

# High-Throughput Analysis of Oligonucleotides using Automated Electrospray Ionization Mass Spectrometry

Mark E. Hail<sup>1</sup>, Brian Elliott<sup>2</sup>, Kerry Nugent<sup>3</sup>, Jeffrey L. Whitney<sup>1</sup>, and  
David J. Detlefsen<sup>1</sup>

<sup>1</sup>Novatia, LLC, Princeton, NJ 08540

<sup>2</sup>Integrated DNA Technologies, Coralville, IA 52241

<sup>3</sup>Michrom BioResources, Auburn, CA 95603

# Introduction

- Tens of thousands of oligos produced daily in HT oligo synthesis labs
- Efficient and reliable confirmation of all synthesized products desired
- To date, MALDI-ToF has been QC method of choice
- Demand for long (e.g., 70-mer) oligos for use in oligo arrays is increasing
- Long and/or fragile oligos have been problematic by MALDI-ToF
- We have developed a totally automated system using LC/MS with:
  - >1000 sample per 24 hr throughput
  - capability to analyze long (>100 mer) or fragile oligos
  - < 100 ppm mass accuracy & precision across entire mass range
  - capability for detailed LC/MS profiling on the same system

# Experimental Systems

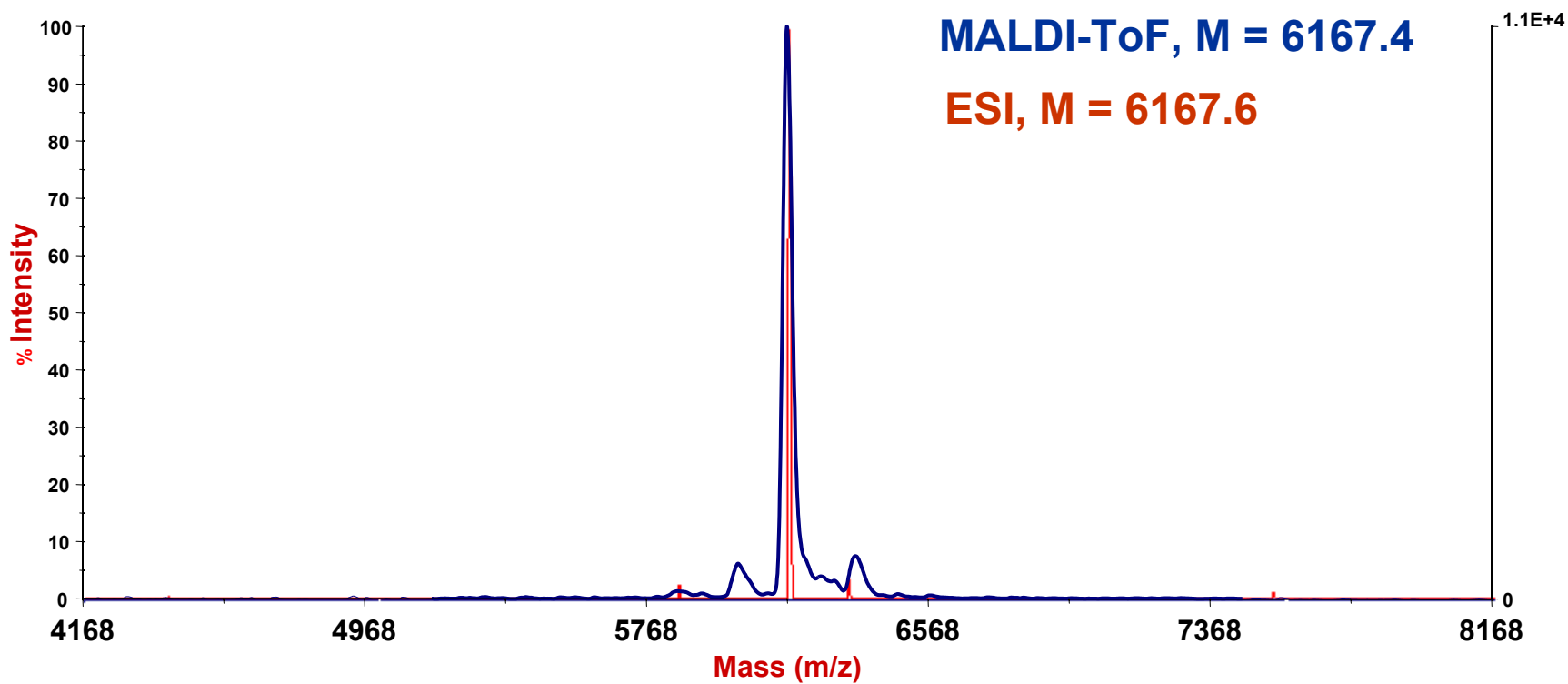
- MALDI-ToF
  - ABI Voyager DE, linear +ve mode, external calibration
  - 3-HPA – ammonium citrate matrix
- LC/MS (Oligo HTCS)
  - ThermoFinnigan TSQ7000, -ve ion mode
  - Xcalibur data system
  - Michrom Paradigm MS4 HPLC
    - HFIP / TEA “lite” mobile phase w/trace EDTA
  - LEAP CTC-PAL autosampler
  - *ProMass* auto biomolecule deconvolution processing software

# *ProMass* Auto ESI Deconvolution Approach

- Uses a simple scoring algorithm to determine the charge of every peak in the raw mass spectrum (Zhang & Marshall, JASMS 1998)
- No deconvolution artifacts
- Incorporates signal processing and score normalization to improve reliability of deconvolution even on very noisy data
- Can confirm presence of target masses

