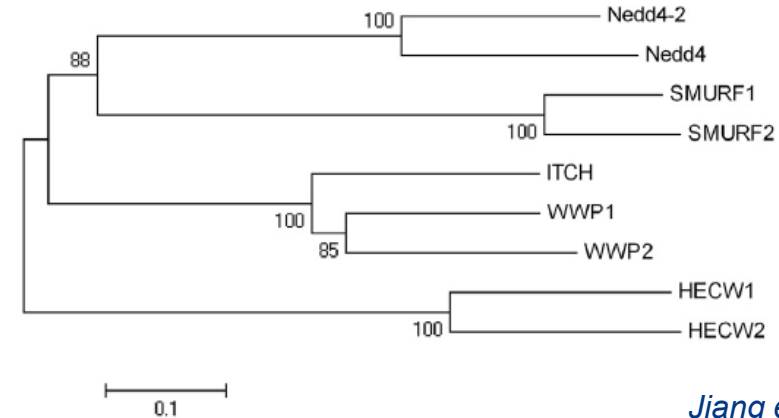
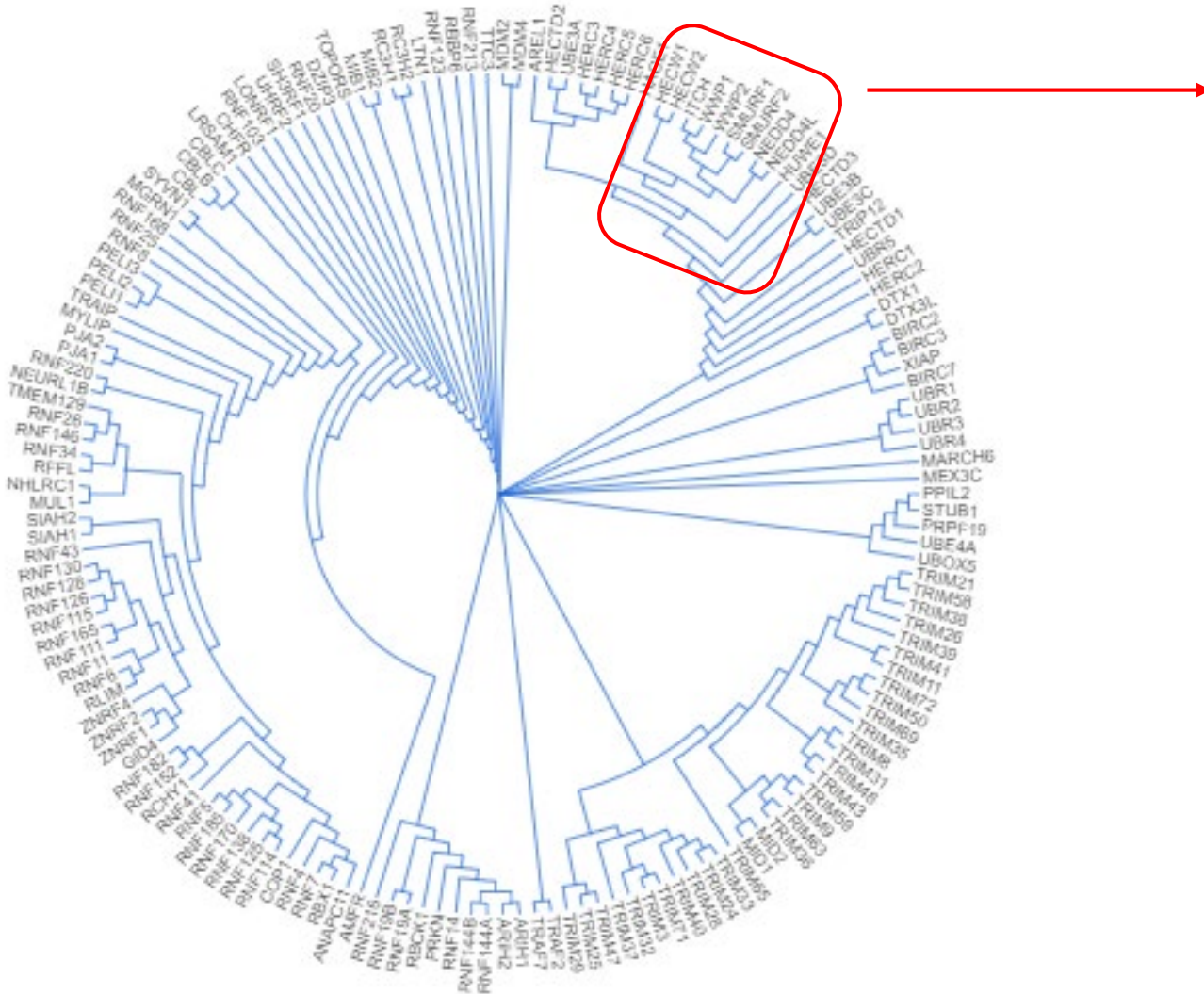
Classification of E3 Ligase Proteins and Complexes



