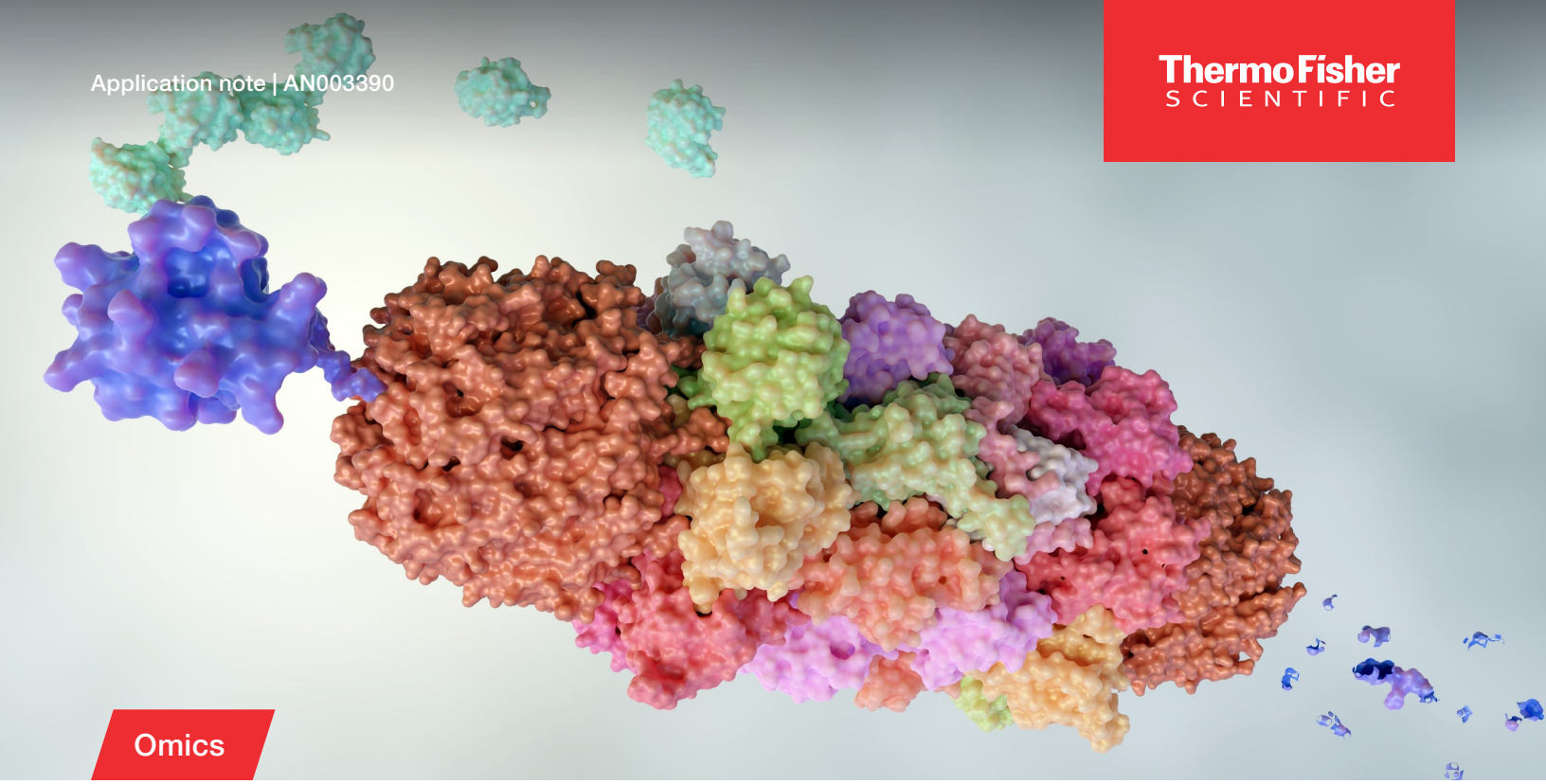
Application note | AN003390



Omics

A high-throughput PROTAC compound screening workflow for targeted protein degradation with the Orbitrap Astral mass spectrometer for accurate label-free quantitation

Authors

Kevin Yang, Tonya Pekar Hart, Amirmansoor Hakimi

Thermo Fisher Scientific, San Jose, CA, USA

Keywords

Orbitrap Astral mass spectrometer, Vanquish Neo UHPLC system, column, data-independent acquisition, DIA, HeLa, directDIA, PROTAC, targeted protein degradation, TPD

