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Technology Transition Workshop| *Steven A. Hofstadler, Ph.D.*

Introduction to Biological Mass Spectrometry (Mass Spectrometry 101)

Disclaimer

- **This presentation covers the basic concepts of mass spectrometry**
- **The goal of this presentation is to give the user a basic understanding of where / how mass spectrometry fits into various SNP genotyping approaches**

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Overview

- Introduction – what is mass spectrometry?
  - Mass spectrometry (MS) and Ibis™ T5000™
- Brief history
- General components
- The "mass" spectrum
  - Definitions and nomenclature
- Ionization sources
  - Matrix Assisted Laser Desorption Ionization (MALDI)
  - Electrospray Ionization (ESI)
  - Others
- Time-of-Flight (TOF) mass analyzers
- ESI-TOF of nucleic acids

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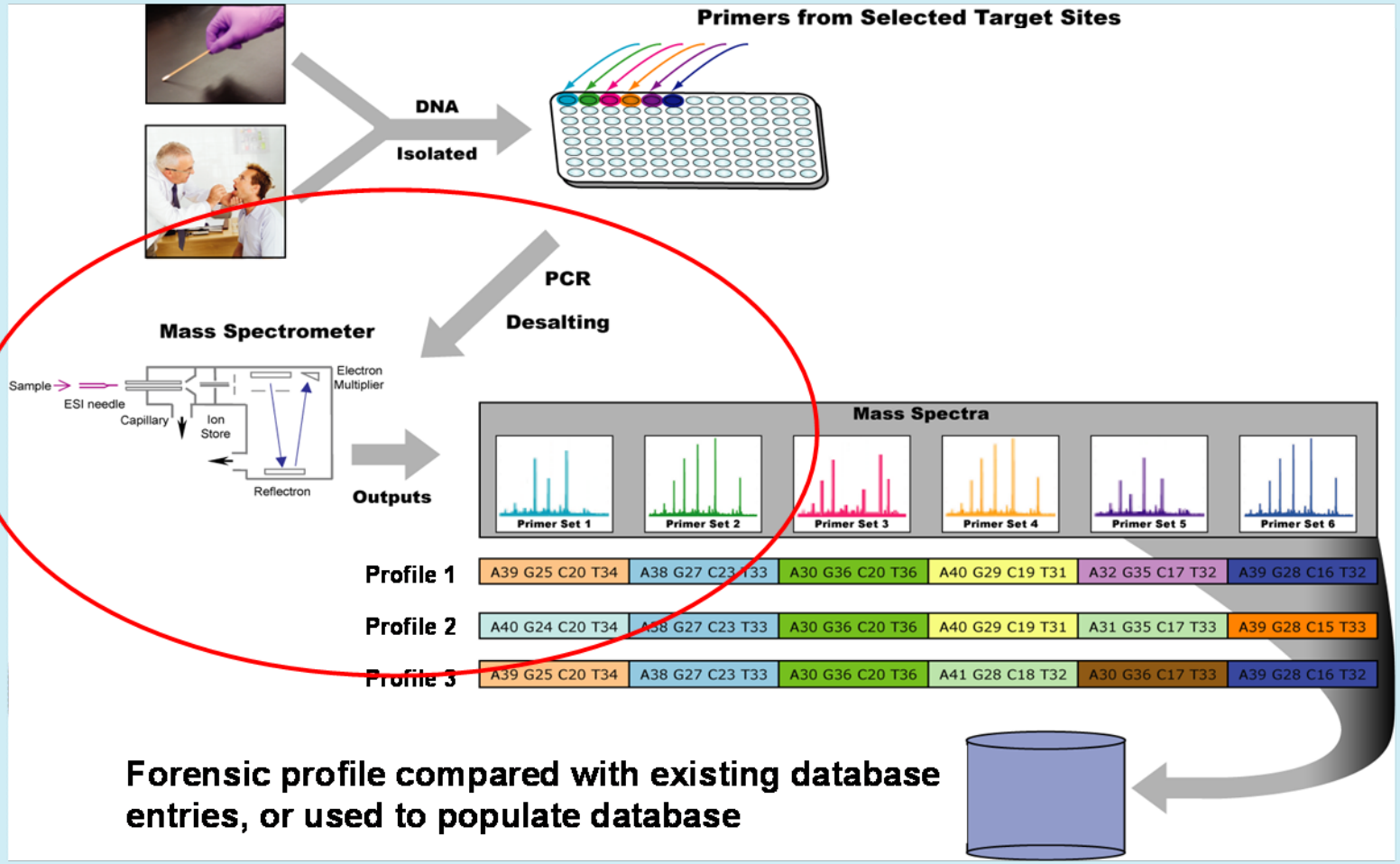
Mass Spectrometry and Ibis™ Platform



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Mass Spectrometry of Nucleic Acids?

- **Information content**
  - **From precise mass measurements unambiguous base compositions are derived [A10 G23 C32 T17] = [10 23 32 17]**
- **Speed**
  - **< 1 minute / sample**
- **Applicability to mixtures**
  - **MS succeeds where sequencing fails (e.g. mixtures)**
- **Automation**
  - **End-to-end process is highly automated (including spectral processing / interpretation)**
- **Sensitivity**
  - **Single copy detection demonstrated with PCR front-end**

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What is a Mass Spectrometer?

- An instrument which measures the mass-to-charge ratio (m/z) of ionized analyte based on its response to applied electric and / or magnetic fields
  - Atoms, molecules, clusters, and macromolecular complexes
- The m/z measurement is converted to a mass measurement
  - m is in atomic mass units or Daltons (Da)
    - 1 Da = 1/12 the mass of a single atom of ^{12}C
    - 1 Da = 1.66×10^{-24} grams
  - z is an integer multiplier of the fundamental unit of charge (q)
    - q = 1.602×10^{-19} Coulombs
  - Mass = m/z X z
- A mass spectrometer is essentially a "molecular (or atomic) scale" that "weighs" analytes of interest

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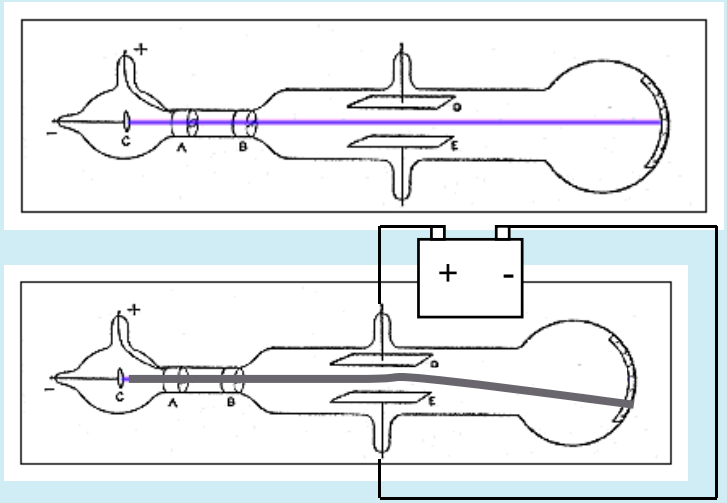
Brief History

- **1897 J.J. Thompson announced the presence of electrons or "corpuscles" based on the deflection of cathode rays by electric and magnetic fields**
- **He later used this "beam-deflection device" to measure the mass of the electron (1906 Nobel Prize)**



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Brief History (continued)

- **1919 F.W. Aston used Thompson's mass spectrometer to measure the atomic masses of 30 gaseous elements and prove the existence of multiple isotopes**
- **Relative abundance measurements were made by recording isotope lines on film**
- **"Mass spectroscopy" (1922 Nobel Prize) (Nature 104, 393 (1919))**
  - **Design principles are the basis of modern electric and magnetic sector instruments**

Aston's original "Positive-Ray Mass Spectrograph"

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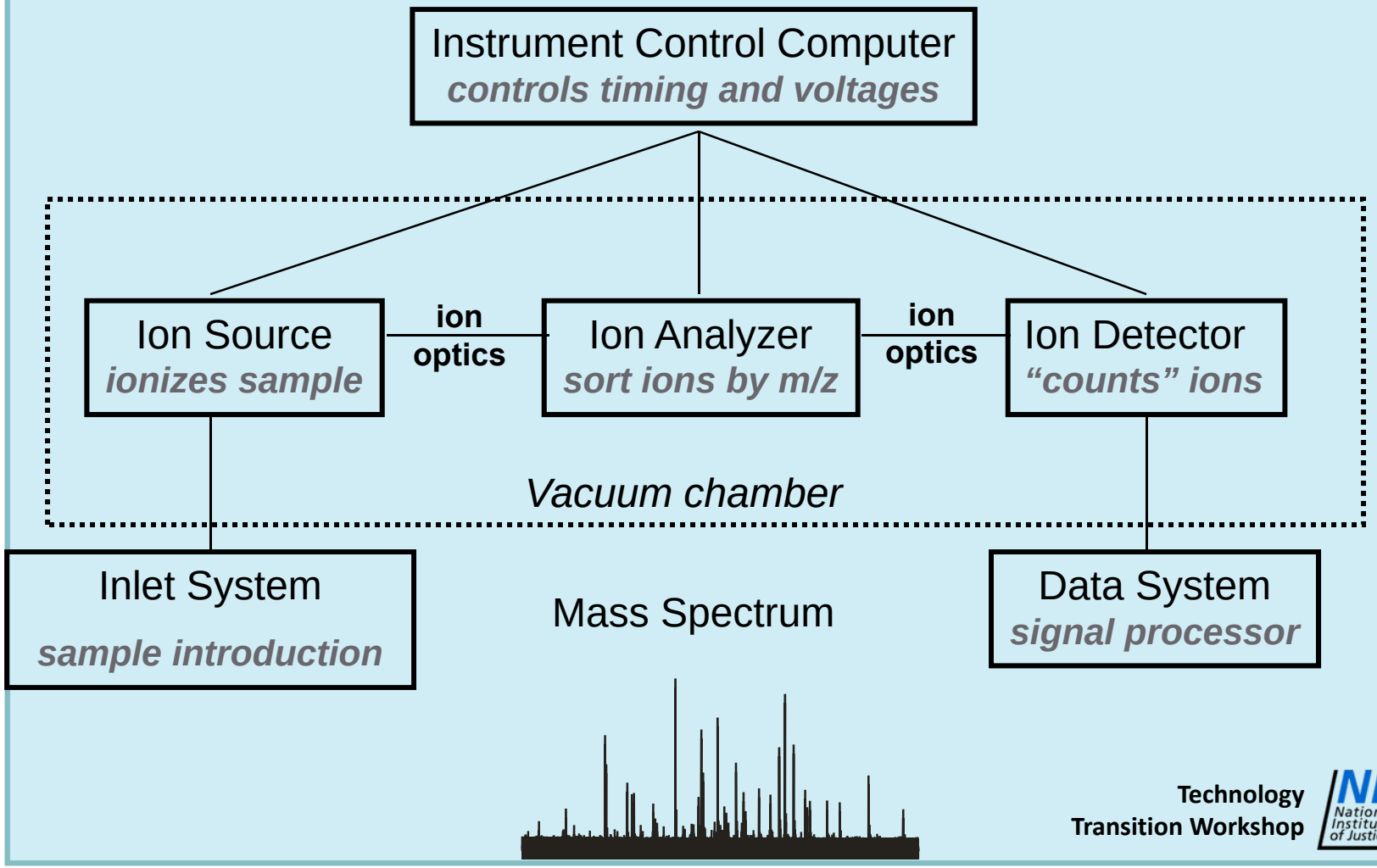
Basic Components



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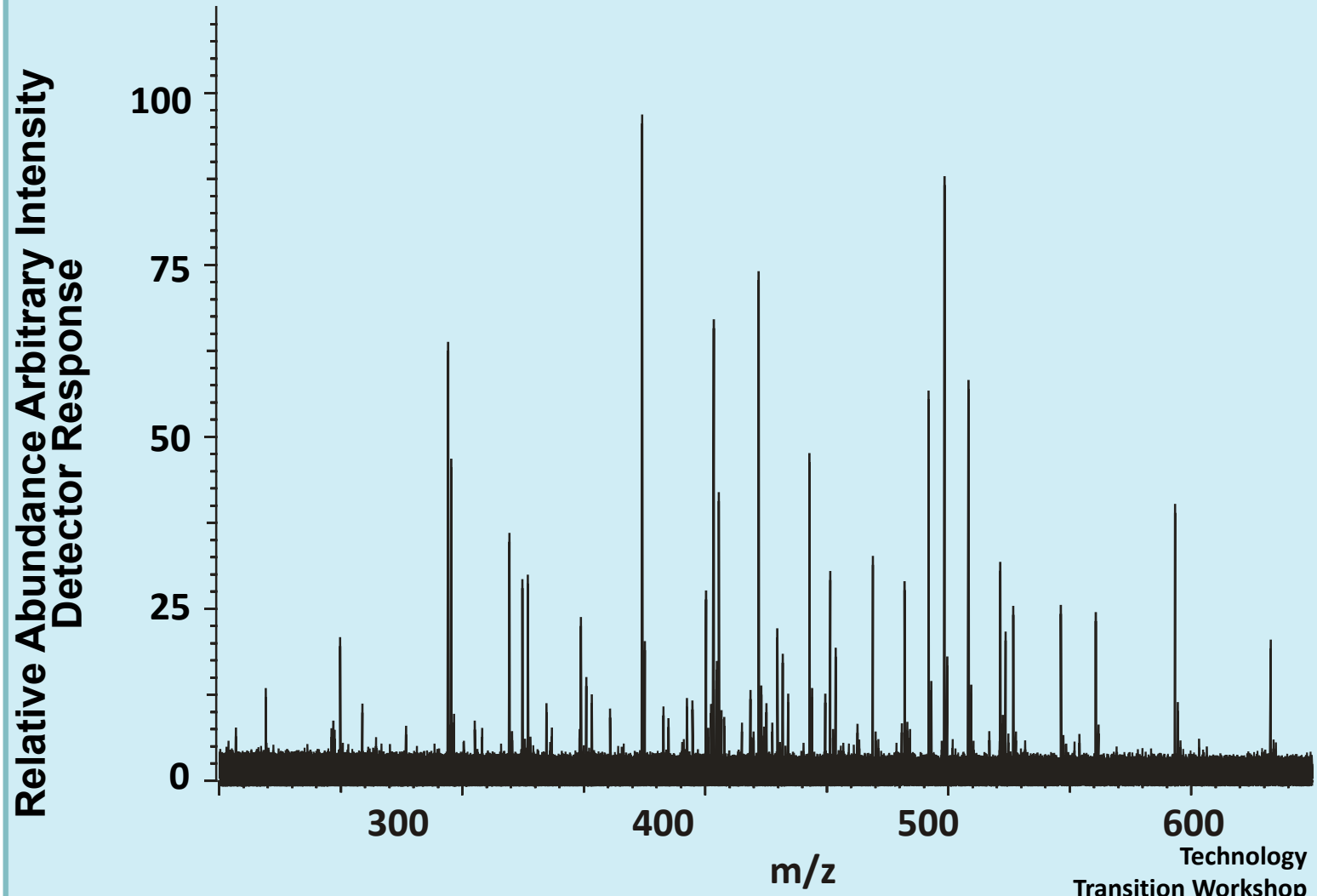
The "Mass" Spectrum



