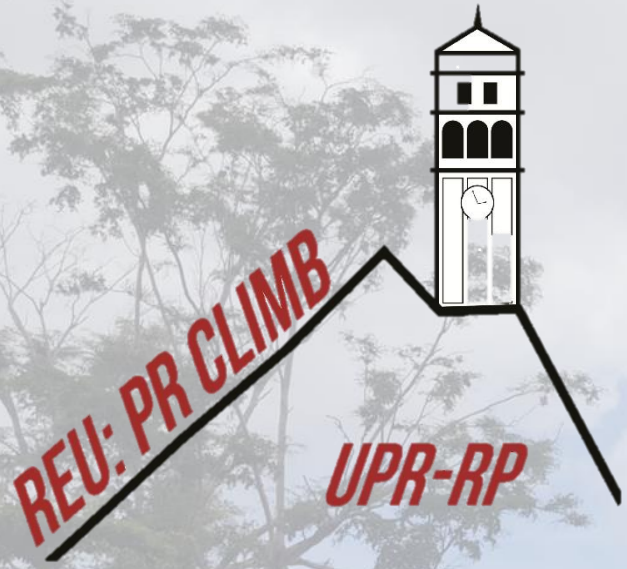


MALDI TOF/TOF Workshop

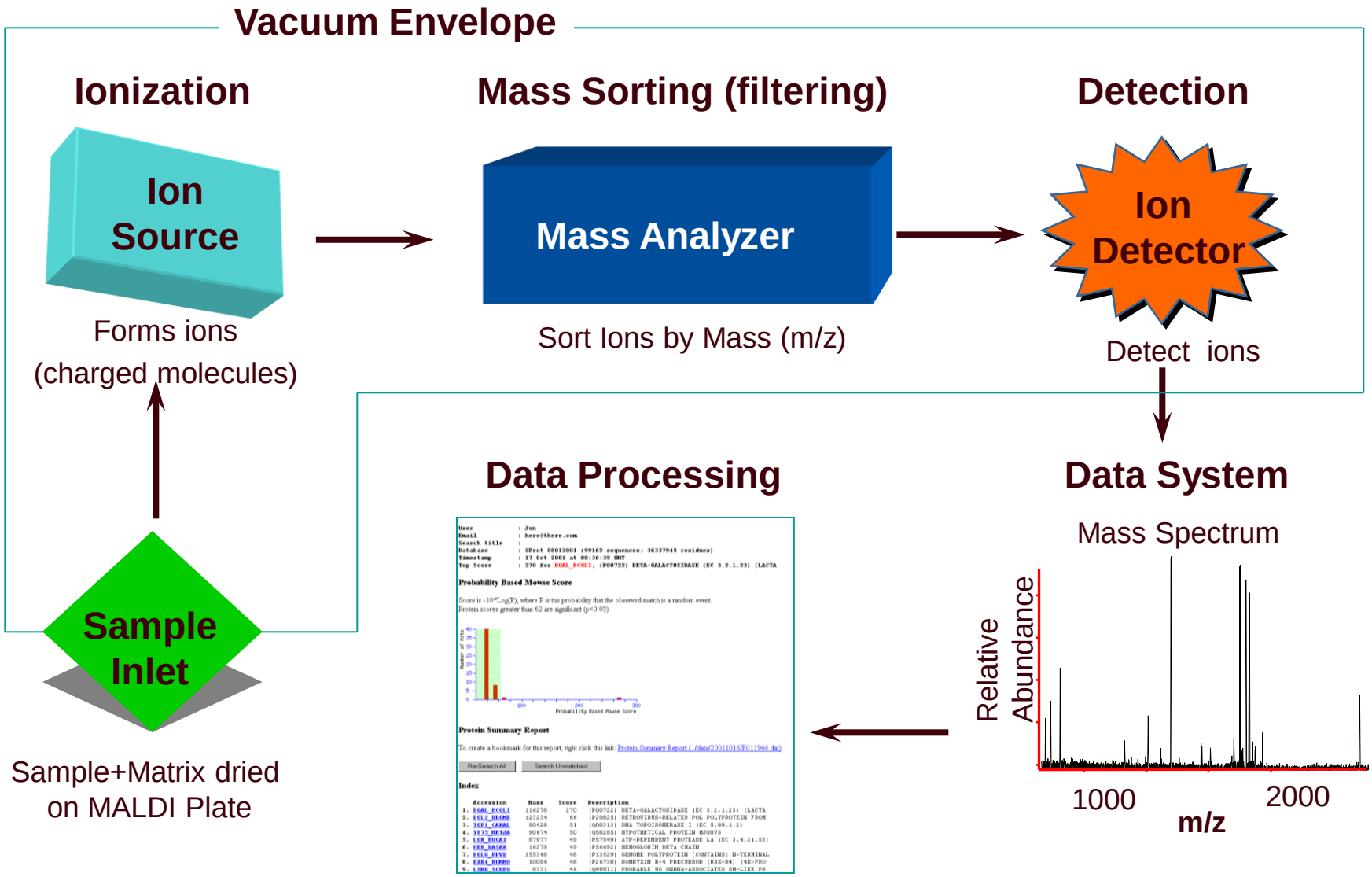
Presented by Dr. Arthur D. Tinoco

Matrix Assisted Laser Desorption Ionization Time of Flight Time of Flight

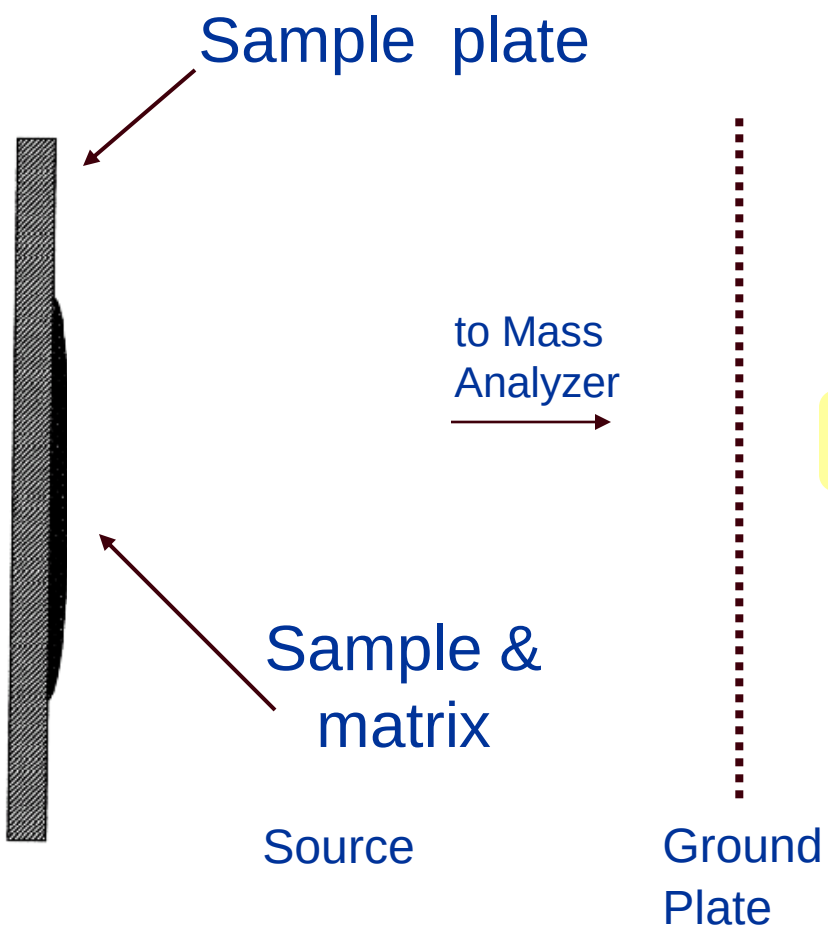
AB SCIEX 4800 MALDI TOF TOF

Concepts and Principles

Basic Components of a Mass Spectrometer



MALDI Source

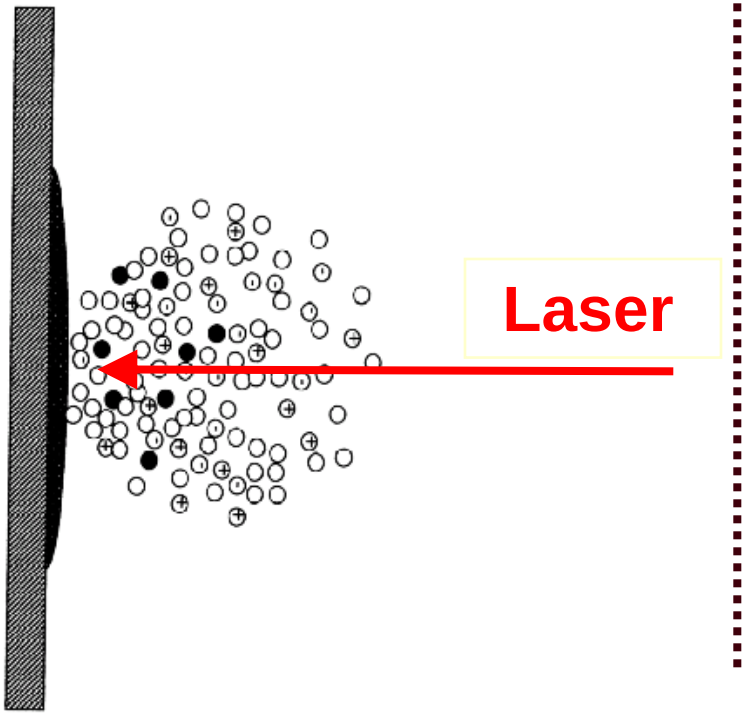


1. **Sample (M)** is mixed with a large excess of **Matrix** (X) and dried on a MALDI plate.

The plate is loaded into the **Ion Source**

Flight Tube

MALDI Laser



2. Laser flash produces matrix neutrals (X), matrix ions $(XH)^+$, $(X\text{-}H)^-$, and sample neutrals (M).

Flight Tube

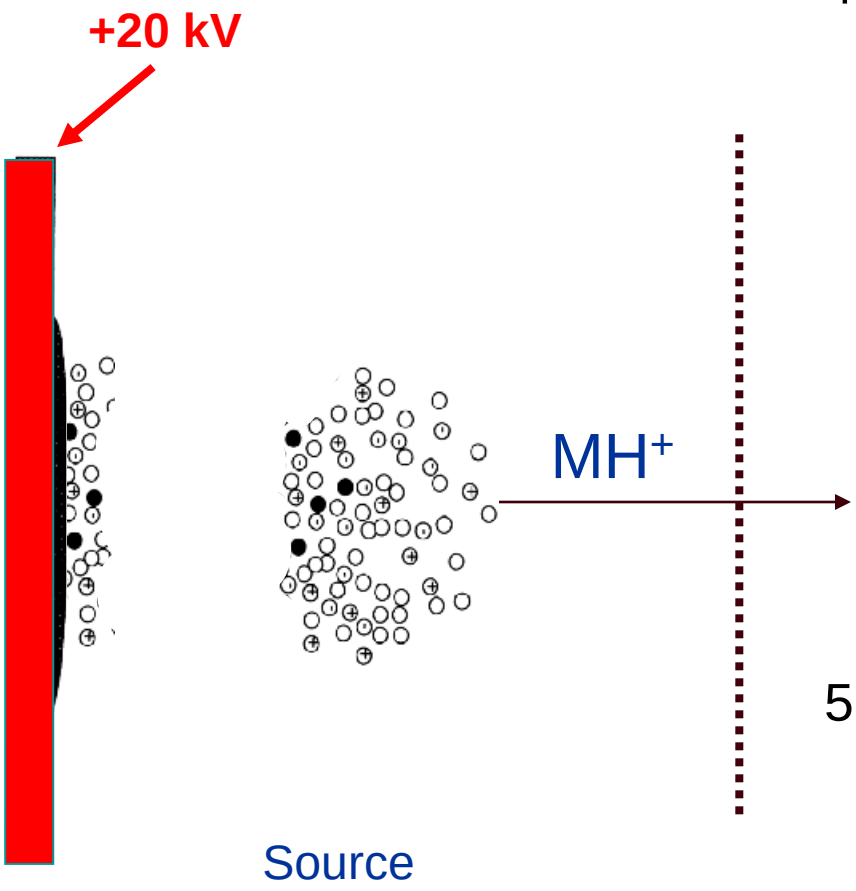
3. Sample molecules are ionized by proton transfer from matrix ions:

$$XH^+ + M \rightarrow X + MH^+.$$

$$X\text{-}H^- + M \rightarrow X + M\text{-}H^-$$

Note: The laser beam is deflected by mirrors such that it strikes the plate at a 90º angle (on-axis laser)

