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High-throughput PROTAC compound screening workflow for targeted protein degradation on an Orbitrap Astral mass spectrometer with accurate label-free quantitation

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Abstract

Purpose: To develop robust mass spectrometry-based proteomics workflows on the Orbitrap Astral mass spectrometer for ultra high-throughput and in-depth compound screening with precise and highly accurate quantitation, thereby supporting drug discovery for targeted protein degradation (TPD).

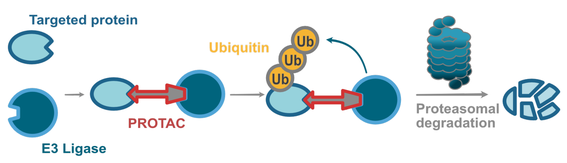
Methods: VCaP prostate cancer epithelial cells were treated with different concentrations of ARCC-4, a proteolysis targeting chimera (PROTAC) protein degrader for androgen receptor. The resulting peptides were loaded onto a 50-cm Thermo Scientific™ EASY-Spray™ PepMap™ Neo column, a Thermo Scientific™ 15-cm EASY-Spray™ PepMap™ or a 5-cm Ionopticks Aurora UHPLC column and separated with nano or capillary flowrate in a trap and elute mode using a Thermo Scientific™ Vanquish™ Neo UHPLC system for a throughput of 24, 60, 180 and 300 samples per day (SPD).

Results: Our data indicate consistent dose-dependent protein degradation of the androgen receptor by ARCC-4, while extended gradients enhance proteome coverage. The results suggest that the Orbitrap Astral mass spectrometer facilitates ultra-high-throughput and in-depth validation of targeted protein degradation compounds such as PROTACs with exceptional sensitivity, reproducibility, and quantitation accuracy, making it an invaluable tool for drug discovery.

Introduction

Targeted protein degradation (TPD) is an emerging and transformative strategy in drug discovery that utilizes cellular protein degradation processes to selectively eliminate deleterious proteins. Through enabling induced proximity to the ubiquitination-proteasome system, the small molecules facilitate the degradation of the disease-causing proteins, opening the possibilities to target the previously undruggable proteins for a wide range of diseases with unprecedented precision and efficacy (**Figure 1**). Directly designing compounds that can promote the induced proximity with selective is challenging in practice thereby compound screening with reliable quantitation accuracy is a crucial phase in drug discovery in the TPD space. The need for screening large numbers of compounds with accurate quantitation makes high throughput mass spectrometry-based workflows an obvious choice for ensuring accurate lead identification.

Figure 1. Schematic diagram of targeted protein degradation.



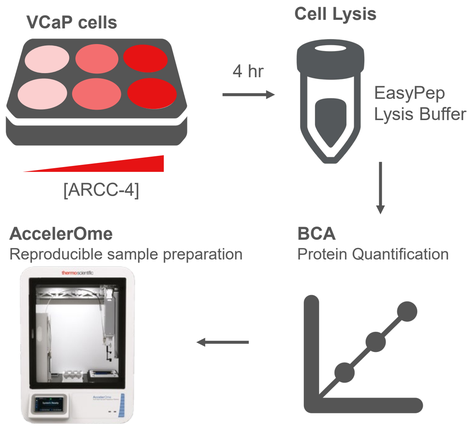
Materials and methods

Sample preparation

VCaP cells were cultured in 6 well plates until ~90% confluent. The resulting cells were treated in triplicate with 5, 50 and 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 BCA assay to measure protein concentration and 15 µg of the proteins were reduced, alkylated, and digested to peptides by using the Thermo Scientific™ AccelerOme™ automated sample preparation platform with Thermo Scientific™ AccelerOme Label-Free MS Sample Prep Kit (Catalog No. A50945) (**Figure 2**).

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



LC-MS/MS Methods

The resulting peptides were loaded in trap and elute mode onto a 5-cm Ionopticks Aurora UHPLC column for 300 SPD, a 15-cm EASY-Spray PepMap column for 60 and 100 SPD, and a 50-cm EASY-Spray PepMap Neo column for 24 SPD using a Vanquish Neo UHPLC system (**Figure 3A-B**).

The eluted peptides were analyzed on a Thermo Scientific™ Orbitrap Astral™ Mass Spectrometer using narrow window DIA analysis (**Figure 3C**).

Data analysis

Data was processed by Spectronaut™ software (Biognosys, v19) using a directDIA approach, DIA-NN (v1.8.1) or Thermo Scientific™ Proteome Discoverer™ software with CHIMERYS™ intelligent search algorithm by MSAID. The tables were imported to Python for downstream data analysis and visualization (**Figure 3A**).