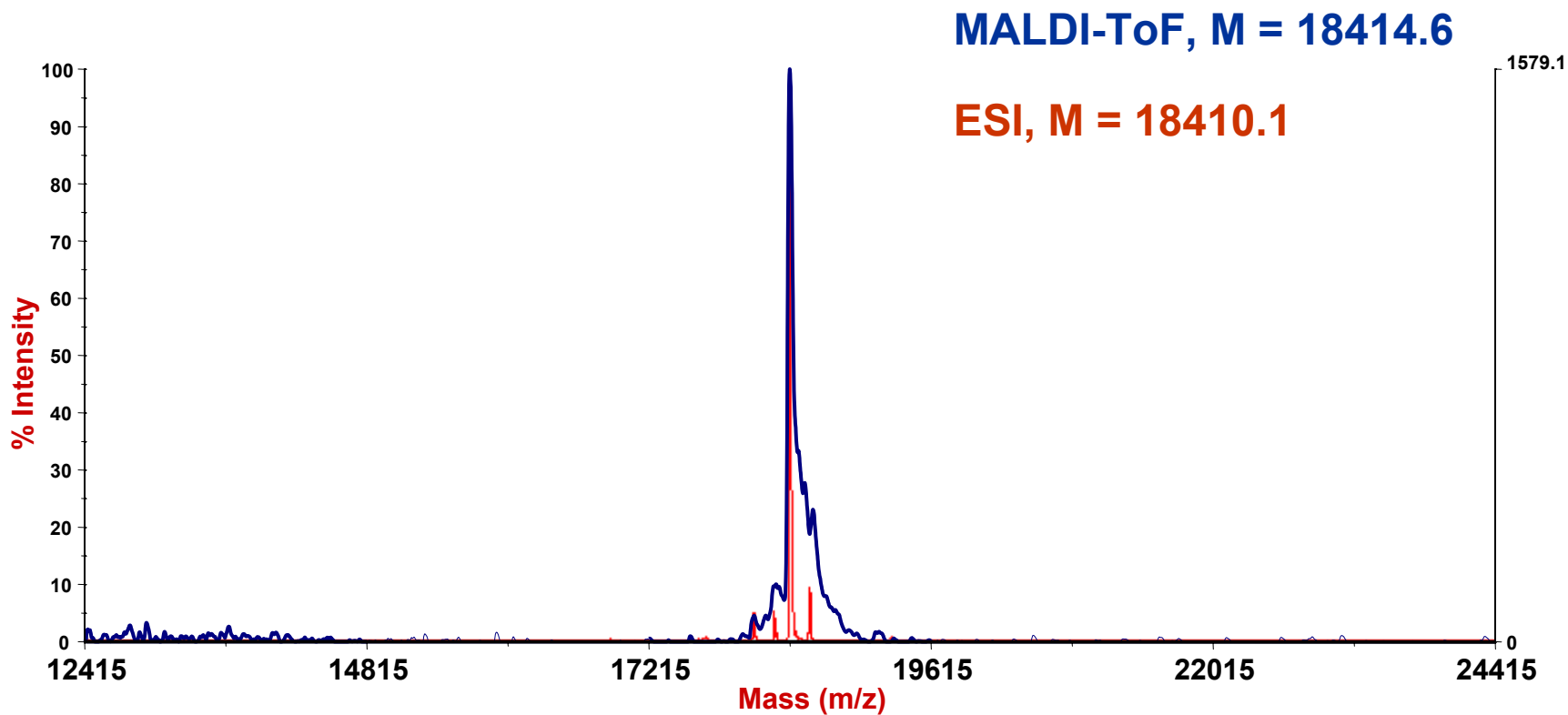
# MALDI and ESI MS Data of a 20-mer Oligo

**M = 6168.4**



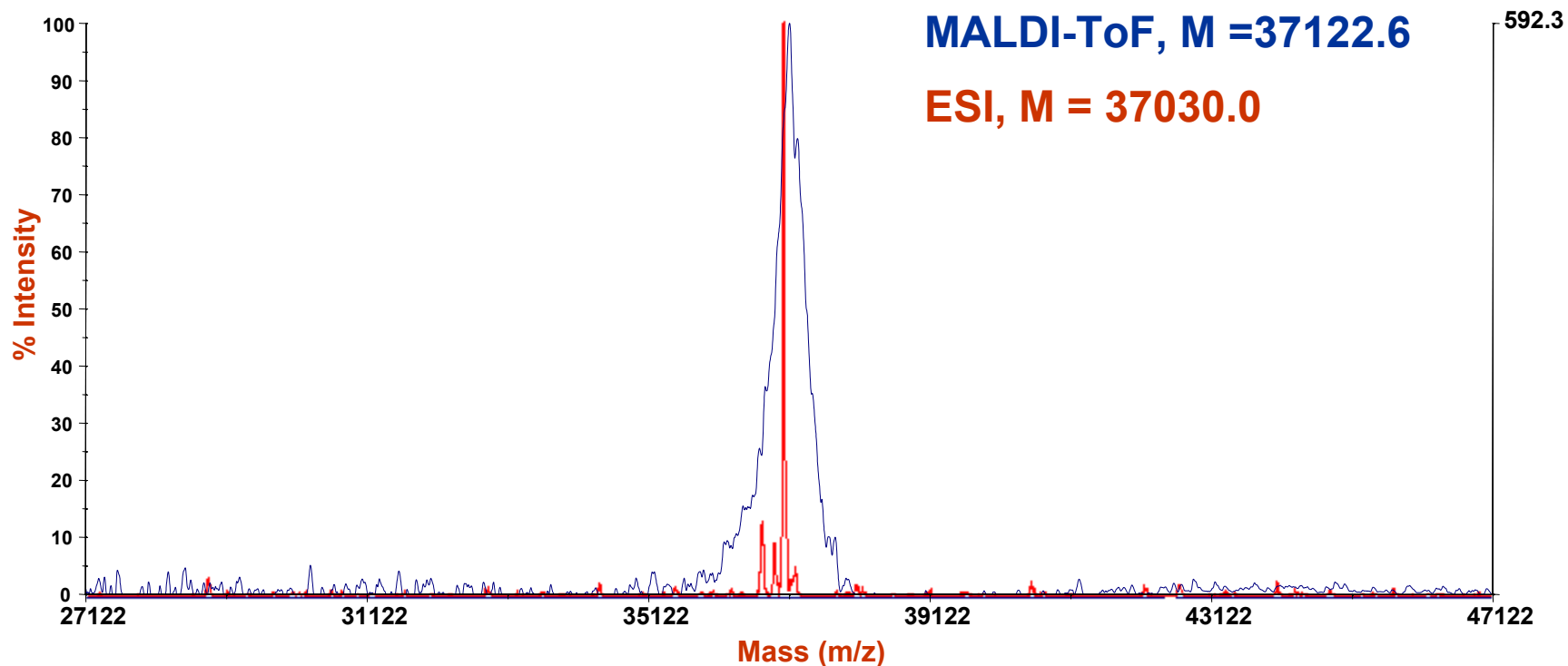
# MALDI and ESI MS Data of a 60-mer Oligo