Ionization



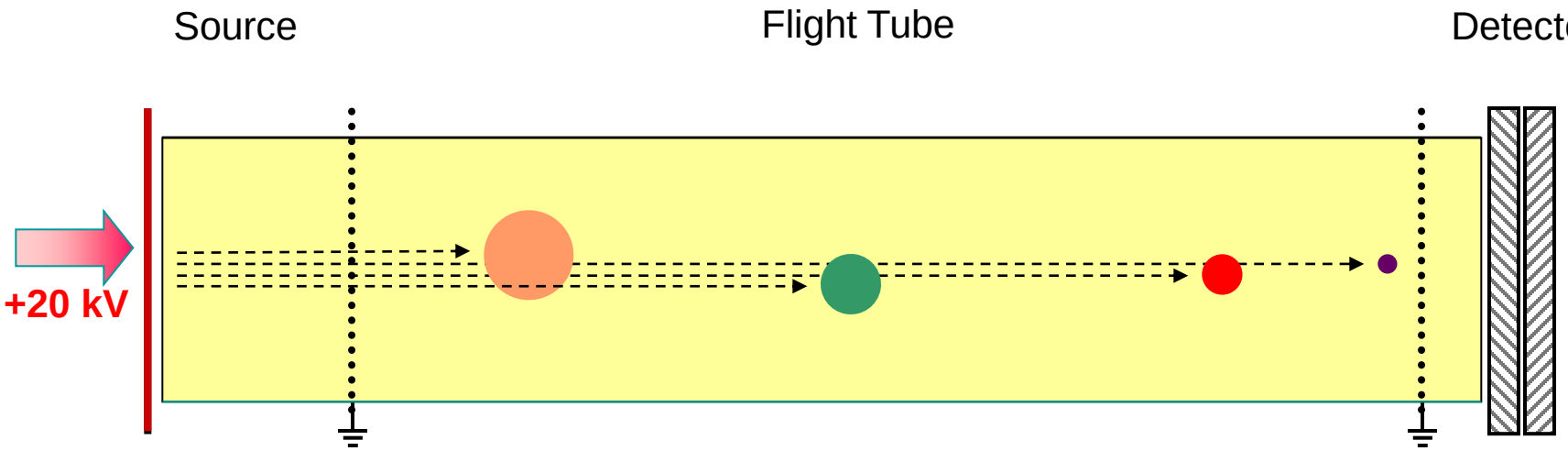
4. **Ion Extraction**: High voltage is applied to the sample plate, accelerating ions of the selected polarity out of the Ion Source into the Flight Tube.

Flight Tube

5. This cycle is repeated up to 1000 times per second.

(Note: Laser repetition rate in the 4800 MALDI TOF/TOF™ system is 200 Hz)

Time-of-Flight Mass Analyzer



Principle: All ions are accelerated with the same potential at a fixed initial point and time, and are allowed to drift (or “fly”) down the Flight Tube

Ions will separate according to their mass-to-charge ratios:
light ions accelerated to a higher velocity that heavy ions.

The lighter ions strike the detector before the heavier ions. The **time of flight** (TOF) can be used to calculate the mass-to-charge ratio.

Important Performance Factors

Mass accuracy: What is the mass assignment of a peak? How accurate is the mass measurement?

Resolution: How sharply defined / focused are the peaks? How well separated are the peaks from each other?

Sensitivity: How small an amount can be detected / analyzed?

Important Performance Factors

Mass accuracy: What is the mass assignment of a peak? How accurate is the mass measurement?

- The mass assignment will depend on whether you are collecting data in the positive or negative ion mode.
- The accuracy will depend on molecular size. Mass accuracy is better for small molecules (<10 kDa) than for big molecules (>10 kDa) and is dependent on instrument calibration.

Important Performance Factors

Resolution: How sharply defined / focused are the peaks? How well separated are the peaks from each other?

- The resolution is excellent for small molecules but poor for big molecules. You should be able to obtain a match for the isotopic distribution of the experimental MS of small molecules with that of the theoretical MS.

- Isotopic peak separation can help you determine the charge of the ion.

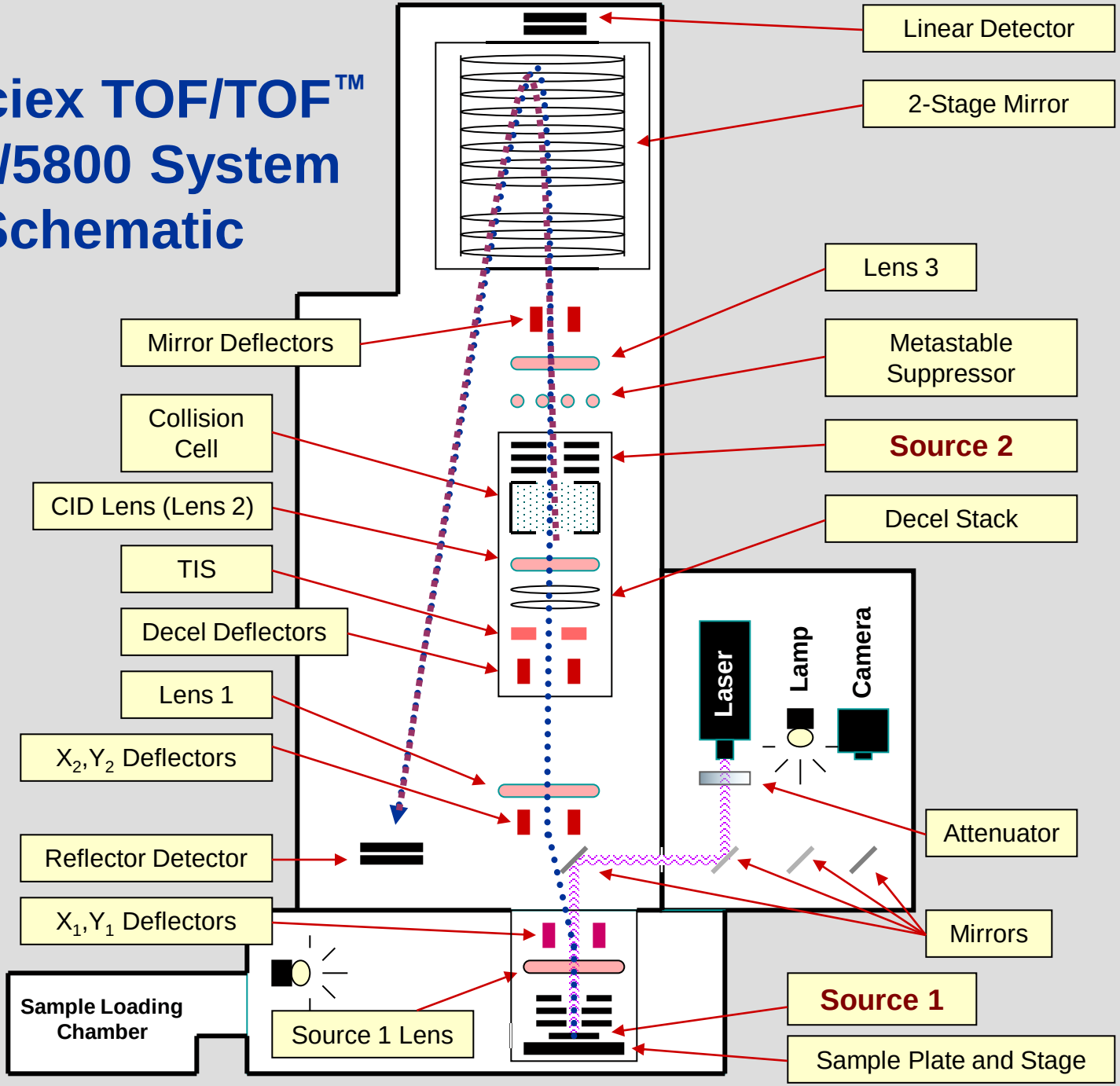
Important Performance Factors

Sensitivity: How small an amount can be detected / analyzed?

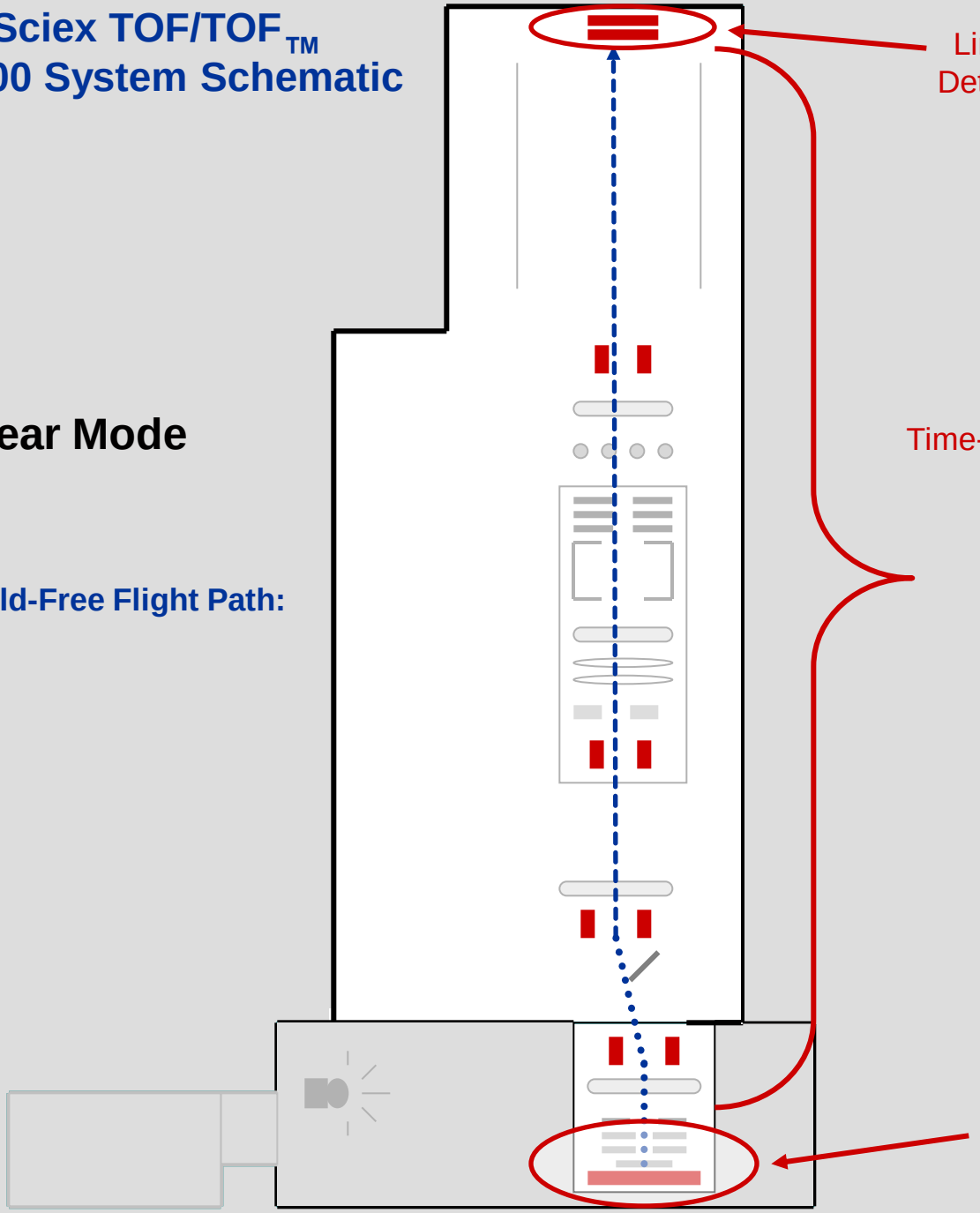
- You should be able to detect small and big molecules down to 1 ppm. This will depend on the quality of your sample and the ionizing ability of the sample as facilitated by the matrix.

