Applications of Mass Spectrometry in Biopharmaceutical Discovery: From Small Molecules to Proteins

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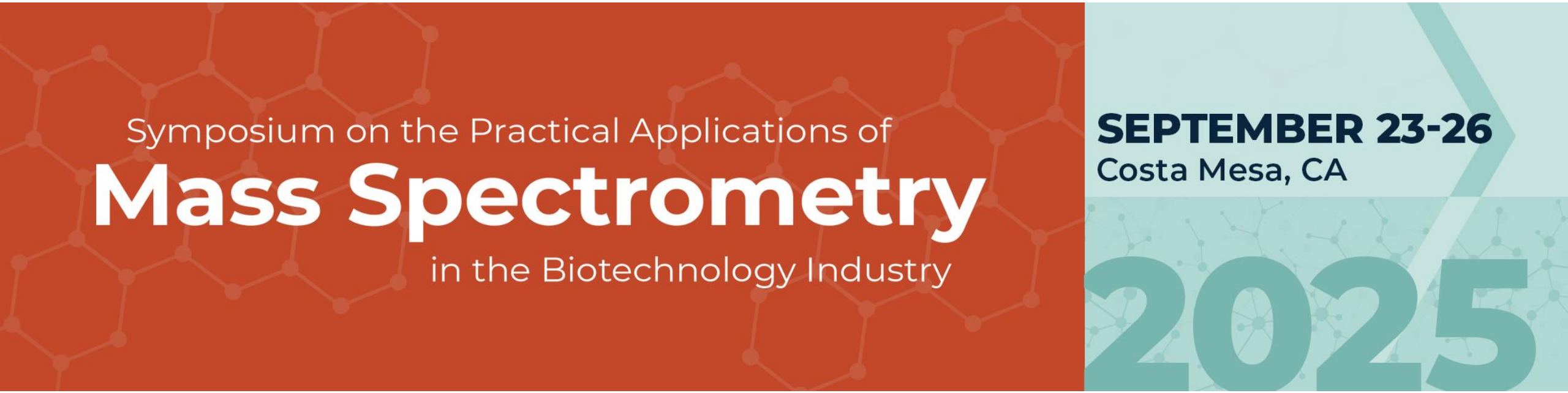
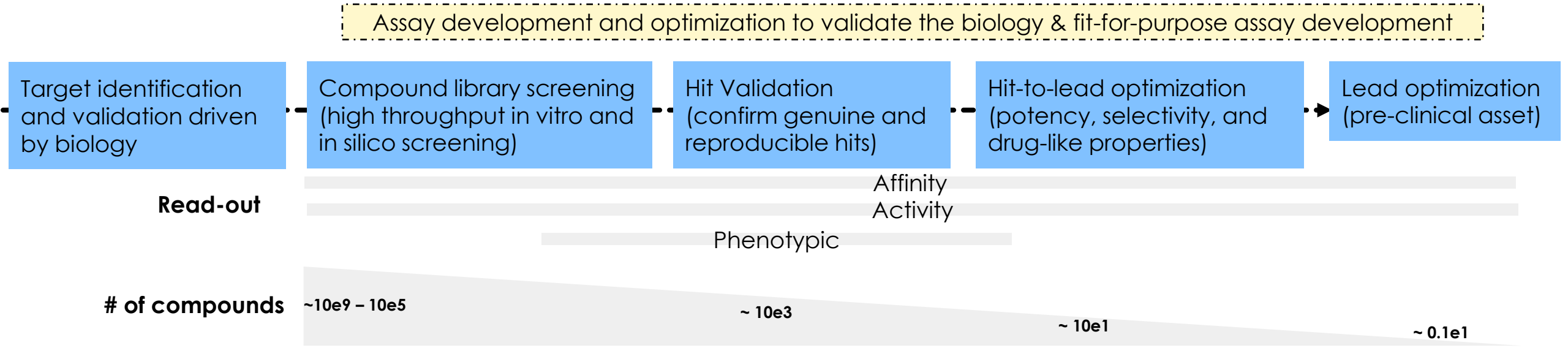
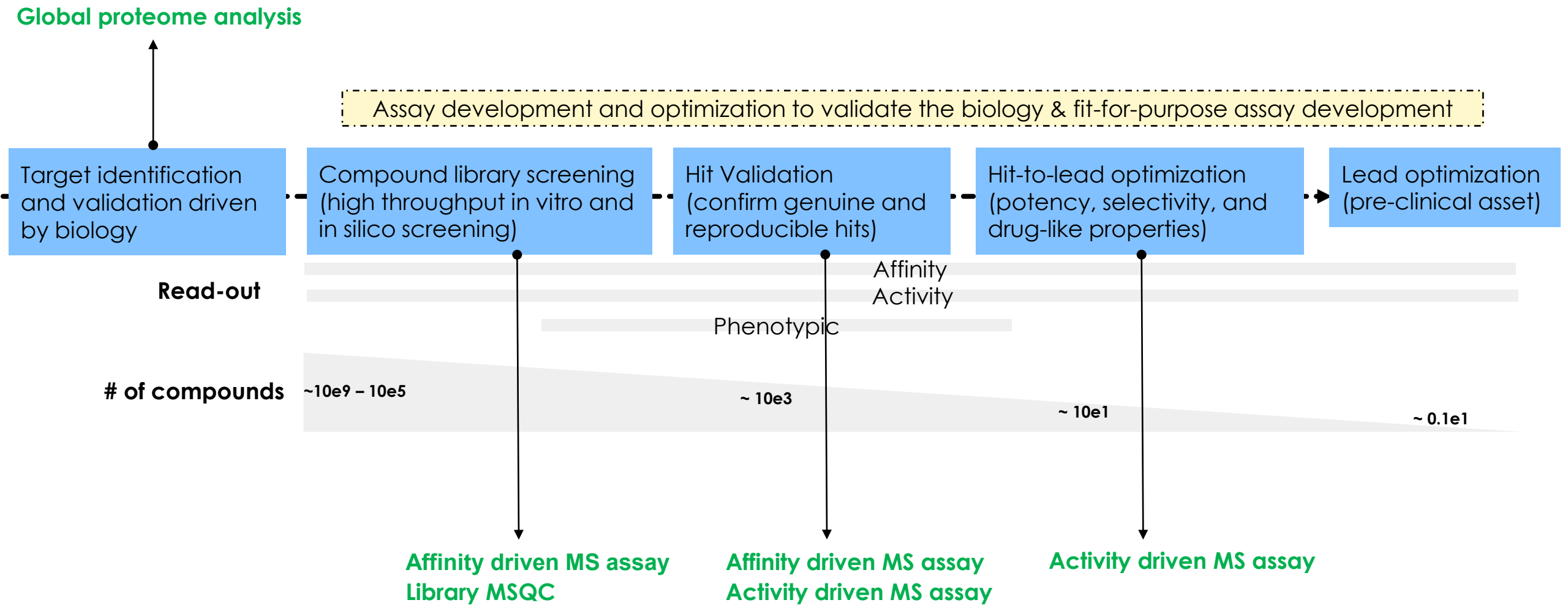


Small molecule discovery funnel



Small molecule discovery funnel

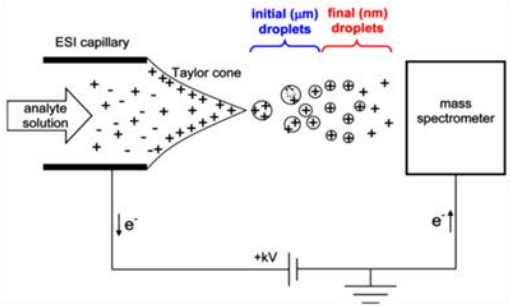


How do we achieve screening large sample space by MS?