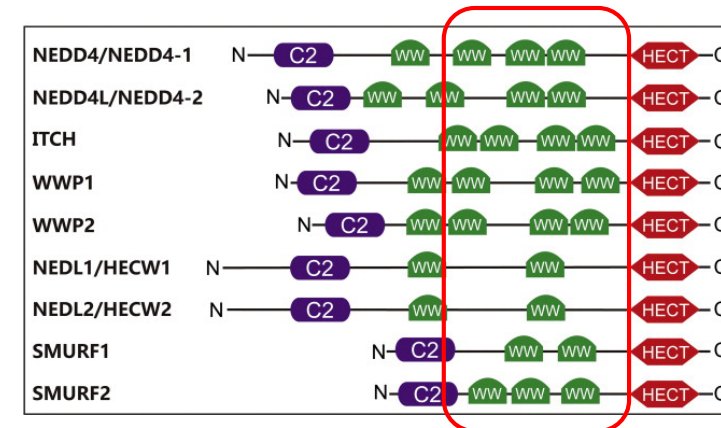
Mészáros et al. 2017, Science Signaling

HECT-NEDD4 E3scan™ Assay Development and Validation

Simple E3 ligases tree



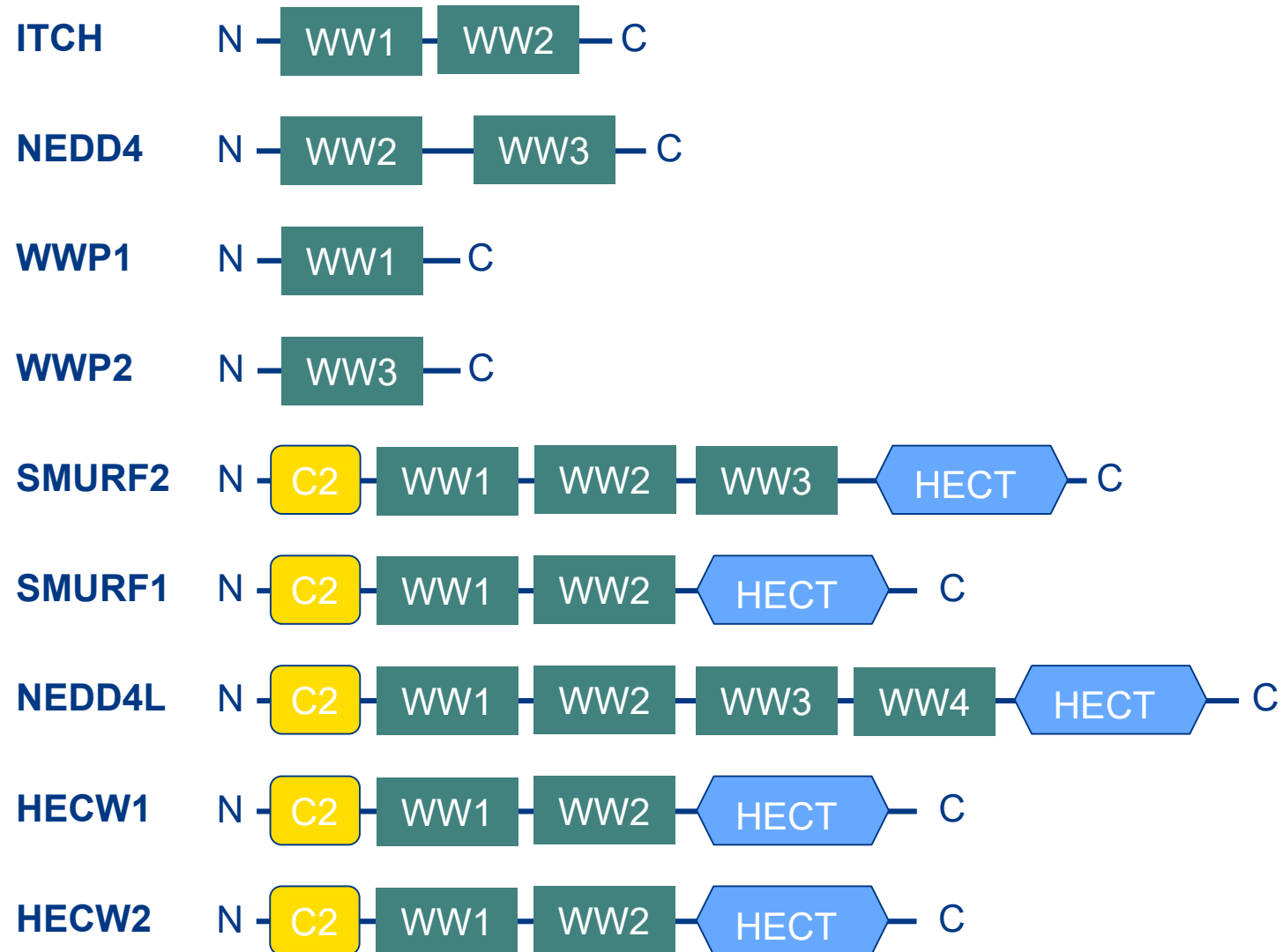
Jiang et al., 2015, FEBS Letters



Zou et al., 2015, BBA

PPxY

HECT-NEDD4 Sub-family Constructs used for E3scan™ Assay Development

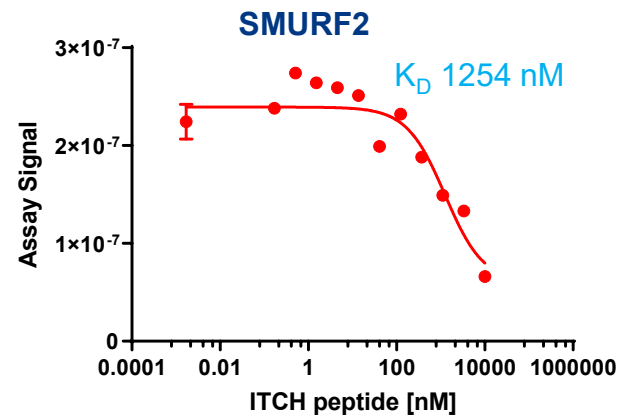
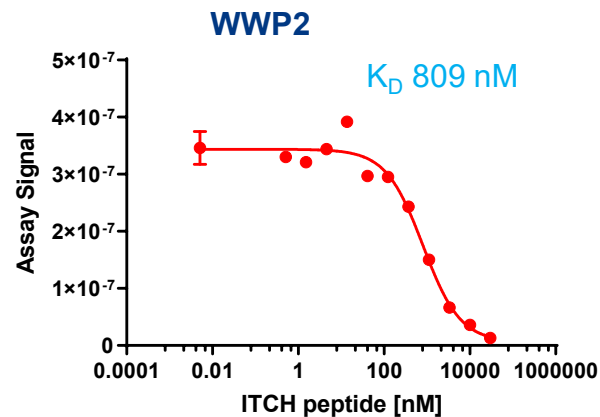
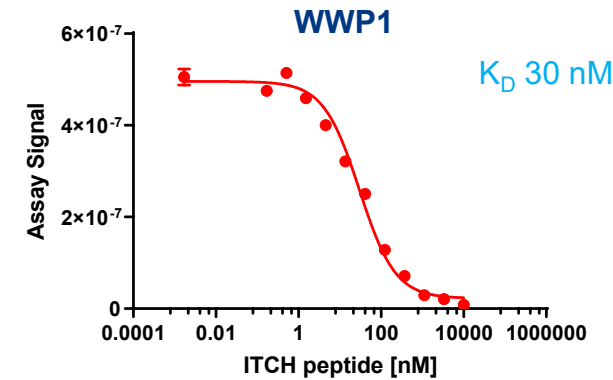
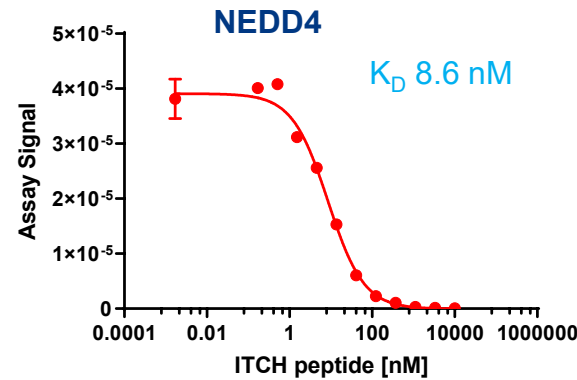
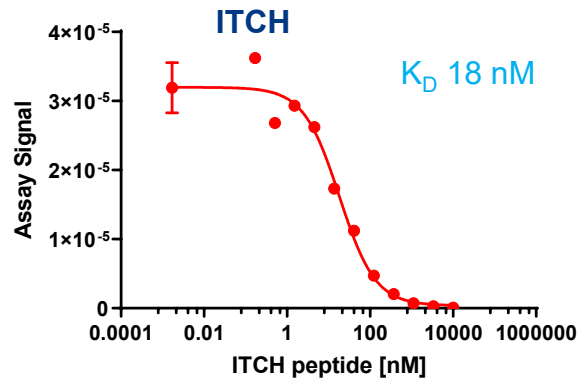


HECT NEDD4 Sub-family E3scan™ Assays: Summary Table

Peptide Name	Derivation	AVG K _D (nM)				
		NEDD4 E3scan	ITCH E3scan	WWP1 E3scan	WWP2 E3scan	SMURF2 E3scan
ITCH	TXNIP substrate of ITCH ¹	5.7	22	24	749	1492
NEDD4-2	ARRDC3 substrate of NEDD4 ²	108	47	54	1819	1597
WBP-1	YAP ligand ³	>10,000	>10,000	1663	>10,000	>10,000
WBP-2A	YAP ligand ³	625	>10,000	3264	>10,000	>10,000
Smad7	Smad7 adaptor of Smurf2 ⁴	1495	629	323	2233	258

1. Liu et al., 2016, Biochem J
2. Qi et al., 2014, JBC
3. Pirozzi et al., 1997, JBC
4. Chong et al., 2006, JBC
5. Hu et al., 2004, Proteomics

ITCH Peptide Across E3scan™ Assays



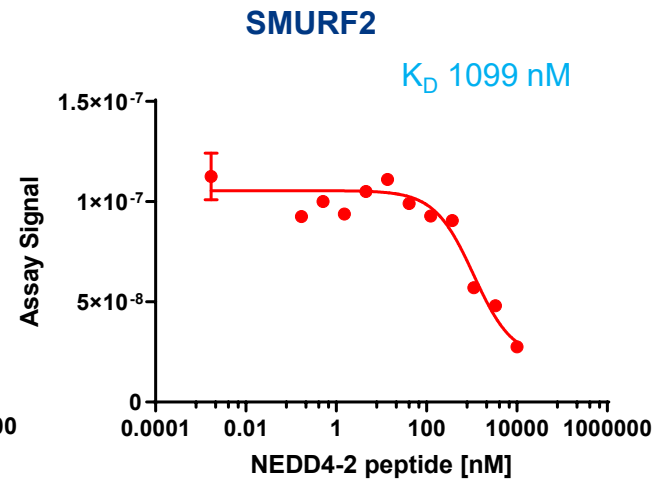
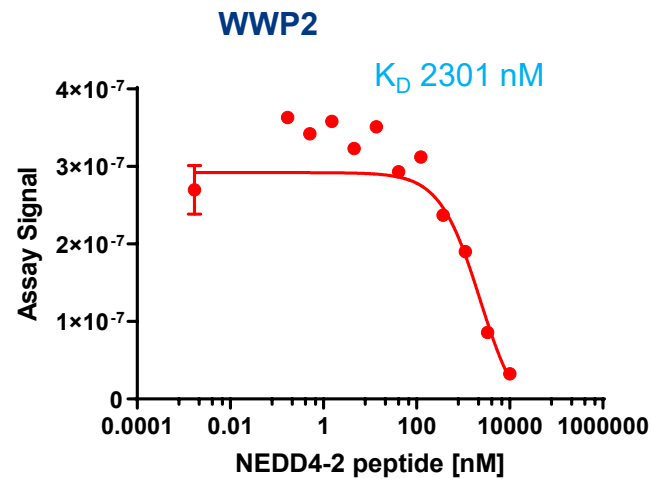
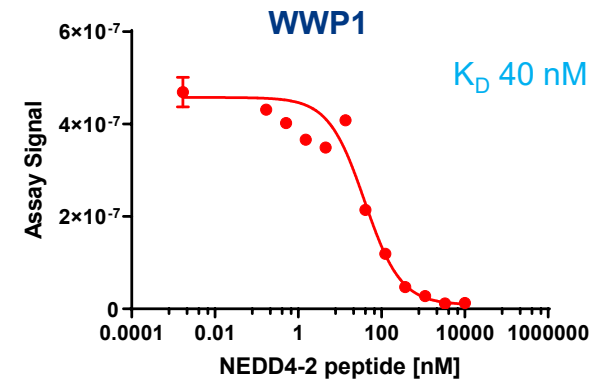
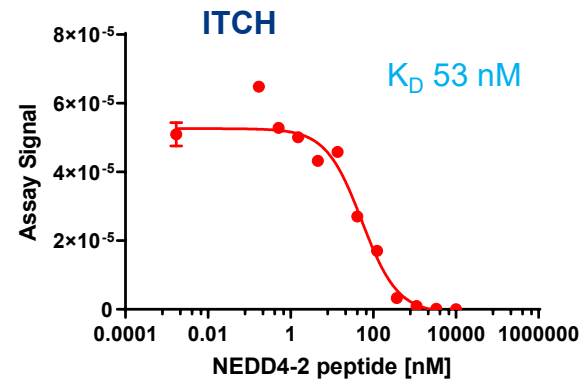
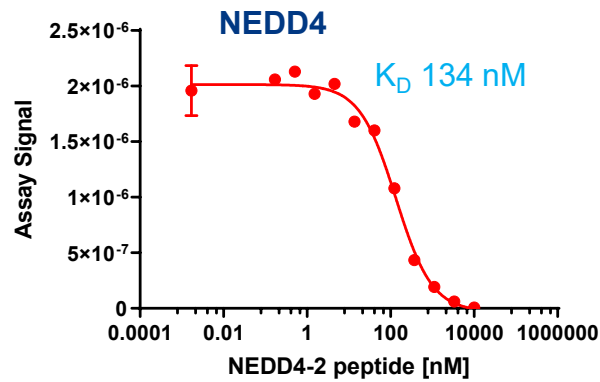
- ITCH peptide containing two PPxY motifs from a substrate of ITCH (TXNIP) was used as reference control peptide
- As expected, ITCH is binding potently to its substrate peptide
- NEDD4 and WWP1 also bind potently to the ITCH substrate peptide, but not WWP2 and SMURF2

