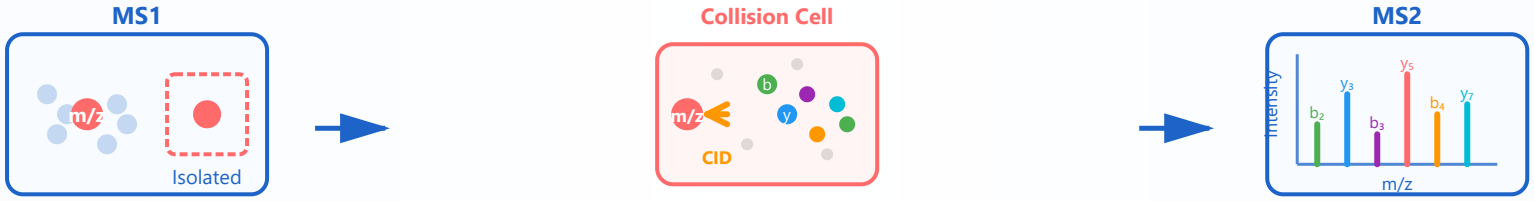
Tandem Mass Spectrometry (MS/MS)



MS1: Select Precursor

Ion Isolation

Fragmentation

CID / HCD / ETD

MS2: Analyze Fragments

Sequence Info

Precursor Selection

- Isolate specific m/z ions
- Top-N data-dependent selection
- Targeted precursor lists

Fragmentation Methods

- CID: collision-induced dissociation
- HCD: higher-energy collisional dissociation
- ETD: electron-transfer dissociation

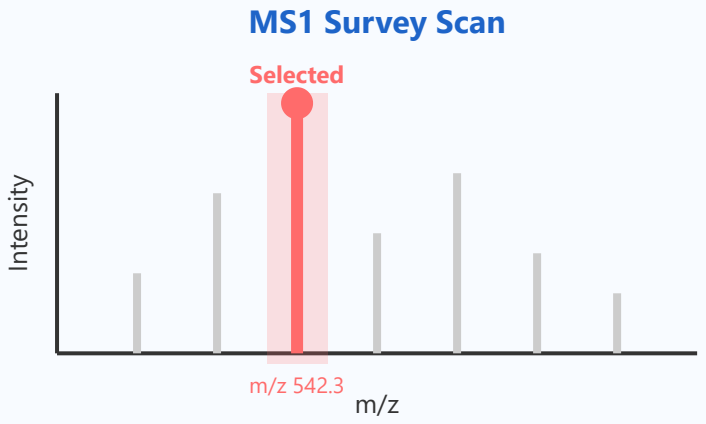
Product Ion Spectra

- b-ions and y-ions from peptides
- Sequence information
- PTM localization

Data Acquisition

- DDA: data-dependent acquisition
- DIA: data-independent acquisition
- Parallel reaction monitoring (PRM)

1 Precursor Selection in Detail



Precursor selection is the critical first step in tandem MS where specific ions are chosen from the complex mixture for further analysis. This process determines which molecules will be fragmented and sequenced.

Selection Strategies

Data-Dependent Acquisition (DDA)
The most common approach where the instrument automatically selects the most intense ions in real-time. Typically operates in "Top-N" mode, selecting the N most abundant precursors from each MS1 scan.

- ✓ **Isolation Window:** Typically 1-3 m/z units wide, ensuring only the target ion enters the collision cell
- ✓ **Dynamic Exclusion:** Prevents reselection of the same ion for a defined time period, increasing proteome coverage
- ✓ **Charge State Selection:** Filters for multiply charged ions (2+, 3+) which fragment more predictably
- ✓ **Intensity Threshold:** Minimum signal required to trigger MS2 acquisition

Targeted Selection

In targeted proteomics, specific m/z values are pre-programmed into an inclusion list. This ensures that peptides of interest are

always selected for MS2, regardless of their abundance. This approach is essential for:

- ✓ Quantifying specific proteins (e.g., biomarkers)
- ✓ Validating protein identifications
- ✓ Monitoring post-translational modifications
- ✓ Clinical diagnostic applications

2 Fragmentation Methods in Detail

Fragmentation is the process of breaking peptide bonds to generate sequence-informative ions. Different fragmentation methods cleave peptides at different locations, providing complementary structural information.

