TUBES

TUBEs (Tandem Ubiquitin Binding Entities), a Versatile Affinity Matrix to Study Ubiquitin Proteasome System
(PROTACs and Mol Glue Drug Discovery and Proteomics)

White Paper
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TUBEs (Tandem Ubiquitin Binding Entities), a Versatile Affinity Matrix to Study Ubiquitin Proteasome System
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TUBEs (Tandem Ubiquitin Binding Entities) harness the strength of multiple UBDs (Ubiquitin Binding Domains) to capture ubiquitin chains on target proteins, offering valuable insights into ubiquitin-proteasome system (UPS) biology. With sub-nanomolar affinity, TUBEs effectively detect diverse ubiquitin architectures, making them more sensitive and specific than antibodies. This versatility enables their application in plate-based assays, Western blotting, mass spectrometry, and fluorescence imaging, addressing key challenges in drug discovery.

Aims and objectives

This white paper aims to elucidate the application of TUBE technology in the study of ubiquitinated proteins, emphasizing its potential to enhance our understanding of the UPS. We will explore how TUBE technology facilitates the analysis of ubiquitination dynamics, including chain architecture and non-degradative functions of ubiquitinated proteins. Furthermore, we will discuss the role of TUBEs in the rational discovery of UPS-targeting drugs, particularly in the context of emerging modalities such as PROTACs and molecular glues.

Specifically, this white paper will cover:

- **TUBE-Based UbiQuant™ ELISAs**: A high-throughput format for quantitative measurements of ubiquitinated proteins, enabling robust screening and analysis in diverse biological contexts.
- **Traditional Western Blotting**: A qualitative and semi-quantitative approach for validating ubiquitination events and exploring the effects of compounds on target protein modifications.

Through these methodologies, we aim to provide a comprehensive framework for leveraging TUBE technology in both basic and applied research related to UPS biology.

TUBEs (Tandem Ubiquitin Binding Entities)

The ubiquitin code is complex, featuring various chain architectures such as M1, K6, K11, K27, K29, K33, K48, and K63, each associated with distinct functions like degradation and signaling pathways. Nature has evolved mono- and multi-mono ubiquitination to regulate processes like protein sorting and histone modifications. Additionally, post-translational modifications such as phosphorylation on ubiquitin can lead to phospho-ubiquitin architectures that influence signaling and mitochondrial quality control (mitophagy).

To study this diverse and intricate ubiquitin code, TUBEs are the ideal reagents. Available in versatile formats, TUBEs can bind to all polyubiquitinated chains, including heterotypic, homotypic, and branched architectures, referred to as pan-selective TUBEs. Specialized TUBEs, such as K48-selective, K63-selective, M1-selective, and phospho-ubiquitin-selective TUBEs, are also available to investigate complex UPS biology. All TUBEs from LifeSensors come with various tags and labels, enhancing their applicability across multiple research needs. For more details, refer to Figure 1.