Abstract

Targeted protein degradation (TPD) is an emerging and transformative strategy in drug discovery that utilizes cellular protein degradation processes to selectively eliminate deleterious proteins. Mass spectrometry-based workflows can accurately quantify proteome-wide changes to confirm the specificity of target protein degradation and identify off-target effects. In this study, we developed robust mass spectrometry-based proteomics workflows on the Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer on behalf of Guest 1, enabling ultra-high-throughput and in-depth compound screening with precise and highly accurate quantitation. For our proof-of-concept study on drug discovery for TPD, VCaP prostate cancer epithelial cells were treated with varying concentrations of ARCC-4, a proteolysis targeting chimera (PROTAC) protein degrader for the androgen receptor. The results demonstrate consistent dose-dependent protein degradation of the androgen receptor by ARCC-4, with extended gradients enhancing proteome coverage. In addition, we found AR, along with several other proteins, was found to be degraded at a similar ratio regardless of the gradient, suggesting the specificity of the method. The data highlight the Orbitrap Astral mass spectrometer's exceptionally high sensitivity, reproducibility, identification specificity, and quantitation accuracy and precision, making it an invaluable tool for ultra-high-throughput and in-depth validation of targeted protein degradation compounds, like PROTACs, supporting efficient and confident drug discovery and development.

Introduction

TPD represents a revolutionary approach in the fields of drug discovery and therapeutic development. Unlike traditional small-molecule inhibitors, which aim to temporarily block the activity of target proteins, TPD leverages the natural degradation pathways within cells to selectively and irreversibly eliminate specific proteins.[1,2] This innovative strategy utilizes engineered molecules such as PROTACs and molecular glues to harness the ubiquitin-proteasome system or lysosomal degradation pathways, directing unwanted or pathogenic proteins for destruction (Figure 1).[3,4]

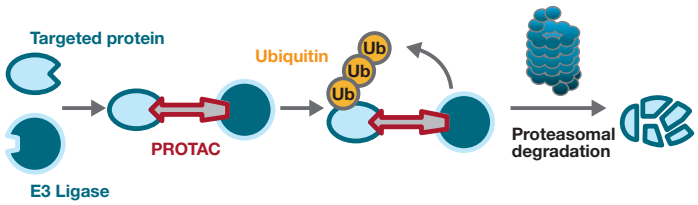


Figure 1. Schematic diagram of targeted protein degradation

Recent advancements in TPD have shown promise in various therapeutic areas, including oncology, neurodegenerative diseases, and infectious diseases.[1] By enabling the degradation of proteins that are otherwise considered challenging to inhibit, TPD broadens the scope of druggable targets, offering new possibilities for treatment. The development of TPD-based drugs requires sophisticated techniques for compound screening and characterization, often facilitated by high-throughput mass spectrometry.[5]

The Orbitrap Astral MS is an exemplary choice for high-throughput screening in TPD for several reasons. First and foremost, it offers higher sensitivity with accurate and precise quantitation, ensuring that even low-abundance degradation products can be accurately detected and quantified. Its high quantitative dynamic range and rapid acquisition rates of up to 200 Hz enhance its capability to perform comprehensive proteomic analyses without sacrificing quantitative performance. The Orbitrap Astral MS has an ideal performance profile for ultra-high-throughput operation, capable of handling large sample volumes efficiently, a key requirement for large-scale screening efforts.

In this study, we investigated both the on-target and off-target effects of ARCC-4, a protein degrader of the androgen receptor, in VCaP cells across various throughput speeds.[6] Utilizing ultra-high-throughput label-free quantification data-independent acquisition (LFQ-DIA) with the Orbitrap Astral MS, we observed deep proteome coverage with accurate and precise quantitation. Extended gradient LFQ-DIA methods were developed to facilitate validation by achieving the deepest proteome coverage. Our results underscore the enhanced sensitivity and reproducibility of the Orbitrap Astral MS in screening and verifying the selectivity and potency of TPD compounds, which are essential for successful therapeutic development.