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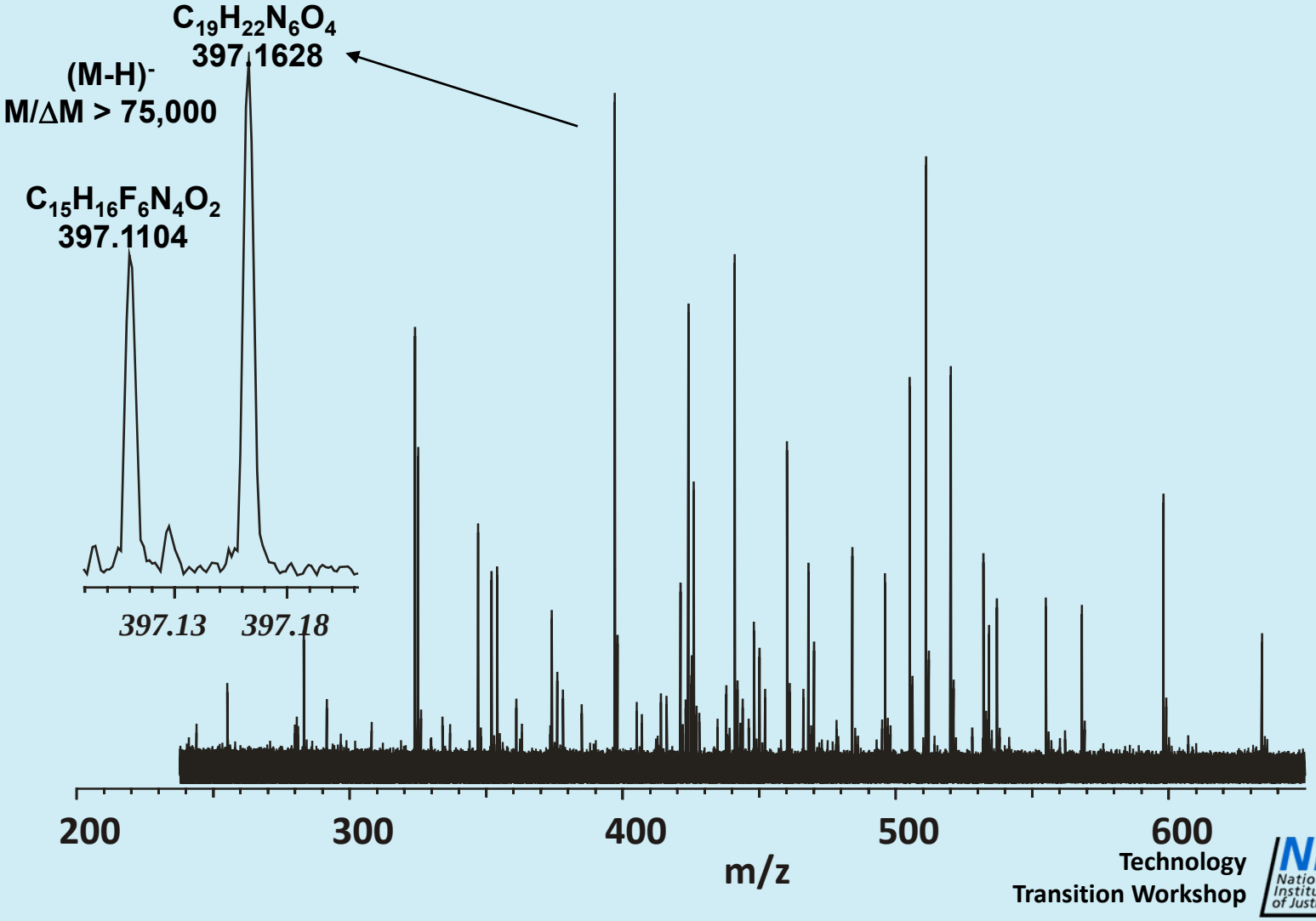
The "Mass" Spectrum



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The Isotopic Envelope

- Most elements have more than 1 isotope
- For a given atom type, different isotopes have different numbers of neutrons
  - e.g., an atom of ^{12}C has 6 neutrons, 6 protons, and 6 electrons
  - an atom of ^{13}C has 7 neutrons, 6 protons, and 6 electrons
- The mass of a neutron is 1.00867 Da
- Each element has different numbers and relative abundances of other isotopes:
  - ^{12}C = 98.90% ^{13}C = 1.10%
  - ^{35}Cl = 75.77% ^{37}Cl = 24.23%
  - ^{19}F = 100%

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The Isotopic Envelope

- **Unless a molecule is composed of only monoisotopic elements, there is a finite probability that it will contain one or more heavy isotopes**
- **The relative abundance of the monoisotopic peak decreases with increasing mass**
- **Observed distribution is the sum of isotopic contributions from all hetero-isotopes**
- **Except in a few cases, "isotopic fine structure" cannot be resolved**
  - **e.g., for an N + 2 peak the contributions from 2 ^{13}C and 1 ^{18}O cannot be resolved**
- **Consider carbon clusters:**

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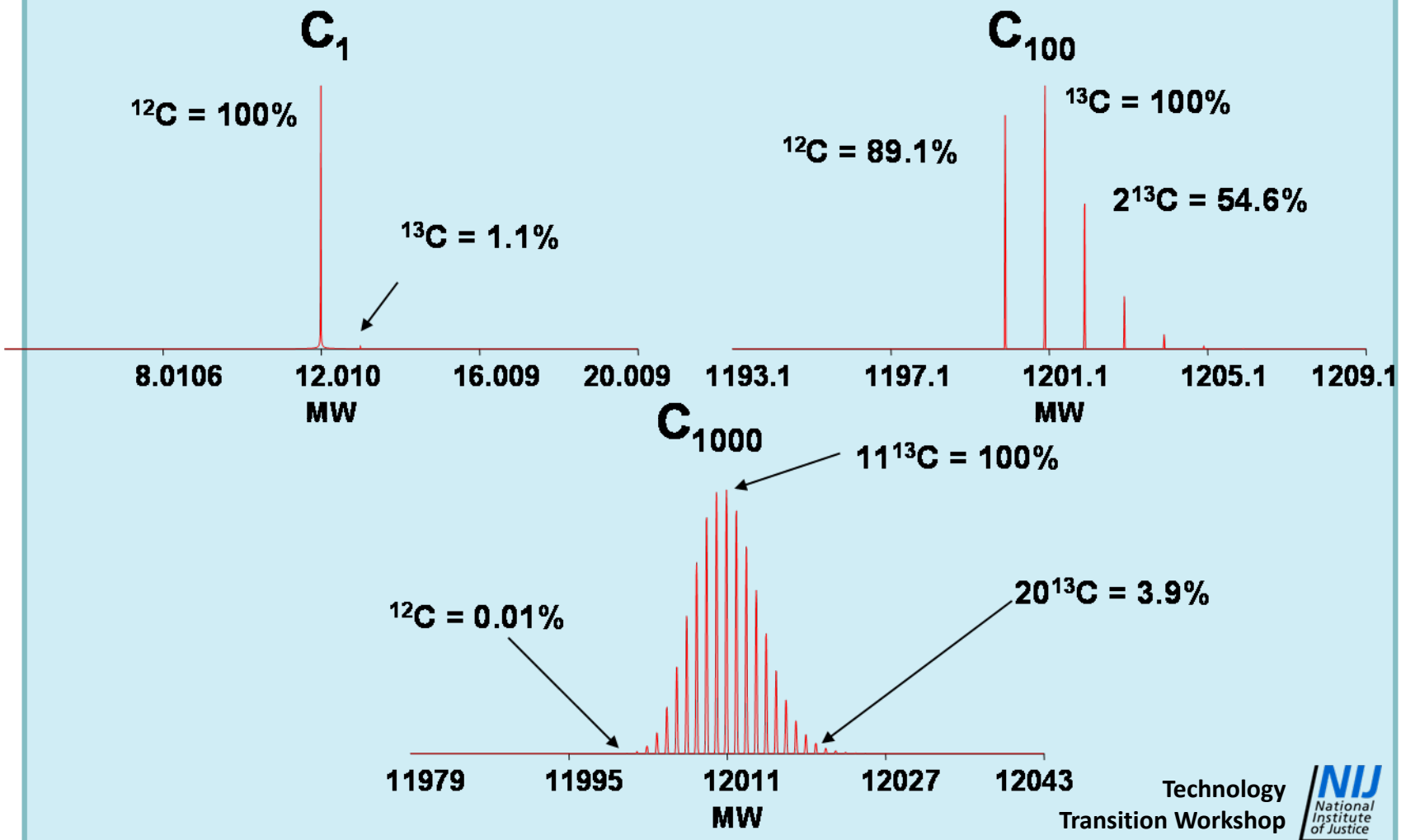
Isotope Distributions of Carbon



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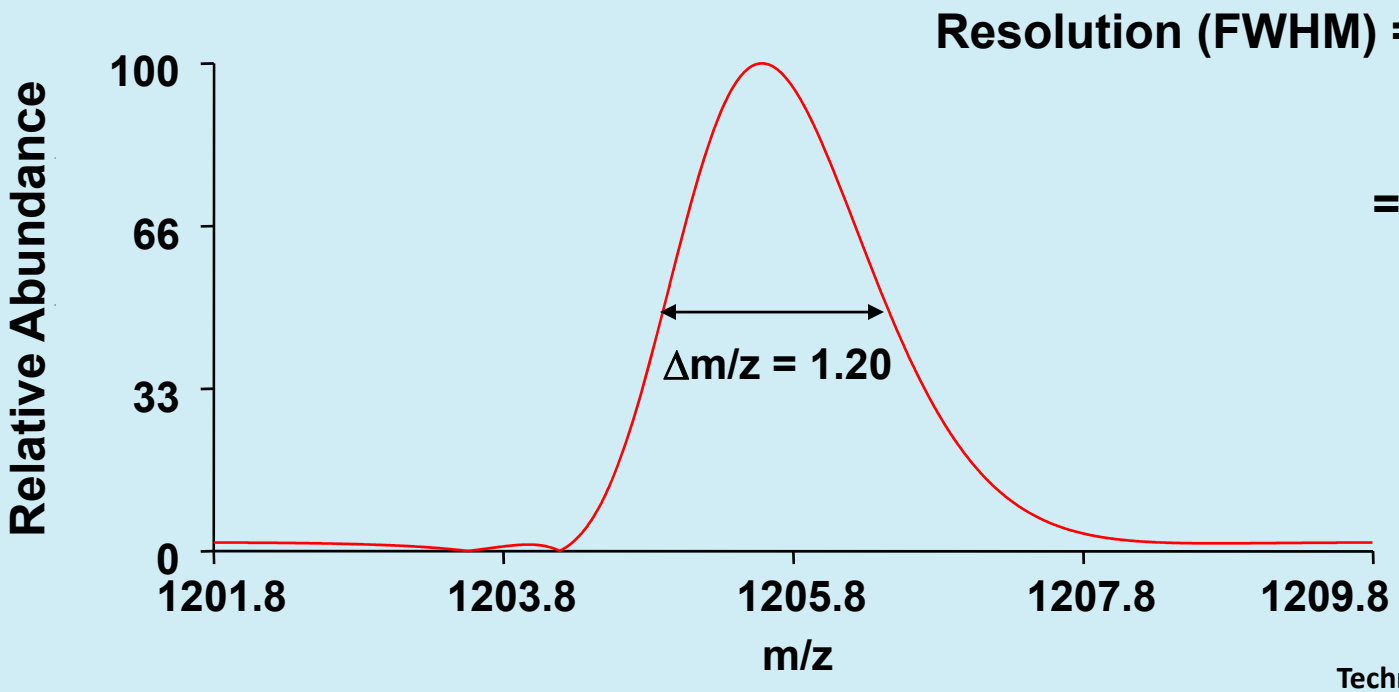
Definitions and Nomenclature

- **Resolution : M/ΔM**
  - **Actually (m/z)/Δ(m/z)**
  - **Δ(m/z) : peak width at full width half maximum (FWHM)**



Resolution (FWHM) = $\frac{1205.8}{1.20}$

= 1005

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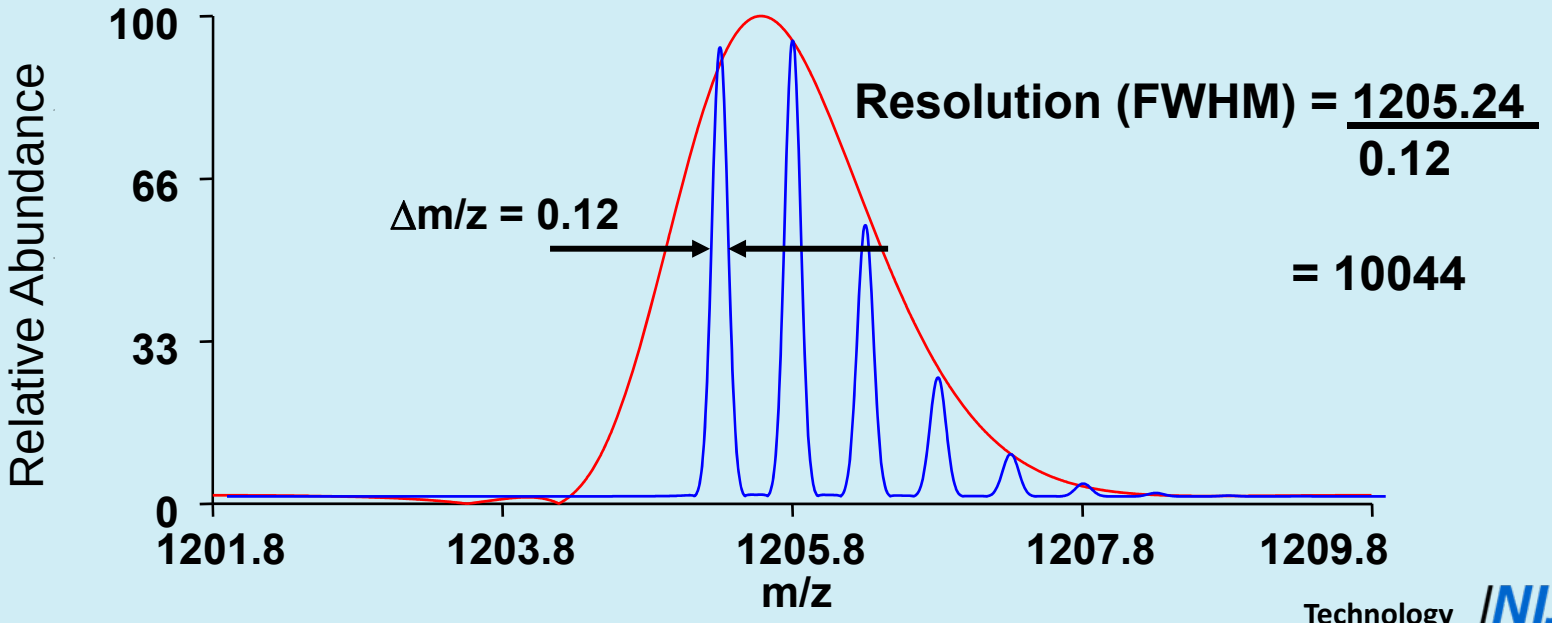
Definitions and Nomenclature