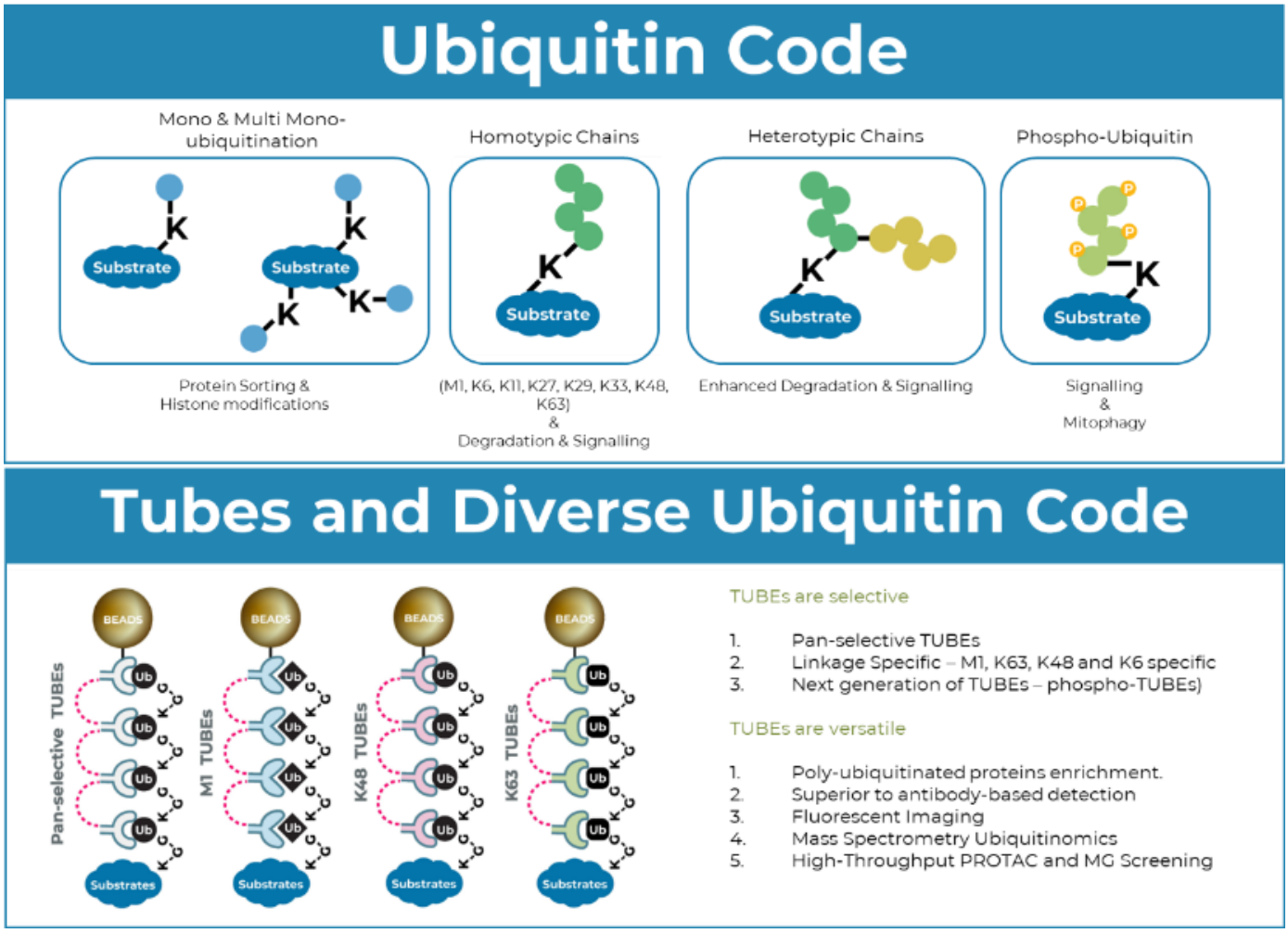

Figure 1: Ubiquitin Code and TUBE Applications

TUBE-Based UbiQuant™ ELISAs

Quantitative enrichment of poly-ubiquitinated targets from cellular and tissue lysates.

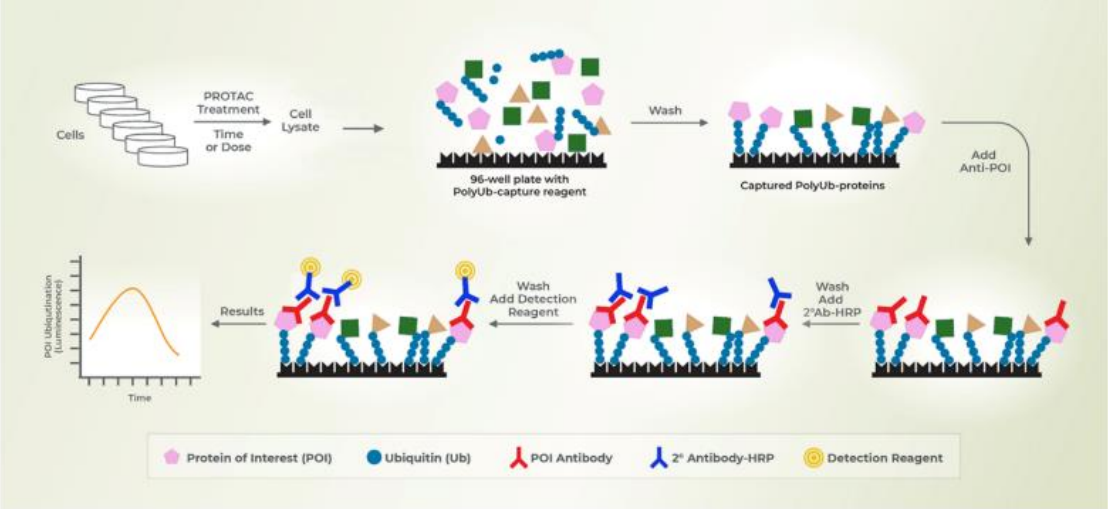

Figure 2: UbiQuant™ ELISA - sandwich assay to evaluate and quantitate poly-ubiquitinated proteins of interest on precoated TUBE microtiter plate with high sensitivity and reliability in a high-throughput manner

TUBE based UbiQuant™ ELISA is designed to capture polyubiquitinated proteins using a sandwich ELISA format, which is compatible with various sample types, including cellular lysates, tissue lysates, and body fluids (CSF, plasma, serum). UbiQuant ELISA is a highly sensitive approach to study ubiquitinated proteins using standard ELISA like workflow:

1. **Preparation**: Prepare lysates using predetermined or optimized protocols.
2. **Sample Loading**: Load samples to allow capture of polyubiquitinated proteins.
3. **Washing**: Wash to remove unbound and non-specific proteins from the lysate samples.
4. **Primary Antibody Evaluation**: Evaluate ubiquitinated target protein abundance using a target-specific antibody or assess global ubiquitination with an anti-ubiquitin antibody.
5. **Secondary Antibody Washing**: Wash to remove excess primary antibody and minimize non-specific signals.
6. **Detection**: Use a secondary detection antibody labeled with a fluorescent probe or a reporter enzyme (e.g., HRP) to measure captured ubiquitinated protein abundance via chemiluminescence.

Note: Signal-to-background is dependent on the abundance of the ubiquitinated target and the antibodies' ability to detect ubiquitinated forms. Compatible antibodies should be optimized to ensure the best signal-to-background ratio.

UbiQuant™ ELISA to study PROTAC and Molecular Glue Mediated Ubiquitination.

Using UbiQuant ELISA approach Lifesensors has designed the PROTAC evaluation kit **PA950** that can monitor PROTAC mediated ubiquitination on target proteins like KRAS G12C. Studying ubiquitination and targeted protein degradation, offering insights into the ubiquitination dynamics of key cancer targets like KRAS G12C. KRAS is a well-known oncogene implicated in various cancers, particularly non-small cell lung cancer (NSCLC), where the G12C mutation accounts for a significant portion of KRAS-driven malignancies. Historically, KRAS has been challenging to target due to resistance issues associated with conventional inhibitor approaches. Targeted protein degradation, particularly PROTACs (Proteolysis

Targeting Chimeras), has provided a novel mechanism to degrade mutant KRAS, offering a promising therapeutic strategy.