- Most MALDI instruments can detect masses no higher than 200 kDa. The lower end limit depends on the matrix and its corresponding ions.

AB Sciex TOF/TOF™ 4800/5800 System Schematic



AB Sciex TOF/TOF™ 4800/5800 System Schematic



Linear Mode

Effective Field-Free Flight Path:
1.5 meters

MS Linear Mode

1. The Linear acquisition mode is used to detect big molecules
2. Sample ionization occurs in the MALDI source (source #1)
3. Ions are accelerated into the field free drift region (TOF)
4. The ion optics steer and focus the ion beam towards the linear detector

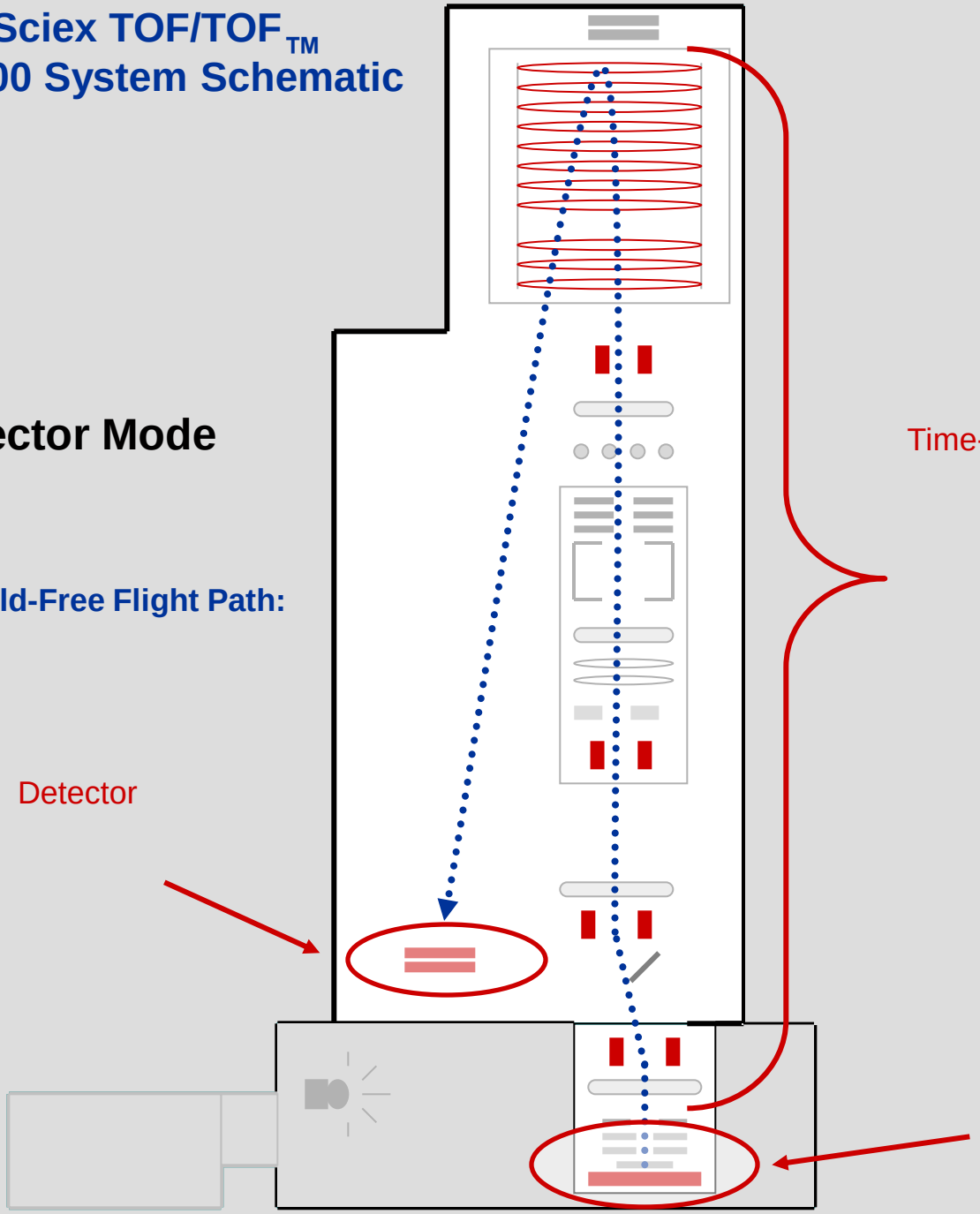
Effective field-free flight path: 1.5 meters

Linear Detector: Dual Microchannel Plate (MCP)

AB Sciex TOF/TOF™ 4800/5800 System Schematic

Reflector Mode

Effective Field-Free Flight Path:
3.0 meters



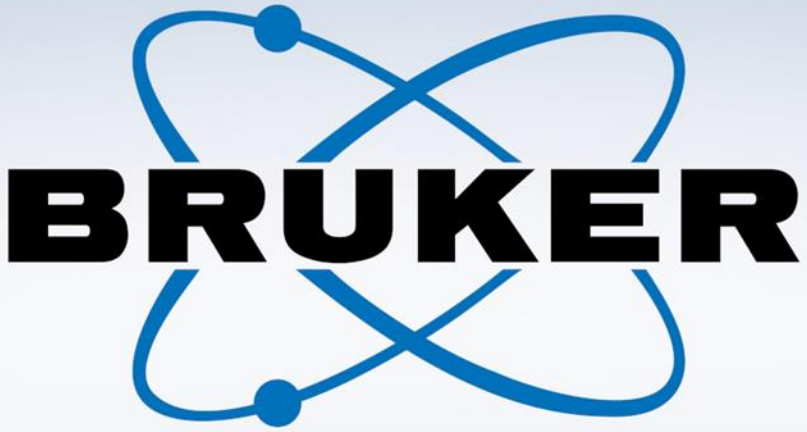
MS Reflector Mode

1. The Reflector acquisition mode is used to detect small molecules
2. Sample ionization occurs in the MALDI source (source #1)
3. Ions are accelerated out of the source into the field free region
4. The ion optics steer and focus the ion beam towards the Reflector entrance
5. The reflectors further focus ions of the same m/z to obtain better resolution and reflect the ion beam towards the Reflector Detector

Reflector: Two stage mirror

Effective field-free flight path: 3.0 meters

Reflector Detector: Dual MCP

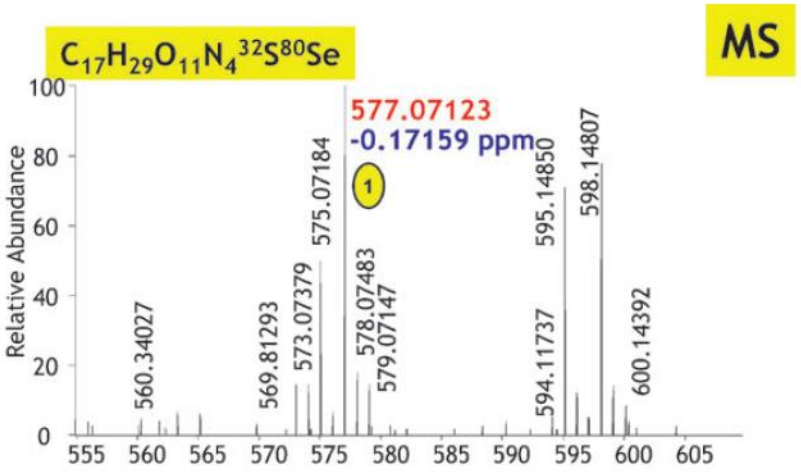


- **YouTube link: https://www.youtube.com/watch?v=0jeFpXHZ8W0**

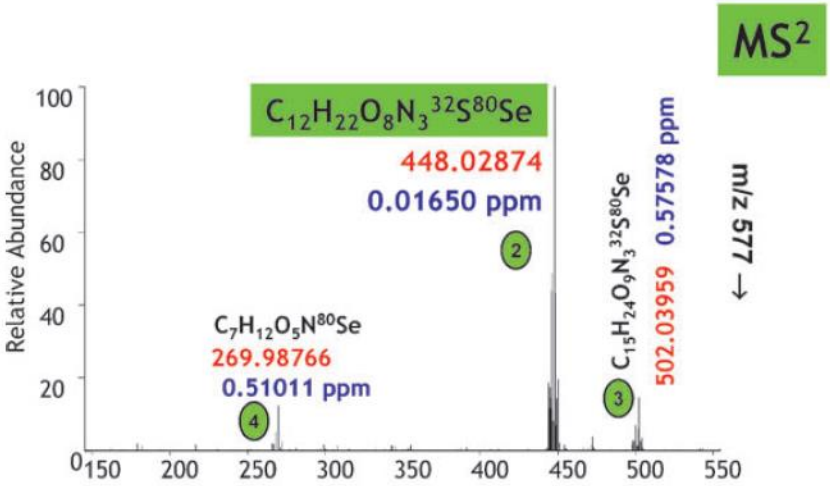
MS/MS Mode

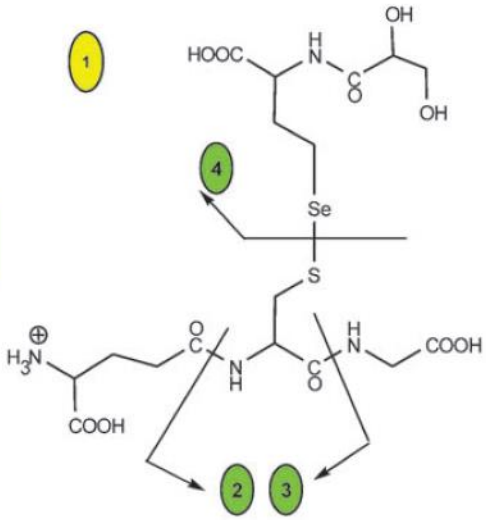
Tandem MS/MS (Fragmentation MS) can be helpful in determining molecular identification.