High Throughput Mass Spectrometry

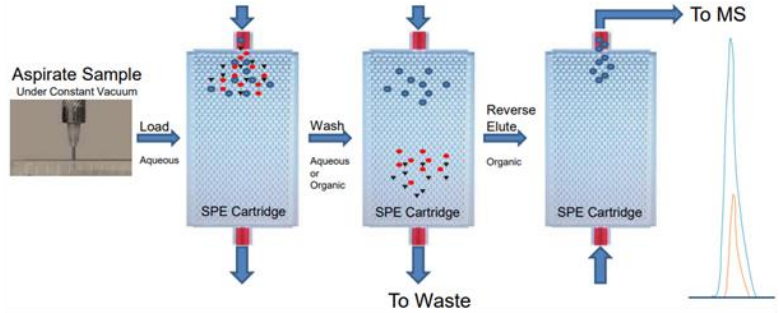
Rapidfire 360 – QTOF 6530

Electrospray ionization (ESI)



- Ionizes small molecules and large molecules
- Relatively high background
- High solvent consumption

Solid-phase extraction (SPE)



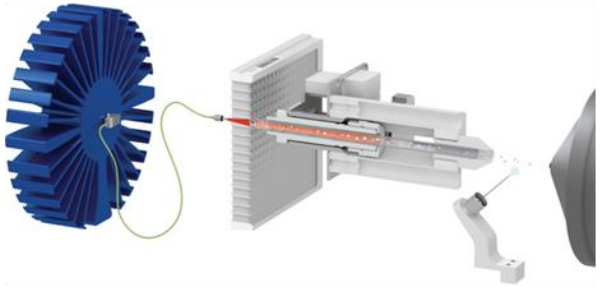
- 8-15 sec/sample; 35-50 µL/sample; ~ 60-80 min/384-well plate
- Multiple SPE cartridges (C4, C8, C18, Cyano, HILIC, etc.)
- Supports 96- and 384-well plate formats

LDTD connected to 5600+ Triple TOF

LDTD; Laser diode thermal desorption

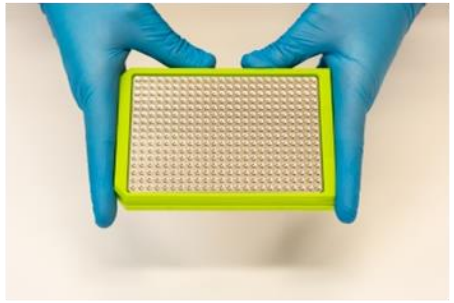
Atmospheric Pressure Chemical ionization (APCI)

LDTD source



- Ionizes small molecules
- Relatively less background
- Low or no solvent consumption

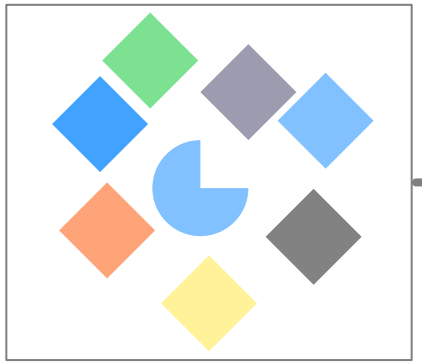
Offline sample dilution in % organic and stamp on DEC plate



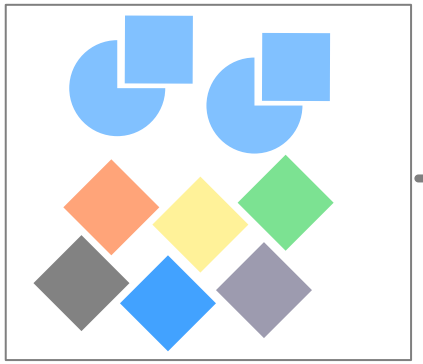
- 3-10 sec/sample; 2 -5 µL/sample; ~ 40-60 min/384-well plate
- Require offline sample dilution in % organic (e.g., 75% MeOH)
- Supports 384-well plate format

Case studies of MS in early drug discovery

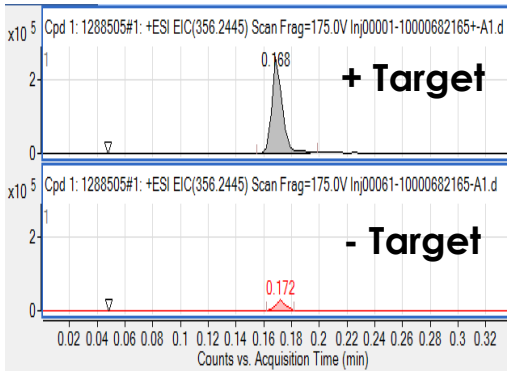
Affinity: Affinity selection Mass spectrometry