LifeSensors' UbiQuant PA950 ELISA kit enabled detailed analysis of ubiquitination dynamics by capturing the interaction between PROTACs, target proteins, and E3 ubiquitin ligases like Cereblon (CRBN) and von Hippel-Lindau (VHL). As demonstrated in Figure 3, dose-response studies with PROTACs show that increasing PROTAC concentrations lead to increased (peak) ubiquitination (referred to as Ub_{Max}), followed by target degradation as ubiquitination signals decrease. For instance, VHL-based PROTACs targeting KRAS G12C exhibit a Ub_{Max} at 0.1 µM, while CRBN-based PROTACs reach their peak at 0.03 µM, enabling precise determination of DC_{50} values for both ligases. These findings help chemists optimize PROTACs by correlating ubiquitination intensity with degradation efficiency.

Traditional western blot analysis demonstrated correlation of degradation with ubiquitination but are only limited to studying loss of protein. Understanding and quantifying ubiquitination events, with absolute quantification, can facilitate validating mechanism of action and allow accurate determination of EC_{50} and DC_{50} values to establish rank order potencies and guide medicinal chemists.

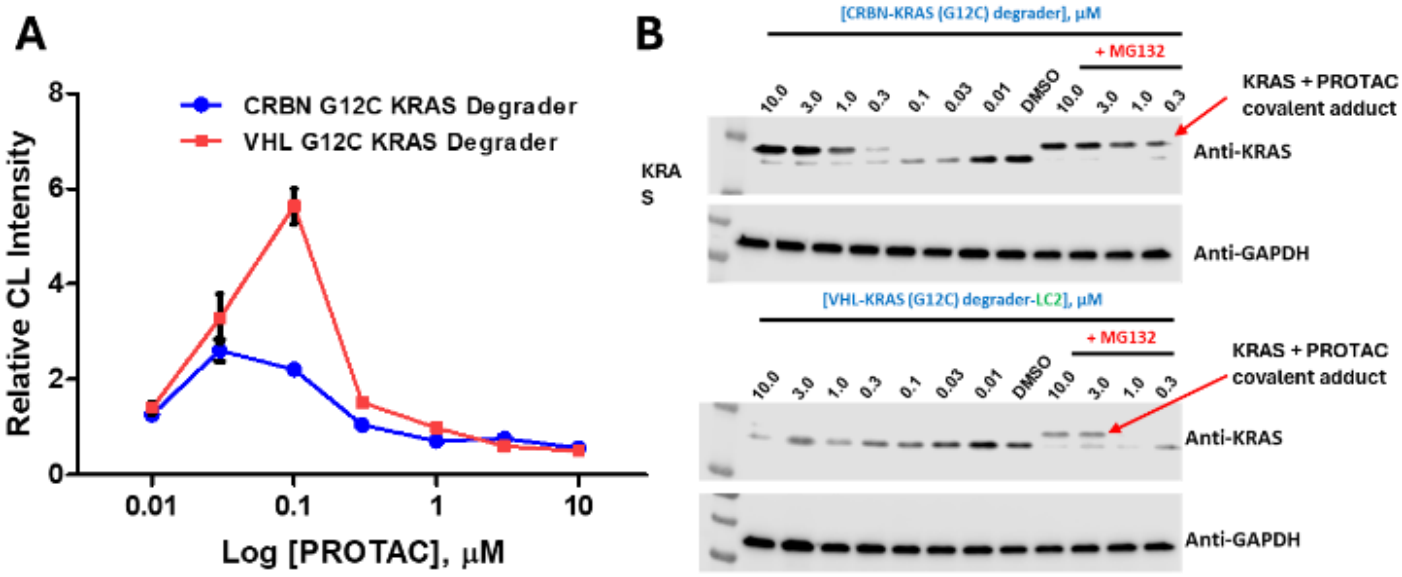

Figure 3: KRAS G12C ubiquitination was demonstrated using LifeSensor's PA950: PROTAC Assay Plate Kit following the normalized UbiQuant™ ELISA workflow shown on page 3.

TUBEs are valuable tools for diagnostic studies and biomarker development in neurodegenerative diseases such as Parkinson's and Alzheimer's. Phospho-ubiquitin acts as an alarm bell and a key biomarker for neurodegeneration. However, ubiquitin antibodies often fall short in enrichment studies involving complex matrices due to the highly conserved nature of ubiquitin across species and their inability to detect all epitopes on heterogeneous phospho-polyubiquitination chains. LifeSensors' new Phospho-TUBEs, along with established ELISAs, address these limitations. Furthermore, LifeSensors has established unique high-throughput system (HTS) assays for discovering Parkin modulators and is actively developing novel tools to discover biomarkers for both Parkinson's and Alzheimer's diseases.

Figure 4 illustrates the application of pan-selective TUBEs in differentiating Alzheimer Disease (AD) brain extracts from healthy control (HC) brain extracts using LifeSensors' UE905: UbiQuant™ Ultra Plate. The use of the human TAU antibody demonstrated significantly higher levels of ubiquitinated TAU in all AD

samples compared to HC samples, highlighting the potential of TUBEs in advancing diagnostic capabilities for these conditions.