Parent ion

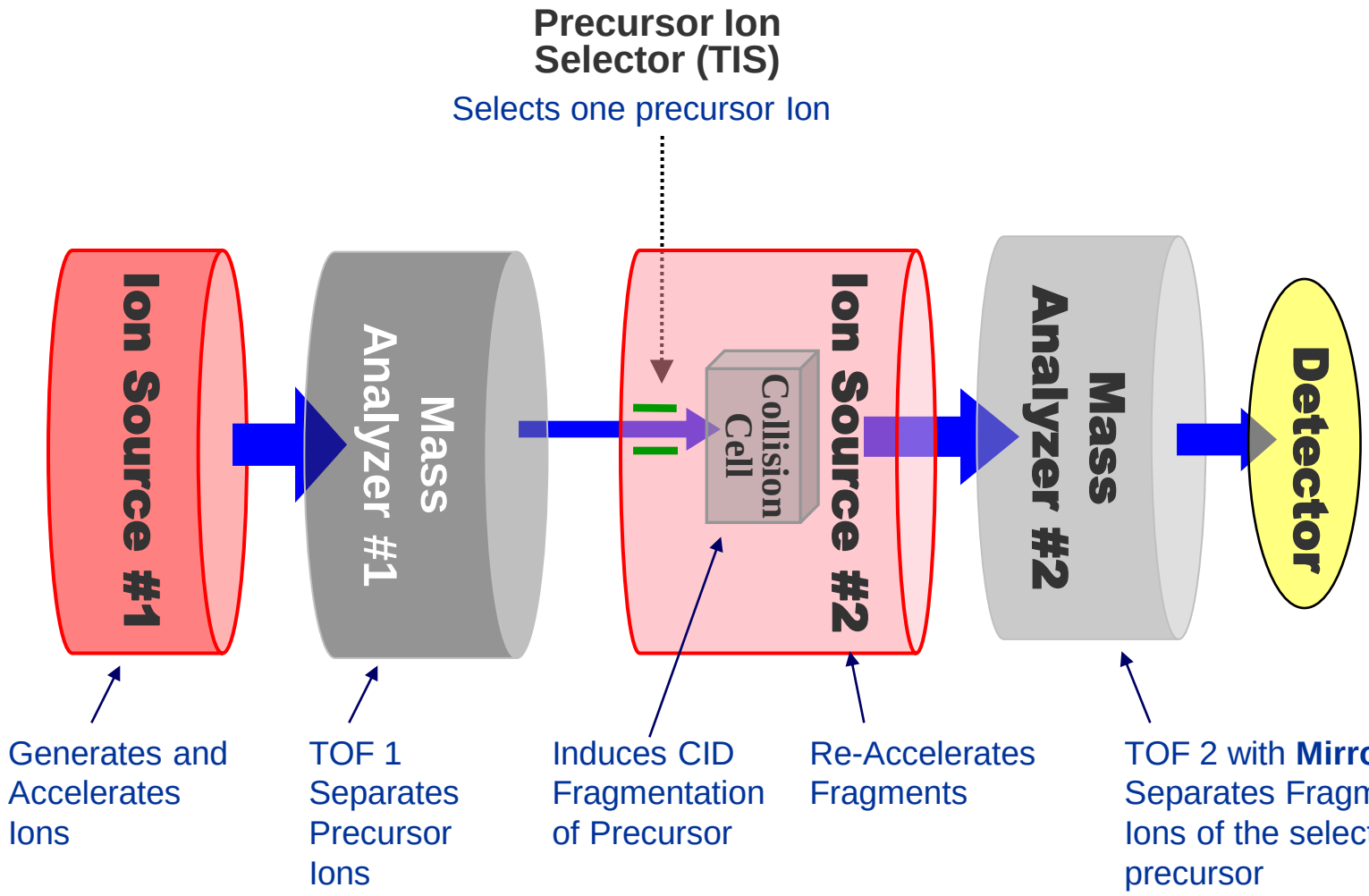


Daughter ions





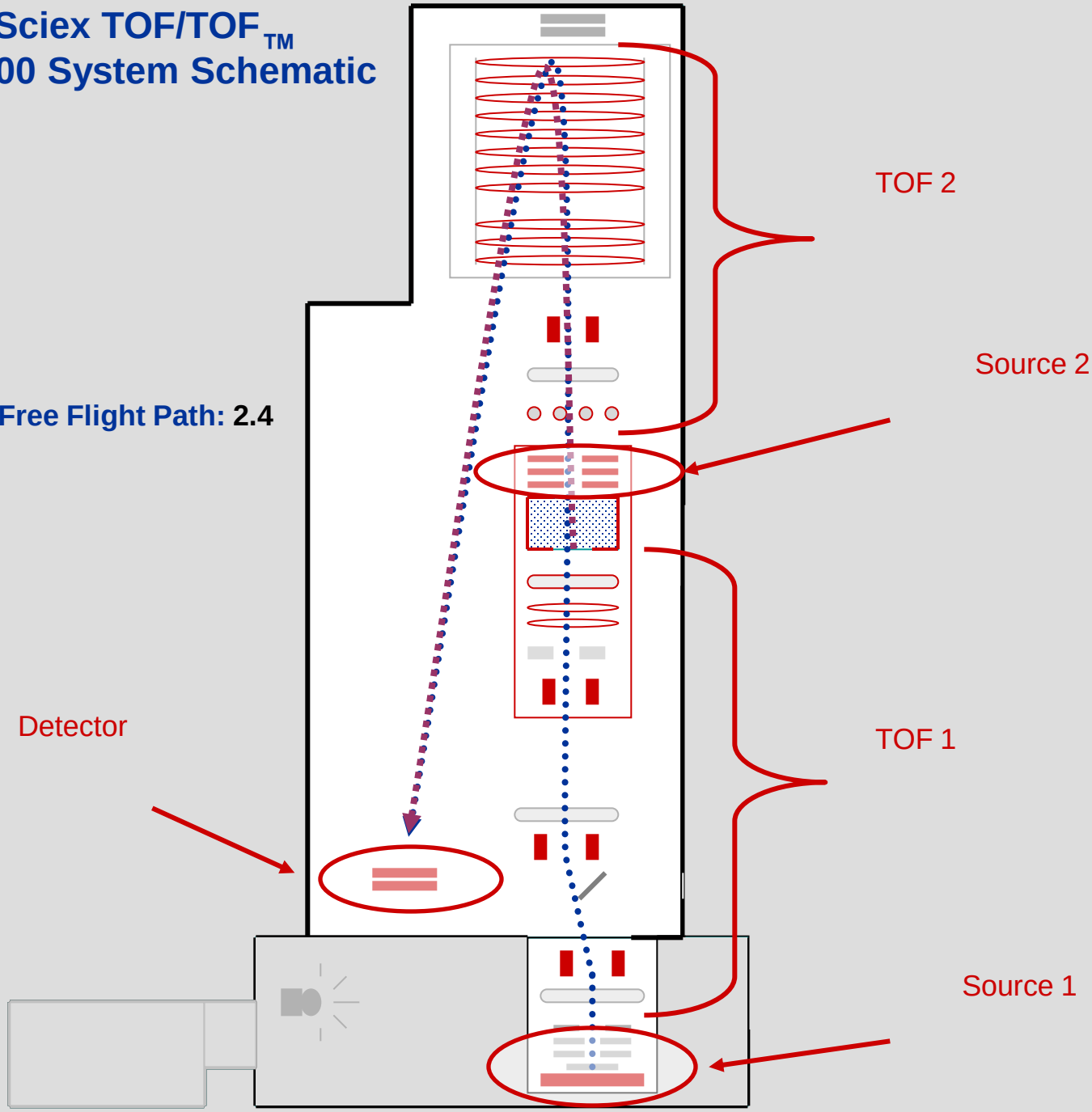
Tandem MS/MS Spectrometer with CID



AB Sciex TOF/TOF™ 4800/5800 System Schematic

Ion Path
MS/MS Mode

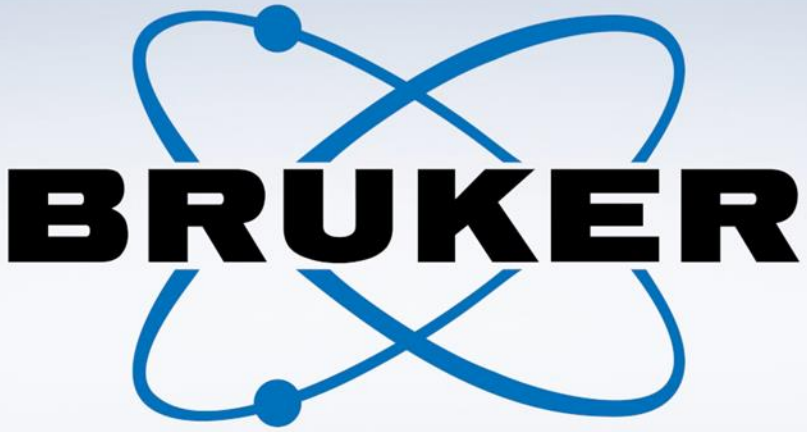
Effective Field-Free Flight Path: 2.4 meters



MS/MS Mode

1. The MS/MS mode is used for molecular identification.
2. Sample ionization via MALDI source (source #1).
3. Ions are accelerated to the first field free region.
4. The ion optics steer and focus the ion beam towards source #2.
5. The precursor ions are selected and transmitted into the collision cell.
6. In the collision cell, precursor ions are fragmented via CID.
7. Product ions are re-accelerated to the second field free region.
8. Ion optics steer and focus the product ion beam towards the reflector region where they are further focused to the Reflector detector.

Reflector:	Two-stage mirror
Effective flight path:	2.4 meters
Reflector detector:	Dual MCP



- **YouTube link: https://www.youtube.com/watch?v=3arO41_edeg**

Sample Preparation

Supplies Needed

- **YouTube link: https://youtu.be/sh2K3cKZlt0**

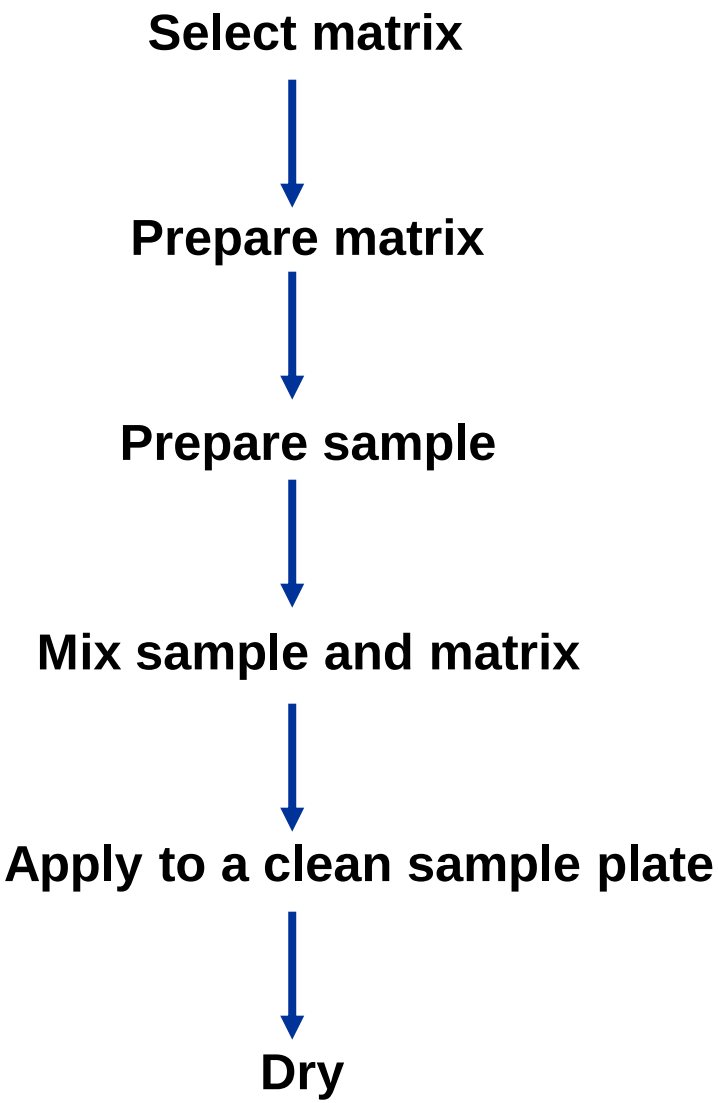
Supplies Needed

1. HPLC grade acetonitrile and water
   - Mass spec grade is better
2. Kim wipes
3. Pipettes and tips
4. Eppies, eppy trays, and markers
5. Access to a weigh balance and weighing supplies
6. Matrix and your sample(s)