- **Resolution (continued)**
  - **Can be limited by the inherent width of the isotope envelope**
  - **Step function to isotopic resolution**
    - **Need M/ΔM > molecular weight for isotopic resolution**



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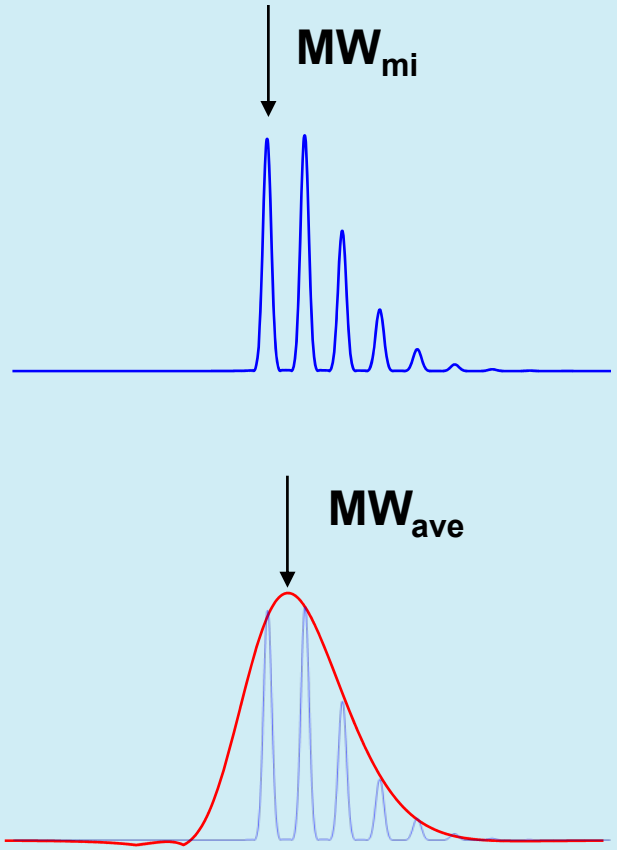
Definitions and Nomenclature: Mass Measurements

Three ways to specify molecular weight

- **Monoisotopic Molecular Weight**
  - **All ^{12}C, ^{14}N, ^{16}O, etc.**
  - **Most accurate method for low MW species**
  - **Monoisotopic peak is base peak (i.e., most abundant peak) up to about 2 kDa**
- **Average Molecular Weight**
  - **Most commonly used**
  - **Few MS platforms can resolve isotopes for analytes > 5 kDa**
  - **Δ between monoisotopic and average increases with increasing MW**



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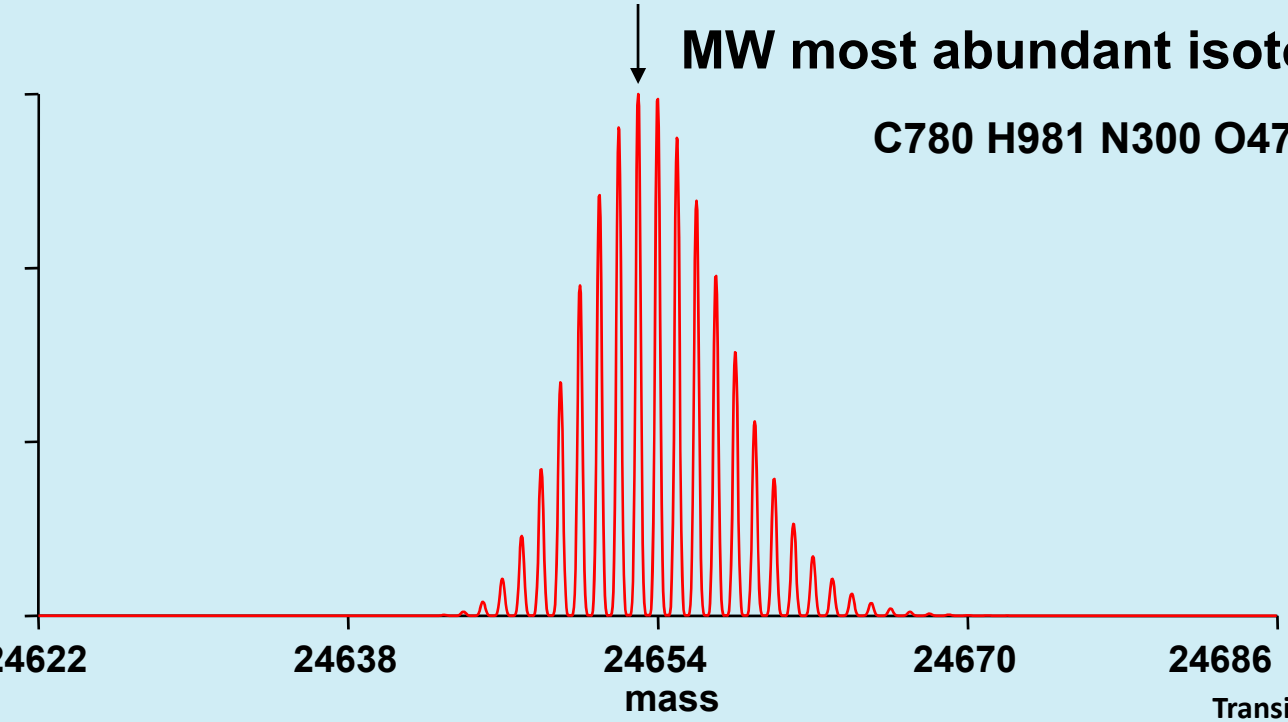
Definitions and Nomenclature: Mass Measurements

Three ways to specify molecular weight

- **Most Abundant Isotope Molecular Weight**
  - **Not widely used**
  - **Convenient for high MW, isotopically resolved species**



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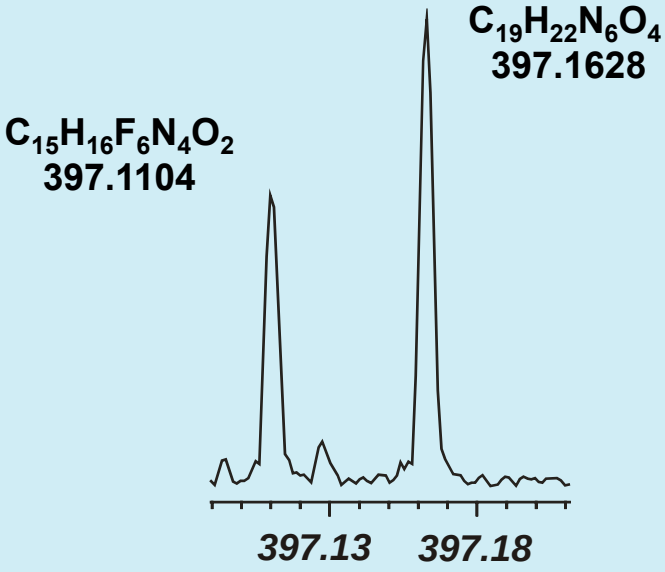
Definitions and Nomenclature

- **Mass Measurement Accuracy**

$$\frac{|(m/z)\text{theory} - (m/z)\text{measured}|}{(m/z)\text{theory}} = \text{Mass Measurement Error (ppm)}$$



$(M-H)^-$
$M/\Delta M > 75{,}000$

cmpd	M_{calc}	M_{meas}	Δm	ppm
$C_{15}H_{16}F_6N_4O_2$	397.1105	397.1104	0.0001	0.25
$C_{19}H_{22}N_6O_4$	397.1630	397.1628	0.0002	0.50

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Mass Spectrometry Nuts and Bolts

- **Ionization Sources**
- **Mass Analyzers**

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Ionization Sources

- **Ionization is the process by which analytes are "charged"**
  - **Adding or removing electrons (e-) (MW = 0.0006 Da)**
  - **Adding or removing protons (H+) (MW = 1.0078 Da)**
- **Several very effective methods for ionizing low molecular weight and / or volatile compounds**
- **Limited MS to analytes with molecular weights under about 1 kDa**
- **In the 1980s, two ionization methods developed for ionizing high molecular weight analytes**
  - **MALDI and ESI**

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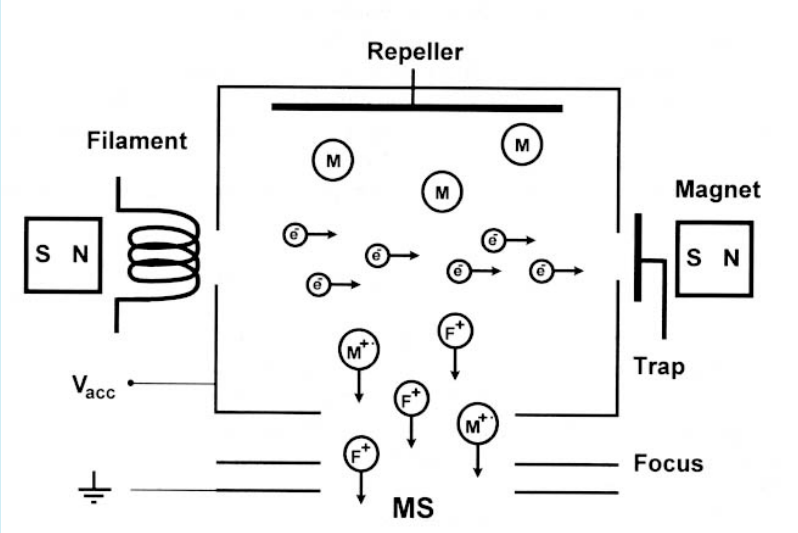
Ionization Sources for Low MW Analytes

- **Atmospheric Pressure Chemical Ionization (APCI)**
  - **Formation of analyte ions through charge exchange with ionized carrier gas**
- **Electron Ionization (EI)**
  - **Generation of ions by bombarding gas phase molecules with high energy electrons**
  - **Analyte must be volatile**
  - **Ionization energy dictates extent of fragmentation**
  - **Still widely used w/ GC**



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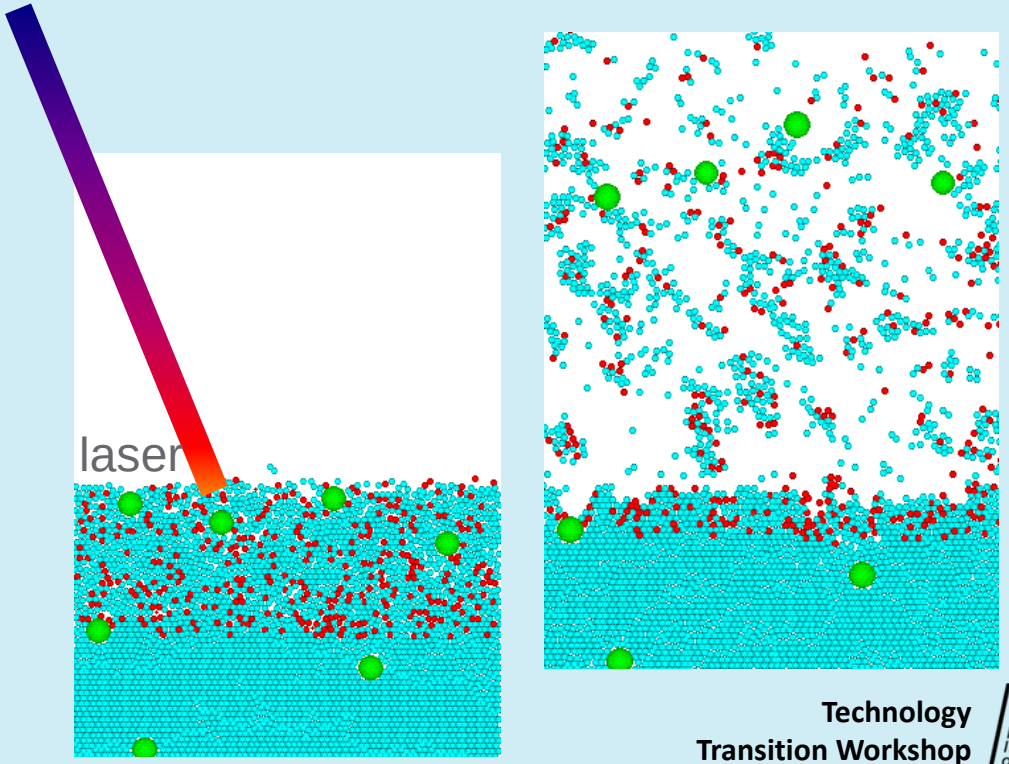
Ionization Sources – MALDI

- **Matrix Assisted Laser Desorption Ionization**
  - **Sample is co-crystallized with a matrix which absorbs photons and creates a desorption plume that ionizes the sample**
  - **Gentle ionization technique (harsher than ESI)**
  - **A pulsed ion source**
  - **Produces singly charged ions**
  - **Relatively salt tolerant**
  - **Effective for wide range of MWs**
  - **Fast and automatable**



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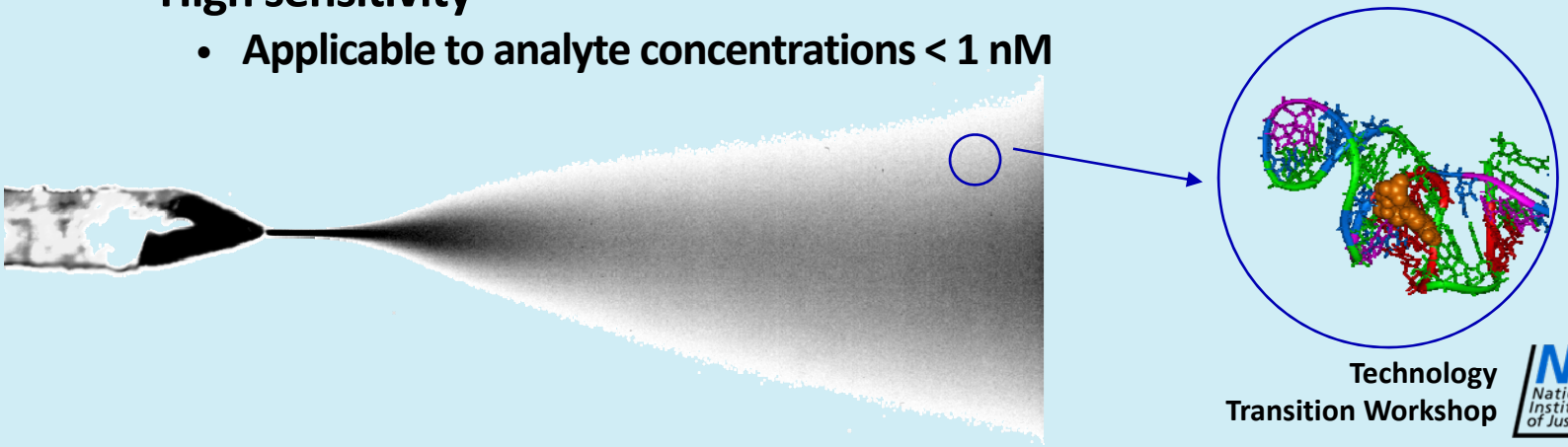
Ionization Sources - ESI