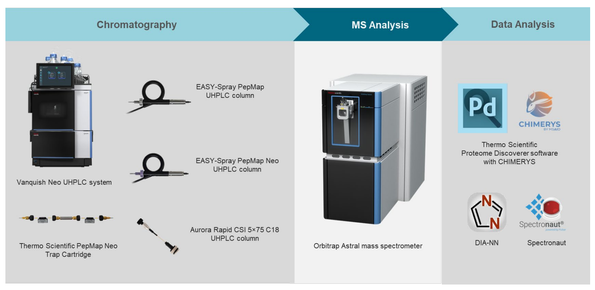
Results

LFQ-DIA workflow on Orbitrap Astral mass spectrometer allows for 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 mass spectrometer for label free quantitation. Here, we developed an LFQ-DIA workflow with various throughputs to meet the needs of different TPD compound screening speeds (**Figure 3**).

Figure 3. LFQ-DIA analysis workflow on Orbitrap Astral mass spectrometer.

(A)



(B)

24 SPD

No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000	Run				
2	0.000	0.000	0.500	8.0	0.00	0.00
3	2.500	2.500	0.500	8.0	1.25	0.84
4	3.000	0.500	0.250	8.0	0.19	0.13
5	37.000	34.000	0.250	28.0	8.50	5.74
6	48.500	11.500	0.250	35.0	2.88	1.94
7	48.900	0.400	0.250	98.0	0.10	0.07
8	48.900	Column Wash				
9	49.000	0.100	0.350	98.0	0.03	0.02
10	54.000	5.000	0.350	98.0	1.75	1.18
11	54.000	Stop Run				
12	54.000	Column Equilibration				

60 SPD

No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000	Run				
2	0.000	0.000	2.000	10.0	0.00	0.00
3	0.300	0.300	2.000	10.0	0.60	0.34
4	0.600	0.300	0.800	10.0	0.42	0.24
5	13.600	13.000	0.800	22.5	10.40	5.86
6	20.500	6.900	0.800	35.0	5.52	3.11
7	20.900	0.400	2.000	55.0	0.56	0.32
8	20.900	Column Wash				
9	21.400	0.500	2.000	99.0	1.00	0.56
10	22.300	0.900	2.000	99.0	1.80	1.01
11	22.300	Stop Run				
12	22.300	Column Equilibration				

180 SPD

No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000	Run				
2	0.000	0.000	2.500	4.0	0.00	0.00
3	0.200	0.200	2.500	8.0	0.50	0.28
4	4.000	3.800	2.500	20.0	9.50	5.35
5	5.800	1.800	2.500	35.0	4.50	2.53
6	5.800	Column Wash				
7	6.200	0.400	2.500	99.0	1.00	0.56
8	6.900	0.700	2.500	99.0	1.75	0.99
9	6.900	Stop Run				
10	6.900	Column Equilibration				

300 SPD

No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000	Run				
2	0.000	0.000	2.000	10.0	0.00	0.00
3	0.050	0.050	2.000	10.0	0.10	0.68
4	0.100	0.050	1.000	12.0	0.08	0.51
5	2.100	2.000	1.000	25.0	2.00	13.51
6	3.400	1.300	1.000	45.0	1.30	8.78
7	3.400	Column Wash				
8	3.500	0.100	1.000	99.0	0.10	0.68
9	3.700	0.200	1.000	99.0	0.20	1.35
10	3.700	Stop Run				
11	3.700	Column Equilibration				

(C)

24-180 SPD

Precursor Mass Range (m/z)	380-980
Isolation Window (m/z)	3
Window Overlap (m/z)	0
Number Of Scan Events	199
Collision Energy Type	Normalized
HCD Collision Energy (%)	25
Detector Type	Astral
TMT	Off
Scan Range (m/z)	150-2000
RF Lens (%)	40
Polarity	Positive
Loop Control	Time
Time (sec)	0.6

300 SPD

Precursor Mass Range (m/z)	580-780
Isolation Window (m/z)	3
Window Overlap (m/z)	0
Number Of Scan Events	66
Collision Energy Type	Normalized
HCD Collision Energy (%)	25
Detector Type	Astral
TMT	Off
Scan Range (m/z)	180-2000
RF Lens (%)	40
Polarity	Positive
Loop Control	All

(A) High-throughput and maximized coverage LFQ-DIA workflow on Orbitrap Astral mass spectrometer for compound screening in targeted protein degradation pipeline. (B) Separation column gradient for 24, 60, 180 and 300 SPD workflow. (C) DIA parameters for 24, 60 and 180 SPD (left panel) or 300 SPD (right panel).