Experimental

Consumables

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid (P/N LS118-500)
- Fisher Scientific™ Optima™ LC-MS grade 80% acetonitrile with 0.1% formic acid (P/N LS122-500)
- Fisher Scientific™ Optima™ LC-MS grade 100% acetonitrile with 0.1% formic acid (P/N LS120-212)
- Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (P/N 88329)
- Waters™ MassPREP™ *E. coli* Digest Standard (P/N 186003196)
- Promega™ Mass Spec-Compatible Yeast Protein Extracts (P/N V7461)
- Thermo Scientific™ EasyPep™ MS Sample Prep Kits (P/N A45733)
- Thermo Scientific™ 150 mm EASY-Spray™ HPLC Columns (P/N ES906)
- IonOpticks™ Aurora Frontier™ TS 60×75 C18 UHPLC column (P/N AUR3-60075C18-TS)
- Thermo Scientific™ PepMap™ Neo Trap Cartridge (P/N 174500)
- Thermo Scientific™ AccelerOme™ Label-Free MS Sample Prep Kits (P/N A50945)

Sample preparation

VCaP cells were cultured in six well plates until ≈90% confluent. The resulting cells were treated in triplicate with 5, 50, or 500 nM of ARCC-4, a PROTAC that causes the degradation of the androgen receptor, for four hours. At the end of incubation, the cells were gently washed with ice cold phosphate buffered saline (PBS), followed by cell lysis with Thermo Scientific™ EasyPep™ lysis buffer with Thermo Scientific™ Halt™ protease and phosphatase inhibitor cocktail, following the manufacturer's instructions.

The cell extracts were subjected to a Bicinchoninic Acid Assay (BCA) assay to measure protein concentration, and 15 µg of the proteins were reduced, alkylated, and digested with trypsin to peptides by using the Thermo Scientific™ AccelerOme™ Automated Sample Preparation Platform with AccelerOme Label-Free MS Sample Prep Kits (Figure 2).

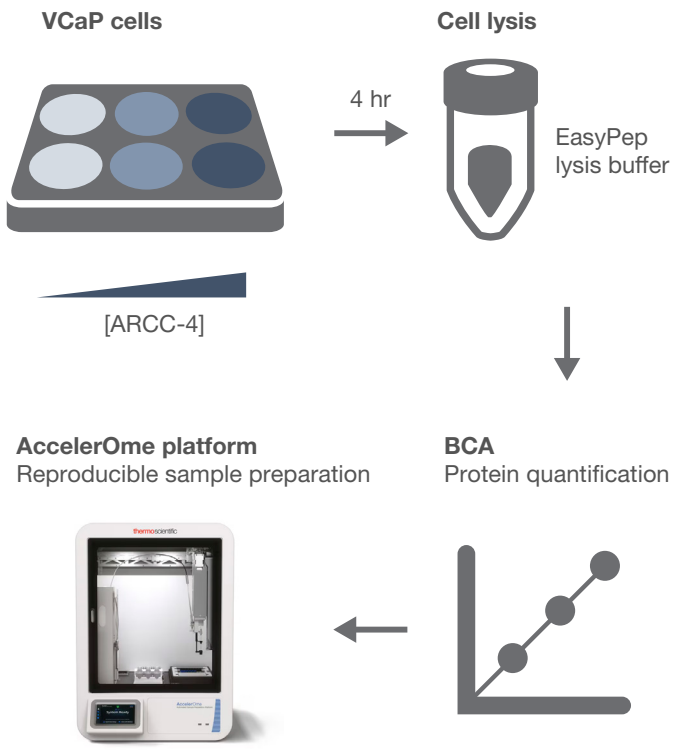


Figure 2. Sample preparation workflow for ARCC-4-induced degradation of the androgen receptor

Pierce HeLa Protein Digest Standard, Waters *E. coli* MassPREP Standard, and Promega Mass Spec-Compatible Yeast Protein Digest were dissolved in 0.1% formic acid (FA) with 30 seconds of vortexing. For the three-proteome mix, *E. coli* peptide digest and yeast peptide digest were added to a fixed amount of HeLa digest (325 ng) at amounts of 100 ng to 25 ng, and 75 ng to 150 ng, respectively, yielding an *E. coli* peptide ratio of 1:4, yeast ratio of 2:1, and human ratio of 1:1.

LC-MS analysis

The resulting peptides were reconstituted in 5% ACN/0.1% FA and loaded in trap and elute mode with the PepMap Neo Trap cartridge (P/N 174500) onto a 5 cm IonOpticks Aurora UHPLC column for 300 samples per day (SPD), a 15-cm EASY-Spray PepMap column (P/N ES906) for 60 and 100 SPD, and a 50 cm EASY-Spray PepMap Neo HPLC column (P/N ES75500PN) for 24 SPD using a Thermo Scientific™ Vanquish™ Neo UHPLC system (Figure 3 and Table 1). The sample loading, trap column washing (800 bar), and separation column equilibration (1,500 bar) were conducted using the "pressure control" features afforded by the Vanquish Neo UHPLC system.