**M = 18410.0**

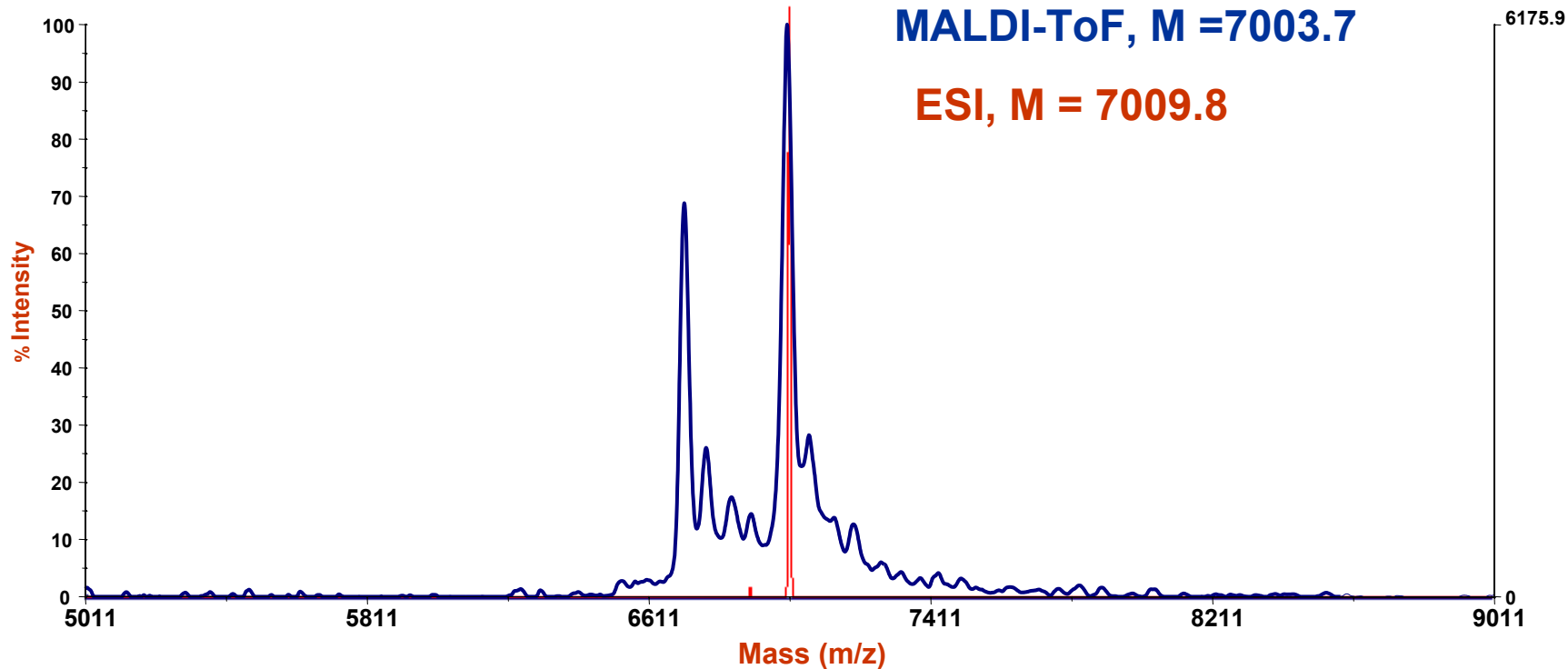


# MALDI and ESI MS Data of a 120-mer Oligo

**M = 37031.0**



# MALDI and ESI MS Data on Fragile Dual-labeled Probe Black Hole Quencher™ M = 7011.8



# Mass Accuracy of 10 to 120-mers

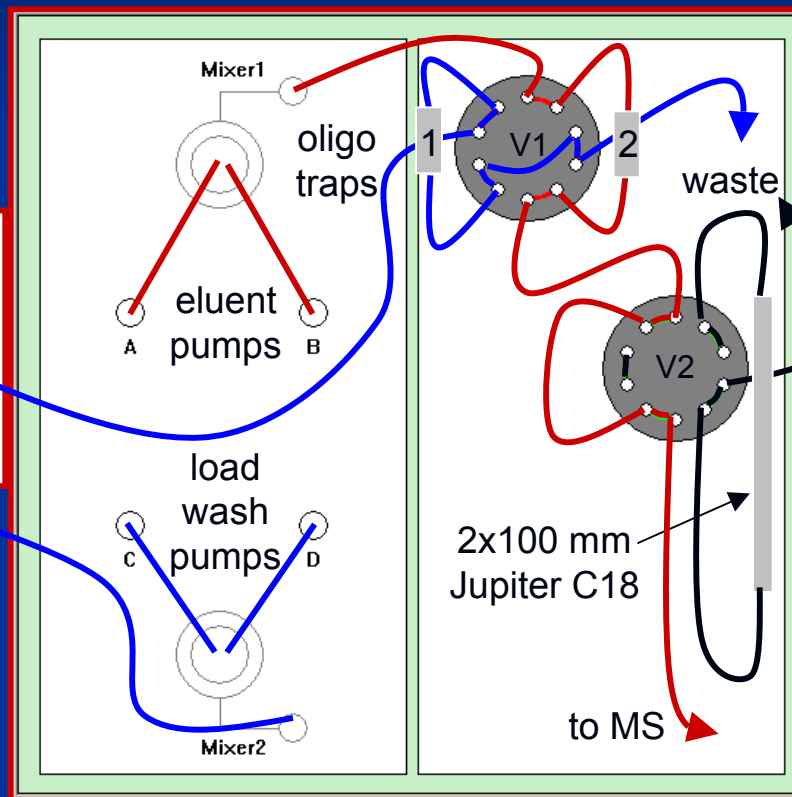
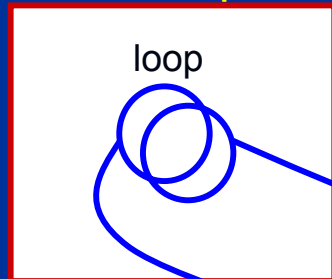
Oligo	MW	MALDI	Error (ppm)	ESI	Error (ppm)
10-mer	2993.0	2992.1	-300.7	2993.1	33.4
20-mer	6168.1	6167.4	-113.5	6167.6	-81.1
40-mer	12300.0	12298.6	-113.8	12299.7	-24.4
60-mer	18410.0	18414.6	249.9	18410.1	5.3
80-mer	24661.1	24664.7	146.0	24661.0	-4.1
100-mer	30951.1	30978.3	878.8	30950.6	-16.2
120-mer	37031.0	37121.6	2446.6	37030.0	-27.0