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5. Hu et al., 2004, Proteomics

NEDD4-2 Peptide Across E3scan™ Assays



- NEDD4-2 peptide containing two PPxY motifs from a substrate of NEDD4 (ARRDC3) was used as reference control peptide
- As expected, NEDD4 is binding potently to its substrate peptide
- ITCH and WWP1 also bind potently to the NEDD4 substrate peptide, but not WWP2 and SMURF2

HECT NEDD4 Sub-family E3scan™ Assays: Summary Table

Peptide Name	Derivation	AVG K _D (nM)				
		NEDD4 E3scan	ITCH E3scan	WWP1 E3scan	WWP2 E3scan	SMURF2 E3scan
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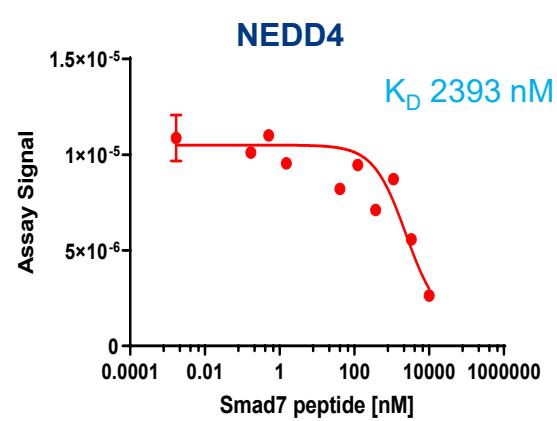
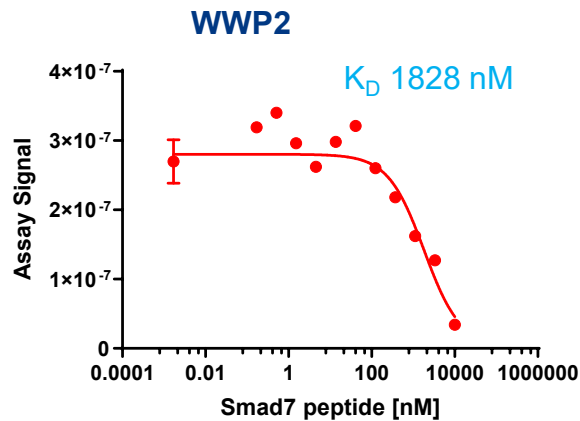
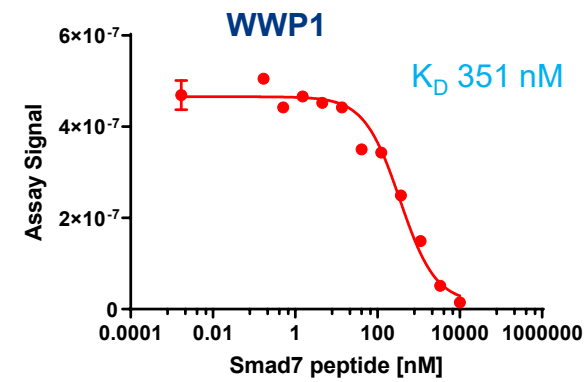
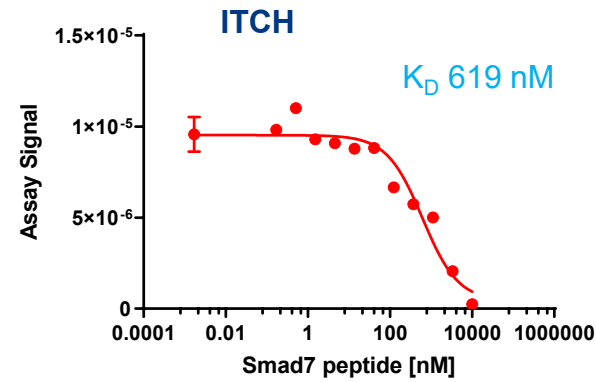
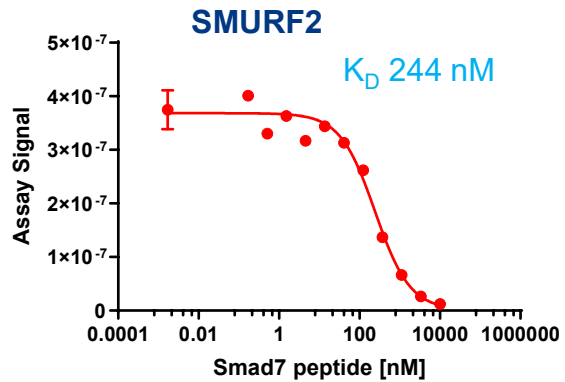
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5. Hu et al., 2004, Proteomics

Smad7 Peptide Across E3scan™ Assays



- Smad7 peptide containing a PPxY motif from an adaptor protein of SMURF2 (Smad7) was used as reference control peptide
- As expected, SMURF2 is binding most potently to its adaptor protein peptide
- WWP1 and ITCH also show some binding to the SMURF2 adaptor protein peptide, but not WWP2 and NEDD4