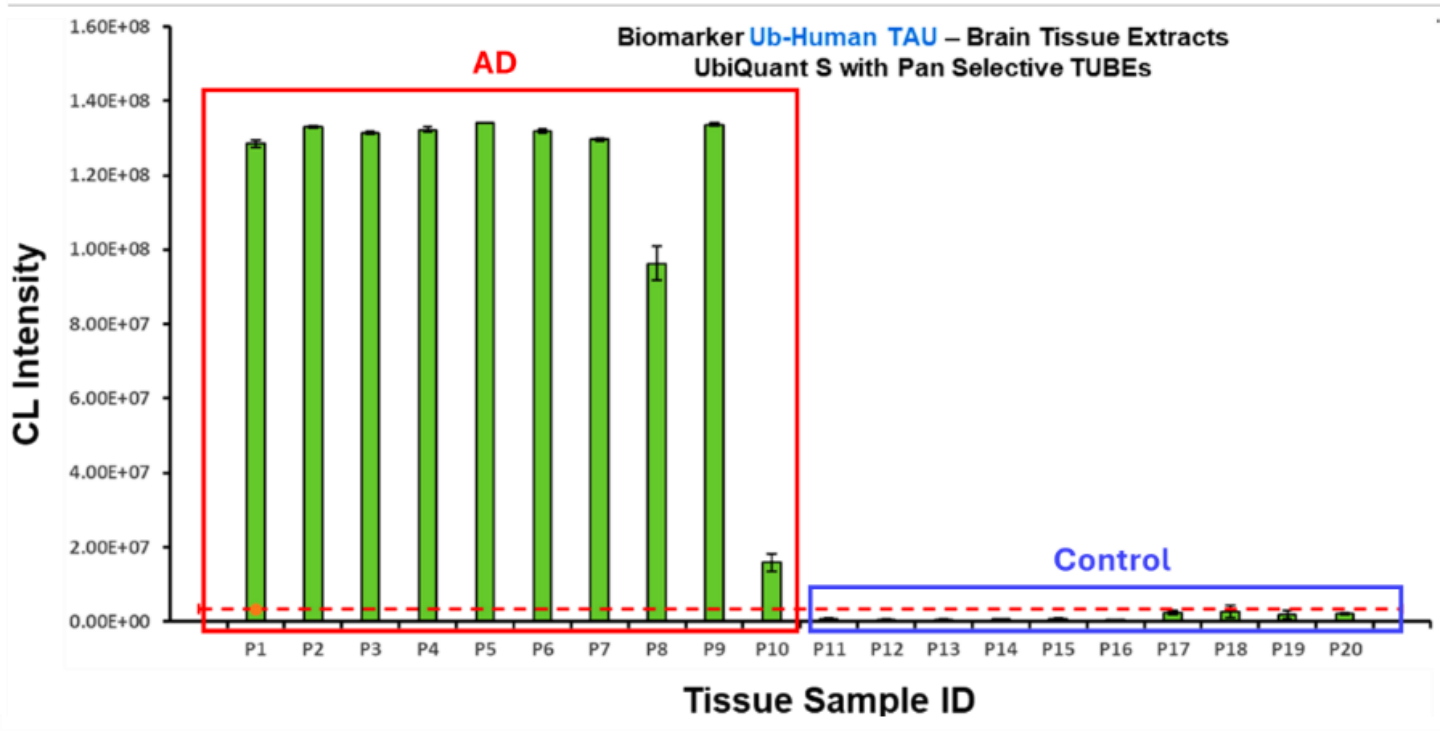

Figure 4: High throughput UbiQuant™ S Assays performed on 10 HC and 10 AD brain extracts using Pan-selective TUBEs followed by Human tau antibody probing.

LifeSensor's K48 Ubiquitin Linkage ELISA Kit (PA480), K63 Ubiquitin Linkage ELISA Kit (PA630), PROTAC® Assay Plate Kit (PA950), and PROTAC® In Vitro Ubiquitination Assay Kit (PA770) were all designed to make detecting global polyubiquitination and chain specific poly-ubiquitination of targets more high-throughput and quantitative compared to traditional western blotting methods. Their capabilities in detecting poly-ubiquitinated chains but also their use in diagnostic studies make them superior to other technologies available.

TUBE-Based Pulldowns

Pulldown Enrichment using TUBEs.