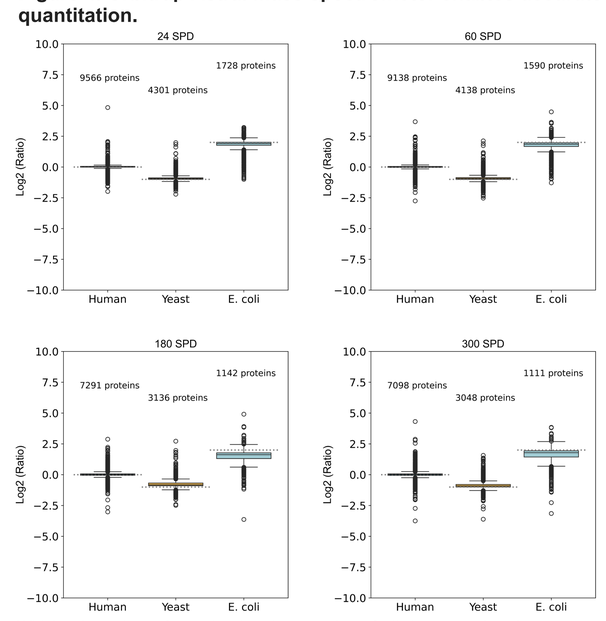
Ultra fast LFQ-DIA workflow for high-throughput TPD compound screening.

To explore the possibility of ultra-fast compound screening, we developed high-throughput methods, including 180 and 300 SPD. Quantitation accuracy was evaluated through three proteome mixtures of human, yeast, and E. coli digests. The accuracy across a wide dynamic range with median values extremely close to the theoretical ratios, as well as a narrow distribution of all data points around the median values, indicating high quantitative accuracy and precision of the workflow (**Figure 4**).

We were able to identify over 8500 protein/130,000 peptide groups and close to 8000 protein/62000 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 mass spectrometer (**Figure 6**).

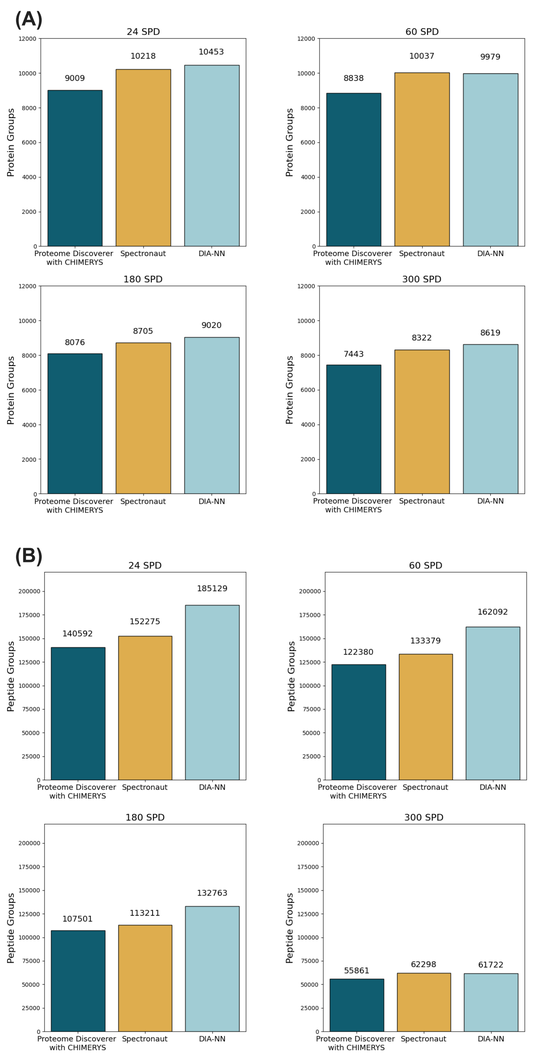
More importantly, AR was the major protein being degraded at the higher concentration of ARCC-4 in both the ultra-high-throughput methods. The results suggest that the advancement of mass spectrometer instrumentation allows for an unprecedented speed of TPD compound screening while achieving good proteome coverage and reliable quantitation for accurate lead identification.

Figure 4. Orbitrap Astral mass spectrometer enables accurate quantitation.