Incubate: Target + small molecule library

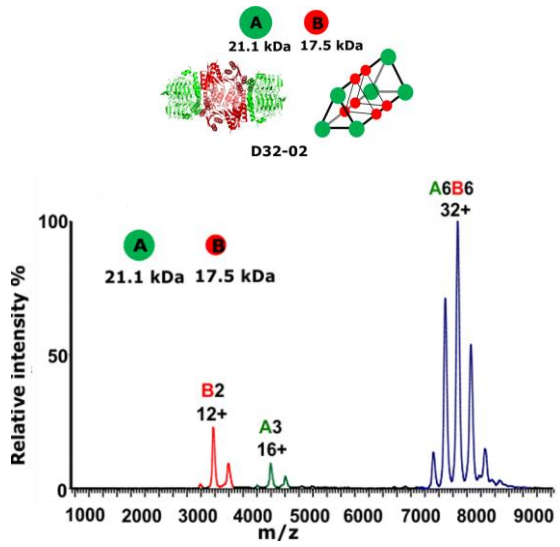


Enrich and separate target-bound small molecules

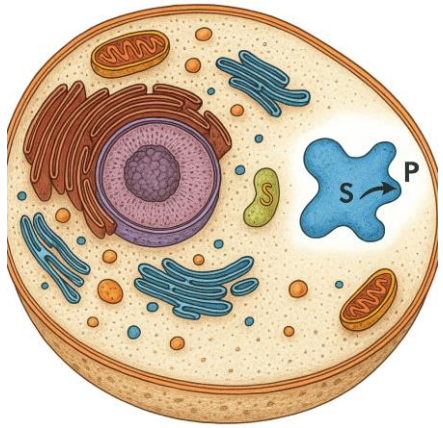


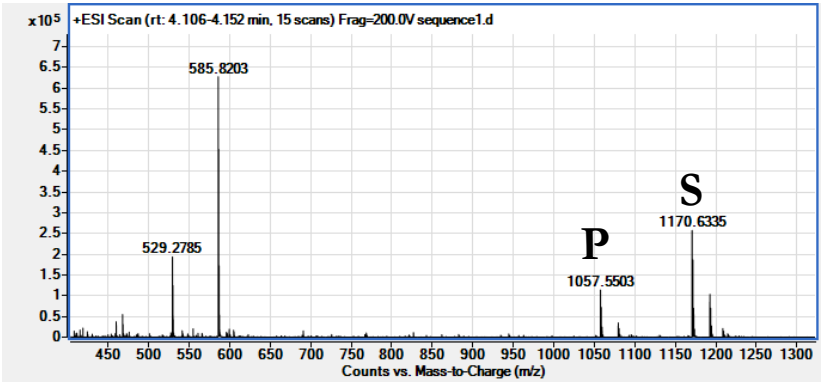
Denature and detect

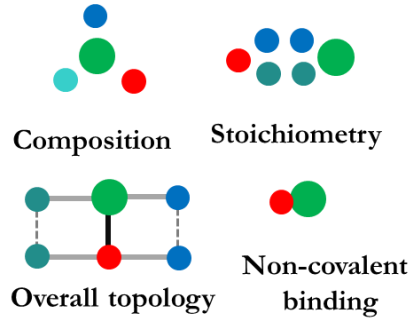
Native MS



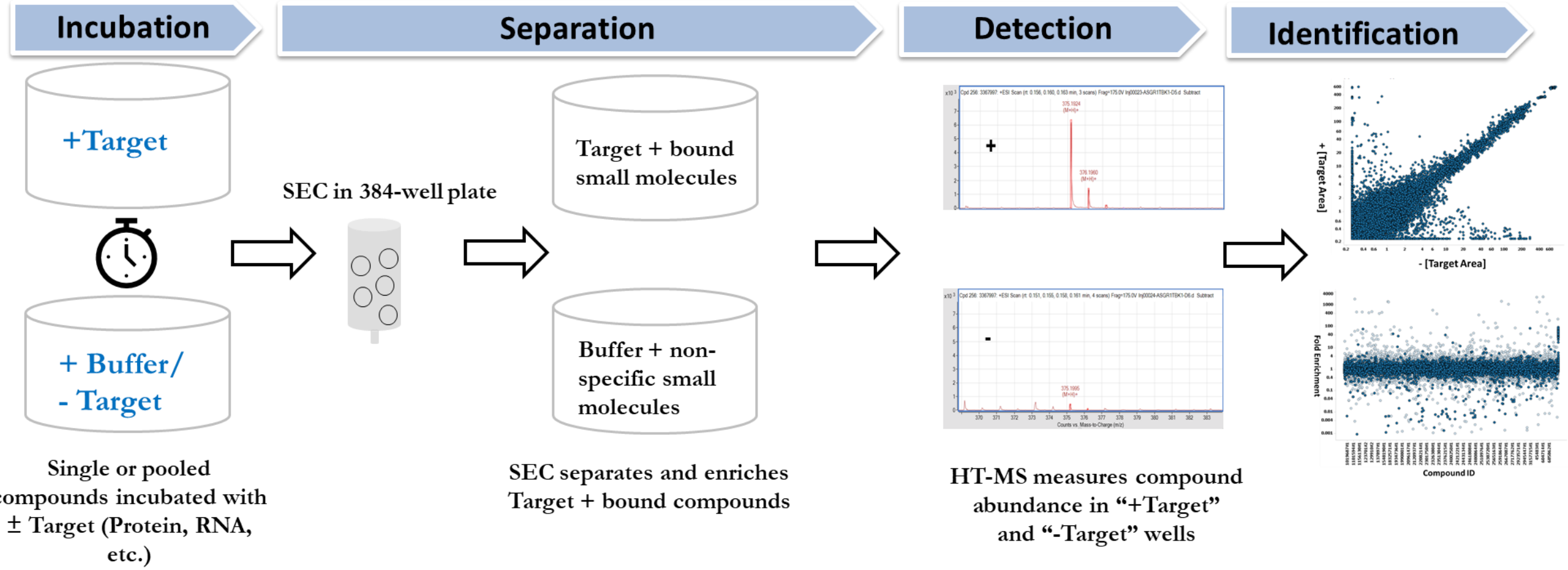
Activity: Cellular enzymatic MS assay







Affinity Selection Mass Spectrometry (ASMS) Outline



Single or pooled compounds incubated with ± Target (Protein, RNA, etc.)

SEC separates and enriches Target + bound compounds

HT-MS measures compound abundance in "+Target" and "-Target" wells

Parameters to consider for successful implementation of ASMS

Molecular library and appropriate controls

384-well format

212-pooled compounds per well at 47.2 µM

Controls
Pooled compounds

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

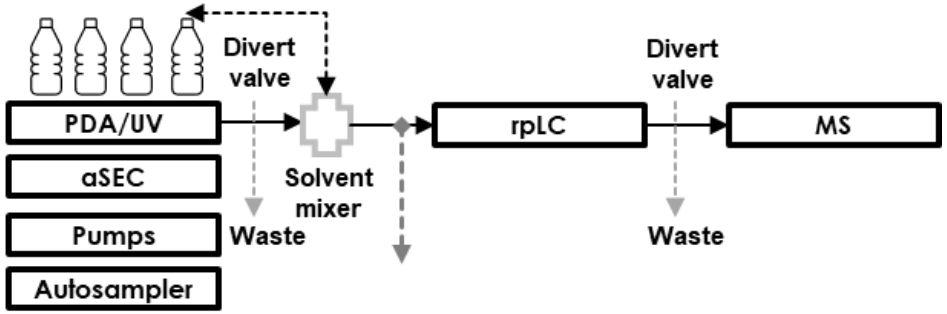
x2 pooled compound plates per assay

1. **+ Target (or + Protein)**
2. **- Target (or + buffer)**

Target-binder enrichment

- **Multi-well gel filtration plate to separate target-bound compound(s) from unbound free compounds.**

- **aSEC followed by RPLC-MS**



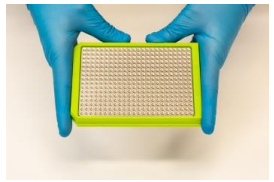
Parameters to consider for successful implementation of ASMS

Method optimization

Sample preparation

Optimized parameters:

- Sample diluent (75% MeOH)
- Sample loading or spotting
- Sample transfer automation (Bravo)

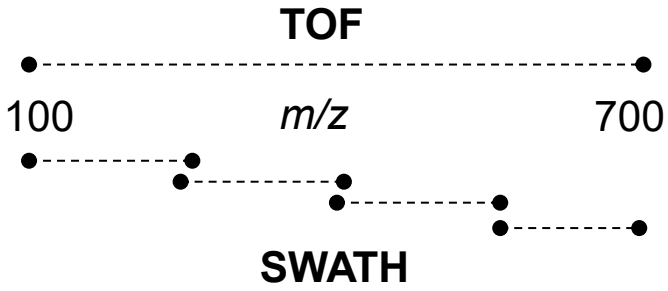


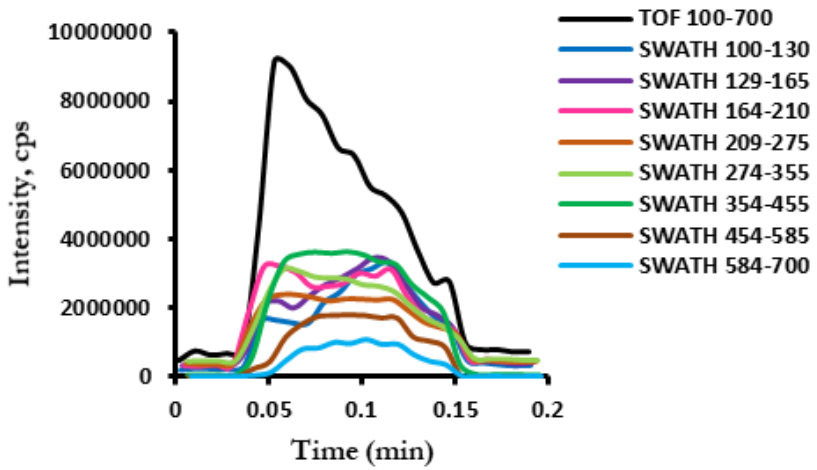
Ionization

Optimized parameters:

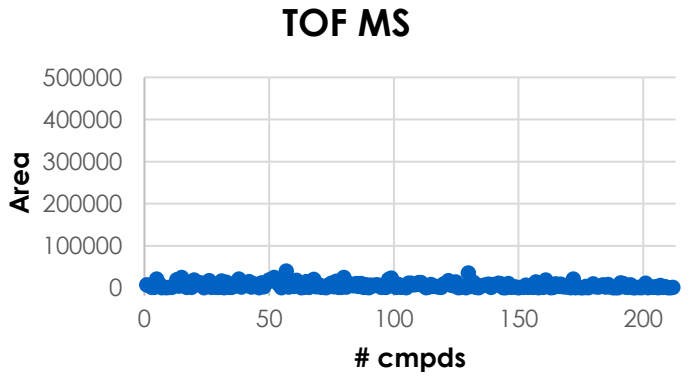
- Laser power
- Addition of start and end delay (0.5 sec)
- Developed 5 sec and 11 sec methods