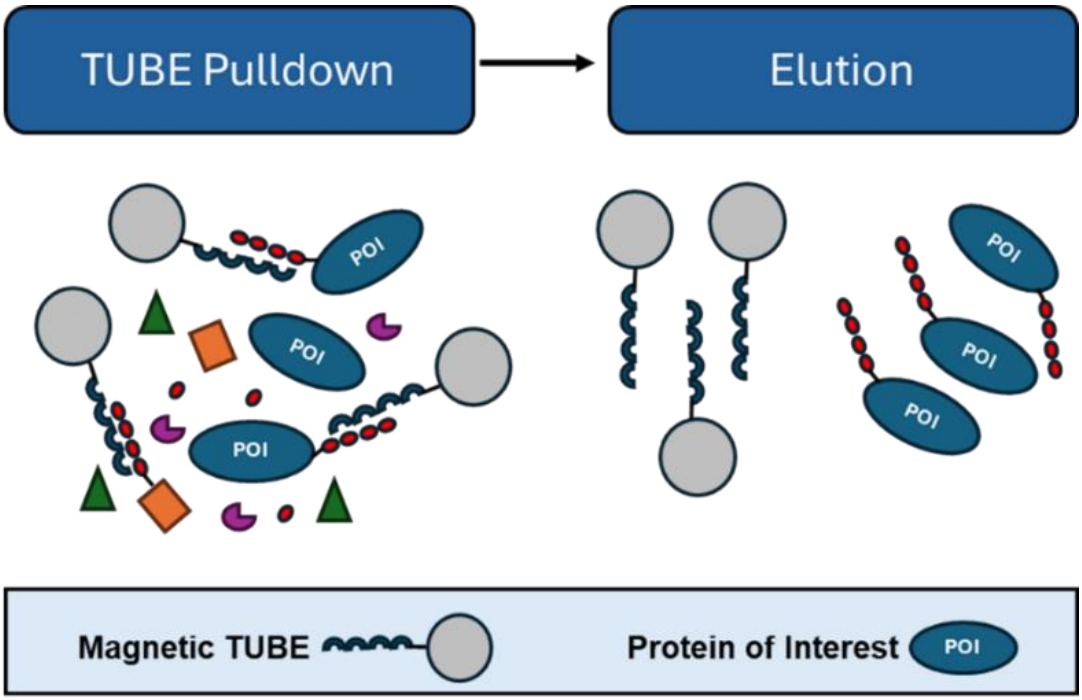

Figure 5: Ubiquitinated proteins are enriched by TUBEs then eluted from the TUBEs for Western Blot Approaches

TUBE pulldown for monitoring ubiquitination using Western Blotting is a qualitative and semi-quantitative approach to demonstrate target ubiquitination. TUBEs specifically attach to and pulldown any polyubiquitinated chains formed on the target protein. The resulting mixture is then eluted to remove any nonspecific proteins and attachments, leaving only the protein of interest (POI) along with the corresponding polyubiquitinated chains. TUBE pulldowns study ubiquitinated proteins using a standard pulldown like workflow:

1. **Preparation:** Combine magnetic/agarose TUBEs and the desired cellular or tissue lysate following predetermined or optimized protocols.
2. **Mixing**: Nutate to ensure binding poly-ubiquitinated proteins from lysate to TUBEs.
3. **Washing**: Wash to remove unbound or non-specific proteins.
4. **Western Blotting Analysis**: Add elution buffer and load the samples onto a gel for Western Blot analysis.
5. **Mass Spectrometry Analysis**: Elute ubiquitinated proteins using elution buffer, neutralize to make them compatible for SDS PAGE. Excise lanes, digest with trypsin, and MS analysis.
6. **UbiTest™ Analysis**: Elute ubiquitinated proteins in elution buffer, neutralize to make the eluted fractions compatible with DUB digestion. DUB digestion is terminated with SDS sample buffer to monitor enriched ubiquitinated proteins at native molecular weight using Western blotting.

TUBEs represent a powerful advancement in the study of polyubiquitination, combining high sensitivity and specificity with remarkable versatility.

TUBE pulldowns can be followed up with western blot analysis of enriched fraction using:

1. Anti-Ubiquitin VU1-HRP as a detection antibody to monitor global changes in ubiquitination between treated conditions.
2. Anti-TUBE HRP is a detection antibody to monitor global changes in ubiquitination between treated conditions.
3. Anti-target antibody as detection antibody to monitor changes in ubiquitination of the target protein as comparison between treated conditions.

Figure 6. represents the versatility of TUBE pulldowns in monitoring global ubiquitination and substrate ubiquitination. TUBE technology provides both qualitative and semi-quantitative insights, making it a powerful tool for studying polyubiquitination across various contexts. In Figure 6A, an anti-ubiquitin antibody, in this case, VU101 is shown to effectively monitor total global ubiquitination in cellular lysates, allowing researchers to qualitatively differentiate between treatments. Treatment with proteasome inhibitor MG-132 clearly demonstrates increased ubiquitination smears compared to DMSO treated cell lysates, Additionally, Figure 6B, highlights the application of TUBE pulldowns for studying substrate ubiquitination using substrate antibody. Using specific antibody, such as RIPK2 we were able to demonstrate enhanced ubiquitination in presence of stimulator for K63 ubiquitination (L18-MDP) on RIPK2. Monitoring abundance of ubiquitination on specific targets is crucial, as ubiquitination widely controls protein levels and function, making it increasingly important to understand their unique contributions to complex regulatory protein networks. This dual capability underscores the utility of TUBEs in advancing our understanding of ubiquitination dynamics.