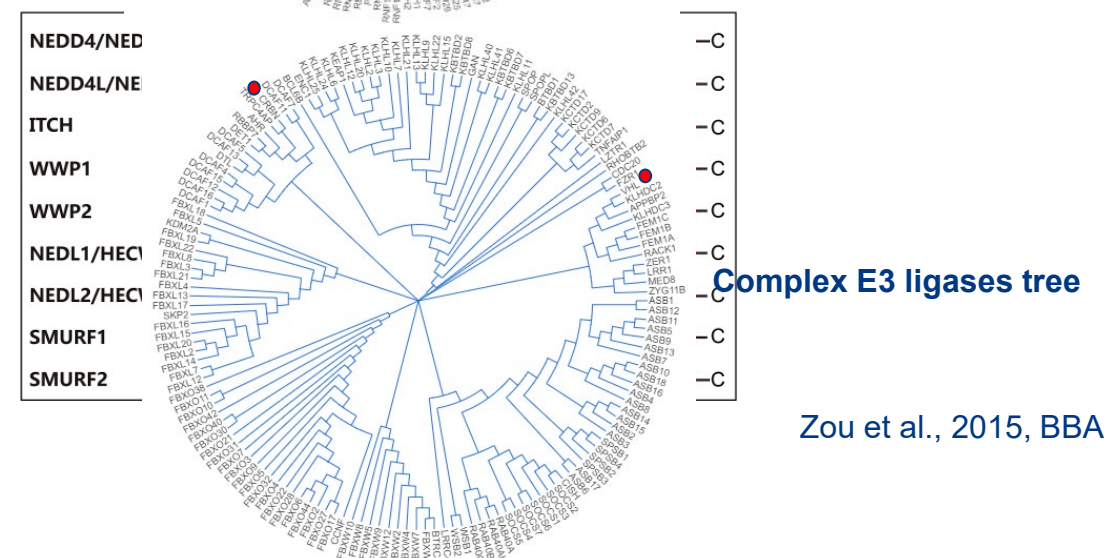
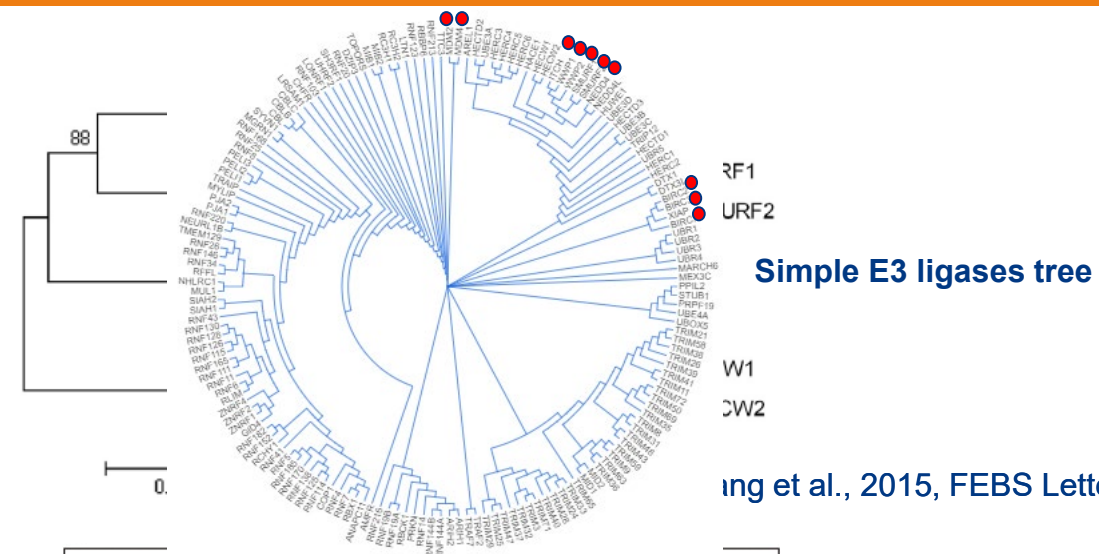
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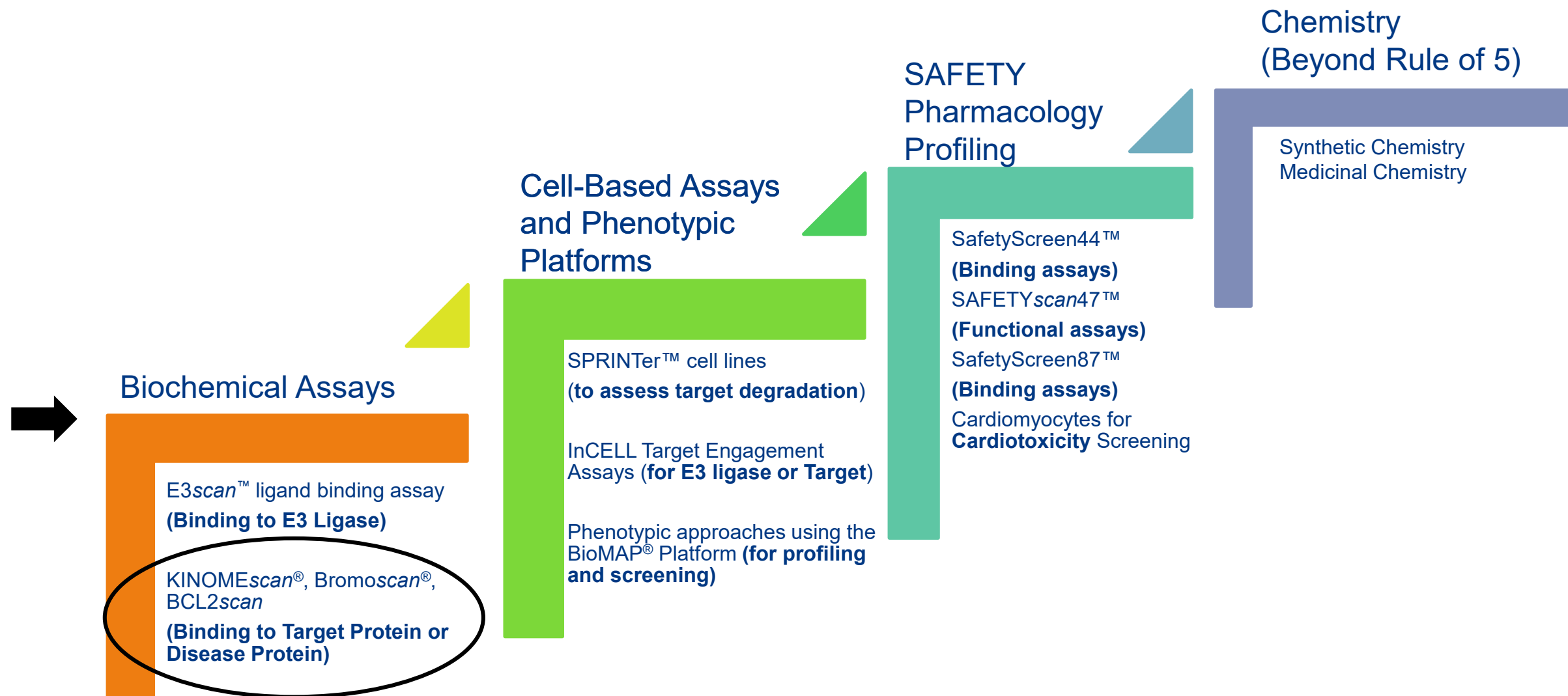
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Take Home Message – E3scan™ Development

- We have developed robust and high throughput **E3scan** assays
- We show selective binding of HECT-NEDD4 family substrates to different members of this E3 ligase family
- These results demonstrate the capabilities of **E3scan** in screening for selective ligands across the E3 ligase family
- More E3 targets in development, not limited by available small molecule ligand



<https://ubihub.thesgc.org/static/UbiHub.html>

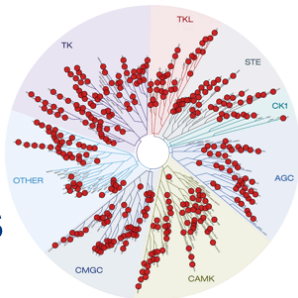


Measure Binding Affinity to Target Proteins using KINOMEScan, BROMOScan, and BCL2scan

- 489 Kinase assays – including clinically relevant mutants
- 40 Bromodomain assays – 60% coverage of the entire family
- BCL2 assays – 5 family members
- Additional custom assay development available (not currently offered in commercial portfolio)
- Potency and selectivity
- Binding mode/Kinetics
- Turn around time: 5 days for weekly submissions, ~20 days for library screen