▸ Collision-Induced Dissociation (CID)

CID is the most widely used fragmentation technique. Precursor ions collide with inert gas molecules (typically nitrogen or argon), converting kinetic energy into internal energy that breaks chemical bonds.

Mechanism: Low-energy collisions (10-50 eV) cause vibrational excitation. The peptide backbone

preferentially breaks at the amide bond, producing b-ions and y-ions.

- ✓ **Advantages:** Well-characterized fragmentation patterns, excellent for peptide sequencing
- ✓ **Limitations:** May lose labile modifications (phosphorylation), produces mainly b/y ions
- ✓ **Best for:** Routine peptide identification and database searching

▸ Higher-Energy Collisional Dissociation (HCD)

HCD uses higher collision energies in a dedicated collision cell, allowing detection of low m/z fragment ions that are lost in traditional ion trap CID.

- ✓ **Energy Range:** Higher than CID (up to 200 eV), causing more extensive fragmentation
- ✓ **Key Advantage:** Can detect immonium ions and reporter ions (e.g., TMT tags)
- ✓ **Applications:** Quantitative proteomics with isobaric tags, small molecule analysis

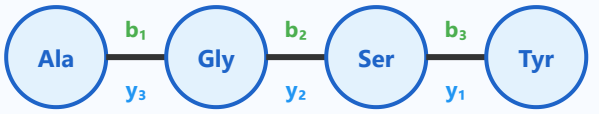
▸ Electron-Transfer Dissociation (ETD)

ETD is a radical-driven fragmentation method where electrons are transferred from reagent anions to multiply charged peptide cations, causing cleavage of N-Cα bonds.

Unique Feature: ETD preserves labile post-translational modifications like phosphorylation, glycosylation, and

Peptide Fragmentation Sites

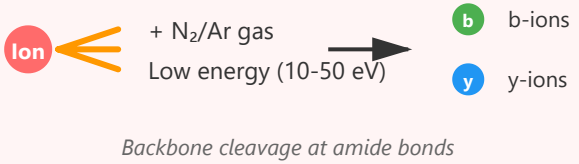
NH_2-Ala-Gly-Ser-Tyr-COOH



Ion Nomenclature

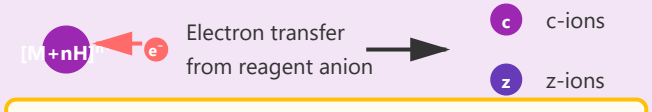
- b-ions: N-terminal fragments (CID/HCD)
- y-ions: C-terminal fragments (CID/HCD)

CID/HCD Fragmentation



Backbone cleavage at amide bonds

ETD Fragmentation



✓ Preserves labile PTMs (phospho, glyco)
✓ N-Cα bond cleavage (different from CID)

Method Comparison

CID/HCD	Best for: Routine identification

sulfation, making it invaluable for PTM analysis.

- ✓ **Fragment Ions:** Produces c-ions and z-ions instead of b/y ions
- ✓ **Optimal for:** Highly charged peptides (≥3+), intact protein analysis
- ✓ **Applications:** Top-down proteomics, PTM localization, disulfide bond mapping

3 Product Ion Spectra Analysis

Product ion spectra (MS/MS or MS2 spectra) contain the fragment ions generated from the selected precursor. By analyzing the mass differences between peaks, we can deduce the amino acid sequence of the peptide.

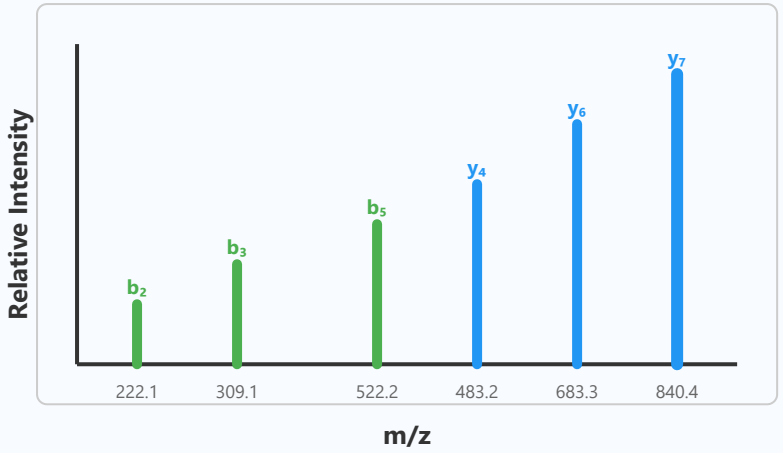
▸ Ion Series Nomenclature

Roepstorff-Fohlmann-Biemann Nomenclature: The standard system for naming peptide fragment ions based on the cleavage site and charge retention.