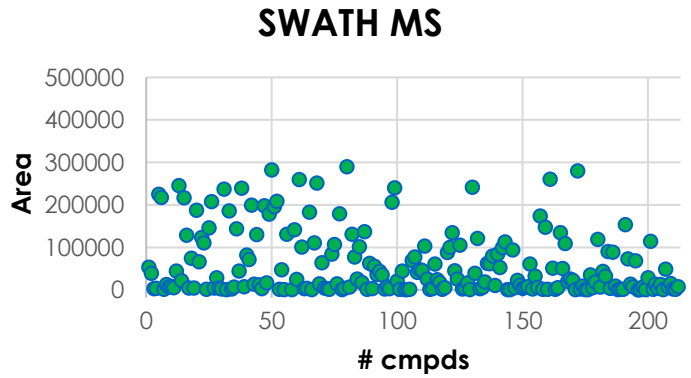
Detection





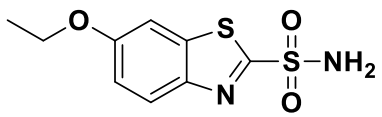
Signal enhancement and improved sensitivity





Compound screening against a protein target carbonic anhydrase II

Example of compound enrichment w/ CA II

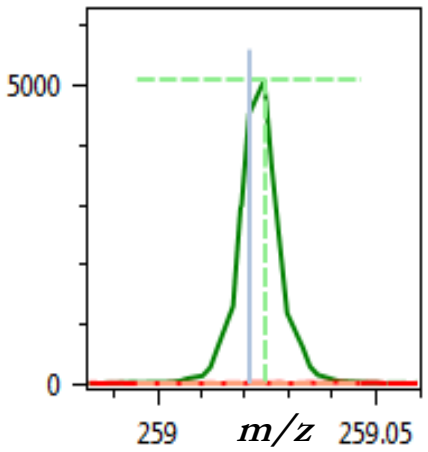


6-ethoxy-1,3-benzothiazole-2-sulfonamide

Positive Control

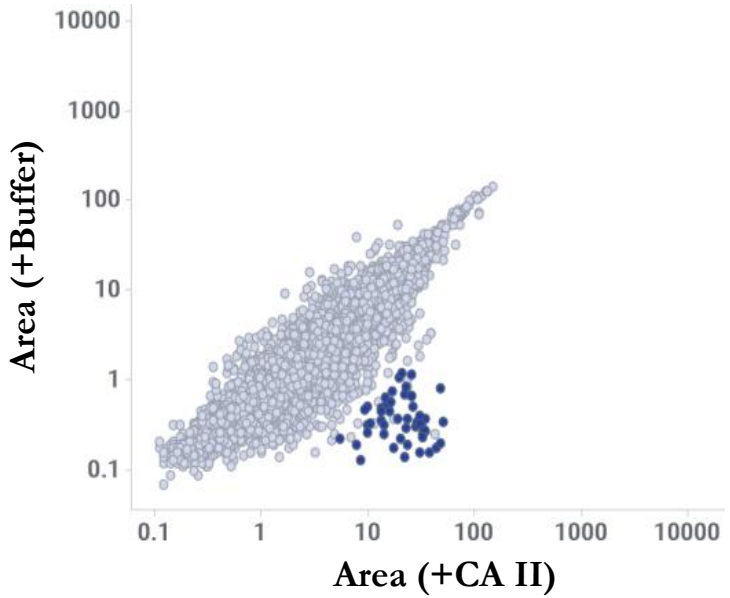
+Target

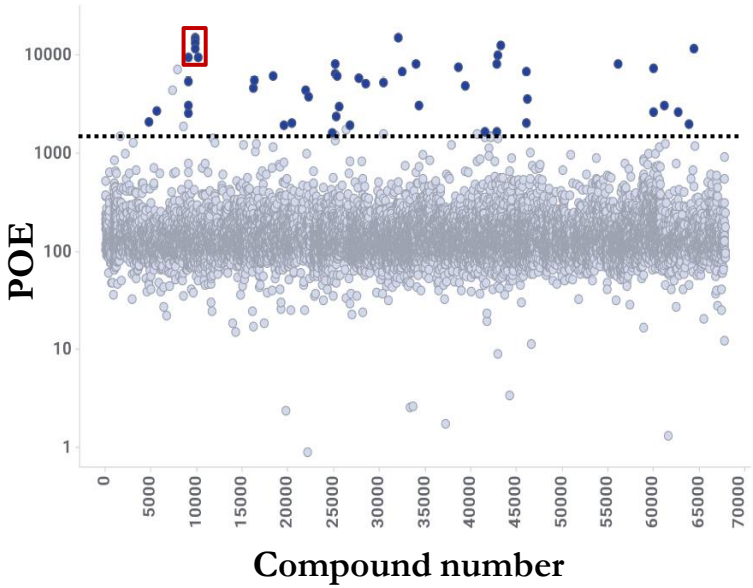
-Target



60k+ compound library screening result

- ❖**Total compounds screened: 67,848**
- ❖**Total hits: 59**
- ❖**% hit: 0.07**
- ❖**Compound selection filter** (dotted line)
  - ➢ Mass accuracy (+CA II): +/- 20 ppm
  - ➢ POE: Mean+3xSD, 1594

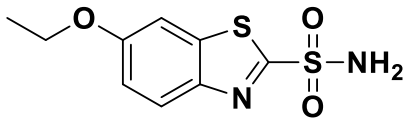




POE: Percent of Enrichment; SD: Standard deviation

Summary

57/59 hits show structural similarity w/ positive control and 97% of the hits contain benzyl-sulfonamide as a common structural feature

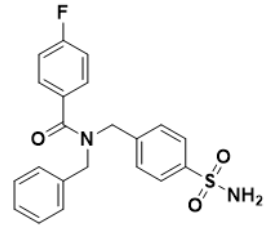


6-ethoxy-1,3-benzothiazole-2-sulfonamide

Substructures of positive control	Compounds	Total hit compounds	% Total hit compounds
Benzothiazoles	2	59	3
Alkyl aryl ethers	13	59	22
Organosulfonamides	**57**	**59**	**97**
Benzenoids	5	59	8
Thiazoles	2	59	3
Azacyclic compounds	31	59	53

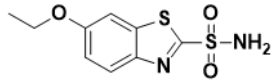
Example of top 4 hits re-confirmed in orthogonal enzymatic assay

1



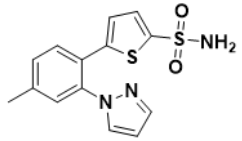
N-benzyl-4-fluoro-N-(4-sulfamoylbenzyl)benzamide

2



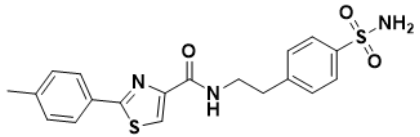
6-ethoxy-1,3-benzothiazole-2-sulfonamide