# Oligo HTCS Plumbing Scheme

Shown in High-throughput QC Mode

## Michrom Paradigm MS4 HPLC

LEAP/CTC  
HTS-PAL  
autosampler



- Dual trap column configuration
- Eluent pumps A/B, 200 uL/min
- Load/wash pumps C/D, 1 mL/min
- V1 toggled for each run
- V2 bypass
- One trap is equilibrated while other is eluting
- 1.4 min/sample = 10 x 96 well plates/day
- 1.7 min/sample = 8 x 96 well plates/day with pre-dilution & mixing steps

# ProMass Automated Data Processing

**Xcalibur Sample Sequence**  
defines samples to be analyzed

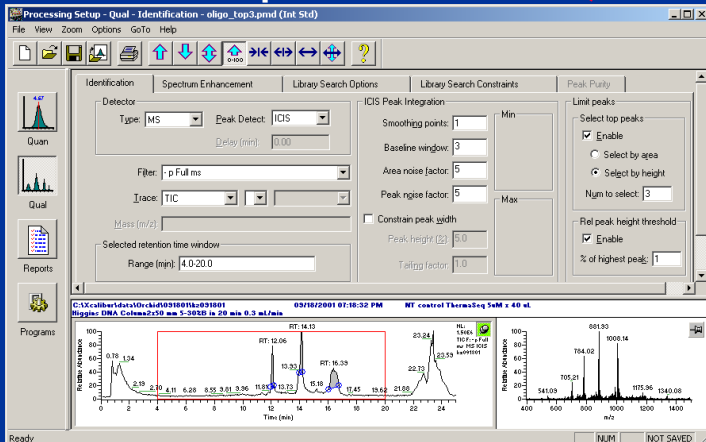
Optional Amino acid or  
nucleotide sequence

**Parameter Setup**  
defines parameters for  
deconvolution

File Name	Proc Meth	ZNova Params	BioSequence
1 1451oflb	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	CATGCATGAAAGTACTTCGGGAGCTA
2 1451ona	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	CACAGCATGCATGAAAGTACTTC
3 637oflb	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	AAGCCATGCATGAAAGTACTTC
4 637ona	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	GAGCATGCATGAAAGTACTTCACA
5 637ona02	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	AACAACCATGCATGAAAGTACTTC
6 7-2probeC3SS	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	TATATACATGCATGAAAGTACTTC
7 7-2probeC6SS	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	CACACATGCATGAAAGTACTTCACA
8 7-1probeC3SS	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	TCCTTCATGCATGAAAGTACTTCAG
9 7-1probeC6SS	C:\Xcalibur\methods\oligo_top3	P'D:\Znova\oligo.params	GCATGCATGAAAGTACTTC

**Xcalibur Processing Method**  
defines how peaks are selected

**Peak picking,  
auto decon-  
volution, and  
report  
generation**



Data File	Sample ID	Sample Comments	Target Masses	Result Code
oligo01	test oligo 01	TEA/HFIPa pH 7.3 / 70% MeOH, PLEP tracers/ab/oligo_0.2 mL/min	[5348.7]	
oligo02	test oligo 02	catCACACACCCAC	[5573.9]	
oligo03	test oligo 03	CCCGCGCTGACCCACACAGC	[5494.6]	
oligo04	test oligo 04	AGCGCTTCCTCCCGCTG	[5387.5]	
oligo05	test oligo 05	TATTTAATATATTACAGACACATTTCCTC	[5916.31]	

Parameter File: C:\Program Files\ProMass\Xcal\Temp.params

Basic Deconvolution | **Advanced Deconvolution** | Results | Reporting

Masses: Input m/z Range (u) From [ ] To [ ]

Output Mass Range (Da) From [ 500 ] To [ 20000 ]

Adduct Ion Mass [ -1.0079 ]

m/z Exclusion List [ ]

Deconvolution: Peak Width [ 2 ], Merge Width [ 0.5 ], Minimum Score [ 2 ], Normalize Scores [ 1 ], Comprehensive Deconvolution [ checked ], Centroid Output [ ]

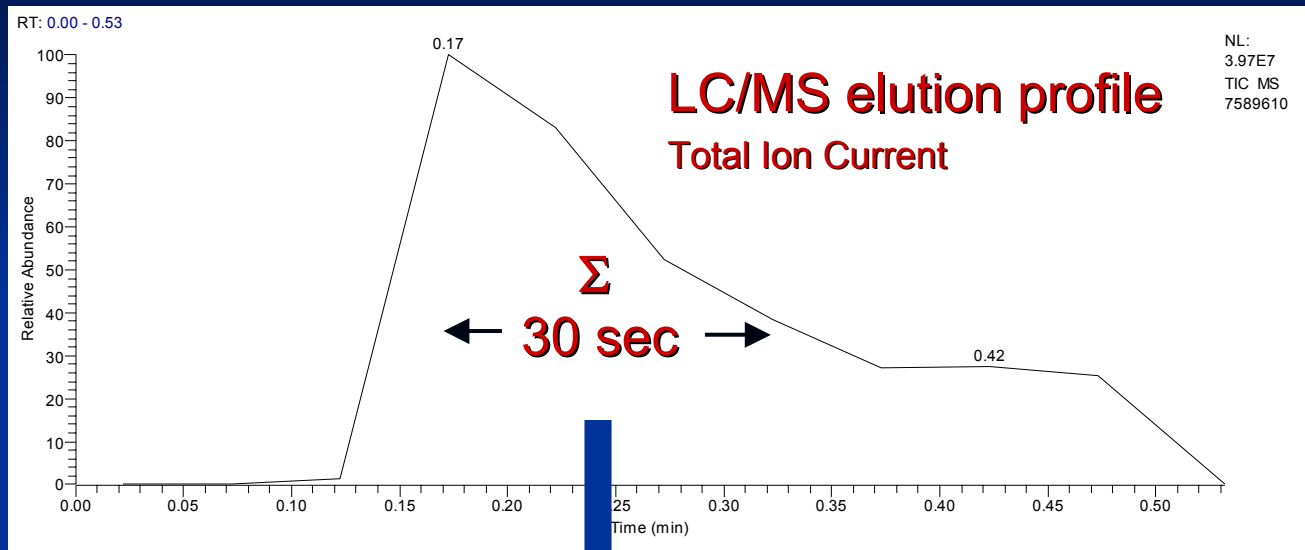
Baseline Removal: [ 0.5 ] [ checked ], On [ ], Low/Normal [ ], Medium [ ], High [ ]