- **Electrospray Ionization**
  - **Ions are desolvated / desorbed from highly charged liquid droplets**
  - **Generates multiple charge states of large analytes**
    - **Results in "folded-over" spectra which can be recorded over narrower m/z range**
  - **Very soft ionization technique**
    - **Applicable to labile molecules and noncovalent complexes**
  - **Low tolerance for nonvolatile salts, buffer additives, and detergents**
    - **Rigorous sample clean-up required for some applications**
  - **High sensitivity**
    - **Applicable to analyte concentrations < 1 nM**



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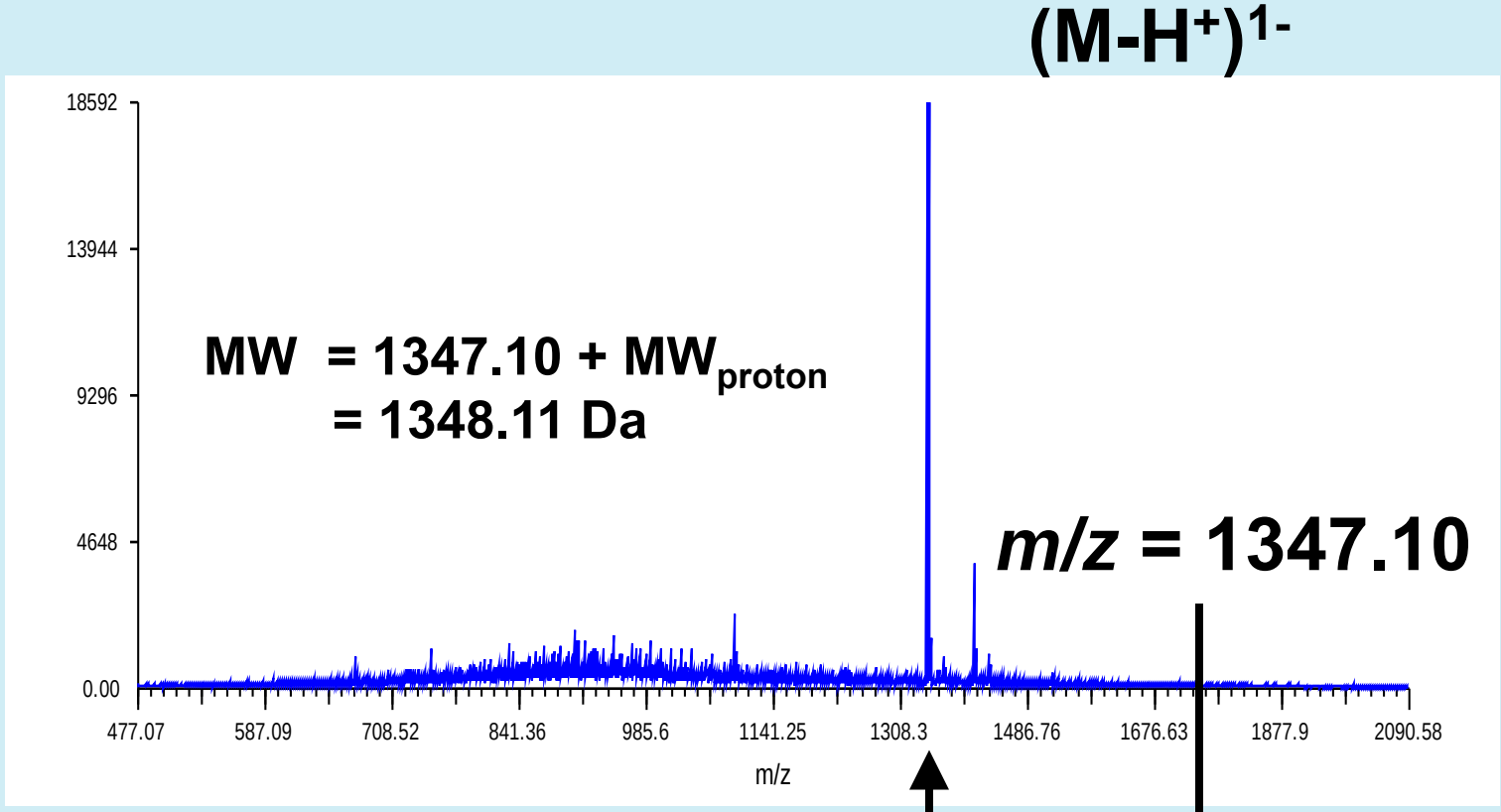
A Negative Ionization Mode ESI Mass Spectrum of a Low MW Analyte: Singly Charged Spectrum



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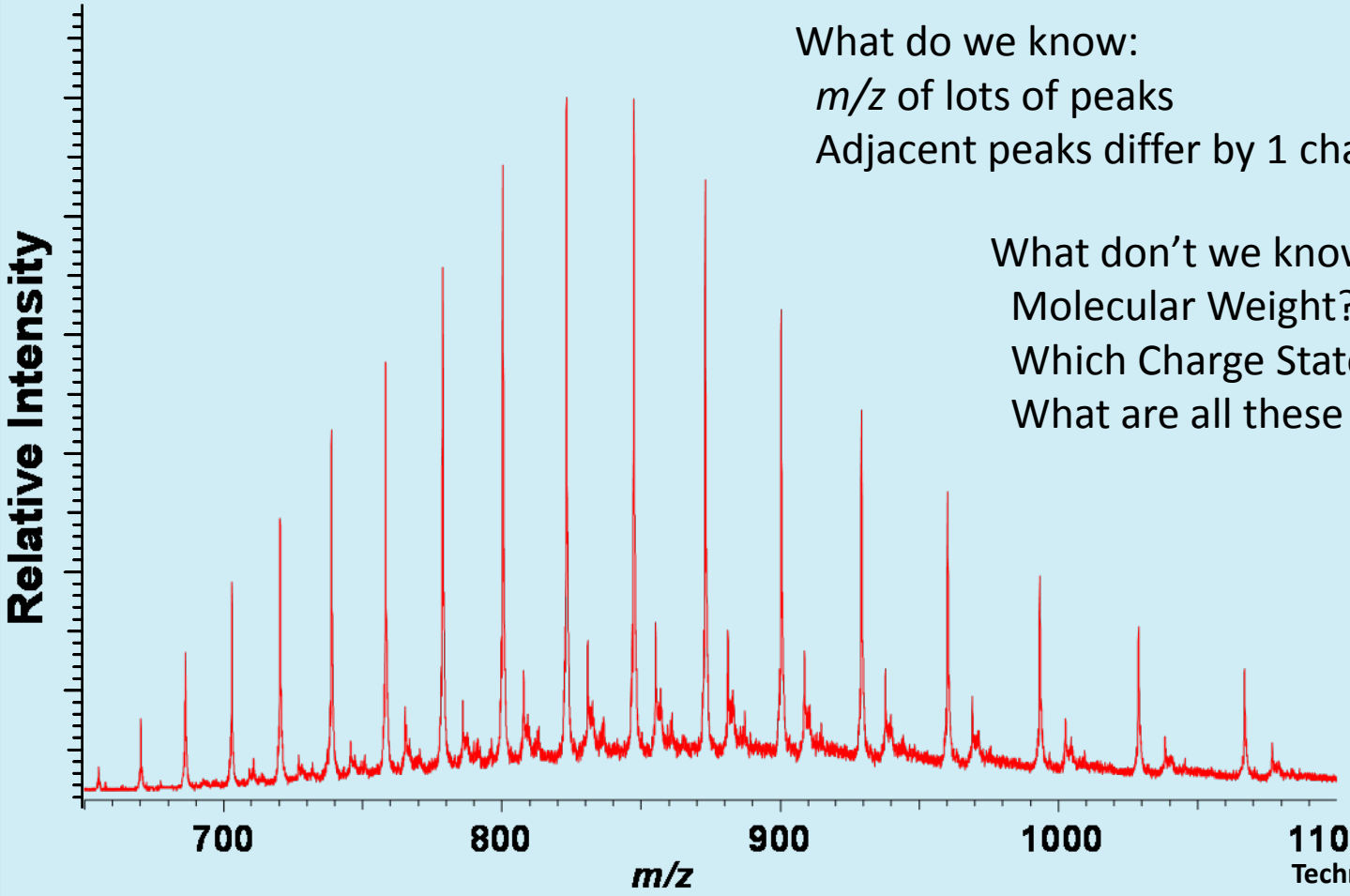
An ESI (+) Mass Spectrum of a High MW Analyte (Carbonic Anhydrase Protein MW = 28,821 Da)



What do we know:

m/z of lots of peaks

Adjacent peaks differ by 1 charge

What don't we know:

Molecular Weight?

Which Charge States?

What are all these peaks?

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Conventional "Deconvolution" of ESI-MS Spectrum

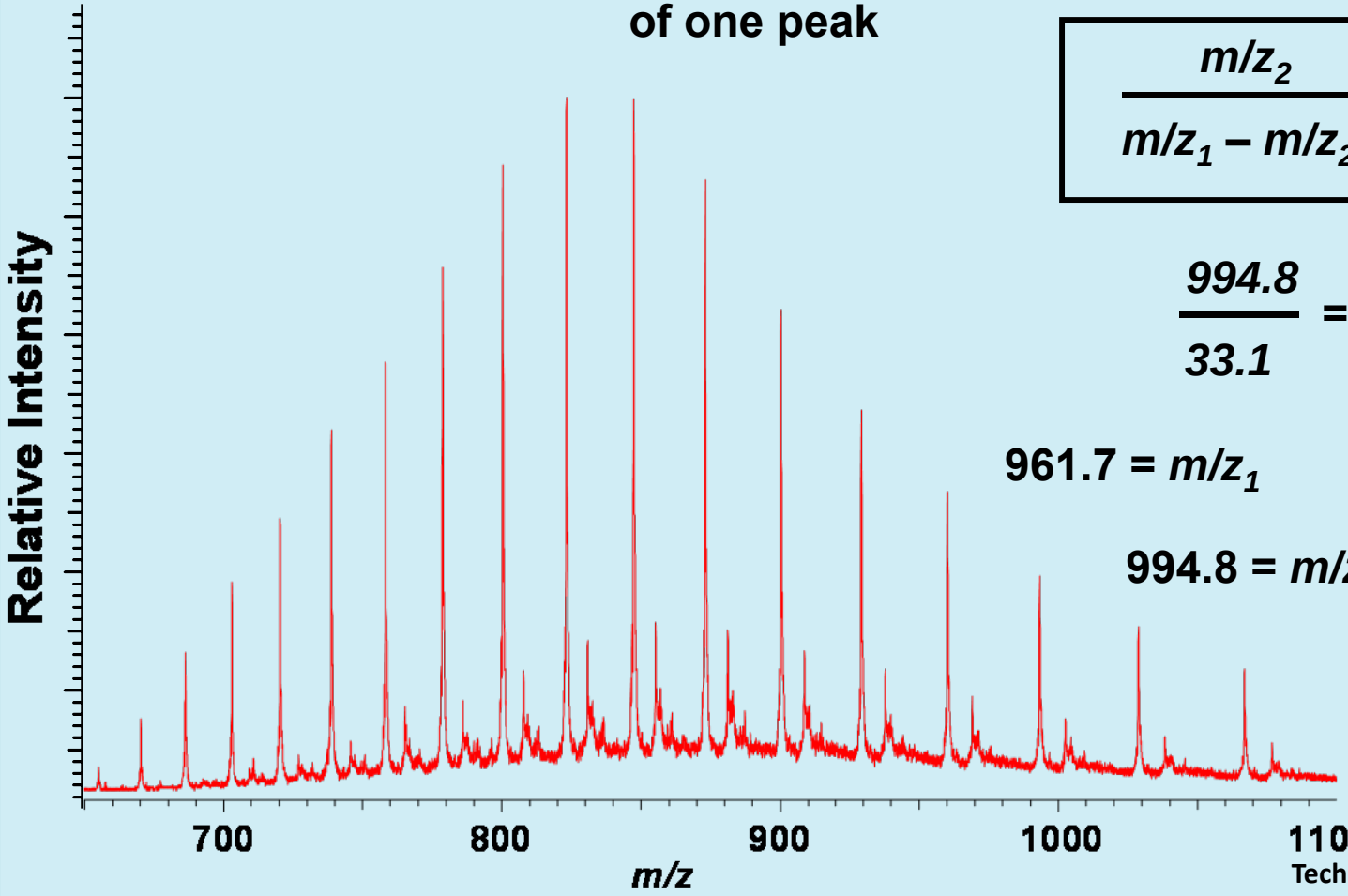
- **From m/z of peaks and Δz, calculate z of one peak**

$$\frac{m/z_2}{m/z_1 - m/z_2} = z_1$$

$$\frac{994.8}{33.1} = 30 = z_1$$

961.7 = m/z_1

994.8 = m/z_2



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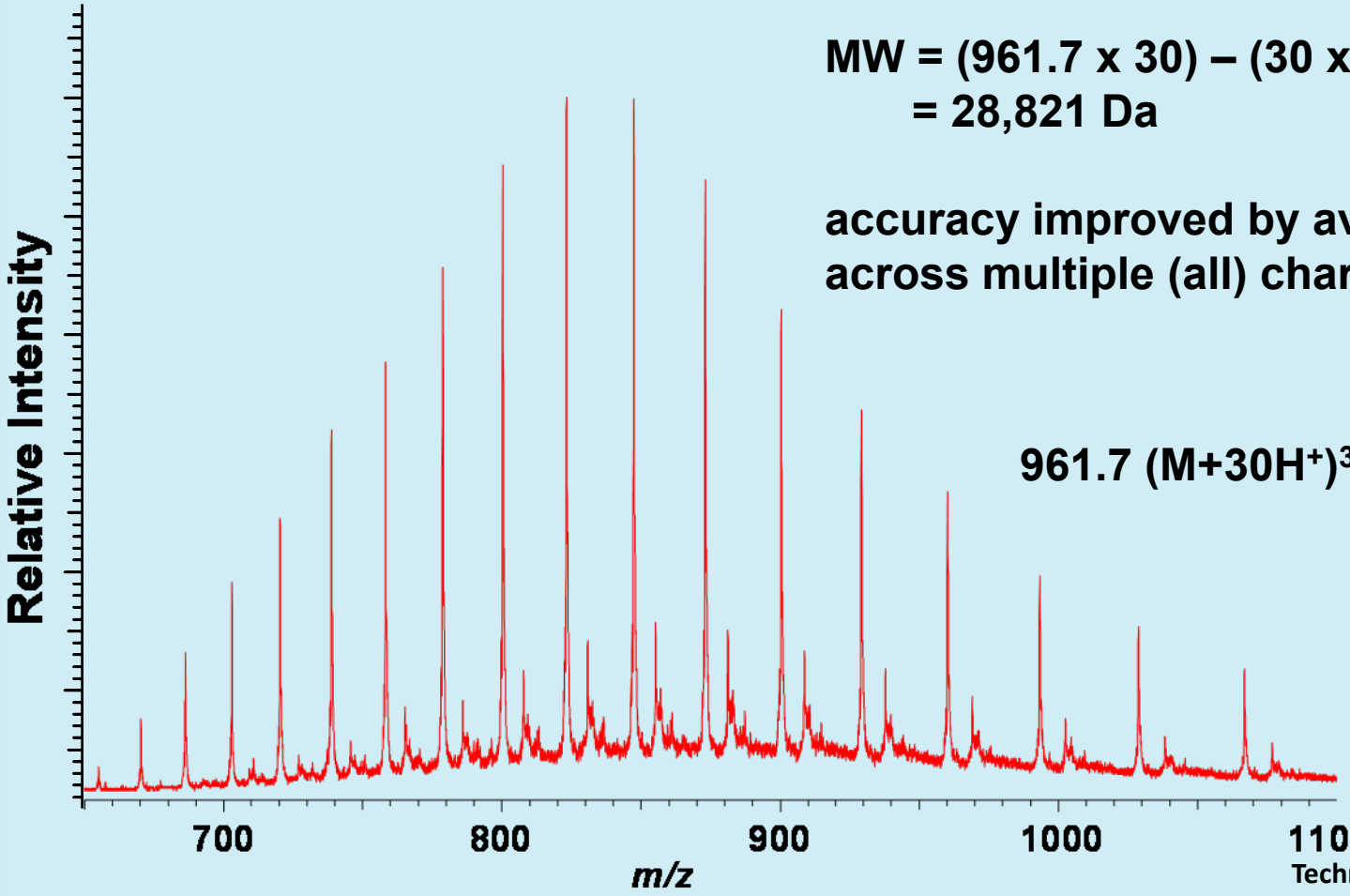
Conventional "Deconvolution" of ESI-MS Spectrum