3

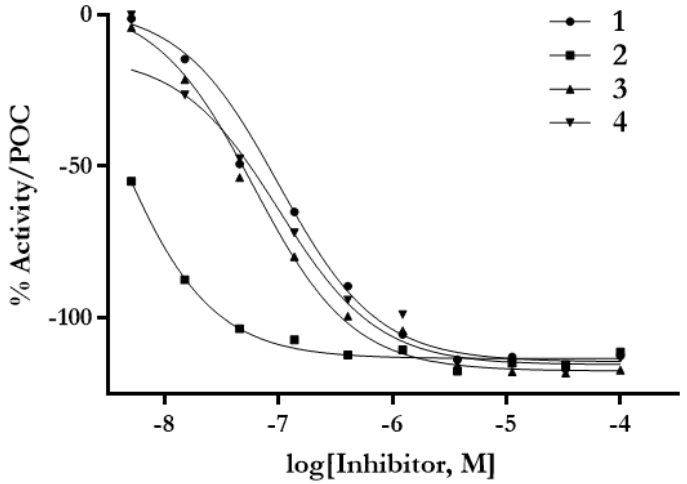


5-(4-methyl-2-(1H-pyrazol-1-yl)phenyl)-2-thiophenesulfonamide

4



2-(4-methylphenyl)-N-(2-(4-sulfamoylphenyl)ethyl)-1,3-thiazole-4-carboxamide

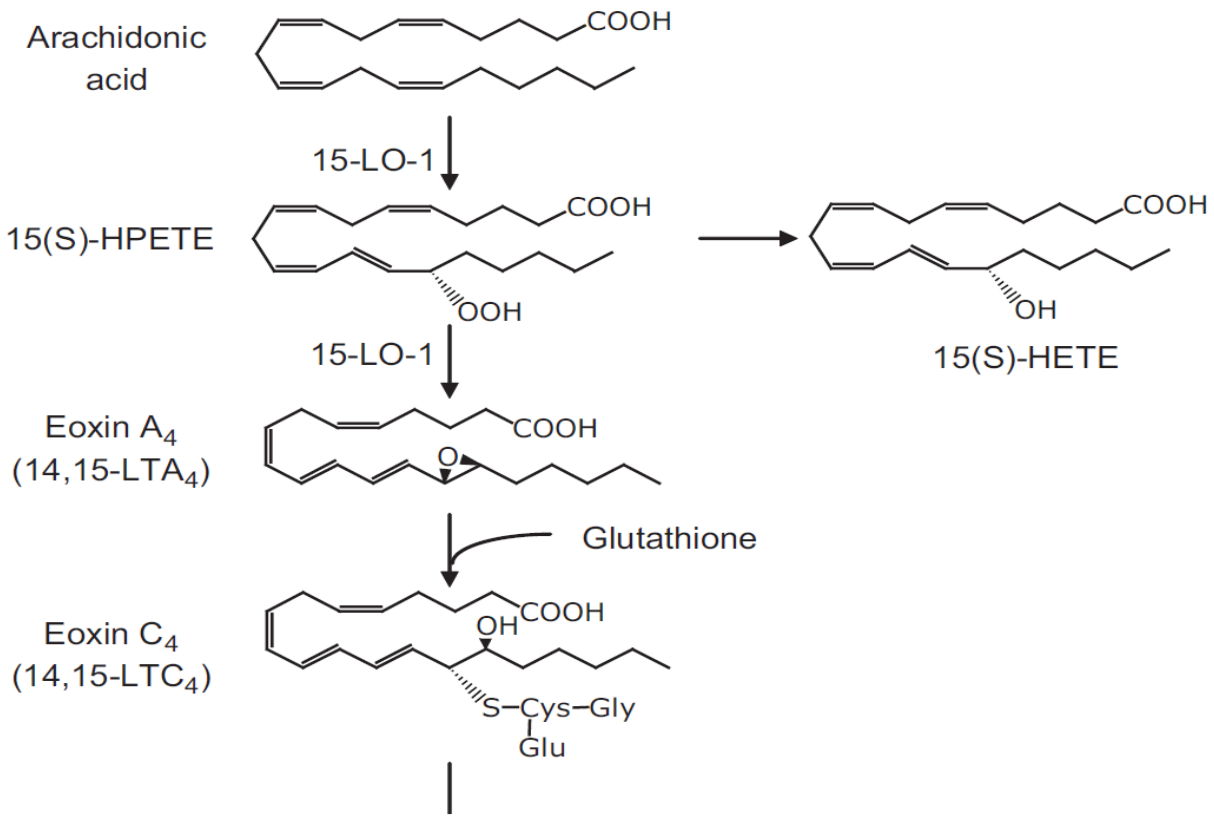


Inhibitor #	TOF POE	SWATH POE	IC50 (M)
1	2159.56	5489.20	100.50×10^{-9}
2	4256.55	13682.44	3.09×10^{-9}
3	1488.64	8398.79	60.55×10^{-9}
4	862.02	8613.24	100.70×10^{-9}

Sahasrabuddhe et al. SLAS Discovery 2021

Cellular target activity assessment by mass spectrometry (where probe-based methods perform poorly)

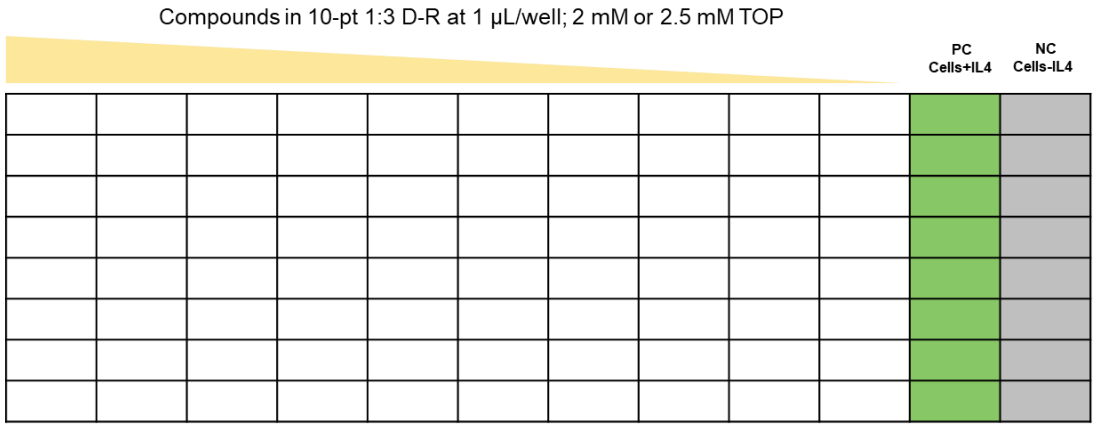
Goal: Cellular enzyme inhibition assay by MS to verify and expand the hit compounds
Target: 15-LO-1
Therapeutic area: Inflammation



- hPBMCs expresses hALOX15 upon +IL4 treatment
- Cellular hALOX15 metabolizes cellular Arachidonic acid to 15(S)-HETE.
- Extract and enrich cellular metabolites.
- LC-MS method selects 319.23 ion (15-HETE) and 327.28 (15-HETE-d8) and tracks MS/MS transitions (or detects signature fragment ion)
- Extract 15-HETE/15-HETE-d8 ratio as a measure of relative intensity of 15-HETE, thereby measuring cellular enzymatic activity.

AMGEN

Cellular enzymatic assay by MS



Day 0

hPBMC in RPMI+10%HI FBS/well; 96-well → Optimize cell density

Add 1uL 200X compound (12.5 uM Top; 1:3) in DMSO (0.5% final) and pre-incubate for 20 min

Add IL-4 (8 ng/mL) and incubate for 48hr → Optimize IL-4 induction and incubation time

Day 2

Centrifuge, remove cell media, and wash cells with 200uL of 150 mM ammonium acetate.

Aspirate the wash, snap freeze cell pellets on crushed dry ice, then store at -80°C until further usage i.e. LC-MS assay

Day 3

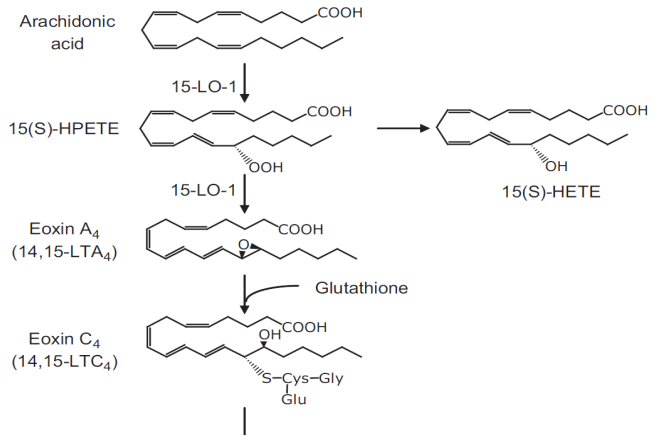
Extract cell pellet with 100 µL 80% MeOH w/15-HETE-d8 (internal standard) and run LC-PRM/MS assay method → Optimize metabolite extraction, LOD, and LC-MS PRM method