Smoothing: Smooth Width [ 3 ], Num of Smooths [ 2 ]

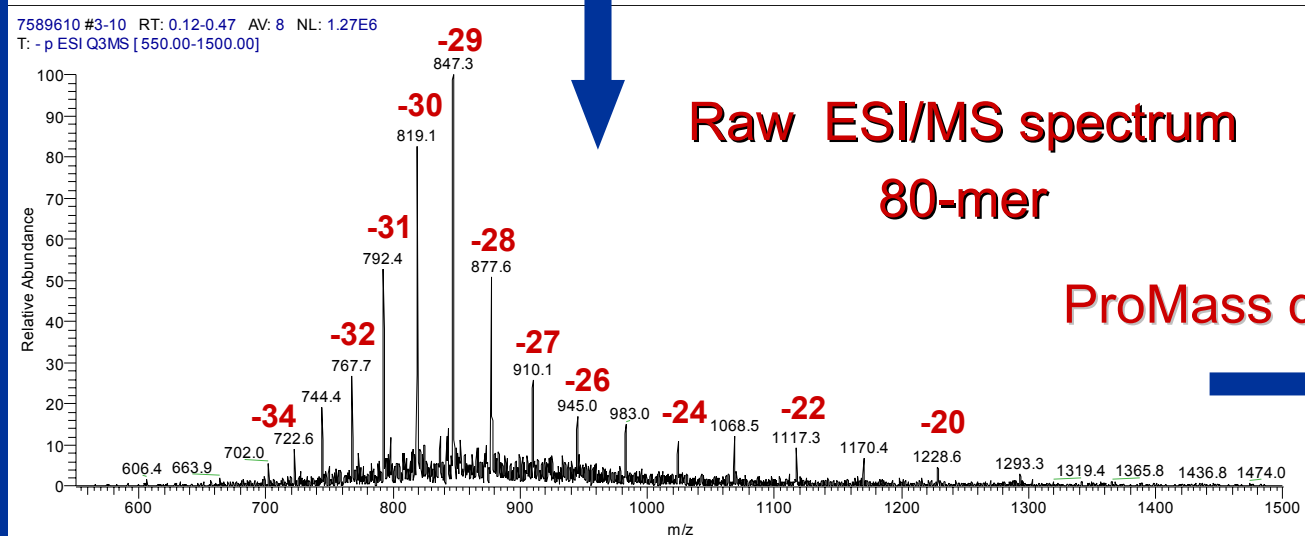
Noise Threshold: Auto [ checked ], %Relative Intensity [ ]

**HTML Results Report**  
including plate view,  
spectra, chromatograms,  
and tabular results

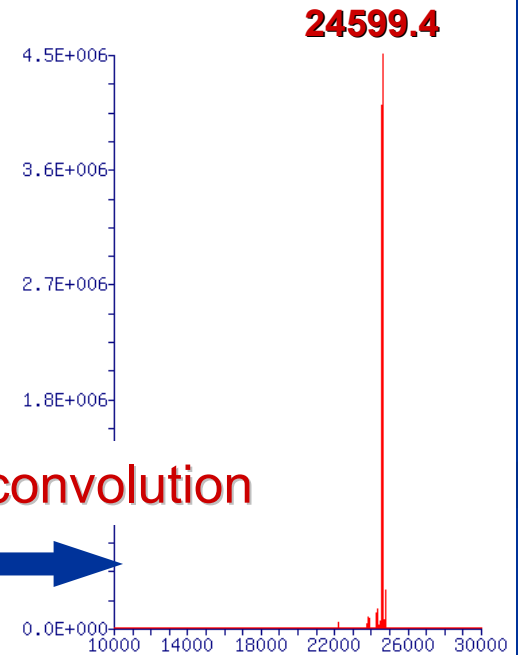
# Automated Oligo ESI Mass Spectral Processing