Three things you need to get Good Data:

1. Good Instrument
2. Good (Acquisition/Processing/Interpretation) Method
3. Good ***Sample Prep***

Sample Preparation



Matrix selection

α-Cyano-4-hydroxy-cinnamic acid (CHCA)	Peptides<10kDa
Sinapinic Acid	Proteins >10kDa
2,5-Dihydroxybenzoic acid (DHB)	Neutral Carbohydrates, Synthetic Polymers
"Super DHB"	Proteins, Glycosylated proteins
3-Hydroxypicolinic acid	Oligonucleotides
2-(4-hydroxy-phenylazo) benzoic acid (HABA)	Proteins, Oligosaccharides
Caution: *Do not use Carbon Nanotubes or similar matrix; these conductive compounds may damage the instrument*	

Matrix selection

α-Cyano-4-hydroxycinnamic acid (α-Cyano, CHCA)

Analytes: peptides, protein digests, small proteins and Molecules below 10,000 Da

Preparation: 5 mg/ml in a solvent that consists of 50% ACN/50% Water/0.1% Trifluoroacetic Acid (TFA)

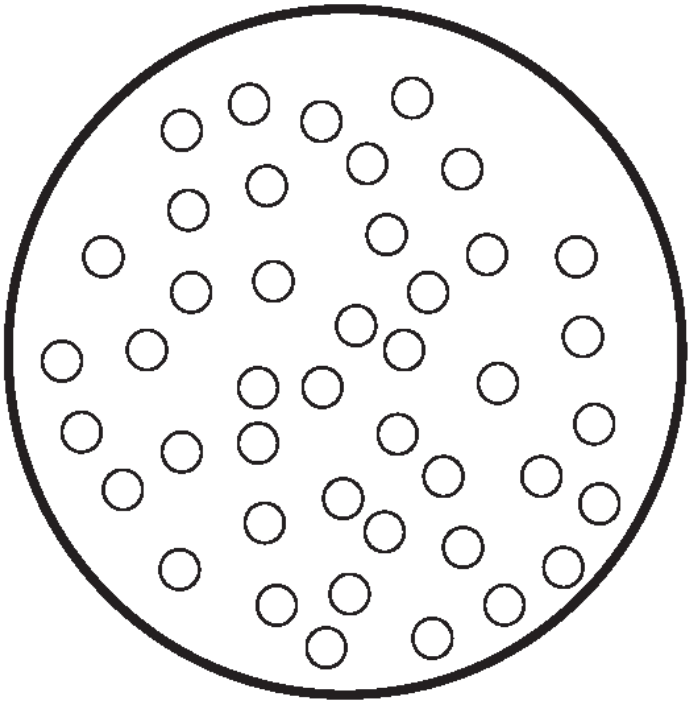
Matrix selection

Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid)

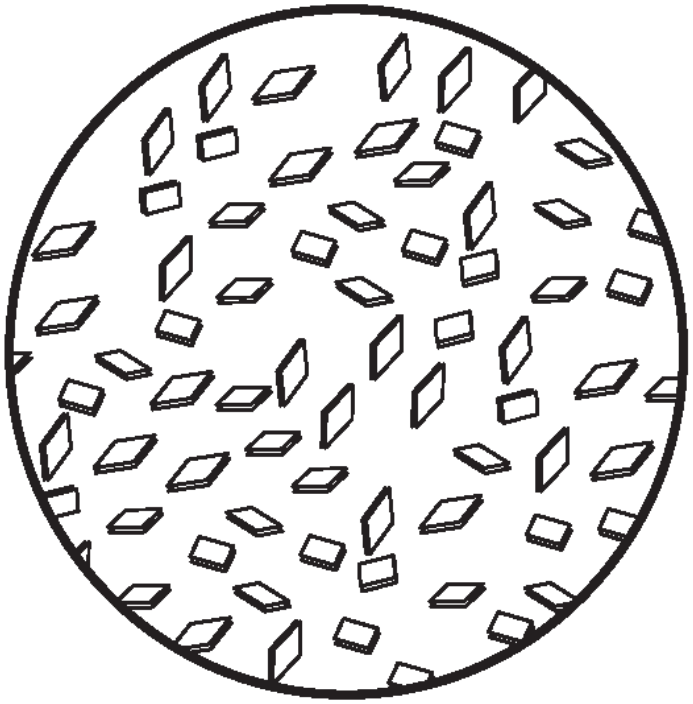
Analytes: proteins, large peptides

Preparation: 10 mg/ml in 50% ACN with 0.1% TFA

Appearance of Matrix Crystals



CHCA
Rounded

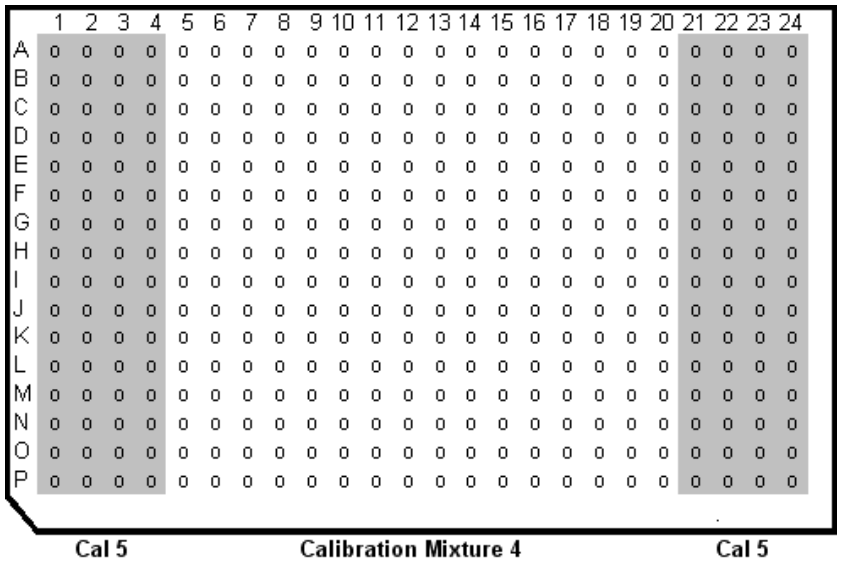


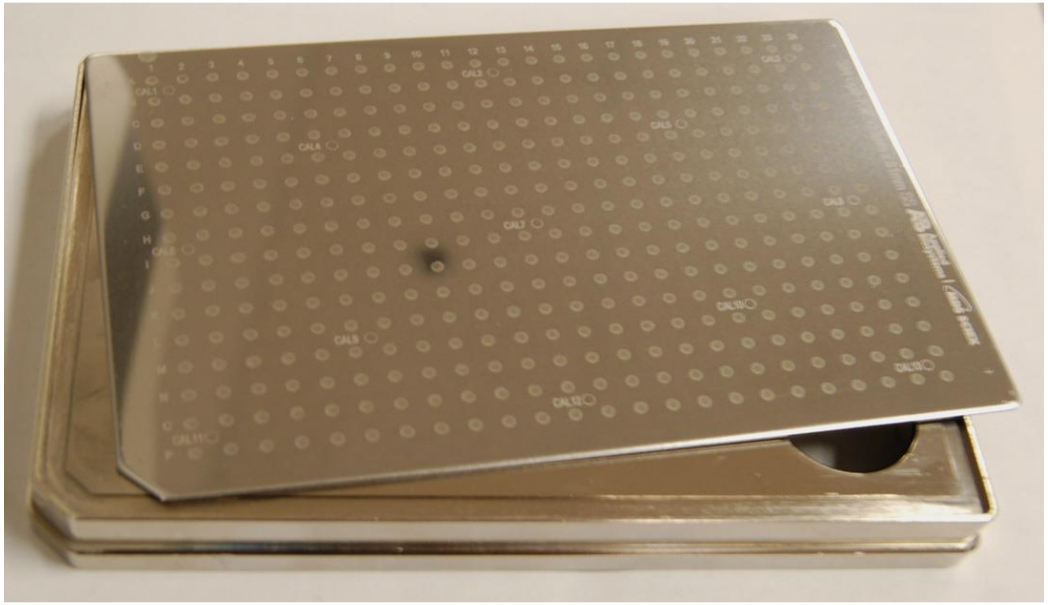
Sinapinic acid
Rhomboid

Note: Sample spots prepared in these matrices should dry to produce evenly distributed crystals with uniform size and shape

Sample Plates

- 3x5 inch plate assembly consists of a **Insert** mounted in a Magnetic **Holder**
- Inserts are **hydrophobically coated** to allow reproducible high density spotting
- Inserts available with 96 or 384 sample wells
- Use Opti-TOF™ Cal Mix plates for calibrating the instrument in the appropriate reflector or linear mode method





Cleaning the MALDI Plate

- **YouTube link: https://youtu.be/vFV_X9PxwSE**

Cleaning the MALDI PLATE -Extra Tip-

- If you prepared your sample using a solvent that is not water or ethanol miscible, then it is a very good idea to add an extra wash step in which you clean the plate with that solvent. The order should be:

1. Water
2. Ethanol
3. Sample solvent
4. Water

Preparing the Matrix Solution

- **YouTube link:** https://youtu.be/k-KwC9IZVXQ

Appearance of Matrix/Sample Spot

Contamination Effects

- Originate from matrix, sample or solvent(s)
- Ring effect around the sample spot
- Clumping of matrix in the well
- Matrix does not crystallize in normal pattern
- Droplet spreads over wide area

Typical contaminants in biological samples

No interference:

TFA, formic acid, b-mercaptoethanol, DTT, volatile organic solvents, HCl, NH_4OH, acetic acid

Tolerable: (< 5 mM)

HEPES, MOPS, Tris, NH_4OAc, octyl glucoside

Note: Minimizing buffer concentrations improves performance.

Use the minimum needed to control pH. Preferable to use an ammonium salt version of a volatile buffer.

Avoid:

glycerol, sodium azide, DMSO, SDS, phosphate, NaCl, 2M urea, 2M guanidine

Sample clean-up

Removal of buffer salts, urea, guanidine, EDTA, glycerol, DMSO, detergents, etc.

- Dilution
- On-plate washing (0.1% TFA in water)
- Cation exchange (replace Na^+ with NH_4^+)
- Pipette tip column chromatography
- ZipTips®

Sample Cleanup by Solid Phase Extraction

ZipTip® - **miniature column chromatography**:

Standard ZipTips have C_{18} solid support

ZipTip C_4 for cleanup of protein samples

Other types available, *e.g.*, Metal Chelating (MC) for concentration of Phosphopeptides

Procedure for peptides/proteins:

- **Condition** the ZipTip with 10 µl of acetonitrile (ACN), then 10 µl of 50% ACN/0.1% TFA, then 2 x 10 µl of 0.1% TFA.
- **Load** the sample onto the ZipTip by pipetting 5-10 µl sample up and down several times and discarding the liquid.
- **Wash** C_{18} tip with 3 x 10 µl of 0.1% TFA to remove salts.
- **Elute** the sample from the ZipTip with 30-70% ACN or elute directly into the matrix (*e.g.* CHCA in 50% ACN/0.1%TFA); minimal volume of ~3 µl can be used.