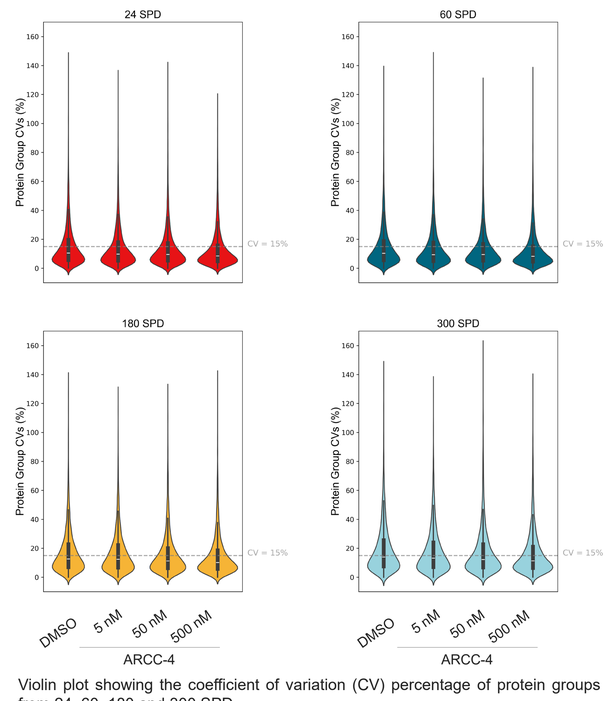
Whisker box plots of protein abundance ratios of all three species demonstrate accurate quantitation by being consistent with theoretical ratios (gray dotted line) for all throughput levels evaluated.

Figure 5. Orbitrap Astral mass spectrometer allows for high-throughput and in-depth LFD-DIA analysis.



Bar charts showing the number of protein groups (A) and peptide groups (B). Data was analyzed with CHIMERYS on Proteome Discoverer, Spectronaut or DIA-NN.

Figure 6. Orbitrap Astral mass spectrometer enables precise quantitation.



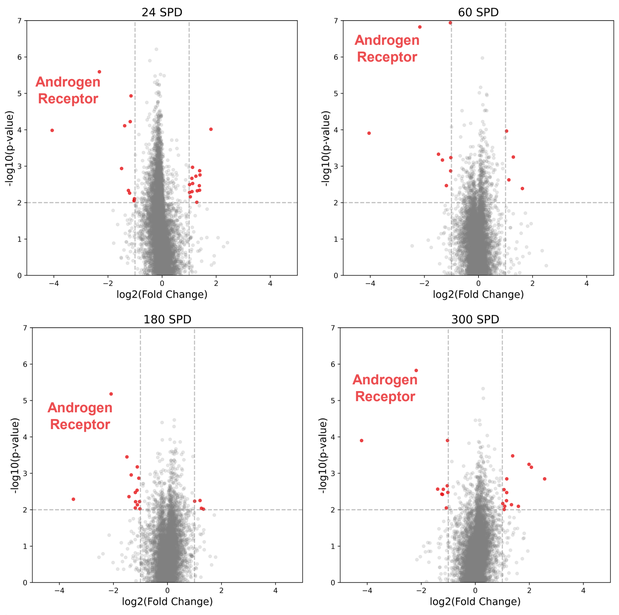
Violin plot showing the coefficient of variation (CV) percentage of protein groups from 24, 60, 180 and 300 SPD.

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. Our results demonstrate that the Orbitrap Astral mass spectrometer provides accurate protein quantitation with extended active gradients, as evidenced by three proteome mixture experiments (**Figure 4**).

We successfully identified, from the ARCC-4-treated VCaP cells, approximately 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 cell. Moreover, the AR protein was observed to degrade with increasing ARCC-4 concentration, demonstrating that our LFQ-DIA workflow on the Orbitrap Astral mass spectrometer provides a comprehensive on-target/off-target validation method.

Figure 7. Androgen receptor identified as the key protein degraded across all throughputs.



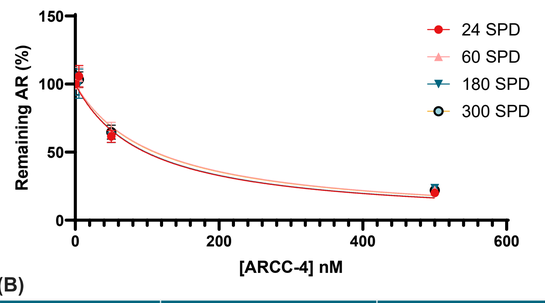
Volcano plots illustrating the differential protein expression in cells treated with 500 nM ARCC-4 compared to DMSO.

Orbitrap Astral mass spectrometer 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. Furthermore, all throughput levels tested in this study revealed a consistent androgen receptor degradation rate. This consistency underscores the capability of the Orbitrap Astral mass spectrometer to be effectively utilized across various phases, from high-throughput screening to downstream in-depth validation.

Figure 6. Degradation rate of androgen receptor across different throughputs on Orbitrap Astral mass spectrometer.

(A)



(B)

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

(A) One-phase decay curve fitting of androgen receptor 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 enable the identification of over 8,500 protein groups, along with accurate quantitation.
- Extended gradients of 24 and 60 SPD on the Orbitrap Astral enable in-depth coverage of over 10,000 protein groups coupled with excellent quantitation accuracy.
- The Orbitrap Astral mass spectrometer enables high-throughput compound screening and in-depth validation for targeted protein degradation.

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