**Expected Mass = 24600 Da**  
**Mass Error = 0.6 Da**

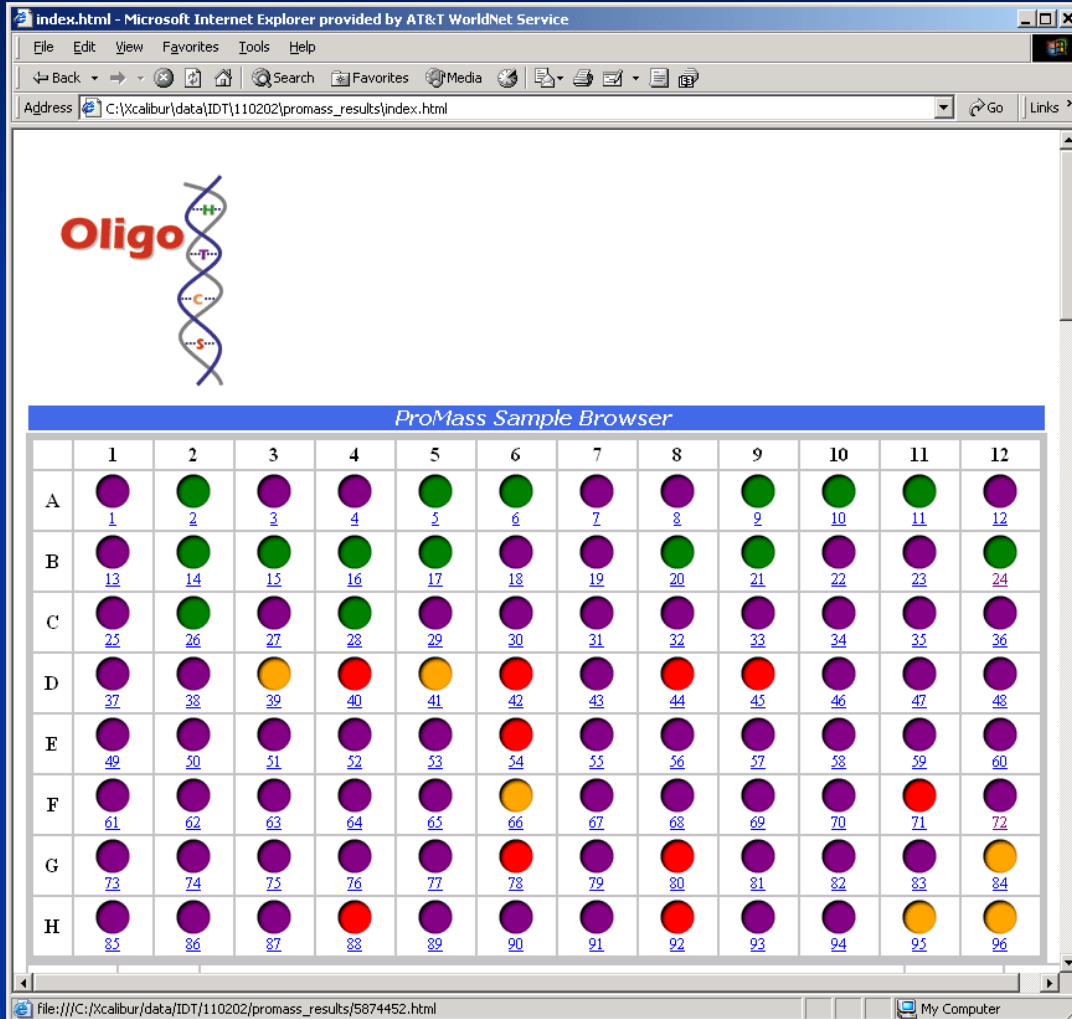


**ProMass deconvolution**







# ProMass Sample Browser



## 96-well analysis of 50-130 mer oligos



- Color codes indicate presence or absence of target masses
- Color-coded wells are hyperlinked to detailed report

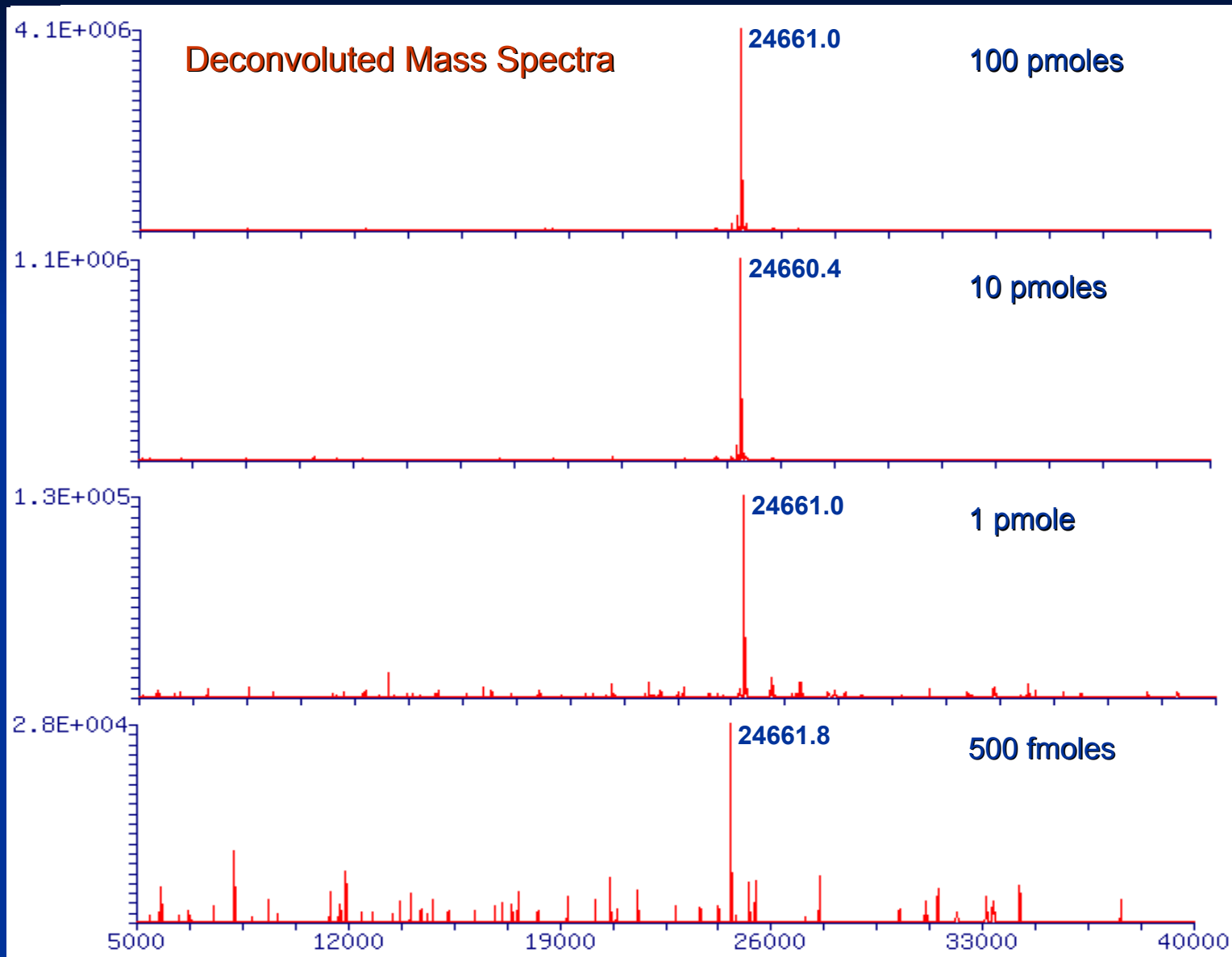
-  Target mass found as major component
-  Target mass found with other significant components
-  Target mass found but not as major peak
-  Target mass not found