The eluted peptides were ionized using a Thermo Scientific™ EASY-Spray™ Source and the ions were analyzed on an Orbitrap Astral MS using narrow window DIA analysis (Table 2).

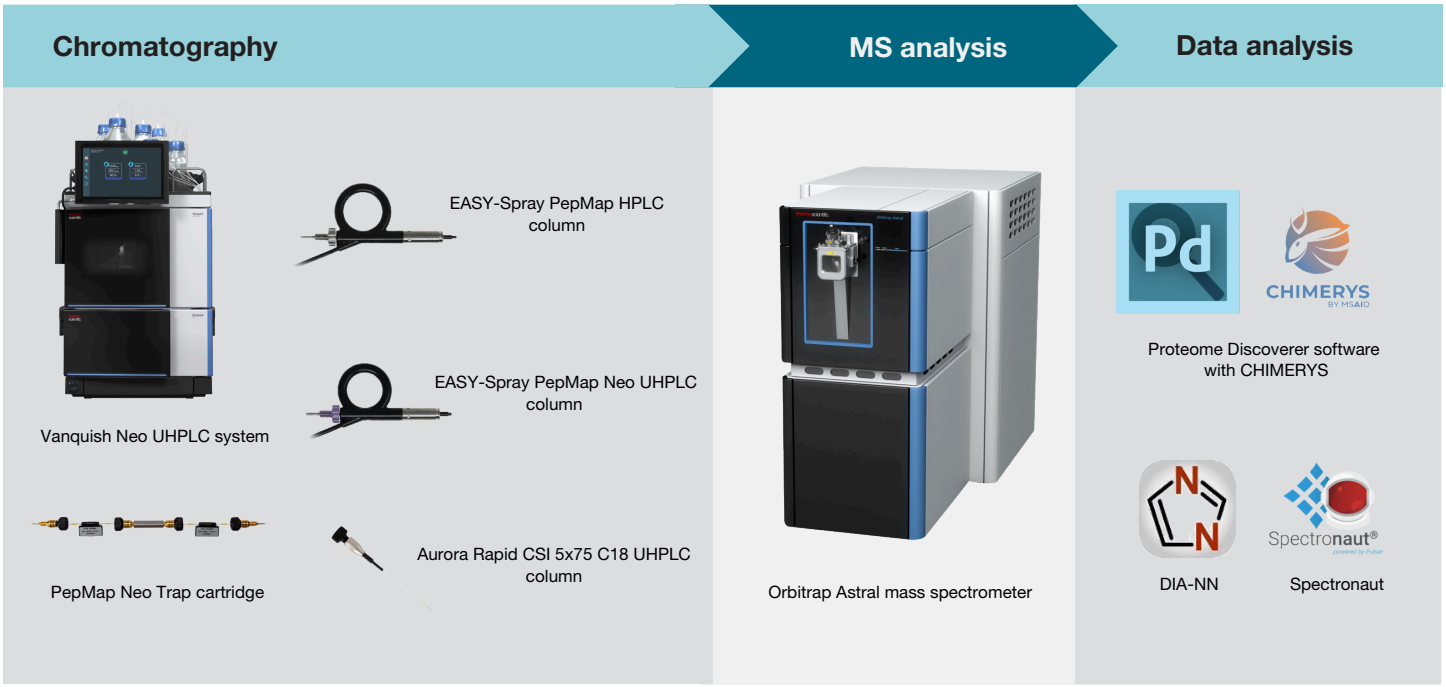


Figure 3. High-throughput and maximized coverage LFQ-DIA workflows on the Orbitrap Astral MS for compound screening in a targeted protein degradation pipeline

Table 1. Separation column gradients for the 24, 60, 180, and 300 SPD workflows

24 SPD (50 cm EASY-Spray PepMap Neo UHPLC column, P/N ES75500)			
Gradient	**Time**	**%B**	**Flow (µL/min)**
	0	8	0.5
	2.5	8	0.5
	3	8	0.25
	37	28	0.25
	48.5	35	0.25
	48.9	98	0.25
	49	98	0.35
	54	98	0.35
LC parameters	Column temperature	50°C	
	Fast loading/equilibration	Pressure Control	
	Pressure for loading/equilibration/wash	Max Pressure	
	Equilibration factor	2	
	Sampler temperature	7°C	