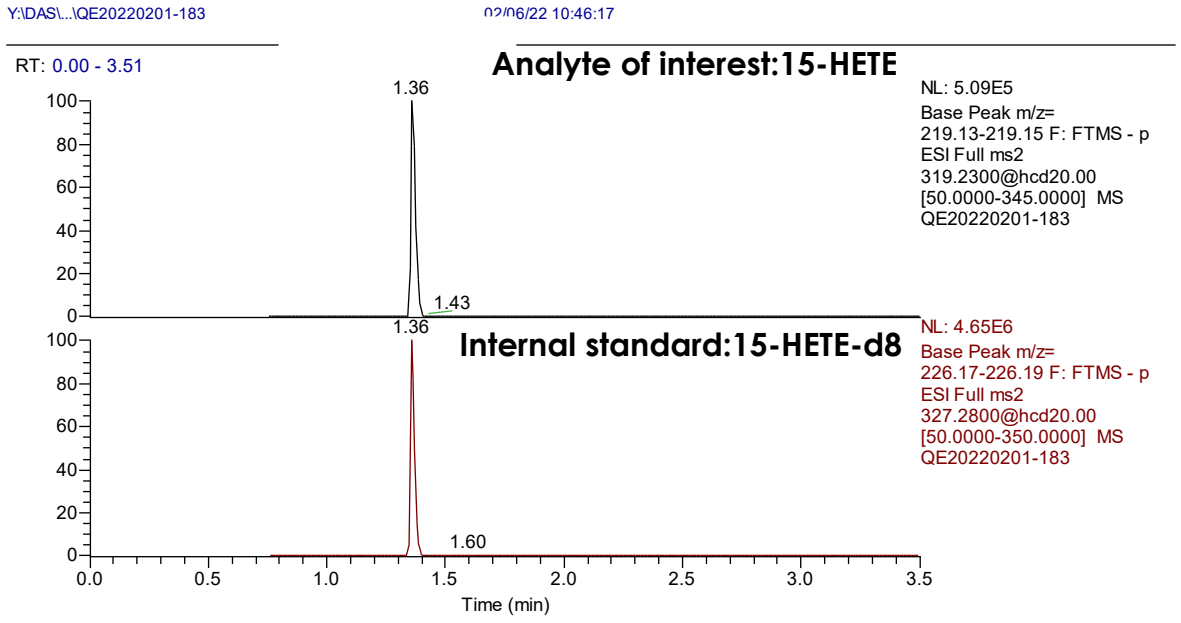
Method parameters

LC	Waters ACQUITY H-Class
Column	Peptide BEH-C18 300Å, 1.7 µm, 2.1 mm X 50 mm
Column Temp	40°C
Solvent A	0.1% formic acid/ H2O
Solvent B	0.1% formic acid/ 99.9% acetonitrile
Injection	20 uL

Time (min)	%B	Flow rate (mL/min)
0	30	0.7
0.9	30	0.7
1	80	0.7
2.8	80	0.8
2.9	30	0.8
3.5	30	0.8

MS	Thermo Q-Exactive Plus
HESI source	
Sheath gas flow rate	58
Aux gas flow rate	16
Sweep gas flow rate	3
Spray voltage (kV)	3.5
Capillary temp (°C)	281
S-lens RF level	50
Aux gas heater temp (°C)	463

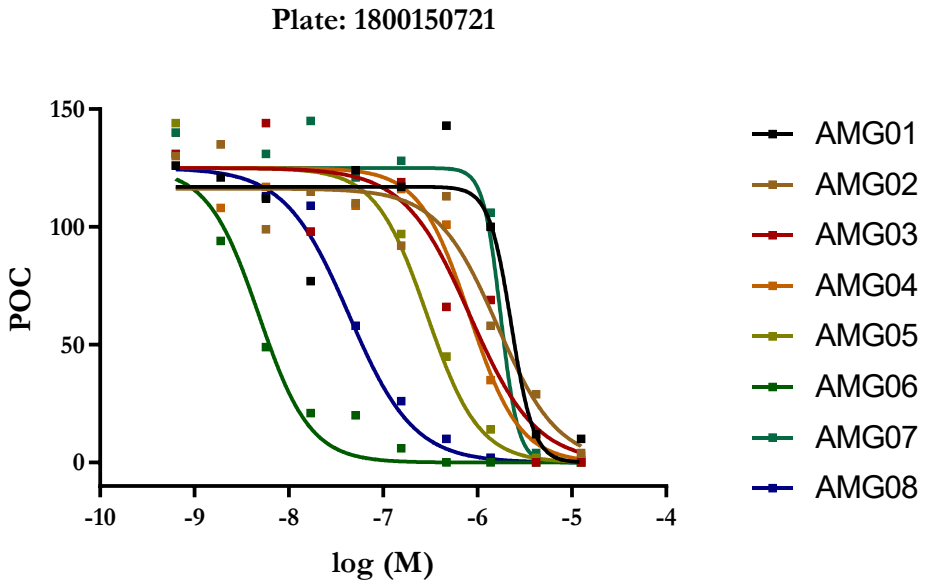




- FTMS - p ESI SIM ms [310.0000-340.0000]
- FTMS - p ESI Full ms2 319.2300@hcd20.00 [50.0000-345.0000] (**15-HETE**)
  Extract the m/z range 219.13-219.15
- FTMS - p ESI Full ms2 327.2800@hcd20.00 [50.0000-350.0000] (**15-HETE-d8**)
  Extract the m/z range 226.17-226.19

Method developed by Chris Spahr

Summary

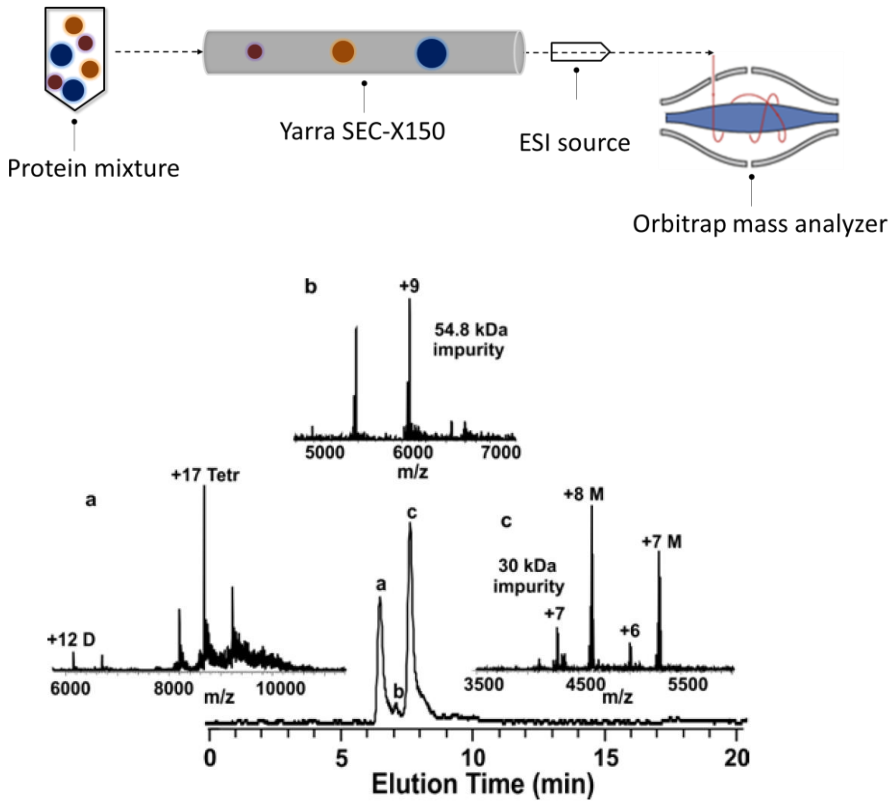


ID	IC50 (μM)	Note
AMG01	2.2840	Pharmacological Control
AMG02	1.6260	
AMG03	**0.8304**	**Inactive VHL based target degrader**
AMG04	0.8224	
AMG05	0.2929	
AMG06	**0.0048**	**Active VHL based target degrader**
AMG07	1.7610	
AMG08	**0.0437**	**Cereblon based target degrader**

	Cellular enzymatic LC-PRM/MS assay	In vitro enzymatic LC-MS assay
ID	IC50 (μM)	IC50 (μM)
AMG03	0.8304	0.1200
AMG06	0.0048	0.0918
AMG08	0.0437	0.0258