# 96-well Plate-Level Mass Accuracy

- Average and Standard Deviation of Green  and Purple  wells from 3 plates run on different days
- **Plate 1 (70-mers):**
  - Average Mass Error: 0.0021% (21 ppm)
  - Standard Deviation: 0.0045% (45 ppm)
- **Plate 2 (70-mers):**
  - Average Mass Error: 0.0000% (0.4 ppm)
  - Standard Deviation: 0.0042% (42 ppm)
- **Plate 3 (50-130-mers):**
  - Average Mass Error: -0.0046% (-46 ppm)
  - Standard Deviation: 0.0046% (46 ppm)

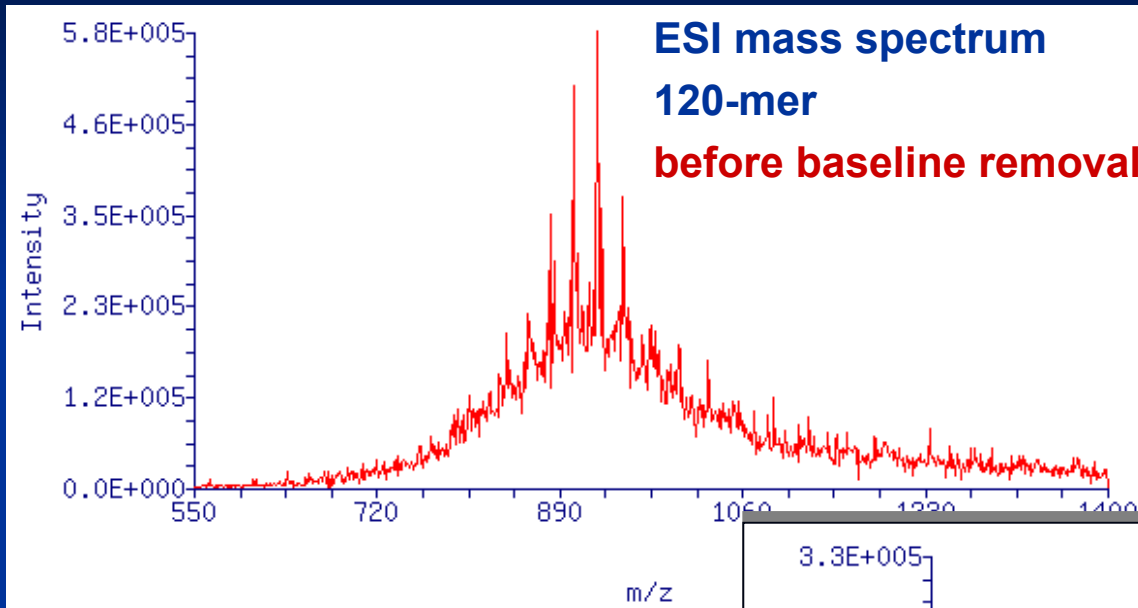
# Oligo HTCS Sensitivity Study – 80 mer oligo

**M = 24661.1**



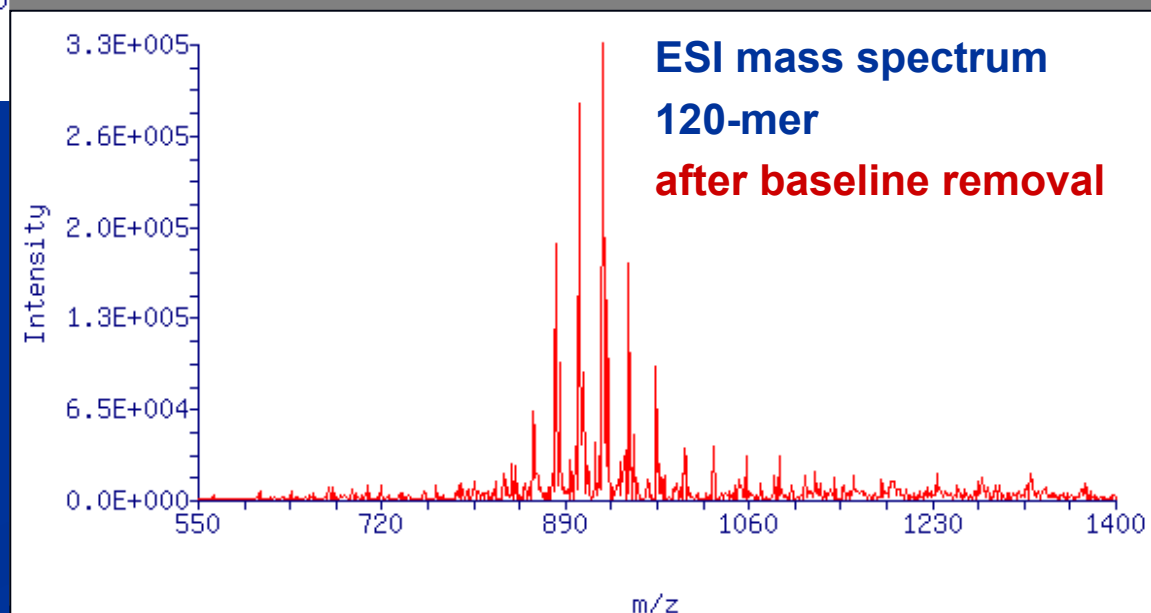
# ESI Mass Spectra of Long Oligos

low purity makes analysis of long oligos challenging



- HT QC of crude synthesis products is highly desirable
- Low purity due to presence of multiple failure sequence products
- Causes baseline “hump” under ESI mass spectrum