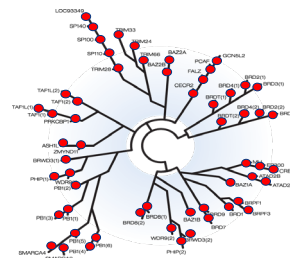
KINOMEScan®

489 assays



BROMOScan®

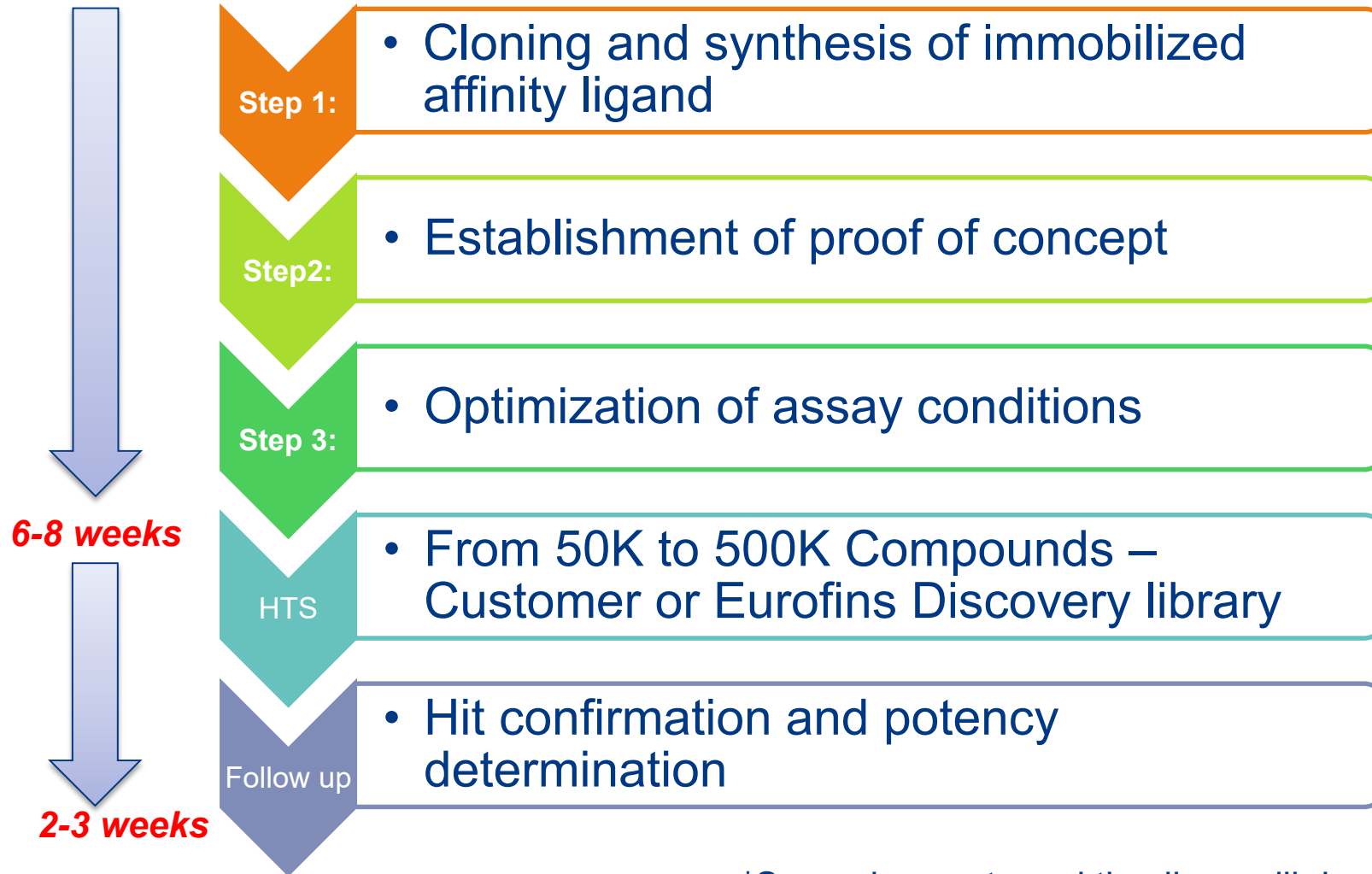
40 assays



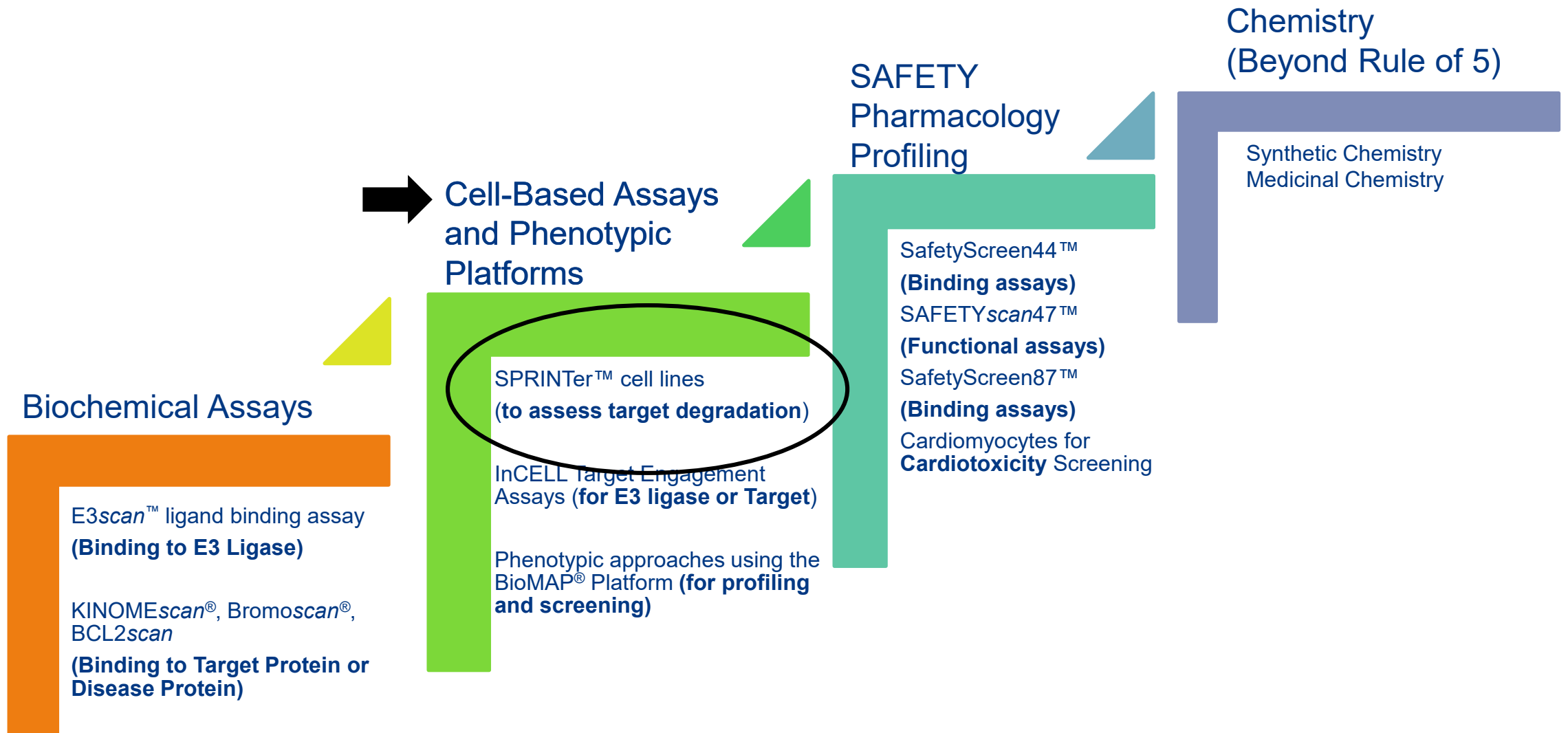
BCL2scan

All 5 anti-apoptotic BCL2 family proteins represented

E3scan™ HTS Standard Flow Chart for Custom Assay Development

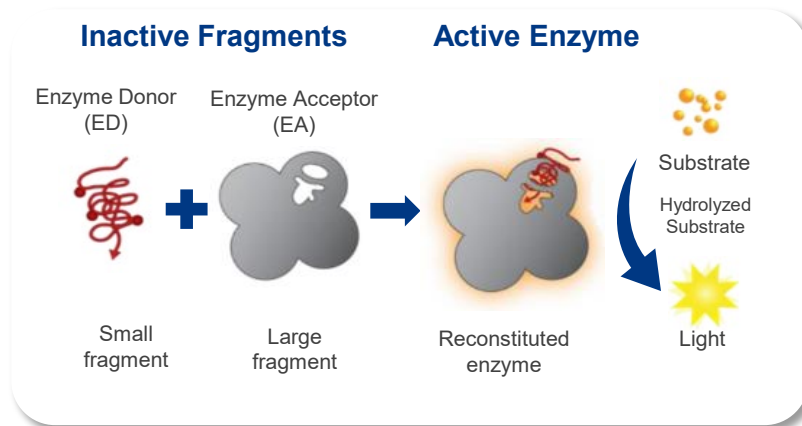


*Screening costs and timelines will depend on assay format and compound numbers



Evaluating On-Target Activity of Targeted Degradator Molecules

- Compatible with disease cell models expressing physiologically relevant levels of the target protein and relevant E3 ligase(s)
 - Over-expression systems incompatible with certain degrader molecules, such as PROTACs
- A sensitive and scalable method for detection of protein turnover
 - Commonly utilized techniques include Westerns (low-throughput), ELISAs, or proliferation assays
- Robust, reproducible, and easy to implement

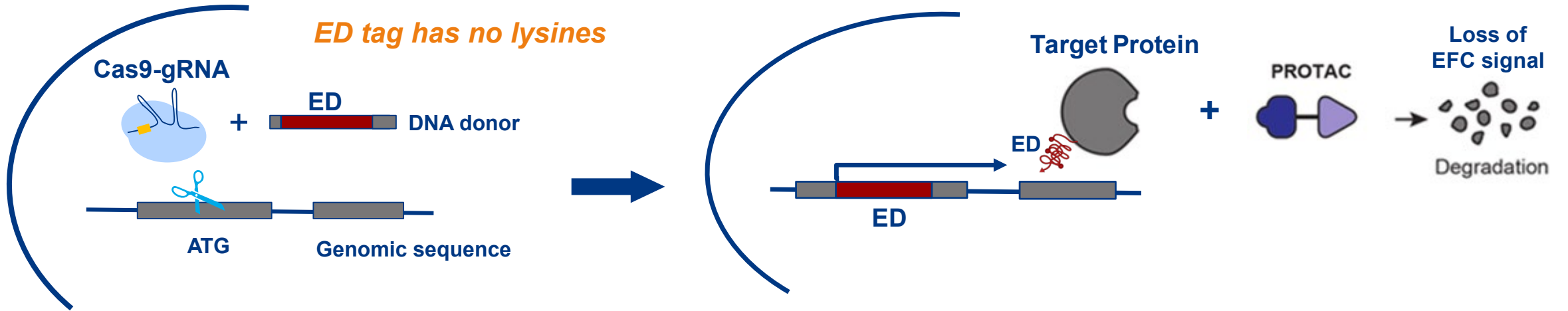


Solution:

Enzyme Fragment Complementation (EFC)

Combines fM reporter sensitivity with ease of implementation (simple, homogeneous assay format)

Assay Concept: Employ CRISPR / Cas9 to Introduce ED tag into Endogenous Target



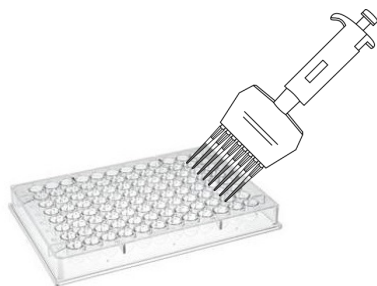
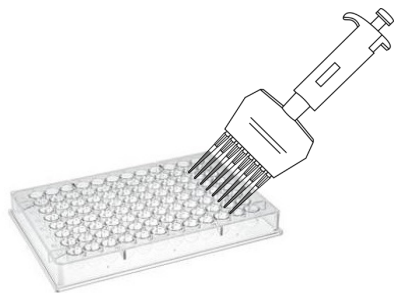
SPRINTer™ Assay Design: A Simple, Rapid and Homogeneous Assay Workflow

Seed stable biosensor cells in 96- or 384-well plate

Add degrader molecule(s)

Add cell lysis reagent and detection reagent containing complementing EA

Read signal on plate reader



37°C



Incubate for 5-72 h



Incubate for 1 h



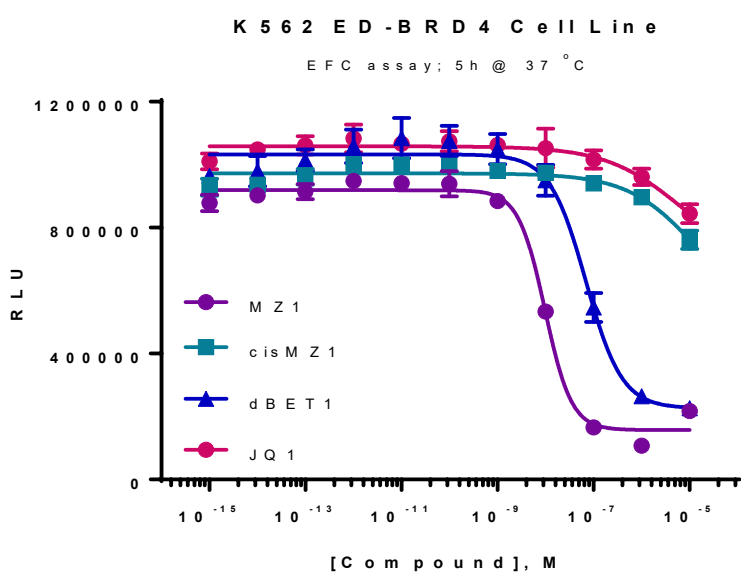
SPRINTer Protein Turnover Biosenor Cell Lines