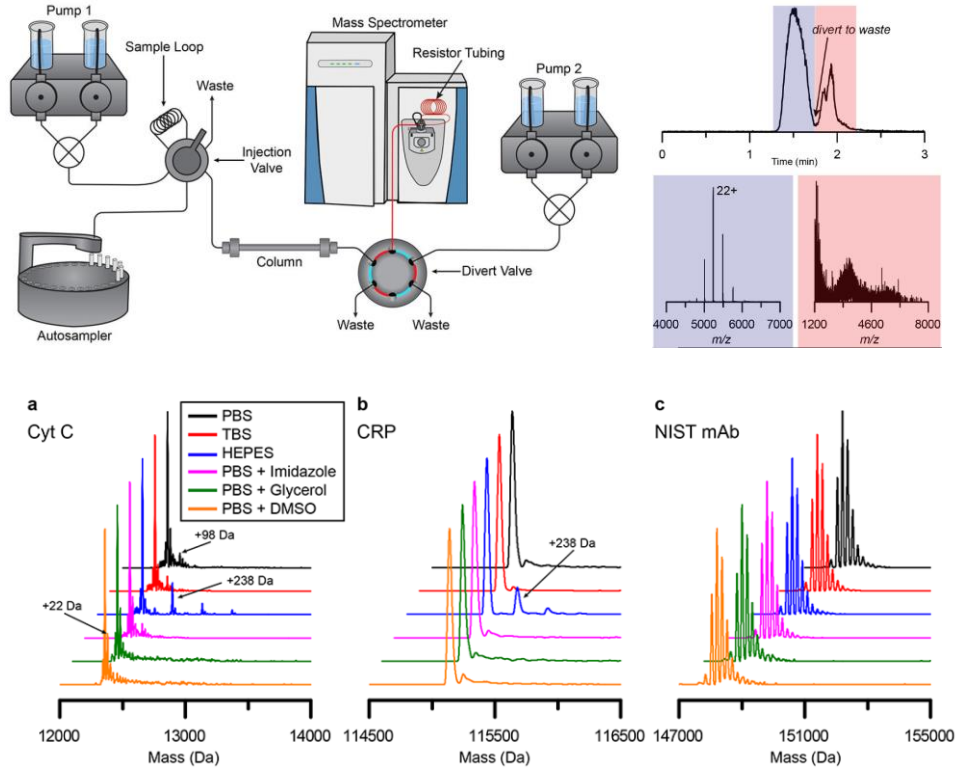
Platforming native MS

Native aSEC-MS



TIC chromatogram for the separation of alcohol dehydrogenase (ADH) by a Yarra X150 SEC-MS

Native OBE-MS



Deconvoluted mass spectra demonstrating the removal of non-volatile components from proteins in common biological buffers by OBE-MS

VanAernum Z.L. et al. Nat Protoc. 2020

Platforming native MS

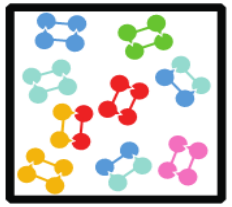
1. Protein mixture

Equimolar mixture of AA', BB',…….GG'

2. Denature

5 M guanidine hydrochloride and incubate at 75°C for 30 minutes

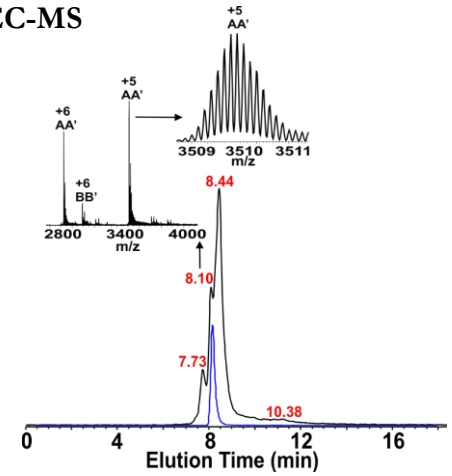
3. Re-equilibrate



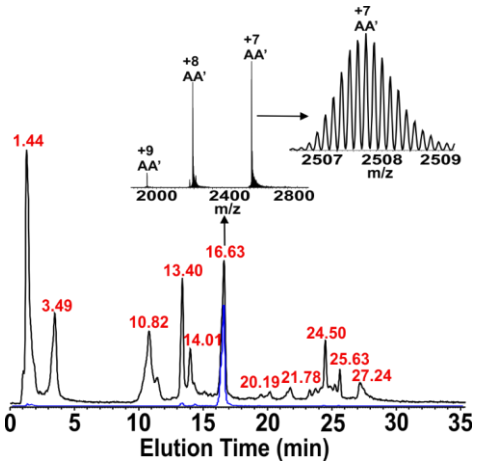
Dialysis in 150 mM ammonium acetate

Native aIEX-MS

SEC-MS



WCX-MS



Non-specific hetero dimer

Specific hetero dimer

		Subunit						
		A	B	C	D	E	F	G
Subunit	A'	■				▒		
	B'							
	C'			■			▒	
	D'	▒			■			
	E'							
	F'			▒			■	
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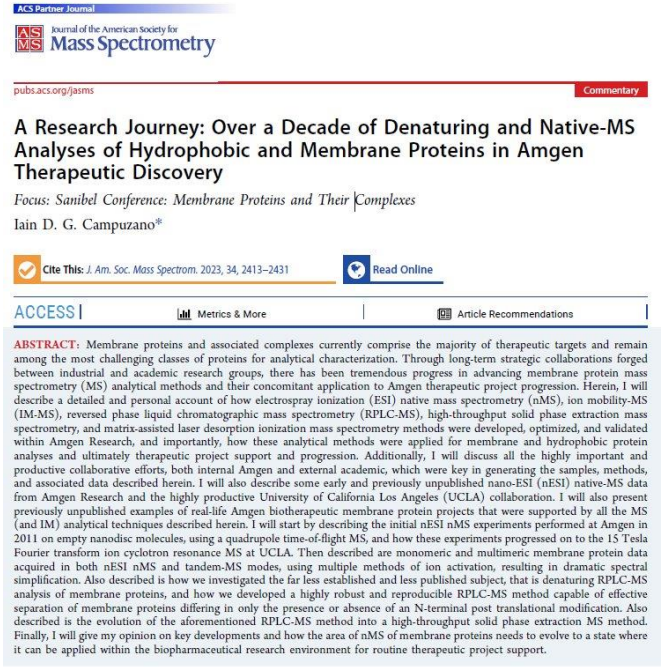
Chen et al. Nature 2019

AMGEN

Where platformed native MS fits into therapeutic research?

- Early discovery reagent protein screening: To check metal ions, cofactor, peptide binding, and oligomerization stoichiometry
- Conjugate characterizations (protein-drug, protein-peptide, protein-oligo, etc.)
- Design-and-test of multispecific modalities (multichain antibodies)
- Protein-small molecule/molecular glue/PROTAC screening



ACS Partner Journal

Journal of the American Society for Mass Spectrometry

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A Research Journey: Over a Decade of Denaturing and Native-MS Analyses of Hydrophobic and Membrane Proteins in Amgen Therapeutic Discovery