- **Use *z* and *m/z* to calculate mass**

MW = (961.7 x 30) – (30 x 1.0078)
= 28,821 Da

accuracy improved by averaging across multiple (all) charge states



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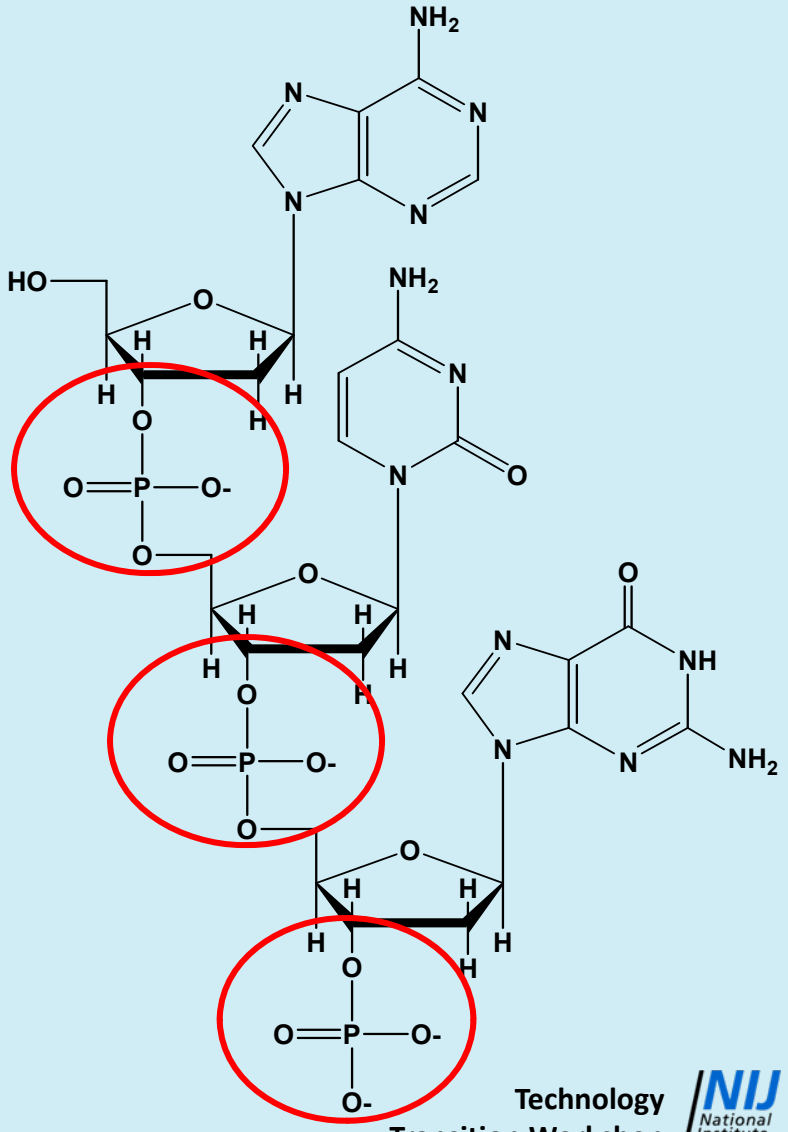
ESI-MS of DNA

- **Phosphodiester backbone is easily deprotonated at high pH**
- **ESI most effective in negative mode (in positive ionization mode basic groups on bases are protonated)**
- **Both backbone and nucleobase linkages to sugar are relatively labile**
- **We have optimized solution and interface conditions for DNA analysis by mass spectrometry over the past 10 years**



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Then and Now…

- **Then (1981)**
  - **Pre-contributions of Fenn, Tanaka, Hillenkamp / Karas**
  - **20-mer DNA**
  - **Cf-252 desorption – TOF**
  - **M/ΔM about 25**
  - **MW = 6301 + 5**
    - **About 800 ppm**

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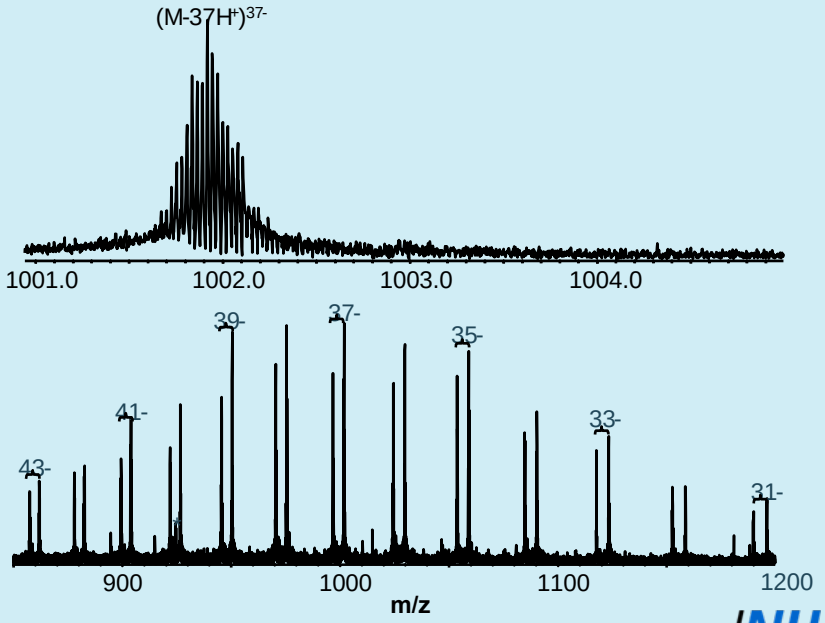
Then and Now…

- **Now**
  - **Additional contributions from Marshall, McLafferty, McLuckey, Smith and others**
  - **120-mer DNA acquired in fully automated modality**
  - **ESI-FTICR**
  - **M/ΔM = 150,000**
  - **MW = 37,091.18 + 0.04**
    - **About 1 ppm**



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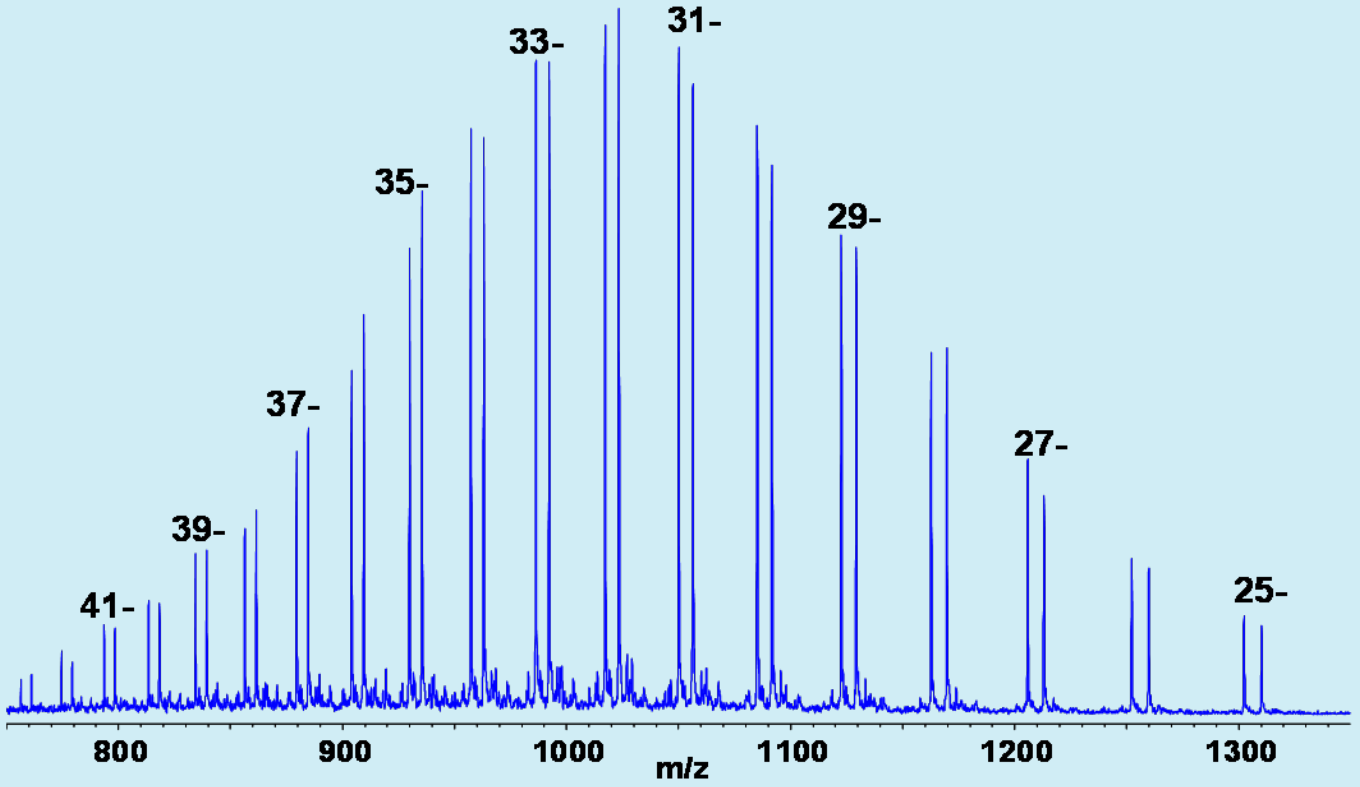
An ESI Mass Spectrum of a PCR Product

- **Doublet peaks at each charge state correspond to forward and reverse strands of amplicon**



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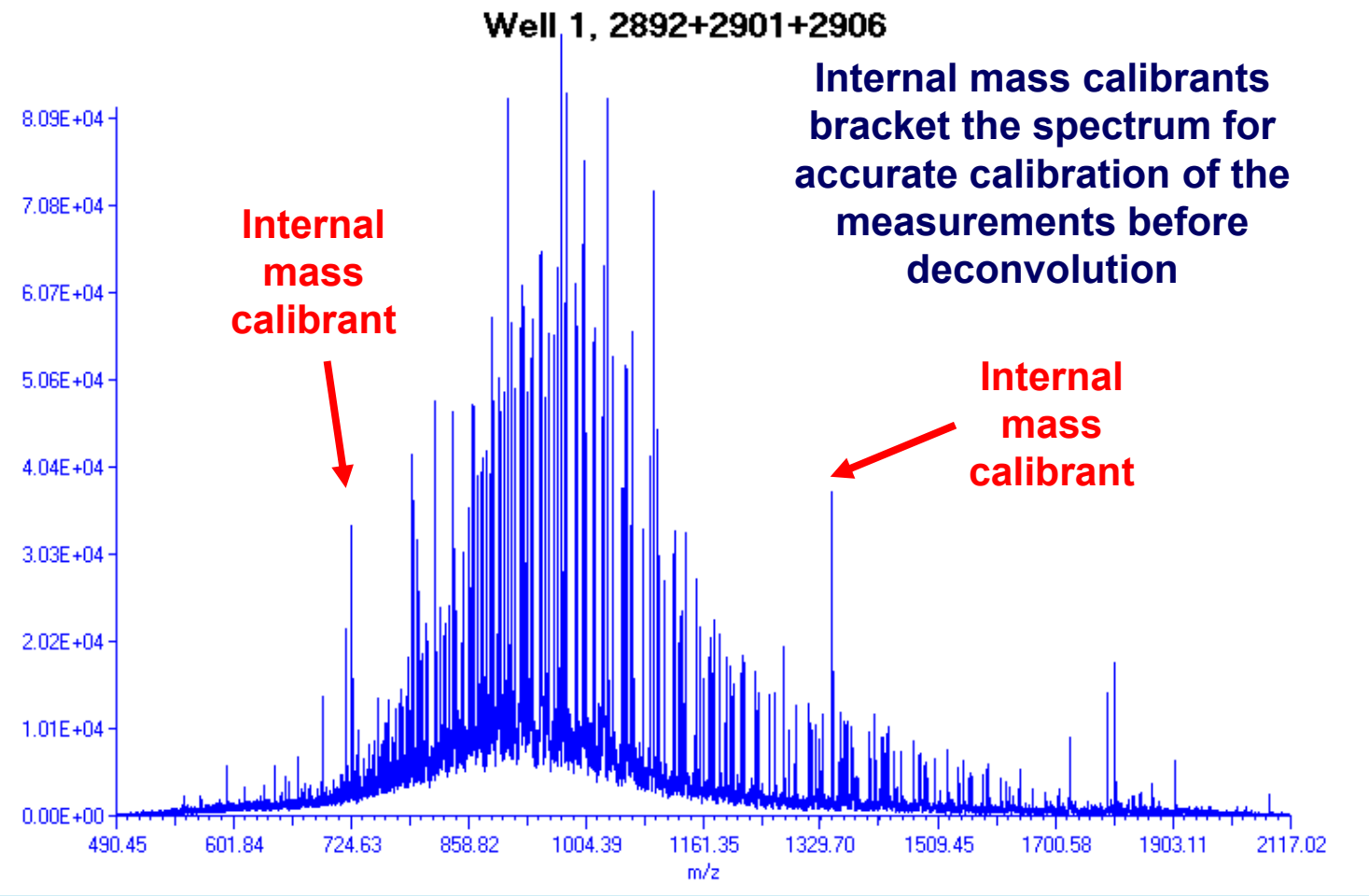
"Raw" ESI-MS Spectrum from 3-plex PCR Mix