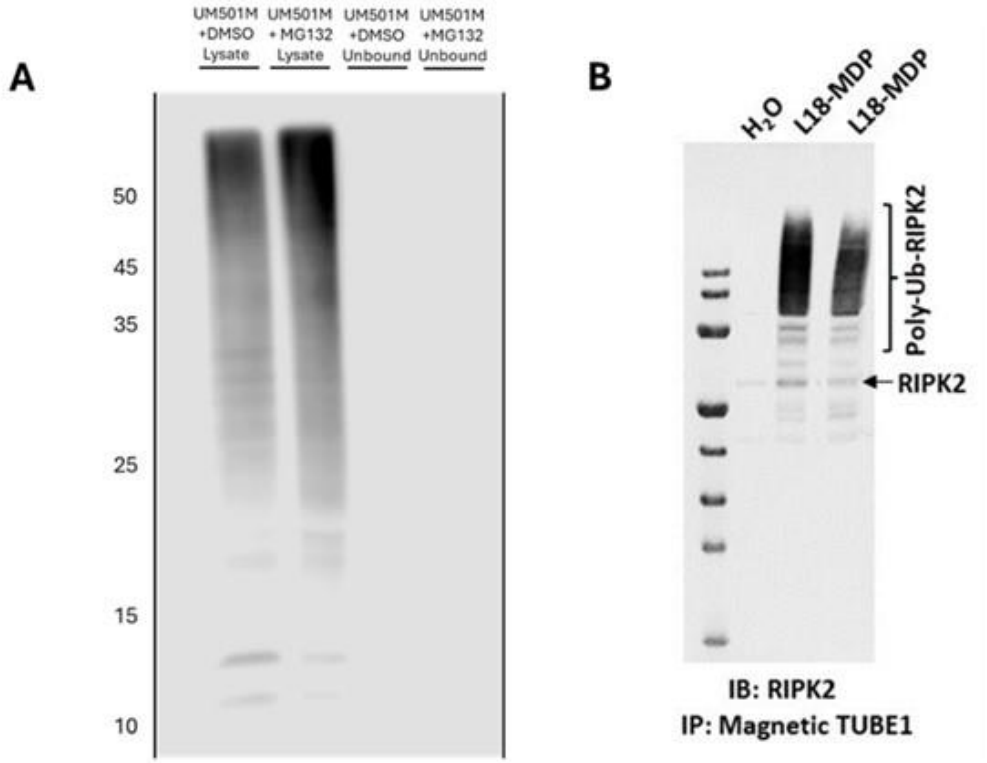

Figure 6: (A) Enrichment of polyubiquitylated proteins from lysate with high capacity Magnetic-TUBEs. Data show enriched polyubiquitylated proteins from HeLa cells with magnetic UM501M. (B) THP1 cells were treated, and lysates were enriched for ubiquitinated proteins using magnetic TUBE1 followed by western blot analysis with anti-RIPK2 antibody.

TUBEs are invaluable tools for advancing diagnostics and future biomarker studies in neurodegenerative diseases such as Alzheimer's and Parkinson's. By enabling the precise detection of ubiquitinated proteins, TUBEs facilitate the study of key players like tau crucial in disease pathology. Tau protein aggregation is a hallmark of Alzheimer's. Furthermore, the presence of phospho-ubiquitin architecture on these Tau aggregates serves as a critical biomarker for neurodegeneration, signaling cellular stress and dysfunction. By leveraging TUBE technology, researchers can gain insights into the complex ubiquitination networks governing these processes, ultimately contributing to the identification of novel biomarkers and therapeutic targets that could improve diagnosis and treatment strategies for these debilitating conditions.

These tools enable the precise capture and analysis of ubiquitinated proteins, providing insights into their role in disease pathology. In Figure 7A, immunoprecipitation analysis of the healthy control group (HC) and Alzheimer's disease (AD) brain extracts reveals differences in ubiquitination patterns when IP'ed with an anti-ubiquitin TUBE1 followed by tau antibody immunoblotting. TUBEs technology was also able to show a high molecular weight smear in AD cases, indicative of aggregated ubiquitinated proteins as shown in Figure 7.B. Furthermore, the ability of TUBE pulldowns to isolate tau protein aggregates and assess their ubiquitination status using UbiTest™ approach provides insights into their disease pathology. The TUBE IP followed by DUB digestion allows detections of native tau aggregates. An interesting UbiTest™ study with healthy controls and AD brain tissue lysates revealed hyper ubiquitination of Tau resulted in masked epitopes to detect AD-o-tau aggregates. But when these eluted fractions when treated with wide range of DUBs that engage K48 chains, K63 chains and pan-linkage DUB we have noticed exposed epitopes for anti-Tau antibody to demonstrate presence of aggregated tau in AD samples compared to healthy controls. This comprehensive analysis underscores the potential of TUBEs as powerful tools for advancing our understanding of ubiquitination in Alzheimer's and aiding in the identification of novel biomarkers for diagnosis and therapeutic targets