Predicting sample ions by generating theoretical mass spectra

Preparing for Data Collection

Before collecting data, make a list of possible ions that may be present in your sample. To do so:

1. The data that you collect are the mass spectra for compounds in ion form and results are reported in the unit of mass/charge (m/z) or (m/e).

2. Determine what sample ions you expect to observe in positive or negative ion mode.
   - Based on your solution conditions and on whether your sample is a neutral or charged molecule, you should expect to see certain ion adducts.

Preparing for Data Collection

3. You will normally observe only +1 or -1 charged ions by MALDI TOF. For large molecules, you may be able to detect +2/-2 charged ions.

4. The signal sensitivity for negative ion linear mode is inferior to positive ion linear mode.

5. When collecting the mass spectra of small molecules, you should be able to obtain excellent mass resolution and observe the isotopic distribution of the ions present in your sample.

Let’s focus on an example:

A Ti(IV) containing compound

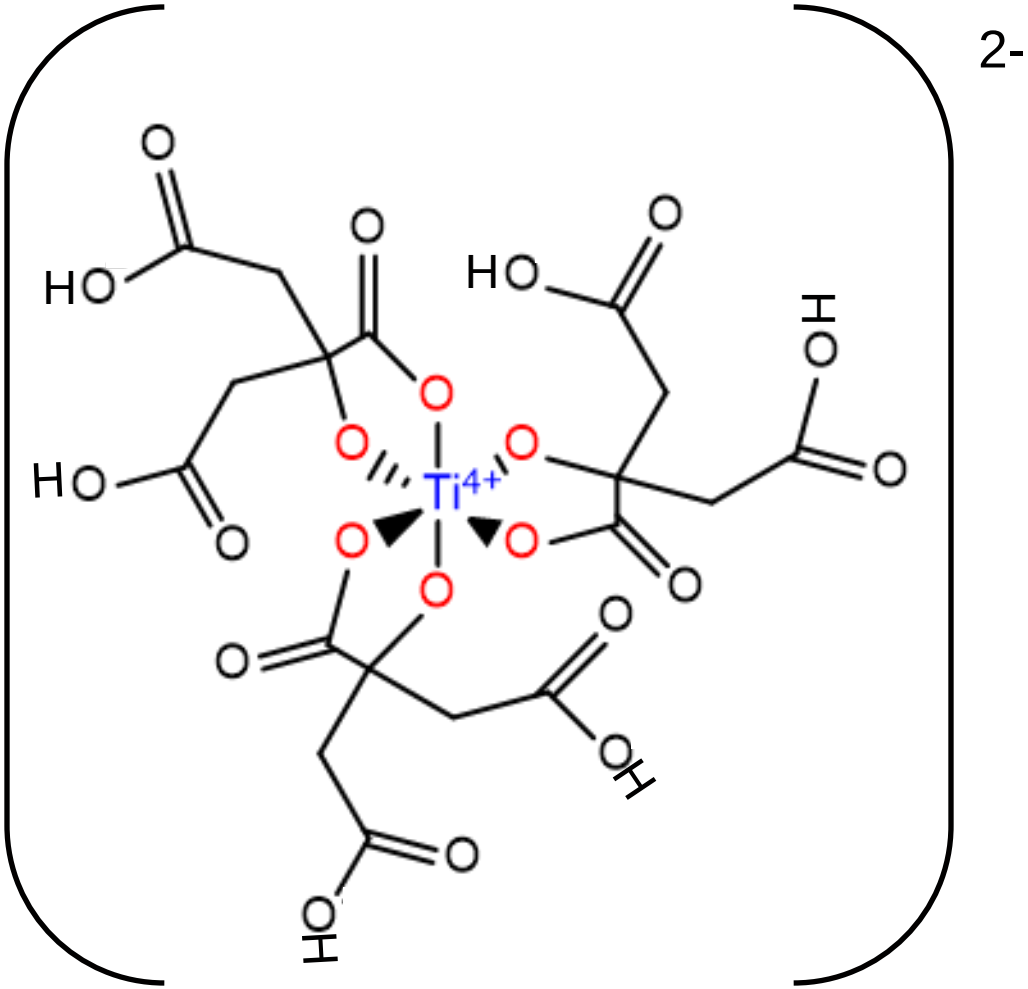
Ti Isotopic Distribution

There are five stable Ti isotopes. Below I report the isotopes and their relative abundance:

Isotope	Abundance
^{46}Ti	8.25%
^{47}Ti	7.44%
^{48}Ti	73.7%
^{49}Ti	5.41%
^{50}Ti	5.18%

- Due to this abundance distribution, the atomic weight of Ti is 47.867.
- The MS of samples containing Ti are dominated by the isotopic distribution of Ti.

Ti(IV) Tricitrate



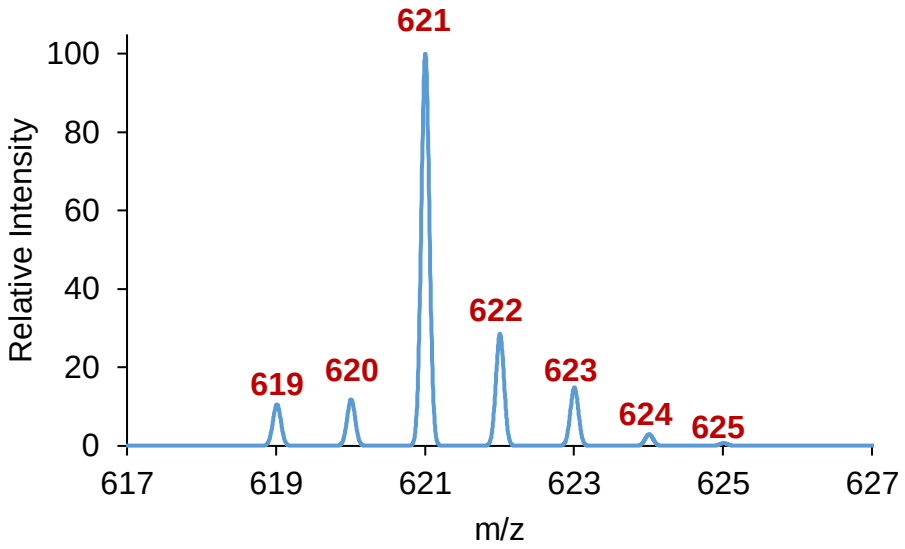
Ti(IV) Tricitrate

Detecting in positive ion mode:

$$[Ti(C_6H_6O_7)_3]^{2-} + 3H^+ \Rightarrow \{(H^+)_3\,[Ti(C_6H_6O_7)_3]\}^+$$

- This is a +1 adduct; 621.00 m/z

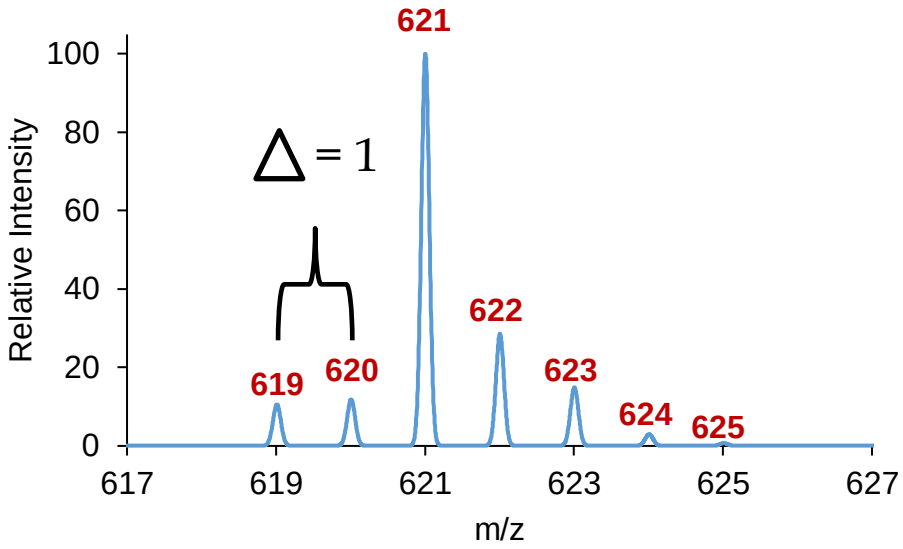
Theoretical Mass Spectrum generated using Isopro3:



Ti(IV) Tricitrate

Note the separation of the isotope ions is a mass unit of 1, which confirms that the species observed is +1.

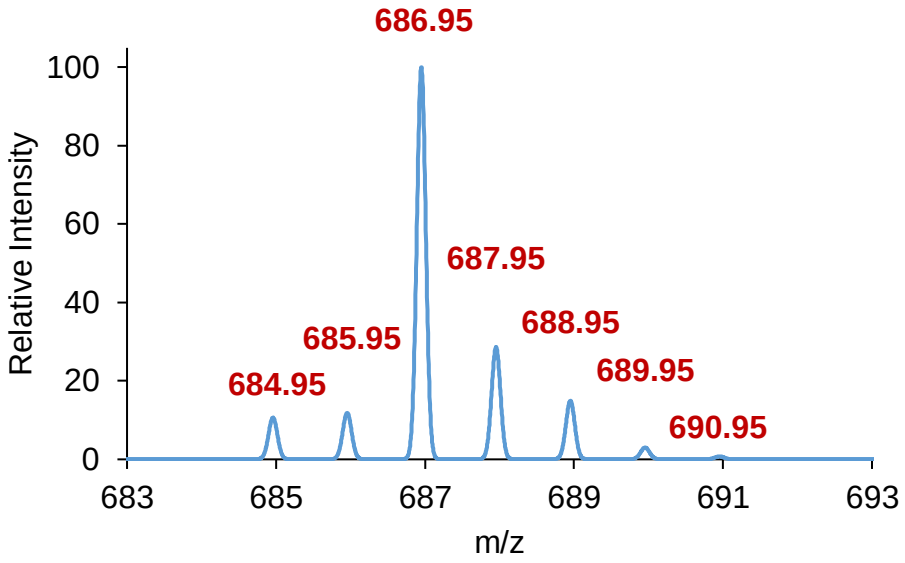
- A separation of 0.5 indicates a charge state of +2.
- A separation of 0.33 indicates a charge state of +3.