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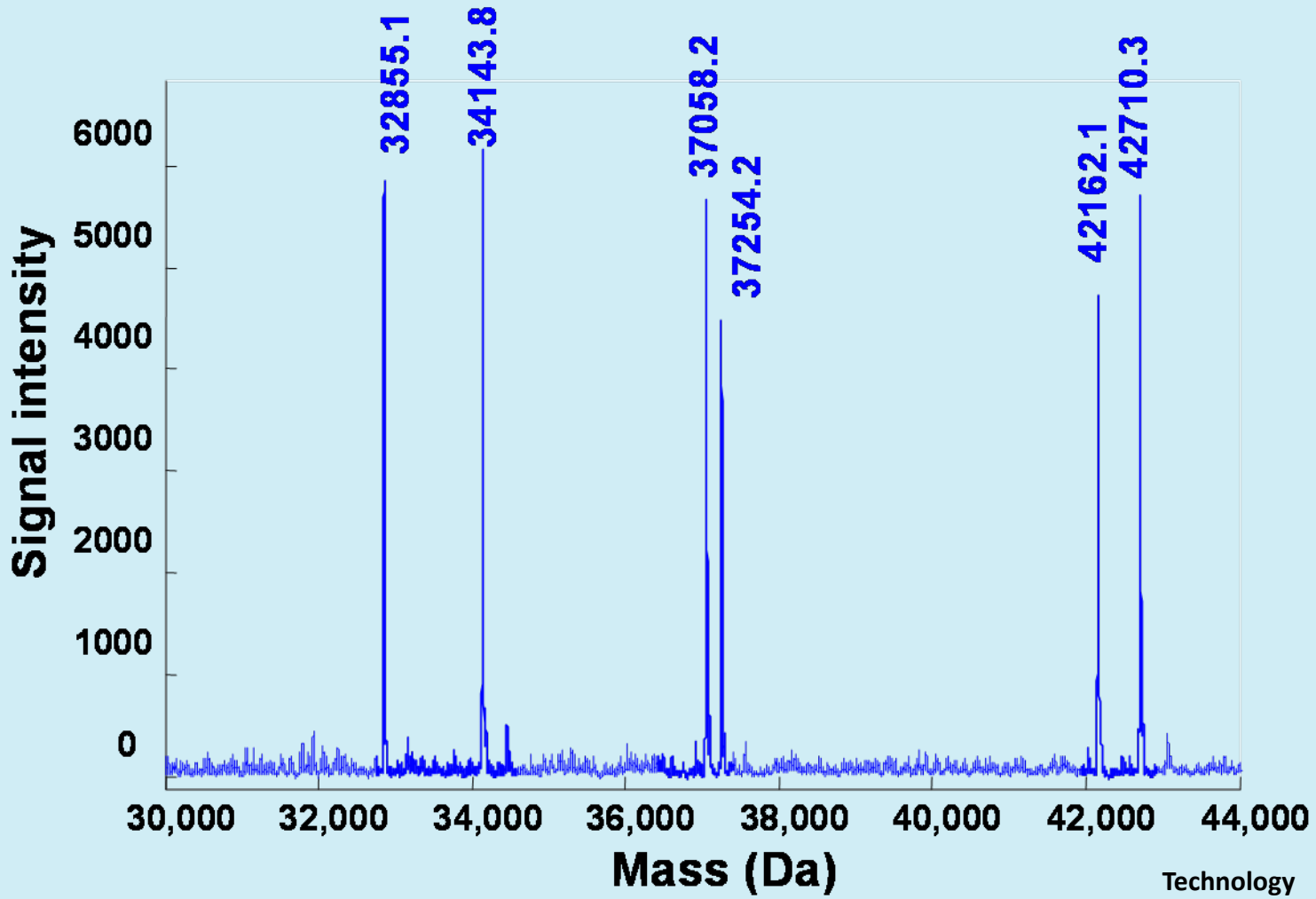
Deconvolved ESI-MS spectrum from 3-plex PCR



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Masses to Base Composition

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Penny = 2.500 g
Nickel = 3.950 g
Dime = 2.268 g
Quarter = 5.670 g

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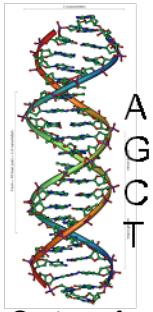
Scale

Weight = 4.6 grams
∴ 2 dimes



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A = 313.0576 amu
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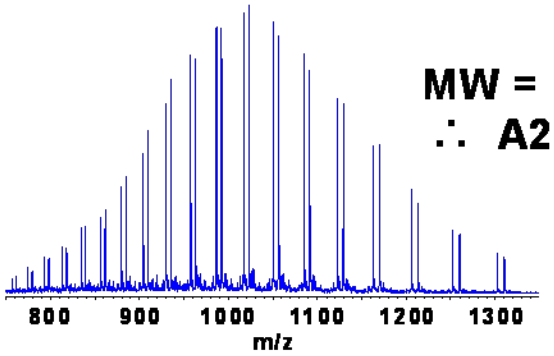

Mass spectrum

MW = 32,588.90 amu
∴ A28 G29 C25 T24

Requires 25 ppm mass measurement error

Math takes into account Watson-Crick base pairing

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Masses to Base Composition

- **Require masses of both strands and fact that the strands are complimentary to determine base composition**

  Single Strand: 32889.450 Da

  (+ 25 ppm or 0.75 Da): 928 base comps

  (+ 1 ppm or 0.03 Da): 82 base comps

  Single Strand: 33071.462 Da

  (+ 25 ppm or 0.75 Da): 948 base comps

  (+ 1 ppm or 0.03 Da): 95 base comps

Da: Dalton (atomic mass unit) **ppm: part per million**

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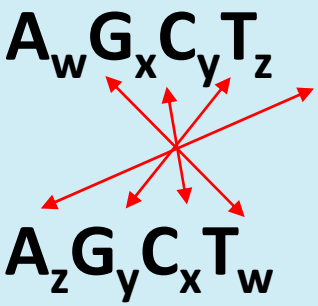
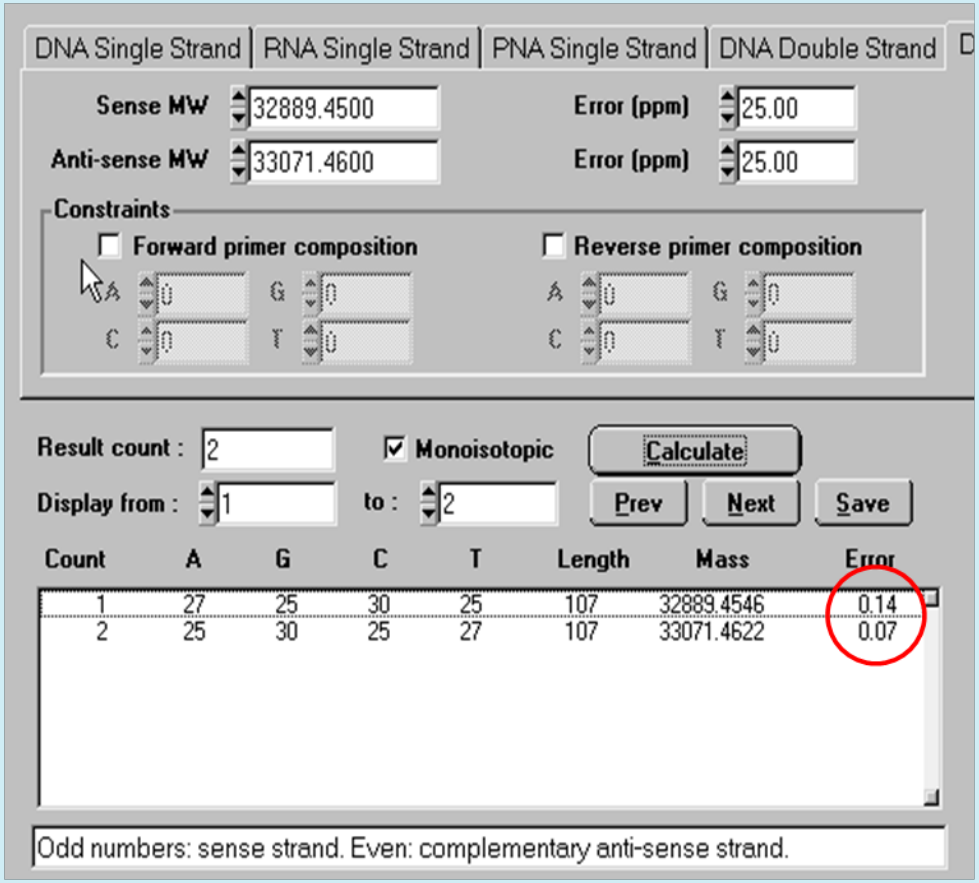
Exact Mass Measurements of Both Strands Facilitates Unambiguous Base Composition Determination

ppm	# comp pairs
0-25	1
50	13
100	66
250	378
500	1447





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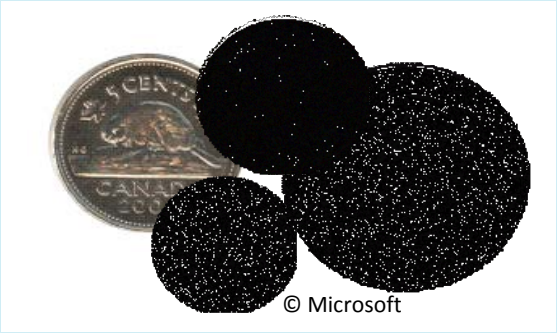
Mass Measurement and the "Canadian Nickel"



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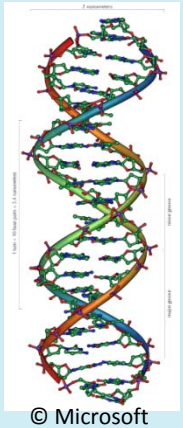
Penny = 2.500 g
Nickel* = 3.950 g
Dime = 2.268 g
Quarter = 5.670 g

- **The "Coins and Scale" analogy doesn't work if using all US coins as a US Nickel weighs 5.000 g**
  - **Thus 5 g could be two pennies or one nickel**
- **Interesting parallel to nucleobases with mass measurement error**
  - **A mass shift of 15 ± 1 Da could be a A → G or a C → T**
  - **A double SNP A →G and T → C would result in a 1 Dalton difference**
  - **A one Dalton uncertainty is consistent with two base compositions**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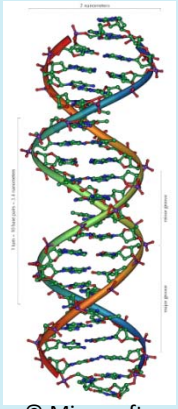
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G = 339.1662 amu
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- **We have a "Canadian Nickel" nucleobase**
  - **^{13}C labeled guanosine shifts the mass by 10 Da per incorporation**
  - **No confusion over which SNP is present**
  - **No uncertainty as to whether the A/G T/C double SNP is present**

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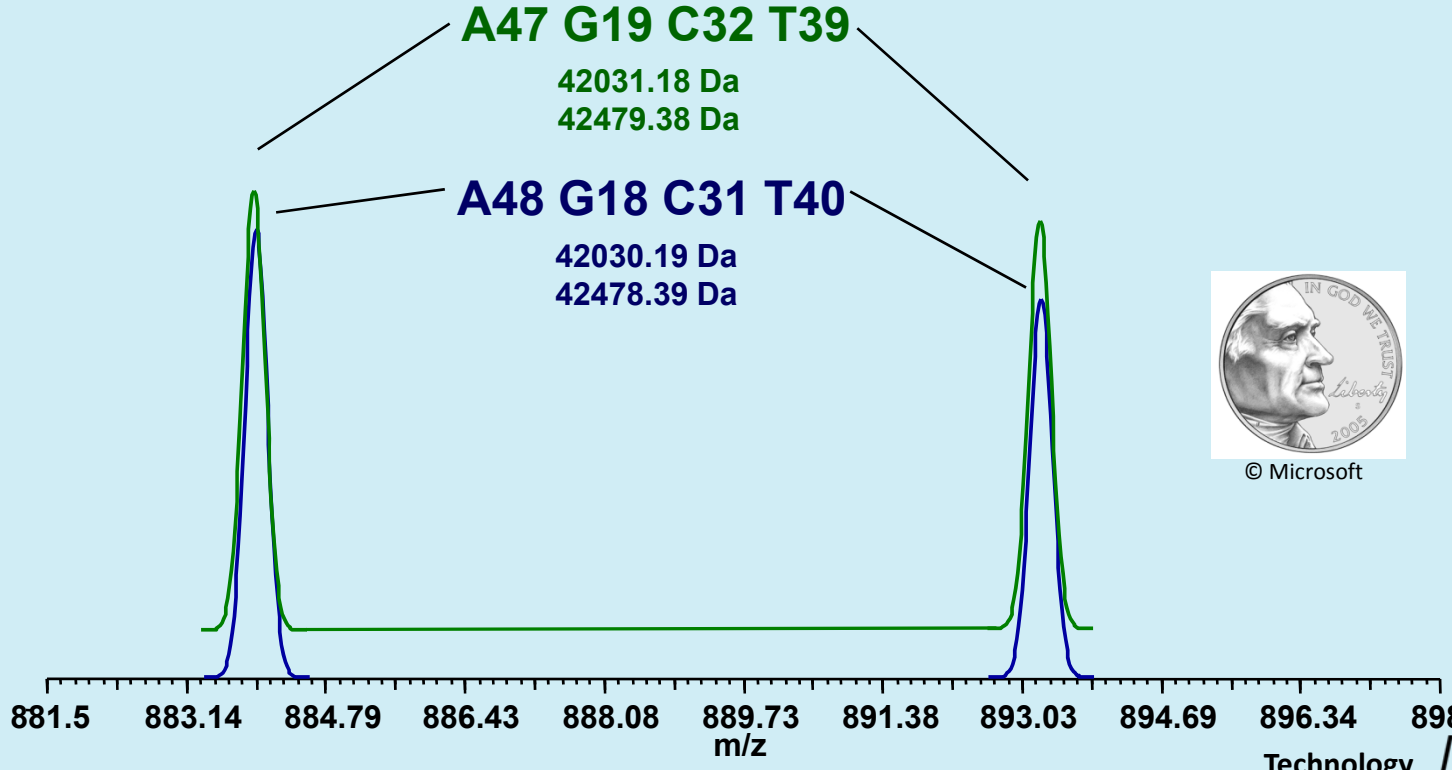
High Mass Precision and Mass Tag Combine to Provide Unambiguous Base Compositions in Routine Operation

Some double SNPs cause small mass differences

Without mass tag:

Product strands differ by 1 Da for two products that differ by a G→A and C→T SNP at the same time.