Focus: Sanibel Conference: Membrane Proteins and Their Complexes

Iain D. G. Campuzano*

Cite This: *J. Am. Soc. Mass Spectrom.* 2023, 34, 2413−2431 Read Online

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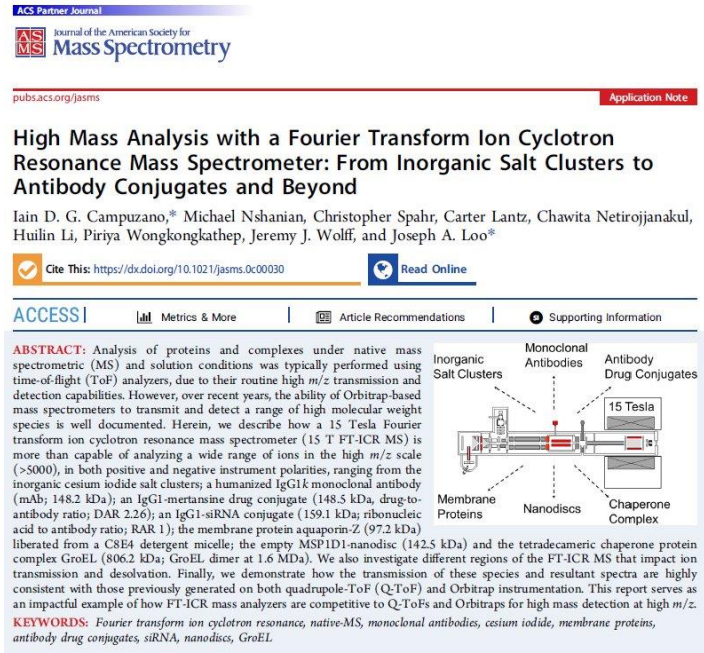
ABSTRACT: Membrane proteins and associated complexes currently comprise the majority of therapeutic targets and remain among the most challenging classes of proteins for analytical characterization. Through long-term strategic collaborations forged between industrial and academic research groups, there has been tremendous progress in advancing membrane protein mass spectrometry (MS) analytical methods and their concomitant application to Amgen therapeutic project progression. Herein, I will describe a detailed and personal account of how electrospray ionization (ESI) native mass spectrometry (nMS), ion mobility-MS (IM-MS), reversed phase liquid chromatographic mass spectrometry (RPLC-MS), high-throughput solid phase extraction mass spectrometry, and matrix-assisted laser desorption ionization mass spectrometry methods were developed, optimized, and validated within Amgen Research, and importantly, how these analytical methods were applied for membrane and hydrophobic protein analyses and ultimately therapeutic project support and progression. Additionally, I will discuss all the highly important and productive collaborative efforts, both internal Amgen and external academic, which were key in generating the samples, methods, and associated data described herein. I will also describe some early and previously unpublished nano-ESI (nESI) native-MS data from Amgen Research and the highly productive University of California Los Angeles (UCLA) collaboration. I will also present previously unpublished examples of real-life Amgen biotherapeutic membrane protein projects that were supported by all the MS (and IM) analytical techniques described herein. I will start by describing the initial nESI nMS experiments performed at Amgen in 2011 on empty nanodisc molecules, using a quadrupole time-of-flight MS, and how these experiments progressed on to the 15 Tesla Fourier transform ion cyclotron resonance MS at UCLA. Then described are monomeric and multimeric membrane protein data acquired in both nESI nMS and tandem-MS modes, using multiple methods of ion activation, resulting in dramatic spectral simplification. Also described is how we investigated the far less established and less published subject, that is denaturing RPLC-MS analysis of membrane proteins, and how we developed a highly robust and reproducible RPLC-MS method capable of effective separation of membrane proteins differing in only the presence or absence of an N-terminal post translational modification. Also described is the evolution of the aforementioned RPLC-MS method into a high-throughput solid phase extraction MS method. Finally, I will give my opinion on key developments and how the area of nMS of membrane proteins needs to evolve to a state where it can be applied within the biopharmaceutical research environment for routine therapeutic project support.



ACS Partner Journal

Journal of the American Society for Mass Spectrometry

pubs.acs.org/jasms Application Note

High Mass Analysis with a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer: From Inorganic Salt Clusters to Antibody Conjugates and Beyond

Iain D. G. Campuzano,* Michael Nshanian, Christopher Spahr, Carter Lantz, Chawita Netirojjanakul, Huilin Li, Piriya Wongkongkathep, Jeremy J. Wolff, and Joseph A. Loo*

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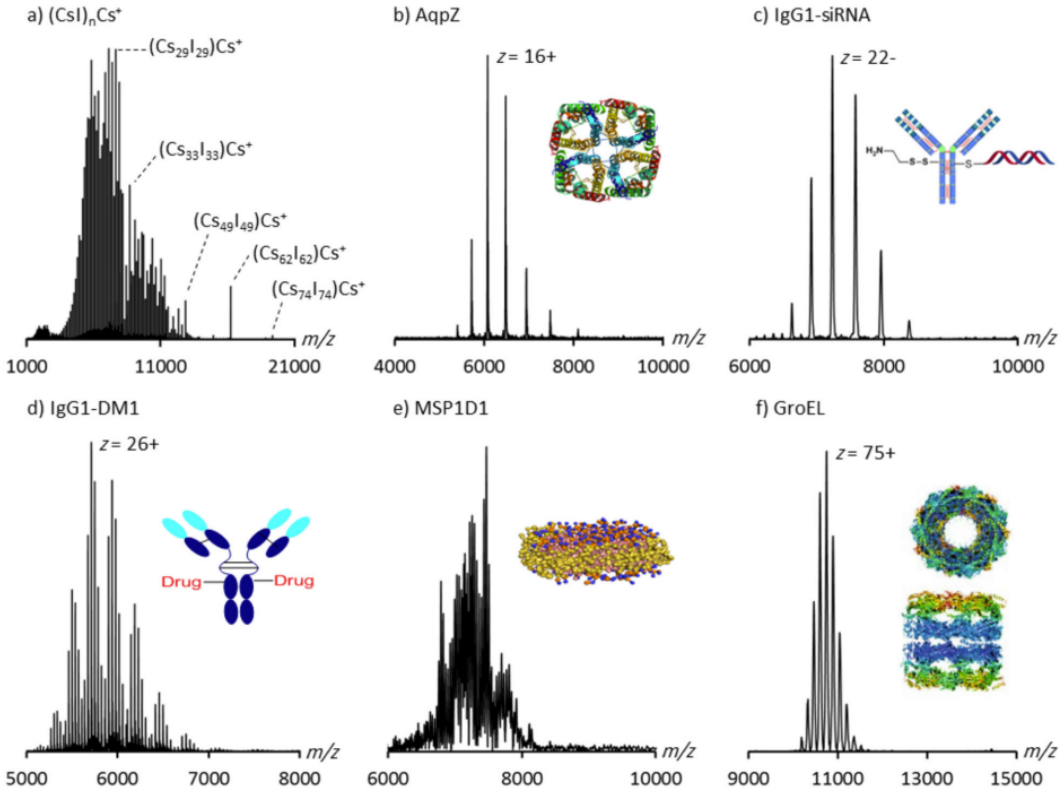
ABSTRACT: Analysis of proteins and complexes under native mass spectrometric (MS) and solution conditions was typically performed using time-of-flight (ToF) analyzers, due to their routine high m/z transmission and detection capabilities. However, over recent years, the ability of Orbitrap-based mass spectrometers to transmit and detect a range of high molecular weight species is well documented. Herein, we describe how a 15 Tesla Fourier transform ion cyclotron resonance mass spectrometer (15 T FT-ICR MS) is more than capable of analyzing a wide range of ions in the high m/z scale (>5000), in both positive and negative instrument polarities, ranging from the inorganic cesium iodide salt clusters; a humanized IgG1k monoclonal antibody (mAb; 148.2 kDa); an IgG1-mertansine drug conjugate (148.5 kDa, drug-to-antibody ratio; DAR 2.26); an IgG1-siRNA conjugate (159.1 kDa; ribonucleic acid to antibody ratio; RAR 1); the membrane protein aquaporin-Z (97.2 kDa) liberated from a C8E4 detergent micelle; the empty MSP1D1-nanodisc (142.5 kDa) and the tetradecameric chaperone protein complex GroEL (806.2 kDa; GroEL dimer at 1.6 MDa). We also investigate different regions of the FT-ICR MS that impact ion transmission and desolvation. Finally, we demonstrate how the transmission of these species and resultant spectra are highly consistent with those previously generated on both quadrupole-ToF (Q-ToF) and Orbitrap instrumentation. This report serves as an impactful example of how FT-ICR mass analyzers are competitive to Q-ToFs and Orbitraps for high mass detection at high m/z.

KEYWORDS: *Fourier transform ion cyclotron resonance, native-MS, monoclonal antibodies, cesium iodide, membrane proteins, antibody drug conjugates, siRNA, nanodiscs, GroEL*



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