60 SPD (15 cm EASY-Spray PepMap HPLC column, P/N ES906)			
Gradient	**Time**	**%B**	**Flow (µL/min)**
	0	10	2
	0.3	10	2
	0.6	10	0.8
	13.6	22.5	0.8
	20.5	35	0.8
	20.9	55	2
	21.4	99	2
	22.3	99	2
LC parameters	Column temperature	50°C	
	Fast loading/equilibration	Pressure Control	
	Pressure for loading/equilibration/wash	Max Pressure	
	Equilibration factor	2	
	Sampler temperature	7°C	

180 SPD (15 cm EASY-Spray HPLC column, P/N ES906)			
Gradient	**Time**	**%B**	**Flow (µL/min)**
	0	4	2.5
	0.2	8	2.5
	4	20	2.5
	5.8	35	2.5
	6.2	99	2.5
	6.9	99	2.5
LC parameters	Column temperature	50°C	
	Fast loading/equilibration	Pressure Control	
	Pressure for loading/equilibration/wash	Max Pressure	
	Equilibration factor	2	
	Sampler temperature	7°C	

300 SPD (5 cm IonOpticks UHPLC column)			
Gradient	**Time**	**%B**	**Flow (µL/min)**
	0	10	2
	0.05	10	2
	0.1	12	1
	2.1	25	1
	3.4	45	1
	3.5	99	1
	3.7	99	1
LC parameters	Column temperature	50°C	
	Fast loading/equilibration	Pressure Control	
	Pressure for loading/equilibration/wash	Max Pressure	
	Equilibration factor	2	
	Sampler temperature	7°C	

Table 2. DIA parameters for the 24, 60, and 180 SPD (left panel) or 300 SPD methods

24 and 60 SPD		
MS1	Resolution	240K
	Scan range (*m/z*)	380–980
	AGC	500%
	Max-IT	5 ms
MS2	Precursor mass range (*m/z*)	380-980
	Scan range (*m/z*)	145–2,000
	Isolation window (*m/z*)	2
	Window placement optimization	on
	AGC	500%
	Max-IT	5 ms
	HCD	25%
	Loop control	Time (0.6 s)
	RF lens (%)	40%

180 SPD		
MS1	Resolution	240K
	Scan range (*m/z*)	380–980
	AGC	500%
	Max-IT	5 ms
MS2	Precursor mass range (*m/z*)	380–980
	Scan range (*m/z*)	145–2,000
	Isolation window (*m/z*)	2
	Window placement optimization	on
	AGC	500%
	Max-IT	3.5 ms
	HCD	25%
	Loop control	Time (0.6 s)
	RF lens (%)	40%

300 SPD		
MS1	Resolution	240K
	Scan range (*m/z*)	580–780
	AGC	500%
	Max-IT	5 ms
MS2	Precursor mass range (*m/z*)	580–780
	Scan range (*m/z*)	145–2,000
	Isolation window (*m/z*)	2
	Window placement optimization	on
	AGC	500%
	Max-IT	3 ms
	HCD	25%
	Loop control	Time (0.6 s)
	RF lens (%)	40%

Data analysis

Data was processed by Spectronaut™ software using a directDIA™ approach (Biognosys AG, v19), DIA-NN (v1.8.1), or Thermo Scientific™ Proteome Discoverer™ (v3.1.0) software with the CHIMERYS™ intelligent search algorithm by MSAID™. Both the peptide-spectrum match (PSM) and proteins were filtered at 1% false discovery rate (FDR). The tables were imported to Python™ software for downstream data analysis and visualization (Figure 3).

Results and discussion

LFQ-DIA workflows on the Orbitrap Astral MS enable in-depth and high-throughput PROTAC compound screening

The tryptic peptides from VCaP cells treated with different concentrations of ARCC-4, a known androgen receptor (AR) degrader with high specificity, were analyzed on an Orbitrap Astral MS for label-free quantitation. Here, we developed LFQ-DIA workflows with various throughputs to meet the needs of different TPD compound screening speeds and proteome depths (Figure 3).