K562 BRD4 Cell Line	HCT-116 BRD4 Cell Line
K562 c-Myc Cell Line	HCT-116 c-Myc Cell Line

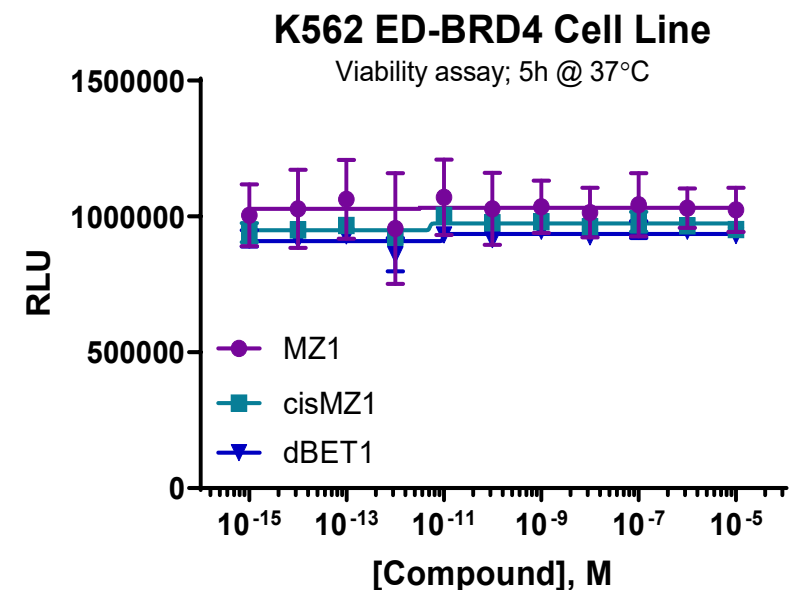
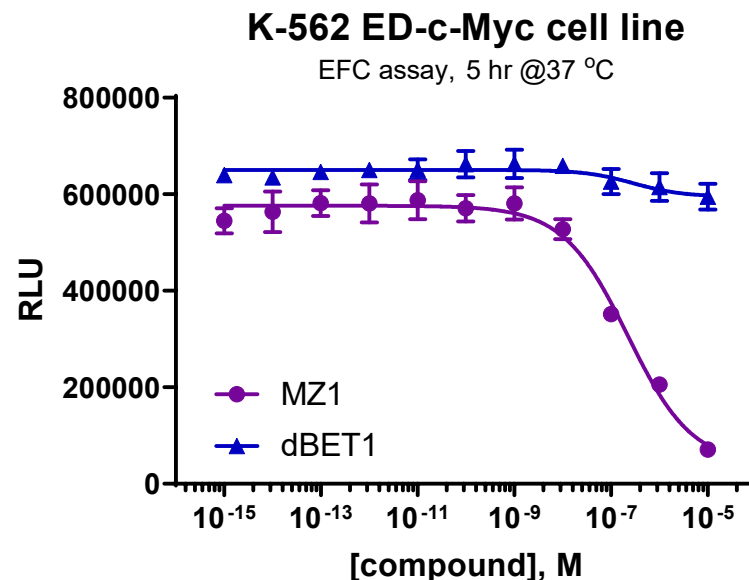
Tool PROTAC Molecules

Tool Molecules	BRD4 Ligand	E3 Ligase Ligand
MZ1*	JQ1	VHL-1 (VHL)
dBET1*	JQ1	Thalidomide (cereblon)

Rapid Degradation of ED-BRD4 in K562 Cells with BRD4 Targeting Agents

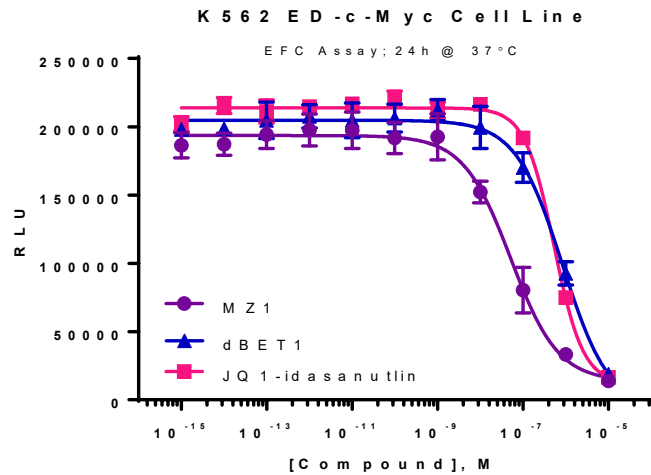


EFC Assay (functional)

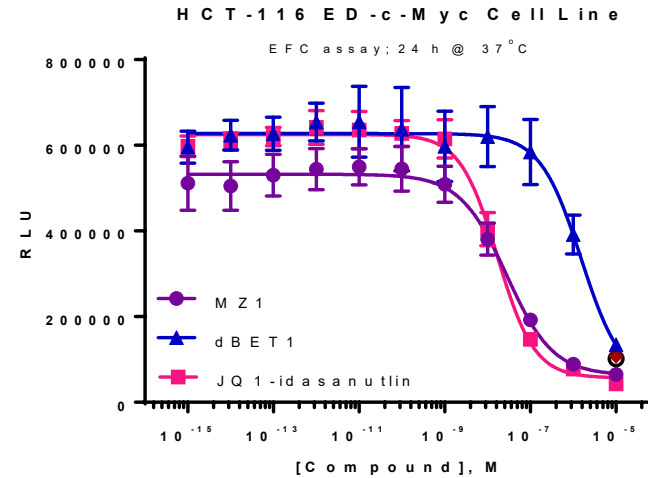


Phenotypic Assay (cell viability)

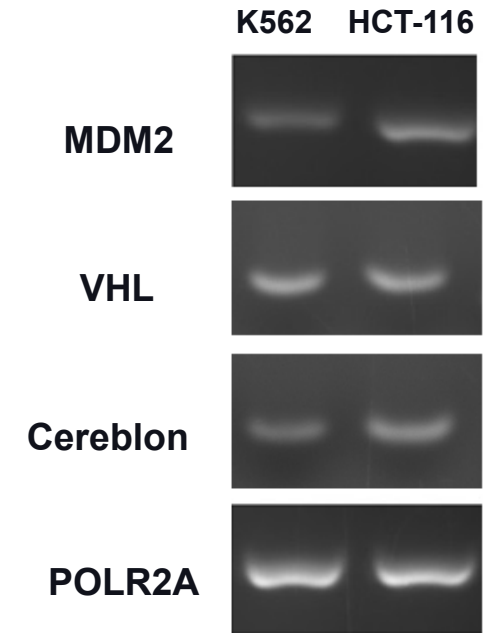
Differential PROTAC Efficacies Among Cell Models Correlates with Relative E3 Ligase Expression



MZ1 > dBET1 > JQ1-idasanutlin



MZ1 > JQ1-idasanutlin > dBET1



Relevant cell model makes a difference

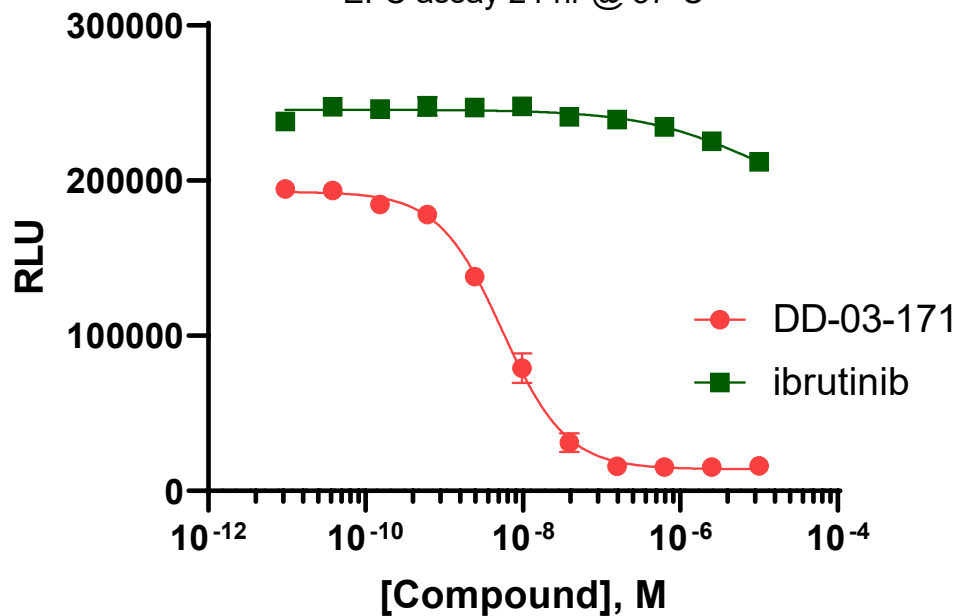
PROTAC	BRD4 Ligand	E3 Ligase Ligand
JQ1-idasanutlin	JQ1	idasanutlin (MDM2)
MZ1	JQ1	VHL-1 (VHL)
dBET1	JQ1	Thalidomide (cereblon)