Ti(IV) Tricitrate

$$[Ti(C_6H_6O_7)_3]^{2-} + 3Na^+ \Rightarrow \{(Na+)_3\,[Ti(C_6H_6O_7)_3]\}^+$$

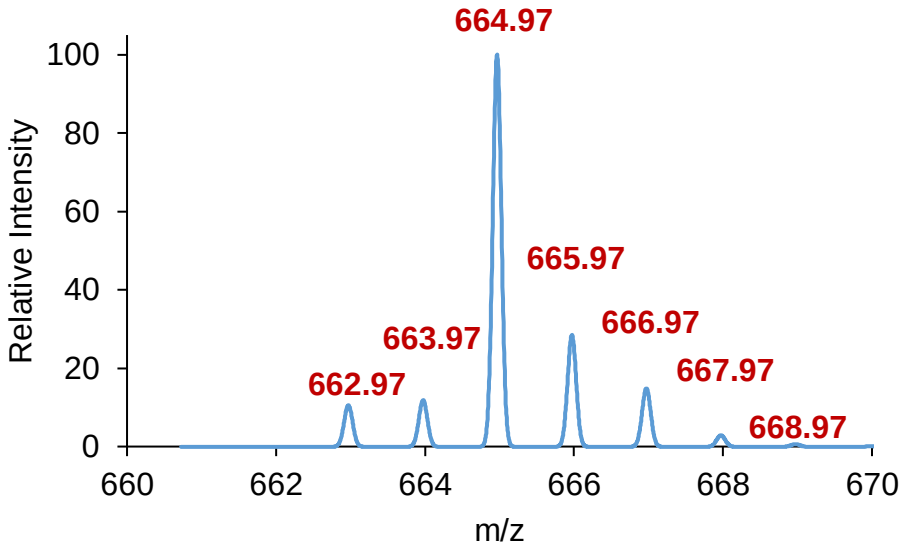
Although your sample may not have any sodium ions in it, the instrument tends to have sodium ions present. It has potassium ions to a lesser extent.



Ti(IV) Tricitrate

$$[Ti(C_6H_6O_7)_3]^{2-} + 1H^+ + 2Na^+ \Rightarrow \{(H^+)_1(Na+)_2\,[Ti(C_6H_6O_7)_3]\}^+$$

Mixed ion adducts are possible.

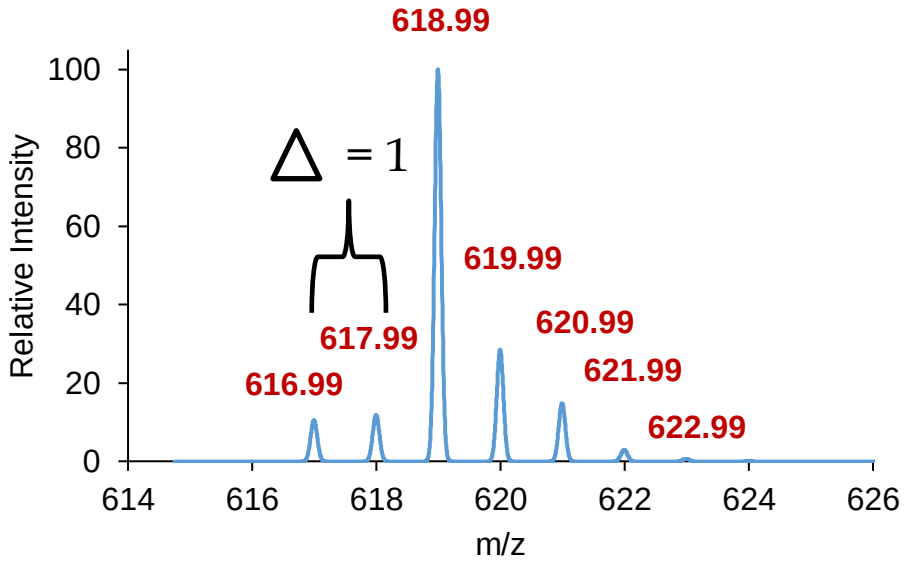


Ti(IV) Tricitrate

Detecting in negative ion mode:

$$[Ti(C_6H_6O_7)_3]^{2-} + H^+ \Rightarrow \{(H^+)[Ti(C_6H_6O_7)_3]\}^-$$

This is a -1 adduct. Note the separation of the ions is a mass unit of 1, which confirms that the species observed is “-1”.



Additional things to be mindful of

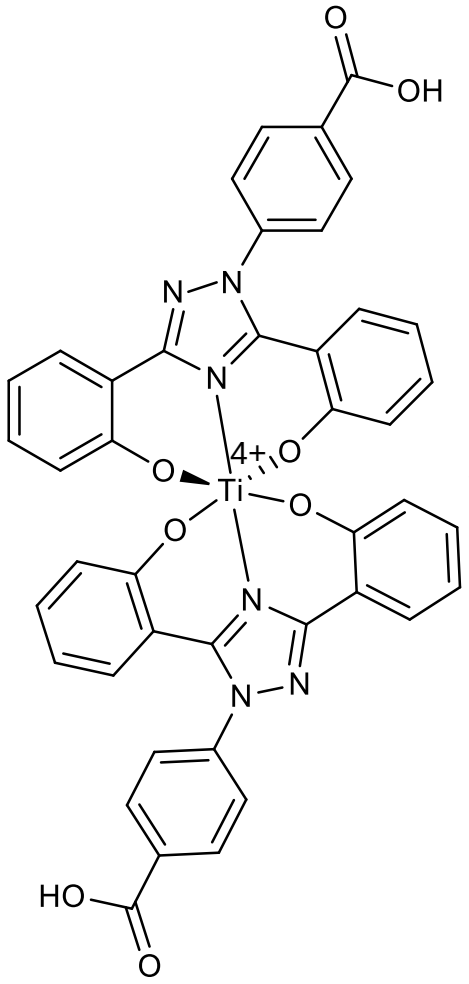
1. When examining a sample for the first time, it is good to prepare your sample at two to three different concentrations. A good rule of thumb is 10, 100, and 1000 micromolar.

2. Try different matrices if you do not detect your sample.

3. If you are interested in doing a pH-dependent study, prepare your sample in ammonium-based buffers. These buffers are relatively friendly to mass spectrometers and certain ones are volatile. The buffer concentration must be at low millimolar concentration.

Let’s collect data on a real sample

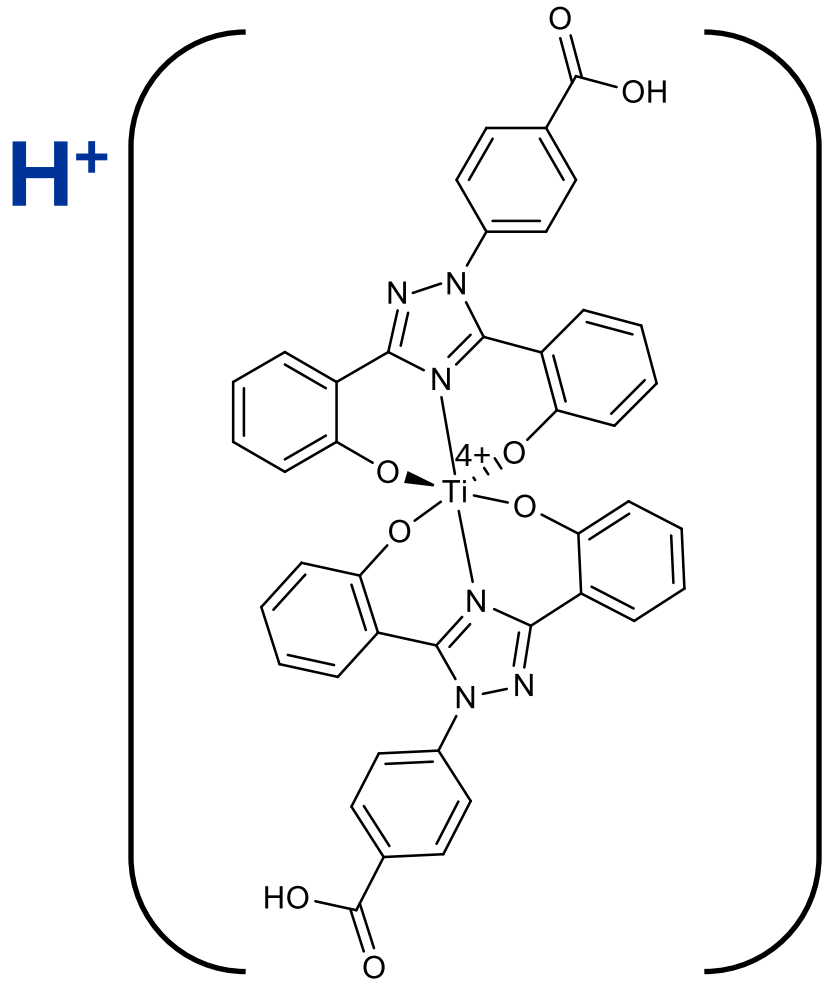
Ti(deferasirox)$_2$



A neutral compound

$Ti(deferasirox)_2$

We will use positive ion reflector mode to detect this compound and cinnamic acid as our MALDI matrix.