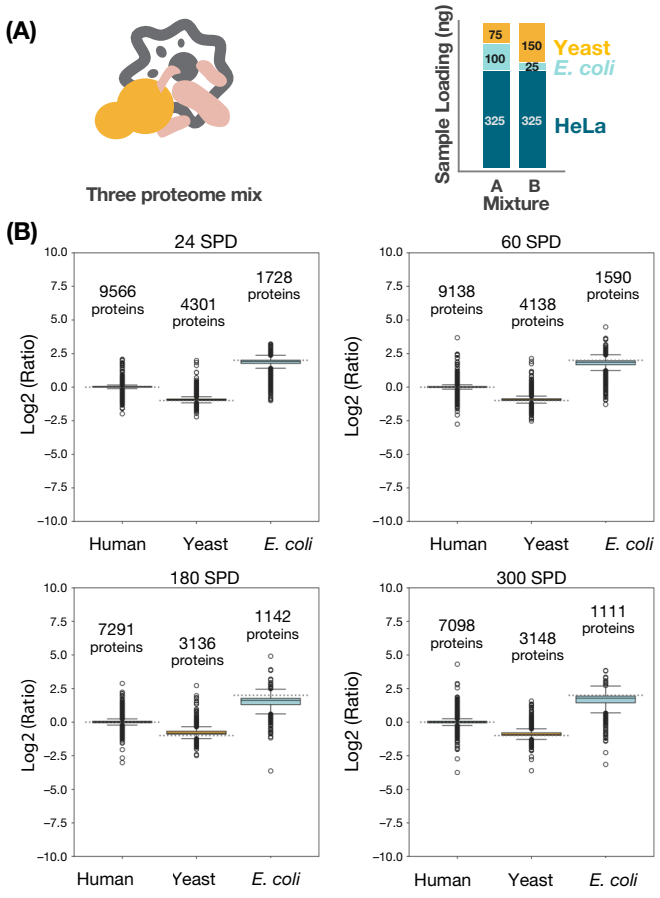


Figure 4. The Orbitrap Astral MS enables accurate and precise quantitation. (A) The three-proteome mix contains a medium human background of 325 ng HeLa peptides together with yeast and *E. coli* peptides digested in ratios of 0.5:1 and 1:4, respectively. (B) Whisker box plots of protein abundance ratios of all three species demonstrate excellent quantitative accuracy and precision by being consistent with the theoretical ratios (gray dotted line).

Ultra-fast LFQ-DIA workflow for high-throughput TPD compound screening

To explore the possibility of ultra-fast compound screening, we developed high-throughput methods of 180 and 300 SPD. Quantitation accuracy and precision were evaluated by using three proteome mixtures of human, yeast, and *E. coli* digests. These experiments demonstrated high accuracy and precision with median values extremely close to the theoretical ratios, as well as a narrow distribution of all data points around the median values (Figure 4).

We were able to identify over 8,500 proteins and 130,000 peptide groups and close to 8,000 protein and 62,000 peptide groups with the 180 SPD and 300 SPD methods, respectively, from the ARCC-4-treated VCaP cells (Figure 5). Additionally, the median coefficient of variation (CV) from biological replicates is approximately 10%, underscoring the precise quantitation achieved with the Orbitrap Astral MS even at ultra-high throughputs (Figure 6).