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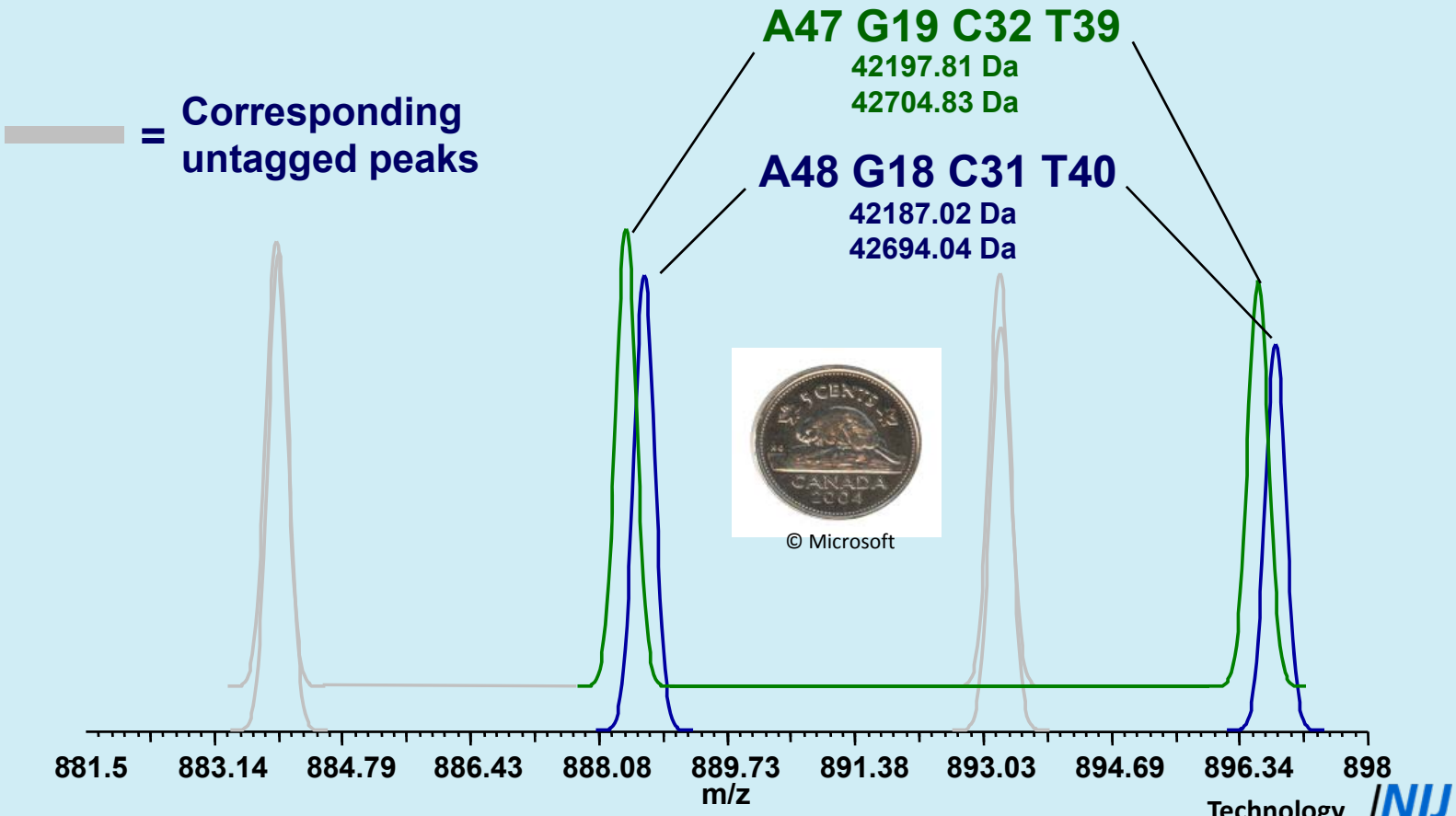
Mass Tag Increases Mass Separation for These SNPs

With mass tag:

With ^{13}C-dGTP, the mass separation increases to ~10 Da for each strand



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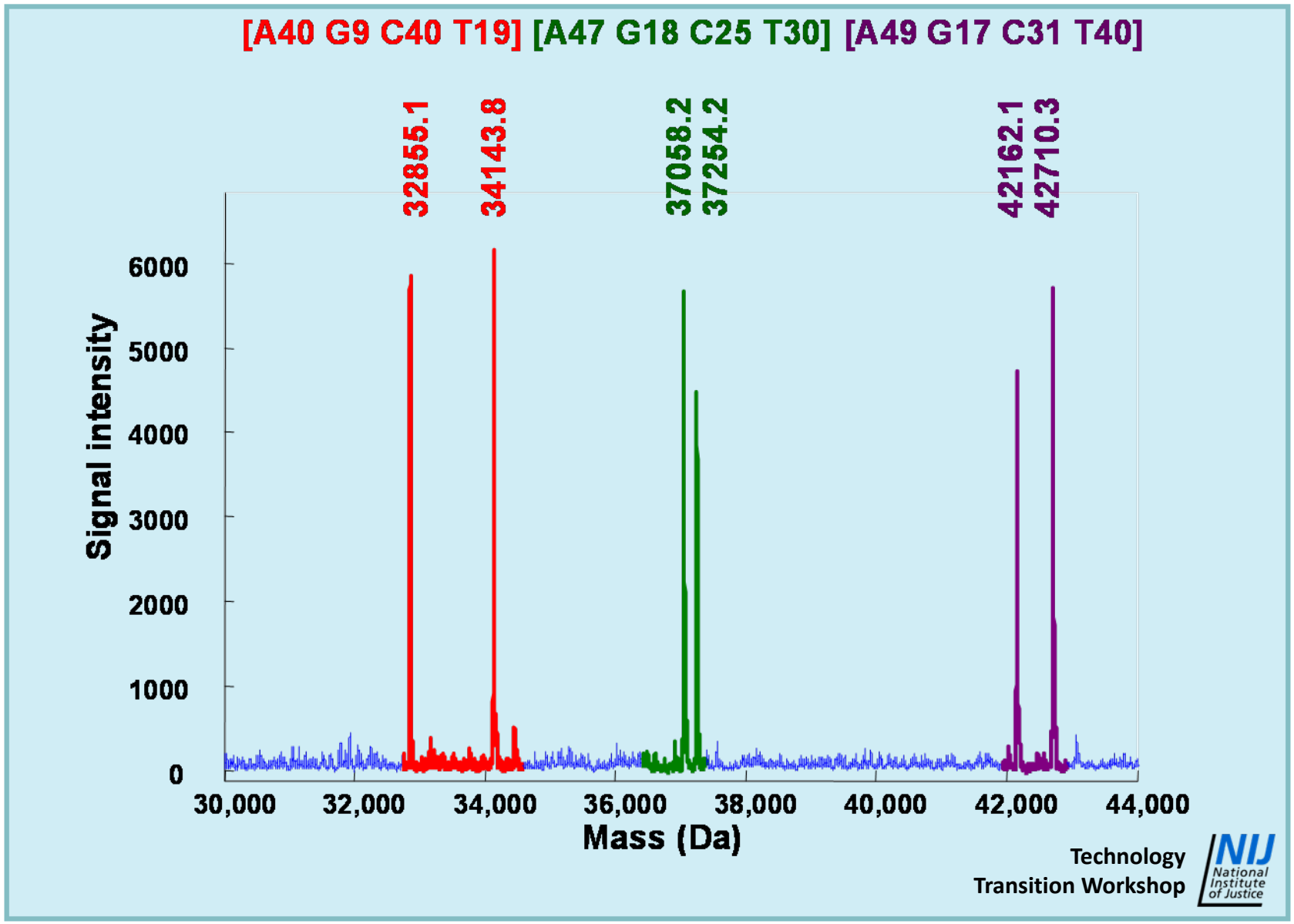
[A40 G9 C40 T19] [A47 G18 C25 T30] [A49 G17 C31 T40]
32855.1
34143.8
37058.2
37254.2
42162.1
42710.3
Signal intensity
6000
5000
4000
3000
2000
1000
0
30,000
32,000
34,000
36,000
38,000
40,000
42,000
44,000
Mass (Da)
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Salt is a Killer…

- **Nonvolatile counterions (e.g., Na^+, K^+, Mg^{2+}) are not removed during desolvation**
  - **High concentrations can preclude the generation of a stable ESI plume**
- **Oligonucleotides are more vulnerable to contamination than proteins**
  - **Phosphodiester backbone is highly anionic**
  - **Larger oligonucleotides more salt intolerant than smaller ones**
- **Effects of salt can be partially mitigated by choice of buffers**
  - **See Griffey et al. RCMS 9, (1995) 97-102**

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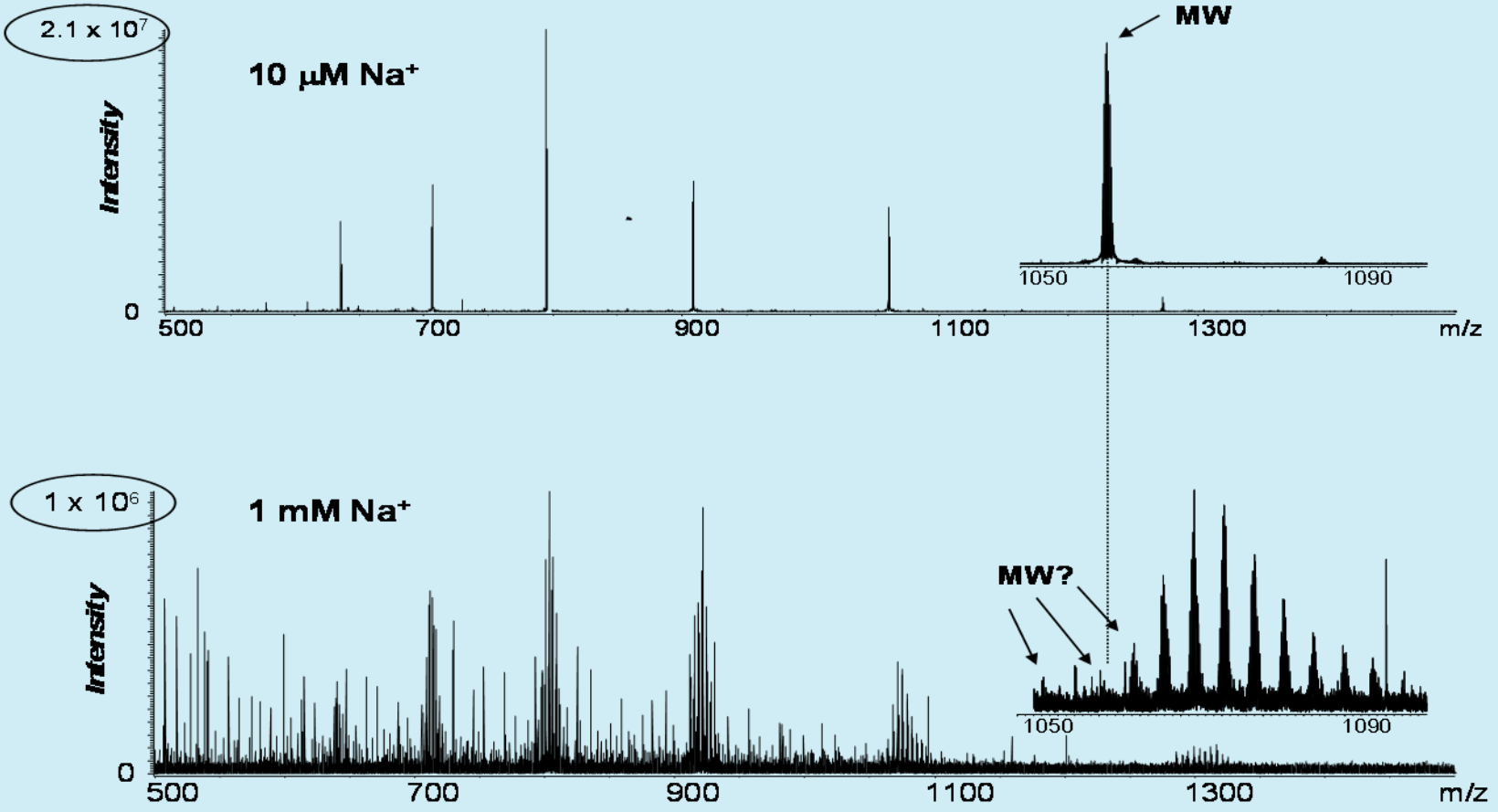
Salt is a Killer…

ESI-MS of 20-mer phosphorothioate oligonucleotide



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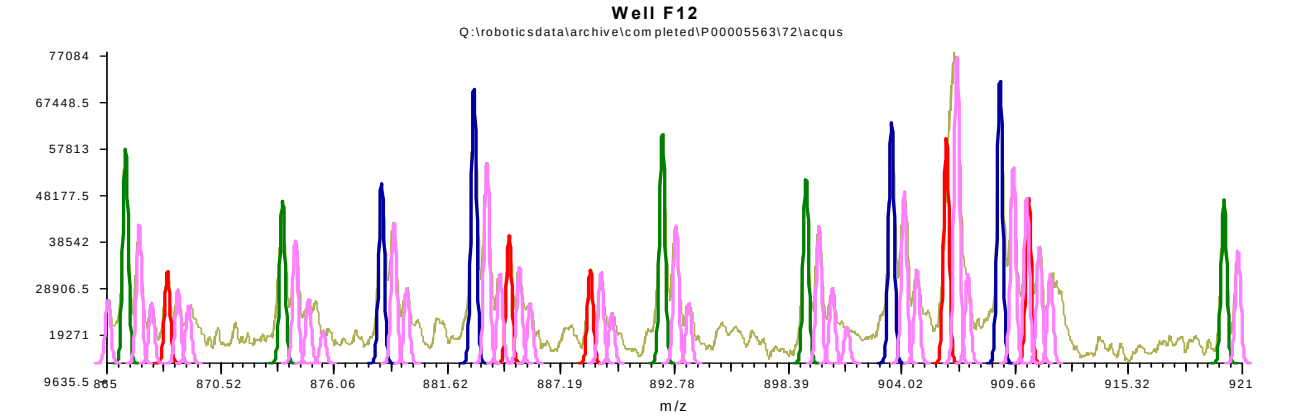
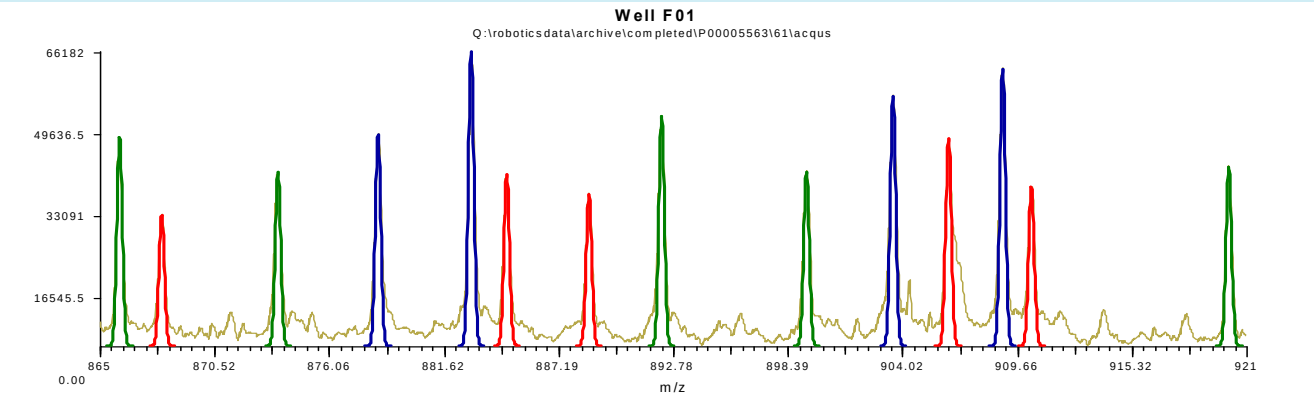
Comparison of Raw Data for Adducted versus Non-adducted Mass Spectrum