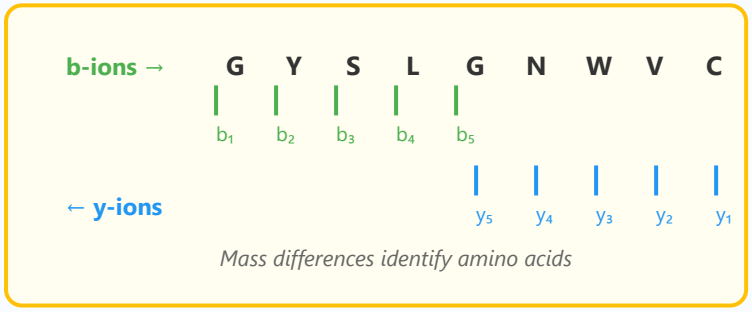
- ✓ **b-ions:** N-terminal fragments retaining the charge on the N-terminus

MS/MS Spectrum Example

Peptide: GYSLGNWVC (m/z 1021.5, 2+)



Peptide Sequence Reconstruction



- ✓ **y-ions:** C-terminal fragments retaining the charge on the C-terminus
- ✓ **c-ions & z-ions:** Produced by ETD fragmentation
- ✓ **a-ions:** b-ions minus CO (carbon monoxide)
- ✓ **Immonium ions:** Single amino acid ions, useful for amino acid composition

▸ Sequence Determination

The process of peptide sequencing from MS/MS spectra involves:

1. **Peak identification:** Assign observed peaks to theoretical fragment ions
2. **Mass ladder construction:** Build a sequence ladder from consecutive mass differences
3. **Sequence coverage:** Aim for at least 70% sequence coverage with both b and y ions
4. **Validation:** Confirm the sequence matches the precursor mass

▸ Post-Translational Modification (PTM) Localization

> **Critical Application:** MS/MS spectra can precisely localize PTMs by identifying mass shifts in specific fragment ions.

For example, a phosphorylated serine (+80 Da) will cause a mass shift in all fragment ions that contain that residue. By

comparing the masses of b and y ions, we can determine exactly which serine is phosphorylated in peptides with multiple potential sites.

- ✓ **Phosphorylation:** +80 Da (or +98 Da loss of H_3PO_4)
- ✓ **Acetylation:** +42 Da
- ✓ **Methylation:** +14 Da
- ✓ **Ubiquitination:** +114 Da (diglycine remnant)
- ✓ **Glycosylation:** Variable mass depending on glycan structure

4 Data Acquisition Strategies

Data acquisition methods determine how the mass spectrometer selects precursors and acquires MS/MS spectra. The choice of strategy significantly impacts proteome coverage, quantification accuracy, and reproducibility.

▸ Data-Dependent Acquisition (DDA)

The Traditional Approach: DDA automatically selects the most intense ions from each MS1 scan for fragmentation in real-time. This is the most widely used method for discovery proteomics.

Workflow:

1. Acquire full MS1 scan (survey scan)
2. Rank all detected ions by intensity
3. Select top-N most intense ions (typically N = 10-20)
4. Isolate each selected ion and perform MS/MS
5. Add fragmented ions to exclusion list
6. Return to step 1 for next cycle

✓ **Advantages:** Simple, effective for abundant proteins, well-established workflows

✓ **Limitations:** Stochastic sampling (low reproducibility), bias toward high-abundance proteins, limited dynamic range

✓ **Best for:** Initial protein discovery, unknown samples, large-scale identification