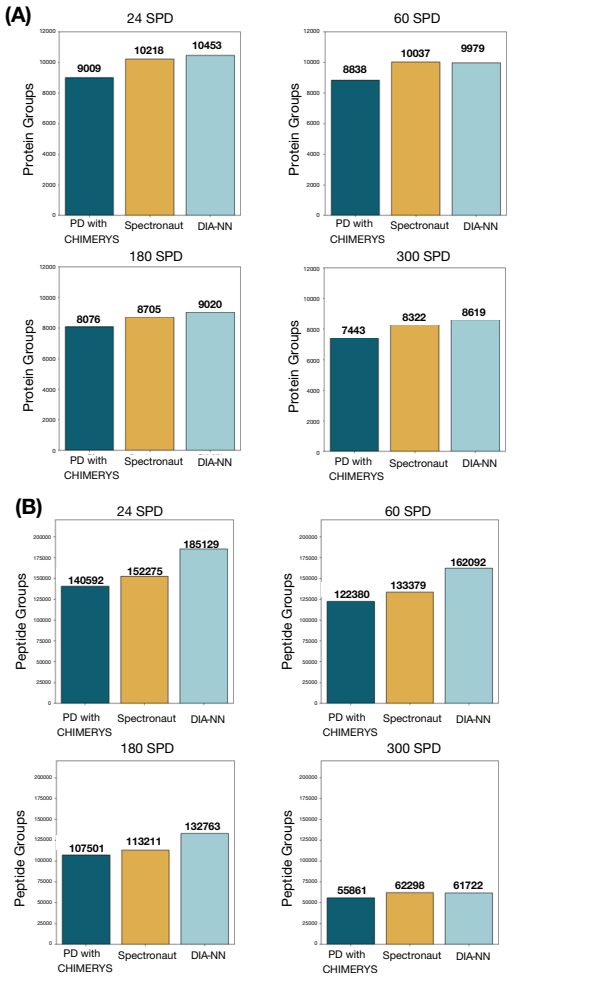


Figure 5. The Orbitrap Astral MS allows for high-throughput and in-depth LFQ-DIA analysis. Bar charts showing the number of protein groups (A) and peptide groups (B). Data was analyzed with Proteome Discoverer software using CHIMERYS, Spectronaut software, and DIA-NN.

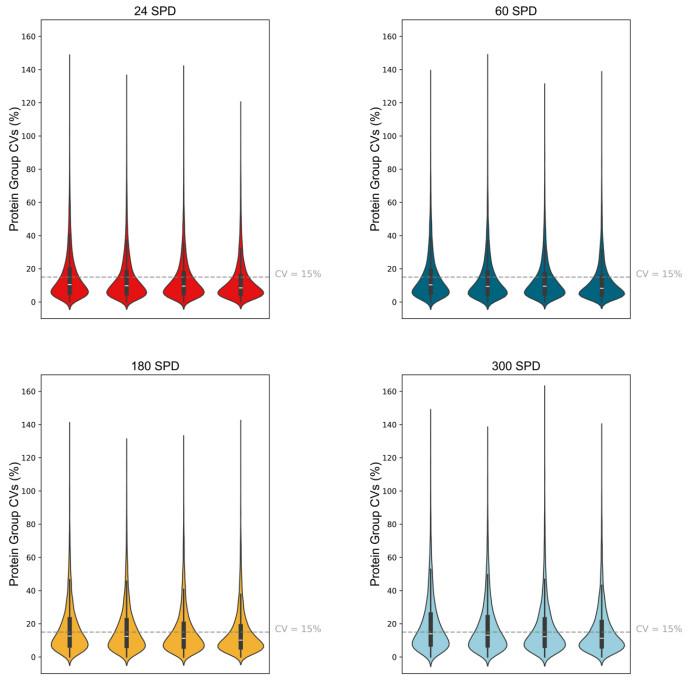


Figure 6. The Orbitrap Astral MS enables precise quantitation. Violin plots showing the coefficient of variation (CV) percentage of protein groups from 24, 60, 180, and 300 SPD.

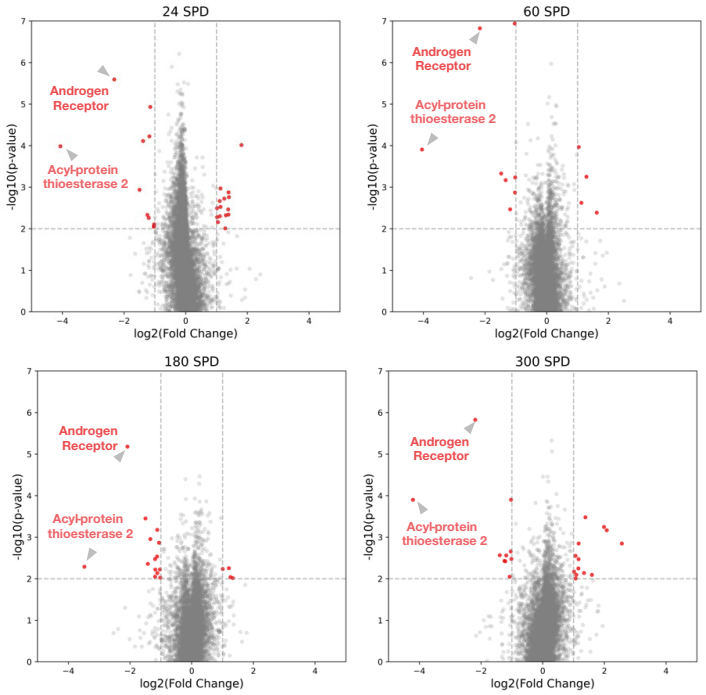


Figure 7. The androgen receptor is identified as the key protein degraded across all throughputs. Volcano plots illustrate the differential protein expression in cells treated with 500 nM ARCC-4 compared to DMSO.