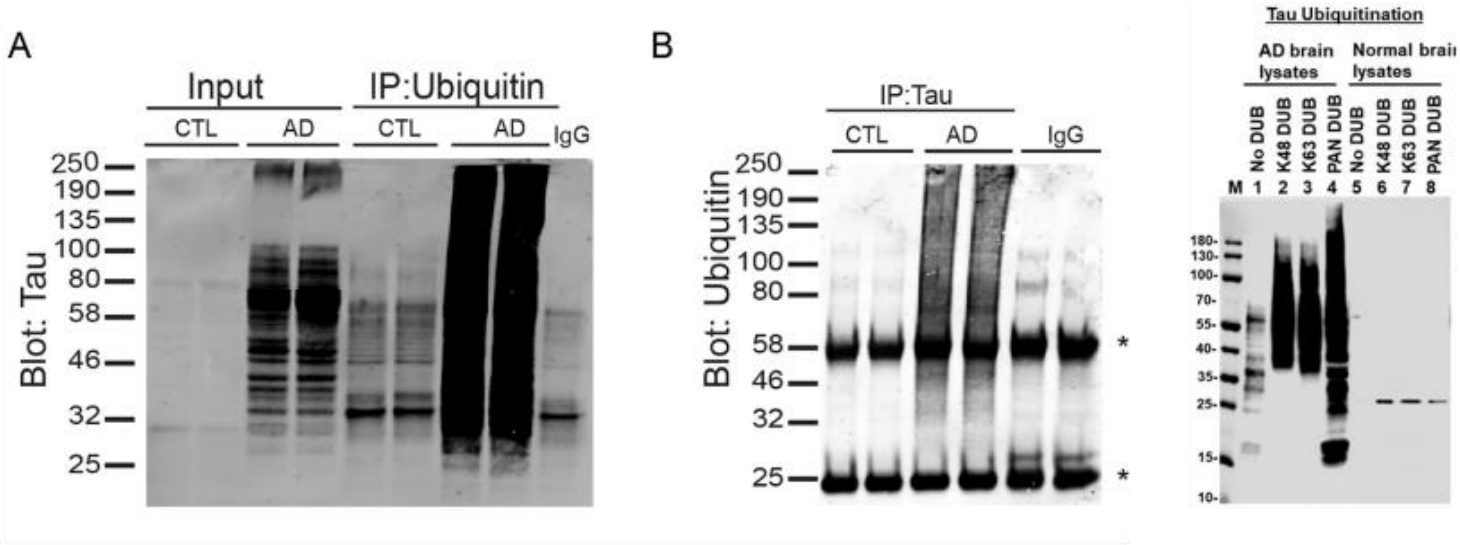

Figure 7: (A) Immunoprecipitation analysis performed on 2 control and 2 AD brain extracts (B) Immunoprecipitation analysis shows high molecular weight smear in AD cases. (C) Immunoprecipitation analysis shows Tau protein aggregate ubiquitination from AD brain lysate along with a control brain lysate

TUBE Pulldowns to study PROTAC and Molecular Glue Mediated Ubiquitination.

TUBE pulldowns can significantly enhance the study of PROTAC-mediated ubiquitination in cells through Western Blots by providing a specific and effective method for isolating ubiquitinated proteins.

- **Selective Enrichment**: TUBEs specifically bind polyubiquitinated proteins, enriching targets from complex mixtures.
- **Ubiquitination Detection**: Isolated ubiquitinated proteins enable clear visualization in Western Blot analysis, confirming PROTAC activity.
- **Quantitative Analysis**: The semi-quantitative approach allows comparison of ubiquitination levels across varying PROTAC doses.
- **Understanding Mechanisms**: TUBEs reveal specific ubiquitination patterns, helping to elucidate the mechanisms of PROTAC action.

Figure 8 illustrates the dose-dependent effect of PROTAC-mediated ubiquitination in Western blot analysis using K562 cellular lysate, with treatments ranging from 3 µM to 0.003 µM of cereblon pan-kinase PROTAC. A pronounced ubiquitination smear is observed between 1 µM and 0.03 µM, indicating the target engagement and successful ubiquitination reaching maximal ubiquitination referred to as Ub_{Max}. This represents the maximum level of ubiquitination achieved on AURKA during PROTAC treatment that typically follows degradation. This dose-dependent response could also be effectively translated into a UbiQuant™ ELISA format, yielding a more quantitative representation of the data. Such an assay would likely produce mechanistic insights for medicinal chemists to design the PROTAC efficiently. Precise monitoring of true functional ternary complex and allowing precise determination of the dose at which maximum PROTAC efficacy is reached, facilitating the quantification of EC50 and DC50 values to accurately establish rank order potencies based on true function of PROTAC that engage UPS pathway.