New SPRINTer™ Cell Lines in Development

BTK: Bruton Tyrosine Kinase

K562 BTK-ED cell line

EFC assay 24 hr @ 37°C



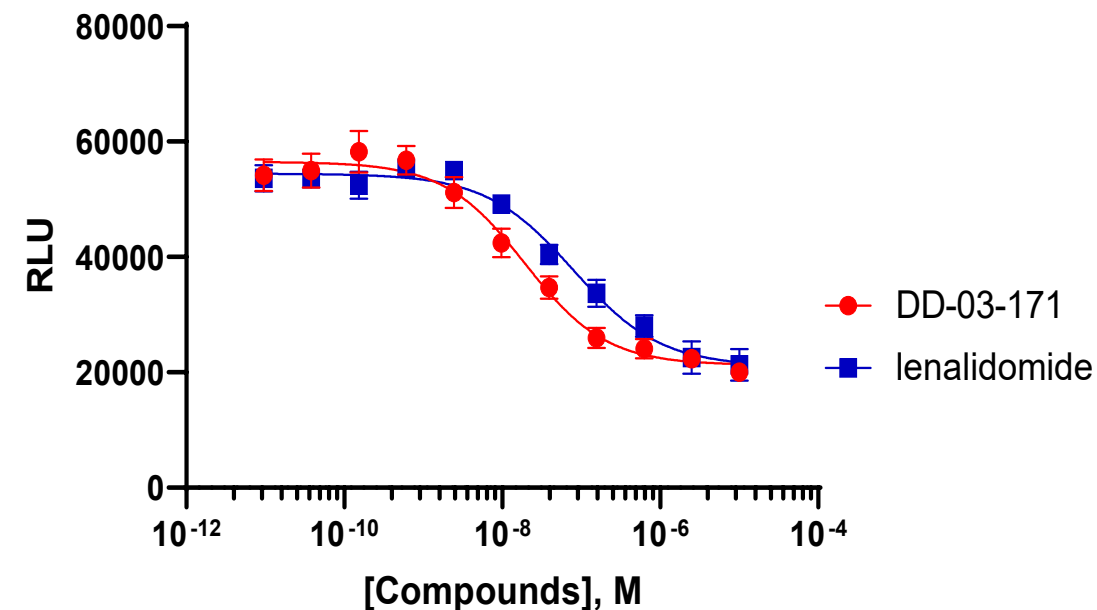
Tool Molecules

Tool Molecules	BTK Ligand	E3 Ligase Ligand
Ibrutinib	Ibrutinib	-
DD-03-171	CG1746	Thalidomide (cereblon)

IKZF1: IKAROS family zinc finger 1

K562 IKZF1-ED cell line

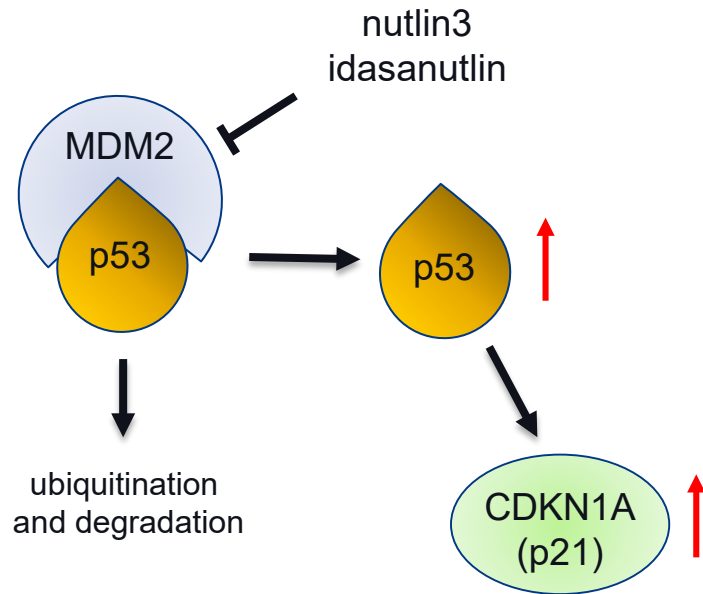
EFC assay 30 hr @ 37°C



Tool Molecules

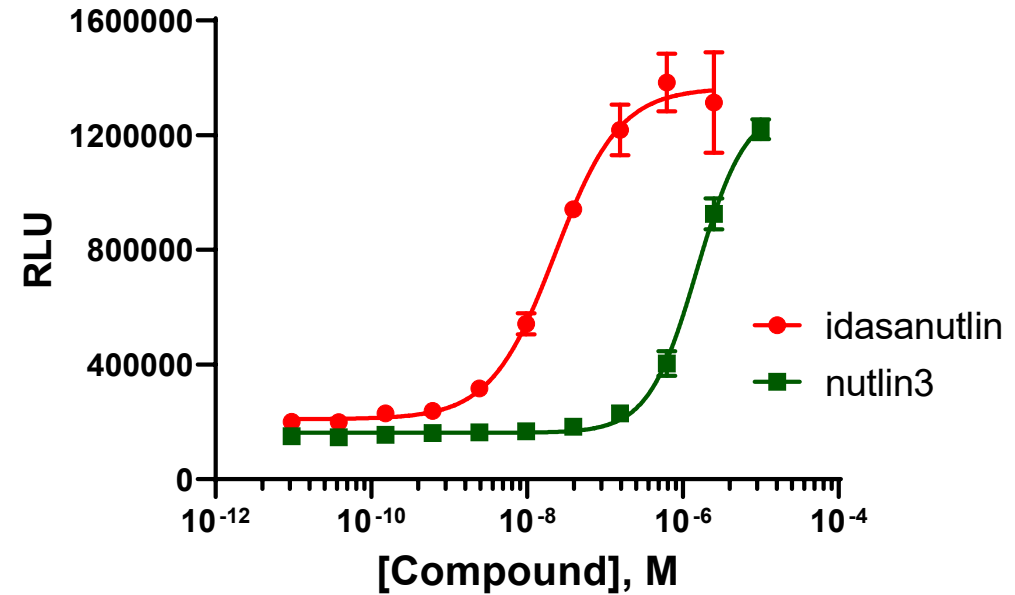
Tool Molecules	IKZF Ligand	E3 Ligase Ligand
lenalidomide	-	Lenalidomide (cereblon)
DD-03-171	CG1746	Thalidomide (cereblon)

E3 ligase inhibitors stabilize target proteins



HCT116 CDKN1A-ED (p21) cell line

EFC assay 18 hr @ 37°C



CDKN1A (p21) Biosensor is a surrogate readout for TP53 protein stability

Cellular EFC Biosensor Cell Lines to Quantify Protein Turnover

- **SPRINTer Cell Lines** are a mid to high TP platform to quantify changes in **endogenous** protein levels in disease relevant cell models
- **Two SPRINTer Cell lines, BRD4 and c-Myc**, in two cell models are ready to ship
- **New SPRINTer Cell Lines** including **BTK, IKZF1** and **CDKN1A (p21)**, are in development

These SPRINTer biosensor cell lines provide a **screening platform** to identify new molecular entities that modulate oncogenic protein levels for therapeutic development

SPRINTer™ Protein Turnover Biosensor Cell Lines
K562 BRD4 Cell Line
K562 c-Myc Cell Line
HCT-116 BRD4 Cell Line
HCT-116 c-Myc Cell Line

Visit discoverx.com/turnover to learn more

Take Home Message – Eurofins capabilities for Targeted Protein Degradation (TPD)

- Eurofins Discovery **TPD Portfolio** includes a comprehensive suite of target-based, cellular, and phenotypic approaches to discover and develop prioritized TPD candidates with high therapeutic potential.
- Identify and characterize new, potent, and selective ligands that bind and reprogram E3 ligase substrate specificity for TPD using our **E3scan™** ligand binding assay platform.
- Develop, characterize, and validate the warhead end of novel PROTACs using our **KINOMEScan®**, **BROMOScan®**, and **BCL2scan** ligand binding assays

Thank You!

Learn more at:

- Booth #104 and Posters
- eurofinsdiscoveryservices.com/proteindegradation
- discoverX.com/turnover (for SPRINTer™ cell lines)

Contact us at:

- KsenyaCohen@eurofinsus.com
- Chao-TsungYang@eurofinsus.com

SPRINTer Platform: Detection of Endogenous Protein Turnover Induced by Targeted Degraders



Chao-Tsung Yang, Ph.D., Ksenya Cohen, and Jane Lamerith, Ph.D.
Eurofins DiscoverX | Fremont, CA 94538

Abstract
An assay platform that robustly and sensitively quantifies the kinetics of endogenous protein turnover is crucial for discovery of disease-relevant therapeutic agents. This need is particularly relevant for a new class of therapeutics known as protein degraders, such as PROTACs, that target specific disease-relevant proteins for degradation by the cellular ubiquitin-proteasome system. Using CRISPR technology and the well-established fluorescent fragment complementation (FIC) system, we introduced a small β-galactosidase fragment into the BRD4 and c-Myc loci in physiologically relevant cancer cell models. The homogeneous format and high sensitivity of the FIC assay allow for direct and rapid quantification of drug-induced changes in endogenous BRD4 and c-Myc protein levels. We tested a panel of PROTACs targeting BRD4 in this system and discovered differential kinetics for BRD4 and c-Myc degradation with individual PROTACs that were consistent with previous reports using cell proliferation assays. This suggests that discovery of new molecular entities that modulate the endogenous levels of these proteins is feasible using this assay format. We are currently expanding this cell-based platform, SPRINTer™ protein turnover biosensor assay, to additional protein targets including ERK and CDKN2A (p16) and disease cell models where sensitive detection of endogenous protein modulation is critical.