More importantly, AR was identified as being significantly degraded with higher concentrations of ARCC-4 with a 5-fold decrease and a p-value less than 1.4 $\times 10^{-6}$ using both the 180 and 300 SPD ultra-high throughput methods (Figure 7). The results suggest that the advancement of MS instrumentation allows for an unprecedented speed of TPD compound screening while achieving good proteome coverage and reliable quantitation for accurate lead identification.

LFQ-DIA with extended gradients enables in-depth TPD compound validation

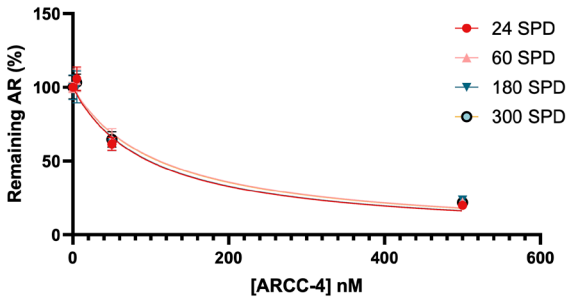
To maximize proteome coverage, which is crucial for the validation phase, we extended this workflow to 24 and 60 SPD methods. Our results demonstrate that the Orbitrap Astral MS provides accurate and precise protein quantitation with deeper proteome coverage when using extended active gradients, as evidenced by three proteome mixture experiments (Figure 4).

We successfully identified, from the ARCC-4-treated VCaP cells, approximately 119,800 and 142,000 peptide groups, which correspond to 10,000 and 10,400 protein groups, using the 60 SPD and 24 SPD methods, respectively (Figure 5). In addition, highly precise quantitation was consistently achieved (Figure 6). Our results suggest near-complete proteome identification within the samples. Moreover, the AR protein was observed to degrade with increasing ARCC-4 concentration, demonstrating that our LFQ-DIA workflow on the Orbitrap Astral MS provides a comprehensive on-target/off-target validation method across nearly the entire proteome.

We identified 14 and 36 AR peptides from the 300 and 180 SPD setups, respectively, highlighting the sensitivity of the Orbitrap Astral MS even in high-throughput analysis configurations. An extended gradient of 60 and 24 SPD allowed for the identification of 41 AR peptides, 13 of which were commonly found across all throughput levels. The increased number of AR peptides suggests improved confidence in quantitation with a longer gradient, which can benefit the validation phase of TPD compound discovery.

The Orbitrap Astral MS unveils true biological insights in targeted protein degradation discover

In the volcano plot analysis, we identified the androgen receptor as the key protein degraded by ARCC-4 (Figure 7). Furthermore, all throughput levels tested in this study revealed a consistent androgen receptor degradation rate (Figure 8). This consistency underscores the capability of the Orbitrap Astral MS to be effectively utilized across various phases, from high-throughput screening to downstream in-depth validation.



SPD	DC50	R^2
24	97.3 nM	0.96
60	111.8 nM	0.96
180	98.4 nM	0.94
300	110.4 nM	0.96

Figure 8. The degradation rate of the androgen receptor across different throughputs on Orbitrap Astral MS. (A) One-phase decay curve fitting of androgen receptor degradation for 24, 60, 180, and 300 SPD. (B) Table displaying consistent half-maximal degradation concentration (DC50) values and R-squared values for curve fitting.

Conclusions

- The ultra-high throughput 180 and 300 SPD methods on the Orbitrap Astral MS enable the identification of over 8,500 protein groups, along with accurate and precise quantitation.
- Extended gradients of 24 and 60 SPD on the Orbitrap Astral MS enable in-depth coverage of over 10,000 protein groups coupled with excellent quantitative accuracy and precision.
- The Orbitrap Astral MS enables high-throughput compound screening and in-depth validation for targeted protein degradation.

References

1. Schapira M. et al., Nat Rev. Drug Discov,. 2019, 18, 949-963
2. Chamberlain P.P., Hamann L.G., Nat. Chem. Biol., 2019, 15, 937-944
3. Lv L. et al., eLife, 2020, 9, e59994
4. Li K., Crews C.M., Chem. Soc. Rev., 2022, 51, 5214-5236
5. Mostofian B. et al., J. Chem. Inf. Model., 2023, 63, 17, 5408-5432
6. Salami J. et al., Commun. Biol., 2018, 1, 100

Learn more at **thermofisher.com/orbitrapastral**