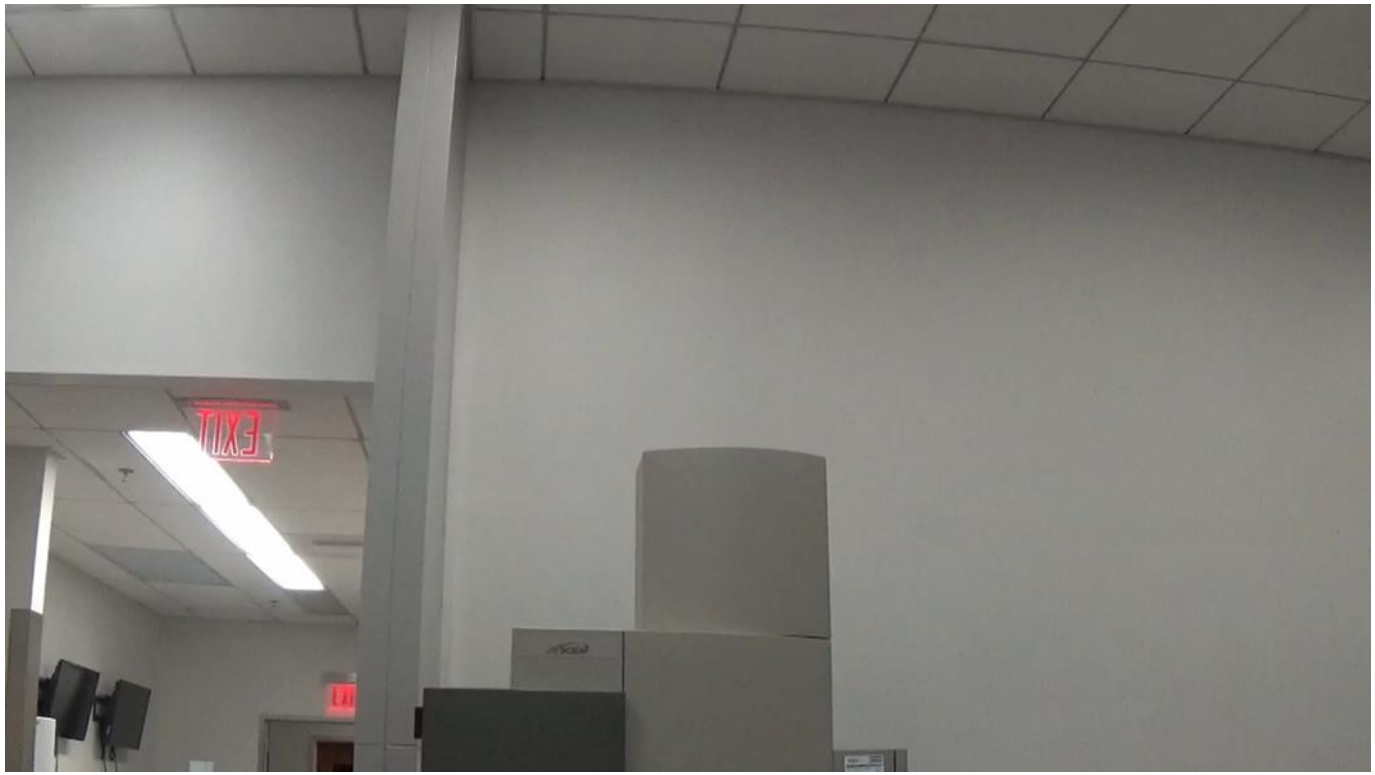


$\{(H^+)Ti(C_{42}H_{26}N_6O_8)\}^+$

Spotting the MALDI Plate

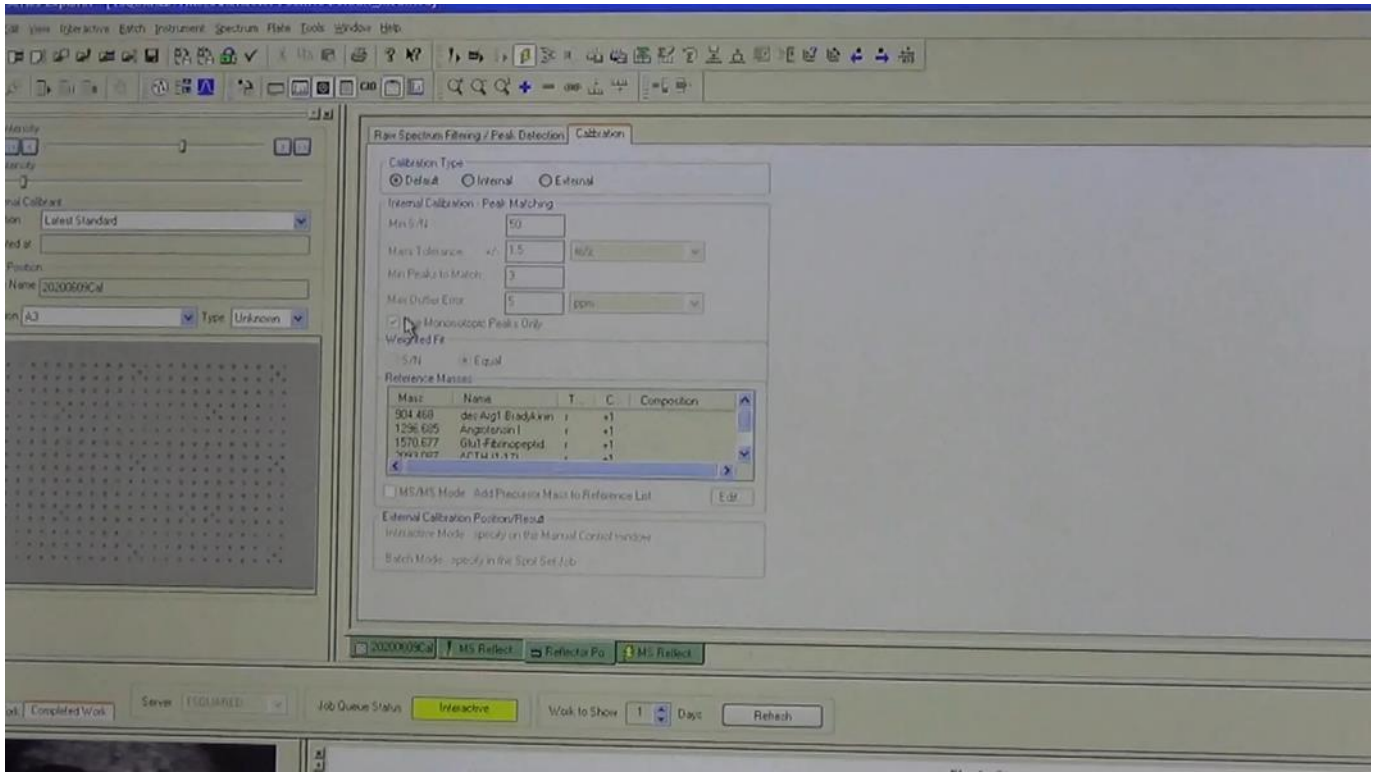
- **YouTube link: https://youtu.be/iE9OYaDUxVg**

Calibrating the MALDI TOF in Reflector Mode



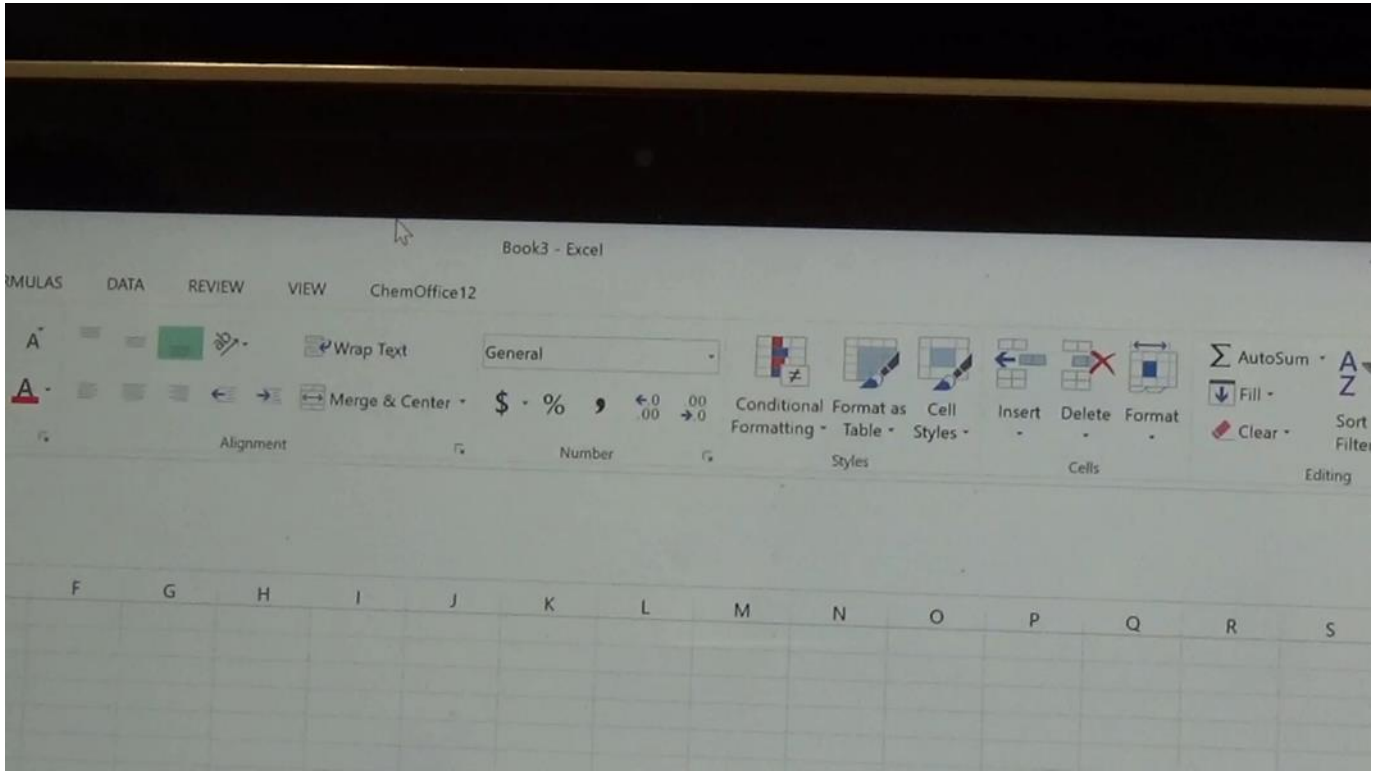
- **YouTube link:** https://youtu.be/7lDOEFv4z64

Collecting MALDI TOF Data



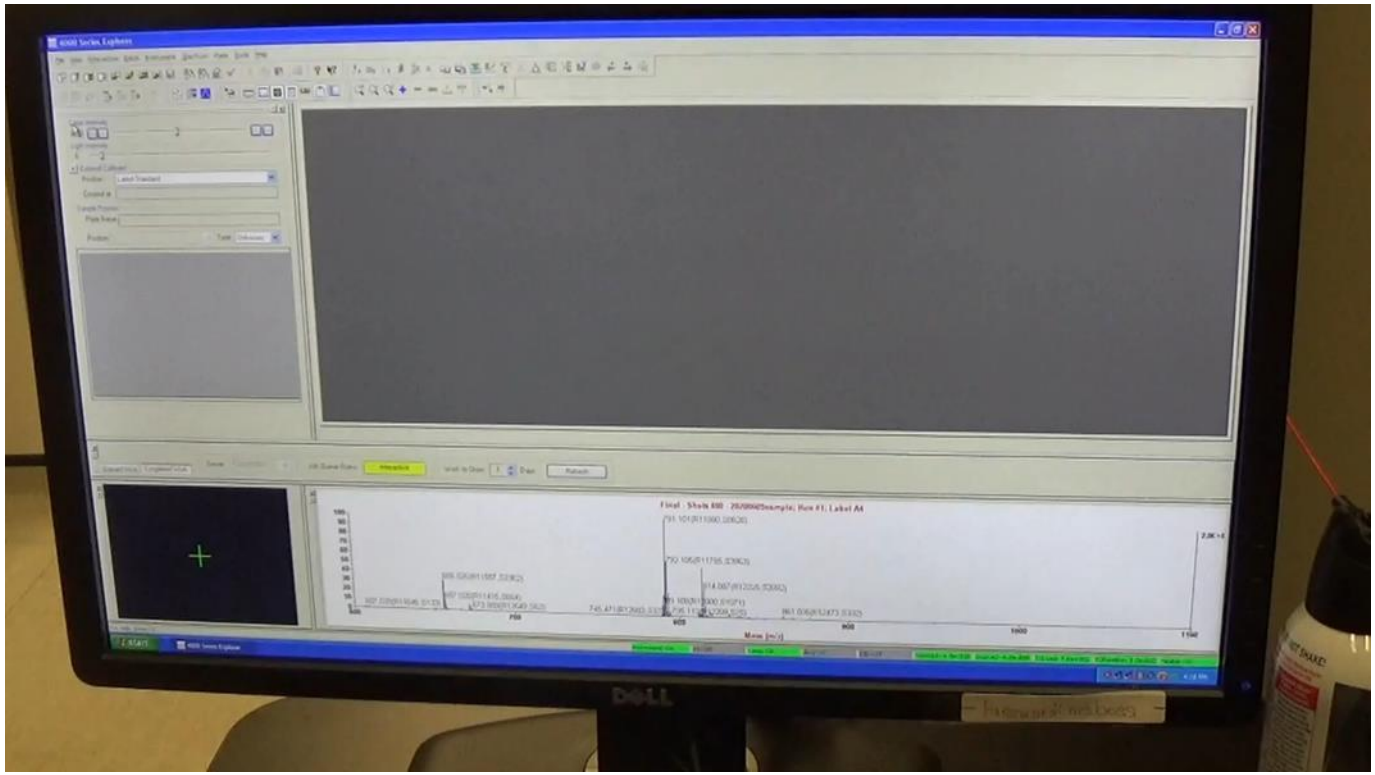
- **YouTube link:** https://youtu.be/bNYdudE_yOg

Analizing MALDI TOF Data



- **YouTube link: https://youtu.be/6-6qGl7N0B0**

Calibrating the MALDI TOF in Linear Mode



- **YouTube link: https://youtu.be/a-sGzsbEm1c**

Quiz

Sample	m/z	Acquisition Mode	Can isotopic distribution be observed?	Detection Level (ppm)	What ion charge is likely to be observed?
Small Molecule					
Big Molecule					

Quiz

Sample	m/z	Acquisition Mode	Can isotopic distribution be observed?	Detection Level (ppm)	What ion charge is likely to be observed?
Small Molecule	<10 kDa	Reflector	Yes	~1	+1/-1
Big Molecule	>10 kDa	Linear	No	~1	+1/-1; +2/-2