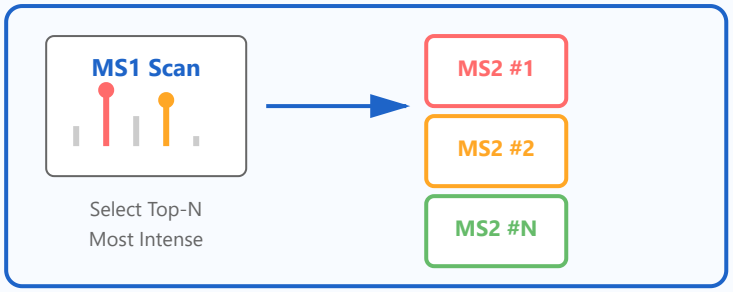
▸ Data-Independent Acquisition (DIA)

> **The Modern Alternative:** DIA systematically fragments all ions within sequential m/z windows, providing comprehensive and reproducible data without precursor selection bias.

How it works: The entire m/z range (e.g., 400-1200) is divided into windows (e.g., 25 Da wide). All ions within each window are co-isolated and fragmented together, creating highly multiplexed spectra.

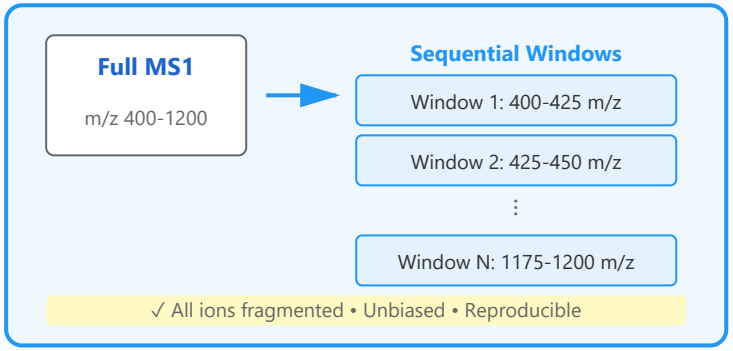
✓ **SWATH-MS:** Sequential Window Acquisition of all THeoretical fragment ions - pioneered by the Aebersold lab

Data-Dependent Acquisition (DDA)

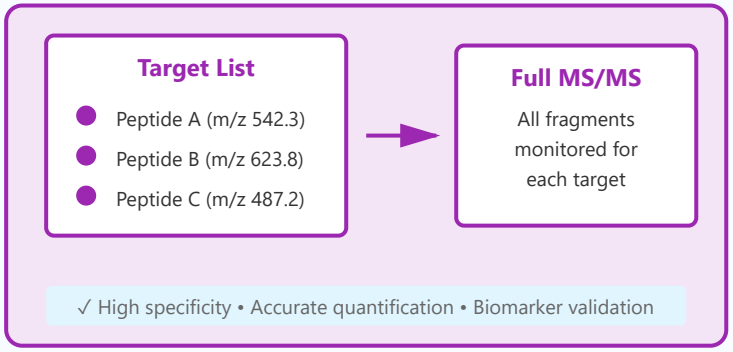


Stochastic selection • High-abundance bias

Data-Independent Acquisition (DIA)



Parallel Reaction Monitoring (PRM)



Method Selection Guide

- ✓ **Reproducibility:** Same ions analyzed in every run, enabling accurate quantification
- ✓ **Sensitivity:** Can detect low-abundance proteins missed by DDA
- ✓ **Challenge:** Complex data analysis requiring spectral libraries or advanced algorithms

▸ Parallel Reaction Monitoring (PRM)

PRM is a targeted quantitative method that combines the selectivity of selected reaction monitoring (SRM) with the high resolution of modern Orbitrap instruments.

> **Precision Quantification:** PRM monitors all fragment ions from selected precursors, providing both identification confidence and accurate quantification.

- ✓ **Targeted Analysis:** Pre-defined list of peptides of interest
- ✓ **Full MS/MS Spectra:** Unlike SRM, acquires complete fragment ion spectra
- ✓ **Multiplexing:** Can monitor 50-100 peptides per run
- ✓ **Applications:** Biomarker validation, pathway analysis, clinical diagnostics

Method	Coverage	Reproducibility	Best Application
DDA	Moderate (stochastic)	Low-Medium (60-70%)	Discovery proteomics, large-scale ID

Method	Coverage	Reproducibility	Best Application
DIA	High (comprehensive)	Very High (>95%)	Quantitative studies, clinical samples
PRM	Targeted only	Excellent (>98%)	Biomarker validation, pathway analysis