Adducted



Not adducted



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Mass Analyzers

- All work by measuring the response of charged particles to electric and / or magnetic fields
- All work at reduced pressure to reduce ion-neutral collisions
  - Want to minimize scatter and / or neutralization
  - Typical operating pressures
    - linear quadrupoles ~ 5 x 10^{-5} torr
    - FTICR < 10^{-9} torr
    - TOF 10^{-5} – 10^{-7} torr

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Highlights of TOF-MS Advantages

- **Simple and rugged benchtop construction**
- **Theoretically unlimited mass range**
- **Adaptable to many ionization sources**
- **Fast acquisition – signal averaging to improve S/N**
- **Mass accuracy rivals that of FTICR**

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Highlights of TOF-MS Disadvantages

- **Limited resolution**
  - **Theoretically limited to detection electronics**
  - **Practically limited by energy and spatial spreads in ions**
- **TOF is inherently pulsed**
  - **Must wait for longest flight time ions before sending next packet of ions (Hz to kHz typical repetition rates)**
  - **Cannot simultaneously measure all m/z values**
    - **This is mitigated by external ion accumulation**

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Time-of-flight (TOF) Mass Analyzers

- **Ions are accelerated by electric field (V/d)**
- **Ions then drift at their final velocity for a fixed distance**
- **Ions impact a detector and their flight time is recorded**
  - **Flight time is :**
    - **Proportional to velocity**
    - **Proportional to the square root of m/z,**

$$K.E. = \frac{1}{2}mv^2 \Rightarrow \frac{1}{2}\left(\frac{m}{z}\right)\vec{v}^2 = \vec{V}/d$$

where ν is velocity, V/d is field strength

$$t = L/\nu = L\sqrt{m/2zV}$$

t: sec L: meters ν: velocity m: kg *z*: Coulombs V: volts

lower *m/z* ions reach higher velocity than higher *m/z* ions

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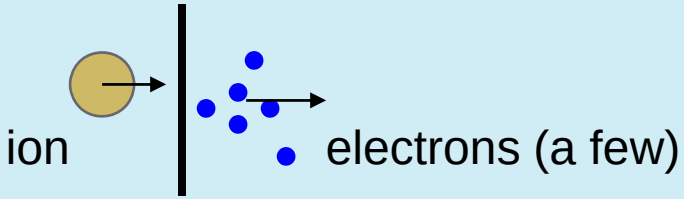
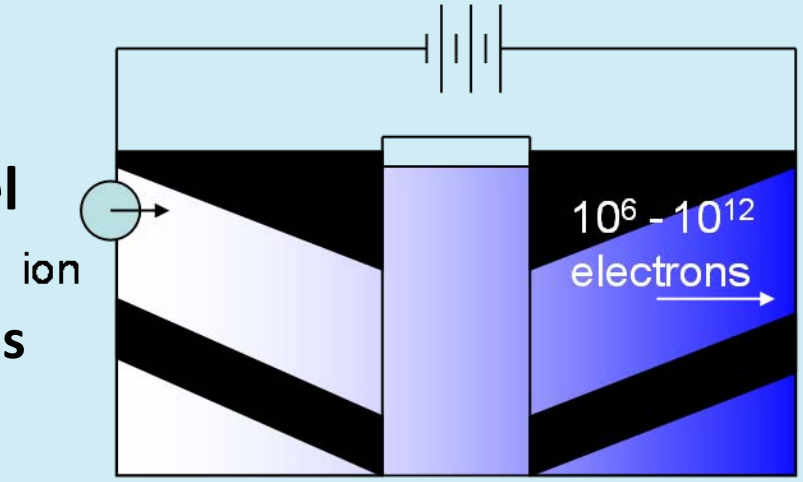
TOF-MS Detection Schemes

- **"Particle impact, electron generation, and detection"**
  - **Electron multiplier**
  - **Microchannel plate**
  - **Hybrids or other particle detectors**
- **Simplest example: metal foil**



- **Most common: microchannel plate**
  - **Array of tilted glass channels**
  - **2 to 10 microns**
  - **Electron cascade = gain**
  - **Also used in night vision**



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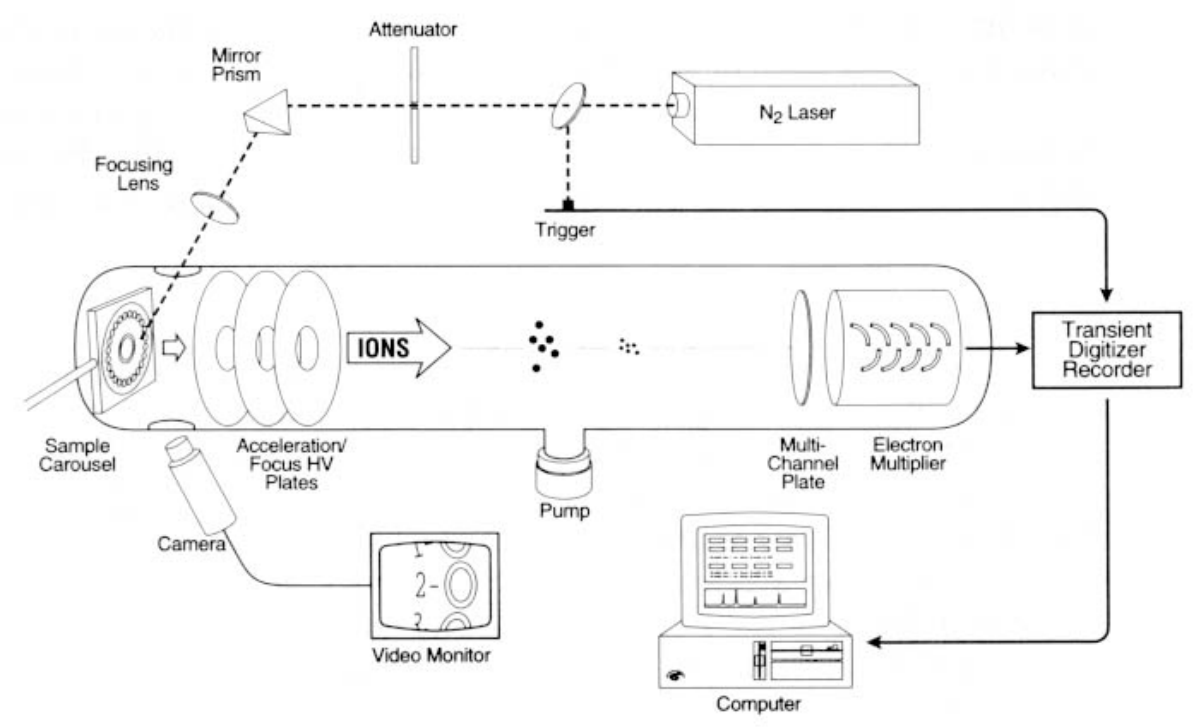
Mass Analyzers – TOF

- **Linear geometry**
  - **Ions drift in field-free region, but energy spread (+DE) leads to time spread (-DT) (more energy gives shorter TOF)**



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Mass Analyzers – TOF

- **Reflectron**
  - **Ions drift, but at ion mirror they turn around**
  - **+ΔE (energy spread) leads to deeper penetration in ion mirror**
  - **Linear config: +ΔE leads to -ΔT**
  - **Reflectron config: +ΔE leads to -ΔT+ΔT (= 0; energy spread eliminated at detector)**

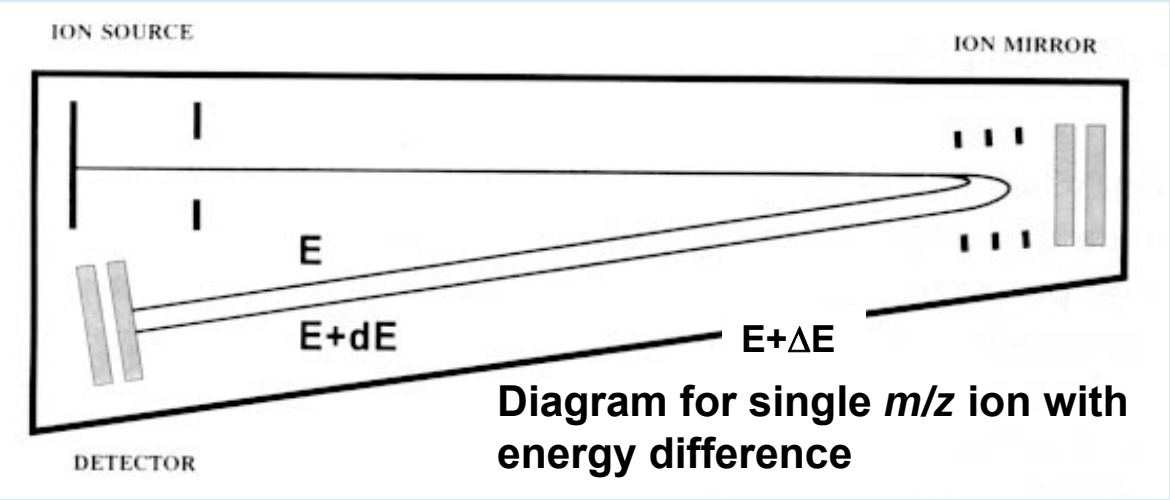

Diagram for single *m/z* ion with energy difference

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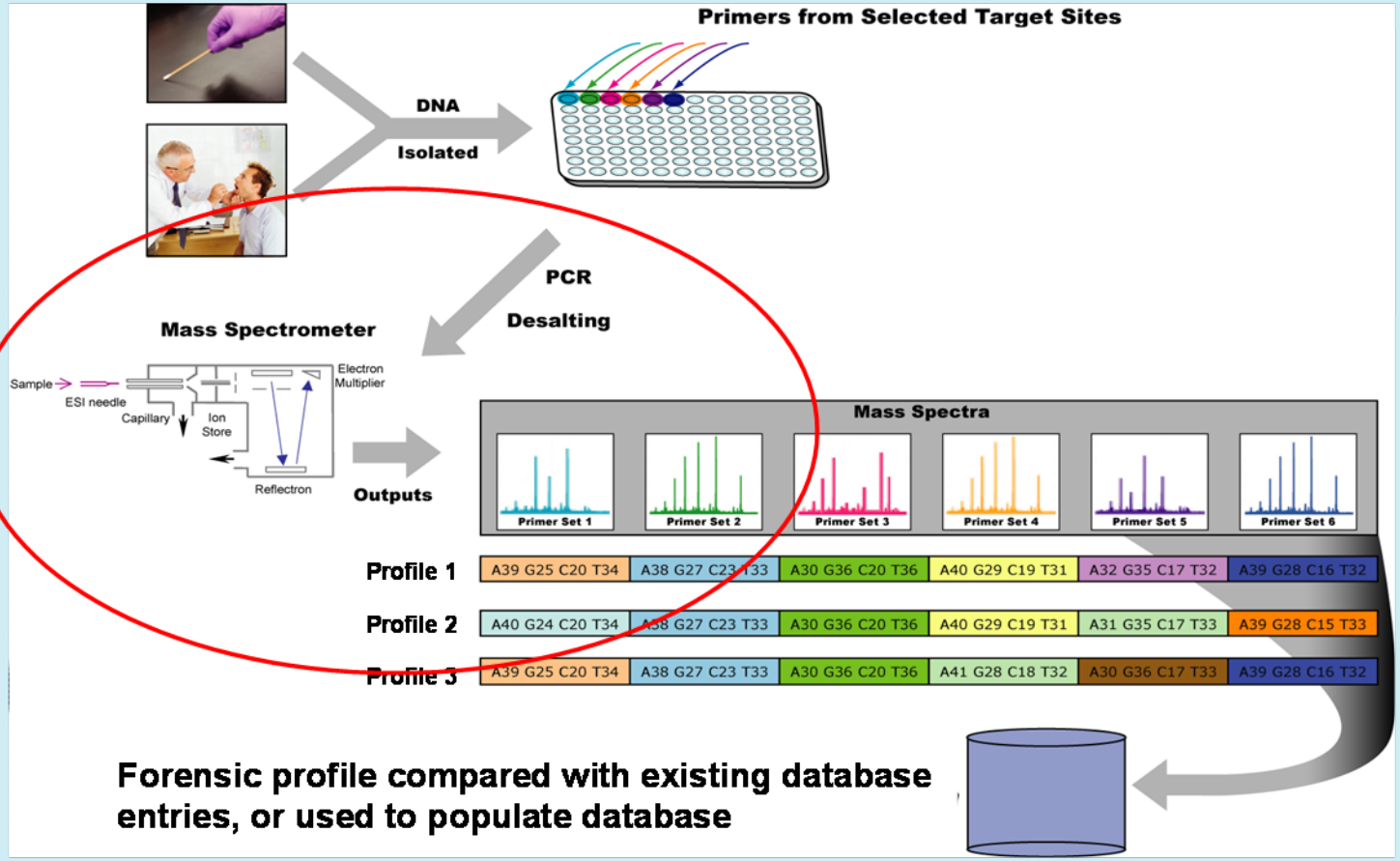
Mass Spectrometry and T5000™



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Conclusions

- **In general, mass spectrometry is used to "weigh" molecular analytes of interest**
- **Electrospray ionization is employed as it can promote large, intact oligonucleotides into the gas phase**
- **Time-of-Flight mass spectrometry is used as it provides accurate molecular weight measurements in a robust, benchtop, instrument format**
- **As part of the Ibis™ process, amplified DNA is "weighed" with enough accuracy to unambiguously determine base composition [AGCT]**
- **Base composition profiles can be compared to other profiles and / or databases**

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Abbreviations and Jargon

APCI	atmospheric pressure chemical ionization
bp	base pair(s)
CAD	collisionally activated dissociation
Da	Dalton = atomic mass unit
DNA	deoxyribonucleic acid
Ds	double stranded (DNA)
EI	electron impact (ionization)
ESI	electrospray ionization
FAB	fast atom bombardment
FD	field desorption (ionization)
FI	field ionization
FTICR	Fourier transform ion cyclotron resonance
FTMS	Fourier transform mass spectrometry
FWHM	full width half maximum (used to specify resolution)
GC	gas chromatography
Hz	Hertz (cycles/second)
IRMPD	infrared multiphoton dissociation
kDa	kilo Dalton(s)
m/Dm	mass divided by peak width (mass resolution)
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption ionization
MSAD	multipole storage assisted dissociation
mtDNA	mitochondrial deoxyribonucleic acid
MW	molecular weight
PCR	polymerase chain reaction
PD	plasma desorption
ppm	parts per million
QIT	quadrupole ion trap
Q-TOF	quadrupole-time-of-flight
rf	radio frequency
SIMS	secondary ion mass spectrometry
ss	single stranded (DNA)
TOF	time-of-flight
TSP	thermospray (ionization)

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Questions?

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