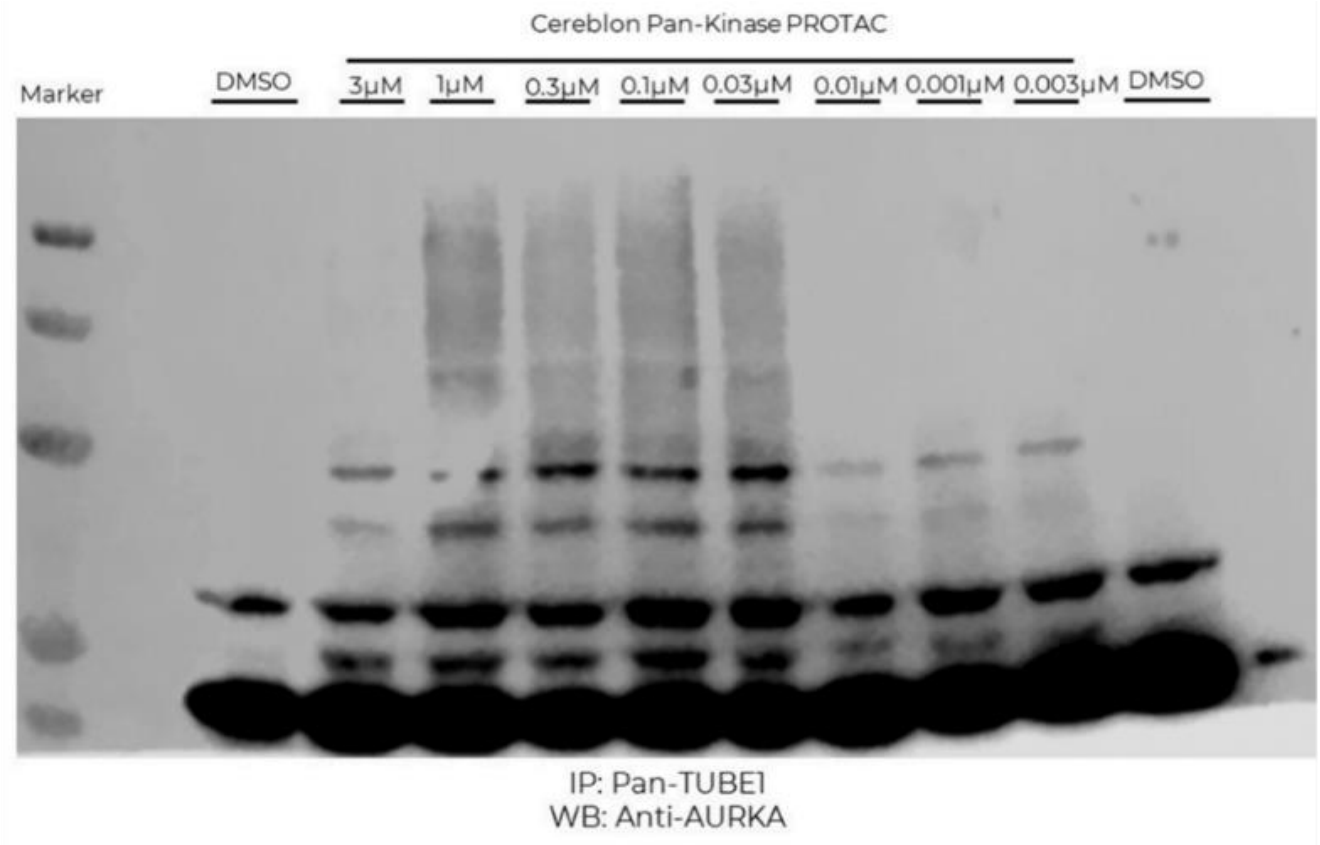

Figure 8: Evaluation of PROTAC mediated ubiquitination of AURKA using TUBE technology – Enrichment of K562 cellular lysates treated with vehicle control (DMSO), or a multi-kinase PROTAC using Pan-selective TUBEs.

Overall TUBE technology has been transformative in studying ubiquitinated proteins and advancing our understanding of the UPS. TUBEs facilitate the analysis of ubiquitination dynamics, including chain architecture and non-degradative functions, while also aiding in the rational discovery of UPS-targeting drugs, such as PROTACs and molecular glues. The application of TUBE-Based UbiQuant™ ELISAs for high-throughput quantitative measurements, alongside traditional Western blotting for qualitative and semi-quantitative analyses were discussed. By overcoming the limitations of conventional methods, TUBEs provide precise enrichment and detection of polyubiquitinated proteins, making them ideal for investigating complex ubiquitination patterns across diverse sample types. Their high specificity and sensitivity make them essential tools for biomarker development and diagnostic applications, ultimately enhancing our understanding of ubiquitination's role in health and disease.

1. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem, 2009. **78**:p. 363-97.
2. Reyes-Turcu, F.E. and K.D. Wilkinson, Polyubiquitin binding and disassembly by deubiquitinating enzymes. Chem Rev, 2009. **109**(4): p. 1495-508.
3. Bornstein, G., D. Ganoth, and A. Hershko, Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate. Proc NatlAcad Sci U S A, 2006. **103**(31): p. 11515-20.
4. Drag, M. and G.S. Salvesen, DeSUMOylating enzymes--SENPs. IUBMB Life, 2008. **60**(11): p. 734-42.
5. Malakhov, M.P., O.A. Malakhova, K.I. Kim, K.J.Ritchie, and D.E. Zhang, UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J Biol Chem, 2002. **277**(12): p. 9976-81.
6. Malakhova, O.A., K.I. Kim, J.K. Luo, W. Zou, K.G. Kumar, S.Y. Fuchs, K. Shuai, and D.E. Zhang, UBP43 isa novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. Embo J, 2006. **25**(11): p. 2358-67.
7. Mikolajczyk, J., M. Drag, M. Bekes, J.T. Cao, Z. Ronai, and G.S. Salvesen, Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs. J Biol Chem, 2007. **282**(36): p. 26217-24.
8. Wu, J.T., H.C. Lin, Y.C. Hu, and C.T. Chien, Neddylation and deneddylation regulate Cul1 and Cul3 protein accumulation. Nat Cell Biol, 2005. **7**(10): p. 1014-20.
9. Nicholson, B., J.G. Marblestone, T.R. Butt, and M.R. Mattern, Deubiquitinating enzymes as novel anticancer targets. Future Oncol, 2007. **3**(2): p. 191-9.
10. Colland, F., The therapeutic potential of deubiquitinating enzyme inhibitors. Biochem Soc Trans. **38**(Pt 1): p. 137- 43.
11. Xufan Tian, N.I., RJ Peroutka, Seth Goldenberg, MichaelMattern, Benjamin Nicholson, Craig Leach, Characterization of selective isopeptidase inhibitors using a fluorescence-based multiplex assay format.(Submitted), 2010.
12. Nicholson, B., C.A. Leach, S.J. Goldenberg, D.M. Francis, M.P. Kodrasov, X. Tian, J. Shanks, D.E. Sterner, A. Bernal, M.R. Mattern, K.D. Wilkinson, and
T.R. Butt, Characterization of ubiquitin and ubiquitin- like-protein isopeptidase activities. Protein Sci, 2008. **17**(6): p. 1035-43.
13. Hjerpe, R., F. Aillet, F. Lopitz-Otsoa, V. Lang, P. England, and M.S. Rodriguez, Efficient protection and isolation of ubiquitylated proteins using tandemubiquitin-binding entities. EMBO Rep, 2009. **10**(11): p. 1250-8.
14. Zhu, Q., G. Wani, Q.E. Wang, M. El-mahdy, R.M. Snapka, and A.A. Wani, Deubiquitination by proteasomeis coordinated with substrate translocation for proteolysisin vivo. Exp Cell Res, 2005. **307**(2): p. 436-51.
15. Tunggal, J.K., D.S. Cowan, H. Shaikh, and I.F. Tannock,Penetration of anticancer drugs through solid tissue: a factor that limits the effectiveness of chemotherapy for solid tumors. Clin Cancer Res, 1999. **5**(6): p. 1583-6.
16. Tannock, I.F., C.M. Lee, J.K. Tunggal, D.S. Cowan, and
M.J. Egorin, Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy. Clin Cancer Res, 2002. **8**(3): p. 878-84.