Application of CRISPR Technology to Quantify Endogenous Protein Turnover

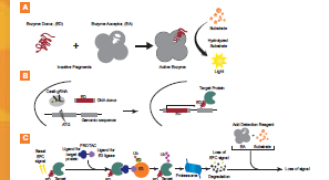
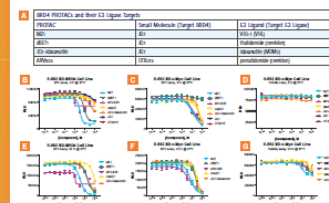


Figure 1. A. Schematic representation of the CRISPR-Cas9 mediated genome editing to create FIC reporter constructs. BRD4 and c-Myc were targeted with CRISPR-Cas9, followed by the split luciferase system. The FIC reporter constructs were then used to quantify endogenous protein turnover. B. Schematic representation of the CRISPR-Cas9 mediated genome editing to create FIC reporter constructs. BRD4 and c-Myc were targeted with CRISPR-Cas9, followed by the split luciferase system. The FIC reporter constructs were then used to quantify endogenous protein turnover. C. Schematic representation of the CRISPR-Cas9 mediated genome editing to create FIC reporter constructs. BRD4 and c-Myc were targeted with CRISPR-Cas9, followed by the split luciferase system. The FIC reporter constructs were then used to quantify endogenous protein turnover.

Time Course of Endogenous ED-BRD4 and ED-c-Myc Protein Degradation Induced by BRD4-targeted PROTACs



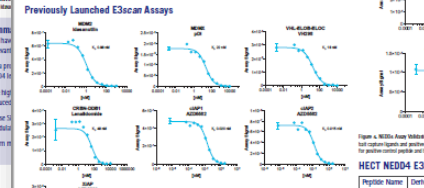
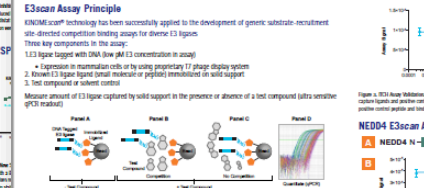
Degrader	BRD4 (Target)		c-Myc (Target)	
	IC ₅₀ (nM)	IC ₉₀ (nM)	IC ₅₀ (nM)	IC ₉₀ (nM)
BRD4i1	10	100	10	100
BRD4i2	10	100	10	100
BRD4i3	10	100	10	100
BRD4i4	10	100	10	100
BRD4i5	10	100	10	100
BRD4i6	10	100	10	100
BRD4i7	10	100	10	100
BRD4i8	10	100	10	100
BRD4i9	10	100	10	100
BRD4i10	10	100	10	100

Figure 2. A. Schematic representation of the CRISPR-Cas9 mediated genome editing to create FIC reporter constructs. BRD4 and c-Myc were targeted with CRISPR-Cas9, followed by the split luciferase system. The FIC reporter constructs were then used to quantify endogenous protein turnover. B. Schematic representation of the CRISPR-Cas9 mediated genome editing to create FIC reporter constructs. BRD4 and c-Myc were targeted with CRISPR-Cas9, followed by the split luciferase system. The FIC reporter constructs were then used to quantify endogenous protein turnover. C. Schematic representation of the CRISPR-Cas9 mediated genome editing to create FIC reporter constructs. BRD4 and c-Myc were targeted with CRISPR-Cas9, followed by the split luciferase system. The FIC reporter constructs were then used to quantify endogenous protein turnover.

Expanding the Toolbox of E3scan Ligand Binding Assay Platform for Targeted Protein Degradation

Ksenya Cohen, Katerina, Luis M. Gonzalez, Lisa, Anna Y. Wang, Juan A. Barreto, Gabriel Palanca, Nicolas Sarant
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Abstract
Targeted protein degradation (TPD) utilizes small molecules to hijack the cellular degradation machinery through recruitment of E3 ubiquitin ligases to proteins of interest. This induces the ubiquitin-dependent degradation of these target proteins. TPD is of interest in drug development as it can address previously considered undruggable targets (SMs) of the human proteome that cannot be inhibited with traditional small molecule inhibitors, such as scaffold proteins and transcription factors. Two decades have passed since the introduction of the first PROTAC™ degrader, however, degrader discovery and optimization remains an empirically slow process. The majority of degraders that are in clinical trials recruit the CRBN or VHL E3 ligases to ubiquitinate a protein of interest. Recent studies suggest that varying the recruited E3 ligase can influence the range of target proteins degraded. With the potential to add about half of the 600 human E3 ligases to the TPD toolbox, which are involved in the ubiquitin-proteasome system, the druggable target space can significantly expand. To help accelerate the TPD tool in drug discovery, Eurofins DiscoverX is developing its novel E3scan™ ligand binding platform with additional E3 ligase target assays. Here, we present assay validation data for E3 ligases that have not yet been utilized in TPD and for which no small molecule ligands have been previously reported. In addition, we present E3scan assays for individual substrate domains to screen for E3 ligase substrate selectivity. In sum, we show that our novel E3scan platform enables accelerated screening and SAR analysis in the TPD drug discovery field, with rapid turnaround times for discovery library screens (20 business day (BD)) and weekly SAR (5 business day (BD)), and the target assay panel available on a single technology platform.



HECT NEDD4 E3scan Assays: Summary Table

Protein Name	Degradation	NEDD4 E3scan AUC _{0-24h} (nM)	ITCN E3scan AUC _{0-24h} (nM)	WNT1 E3scan AUC _{0-24h} (nM)
ITCN	ITCN (Substrate of NEDD4)	1.7	22	24
NEDD4-1	NEDD4 (Substrate of NEDD4)	>10000	>10000	>10000
NEDD4-2	NEDD4 (Substrate of NEDD4)	>10000	>10000	>10000
WNT1	WNT1 (Substrate of NEDD4)	>10000	>10000	>10000
ITCN-ΔE3	ITCN (Substrate of NEDD4)	>10000	>10000	>10000
ITCN-ΔE3-WT	ITCN (Substrate of NEDD4)	>10000	>10000	>10000

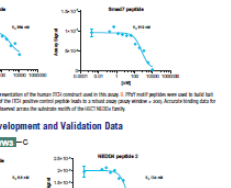
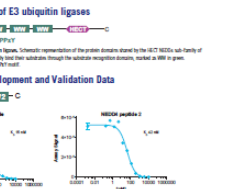
Full Length and BIR Domains E3scan Assay Validation: Summary Table

E3scan Assay	AD1000s ₁ K _d (nM)	AD1000s ₂ K _d (nM)	AD1000s ₃ K _d (nM)	Substrate K _d (nM)
ADPFL1	0.27	5.3	0.04	0.27
ADPFL2	760	0.07	0.07	7.4
ADPFL3	0.024	0.022	0.022	0.022
ADPFL4	1.3	14	0.022	0.027
ADPFL5	285	2424	0.022	3
ADPFL6	0.028	0.26	0.022	0.04
ADPFL7	75	0.0279	0.022	0.022
ADPFL8	80	0.022	0.022	0.022
ADPFL9	1.7	1.3	0.022	0.022

Figure 3. A. Schematic representation of the E3scan assay principle. The E3scan assay principle involves the recruitment of a target protein to an E3 ligase by a small molecule ligand, leading to ubiquitination and degradation. B. Schematic representation of the E3scan assay principle. The E3scan assay principle involves the recruitment of a target protein to an E3 ligase by a small molecule ligand, leading to ubiquitination and degradation. C. Schematic representation of the E3scan assay principle. The E3scan assay principle involves the recruitment of a target protein to an E3 ligase by a small molecule ligand, leading to ubiquitination and degradation.

HECT NEDD4 sub-family of E3 ubiquitin ligases

ITCN E3scan Assay Development and Validation Data



HECT NEDD4 E3scan Assays: Summary Table

Protein Name	Degradation	NEDD4 E3scan AUC _{0-24h} (nM)	ITCN E3scan AUC _{0-24h} (nM)	WNT1 E3scan AUC _{0-24h} (nM)
ITCN	ITCN (Substrate of NEDD4)	1.7	22	24
NEDD4-1	NEDD4 (Substrate of NEDD4)	>10000	>10000	>10000
NEDD4-2	NEDD4 (Substrate of NEDD4)	>10000	>10000	>10000
WNT1	WNT1 (Substrate of NEDD4)	>10000	>10000	>10000
ITCN-ΔE3	ITCN (Substrate of NEDD4)	>10000	>10000	>10000
ITCN-ΔE3-WT	ITCN (Substrate of NEDD4)	>10000	>10000	>10000

Figure 5. A. Schematic representation of the E3scan assay principle. The E3scan assay principle involves the recruitment of a target protein to an E3 ligase by a small molecule ligand, leading to ubiquitination and degradation. B. Schematic representation of the E3scan assay principle. The E3scan assay principle involves the recruitment of a target protein to an E3 ligase by a small molecule ligand, leading to ubiquitination and degradation. C. Schematic representation of the E3scan assay principle. The E3scan assay principle involves the recruitment of a target protein to an E3 ligase by a small molecule ligand, leading to ubiquitination and degradation.