Baseline removal is  
key for reliable  
deconvolution

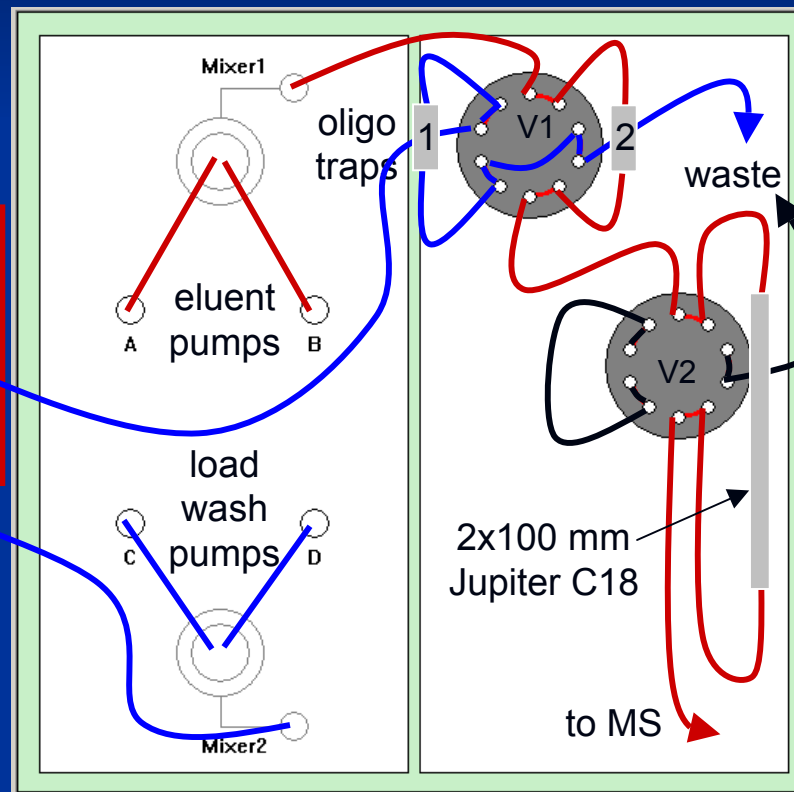
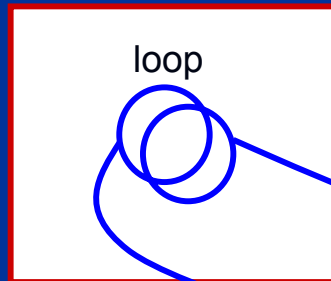


# Oligo HTCS Plumbing Scheme

Shown in Detailed LC/MS Profiling Mode

## Michrom Paradigm MS4 HPLC

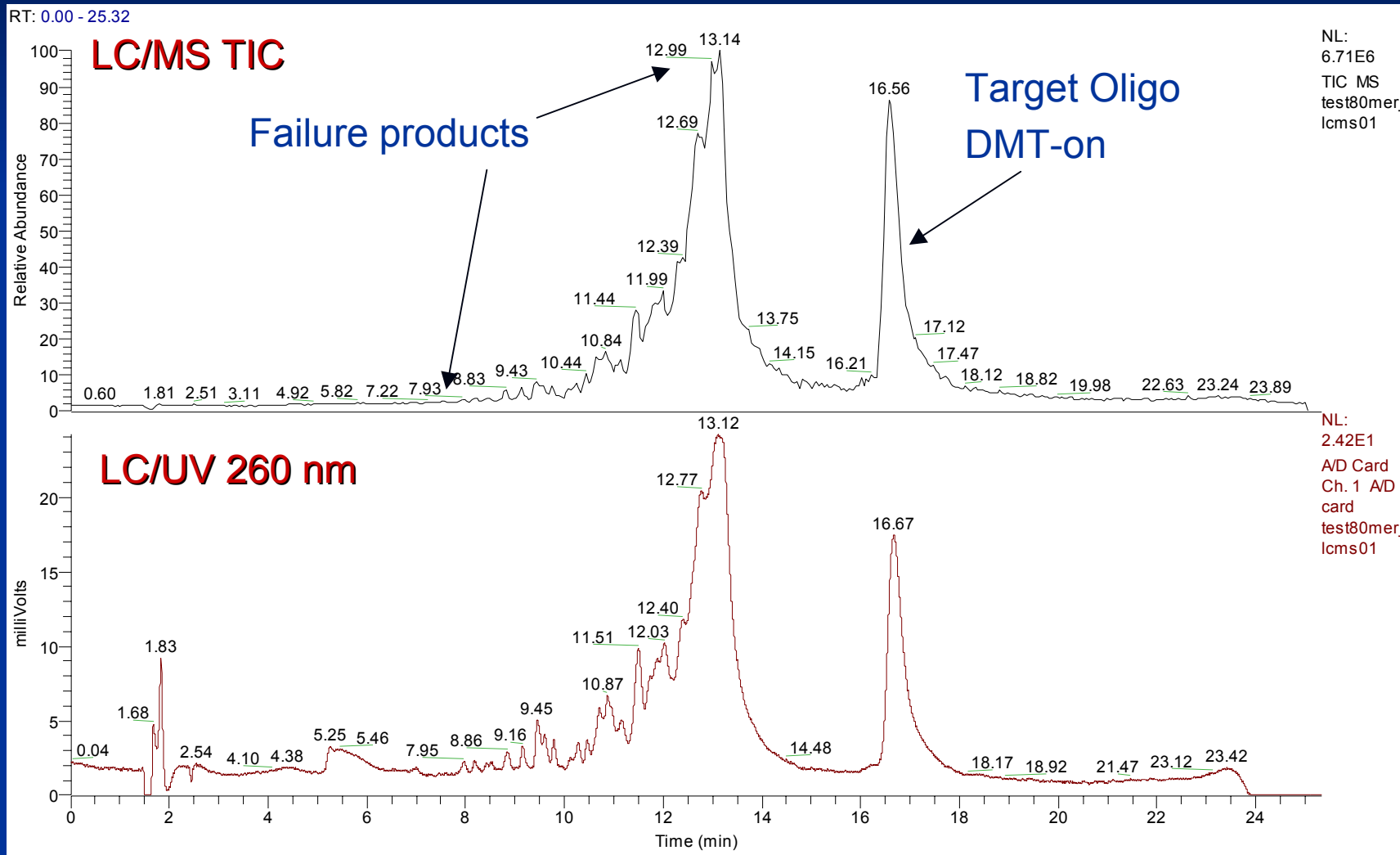
LEAP CTC-PAL  
autosampler



- Switch from HT to detailed mode automatically
- V2 activates 2 x 100 mm C18 column
- Column wash w/ 100% D
- Useful for detailed profiling
- 5-40% B using 20 min gradient

# LC/MS Analysis of Crude 80-mer Oligo

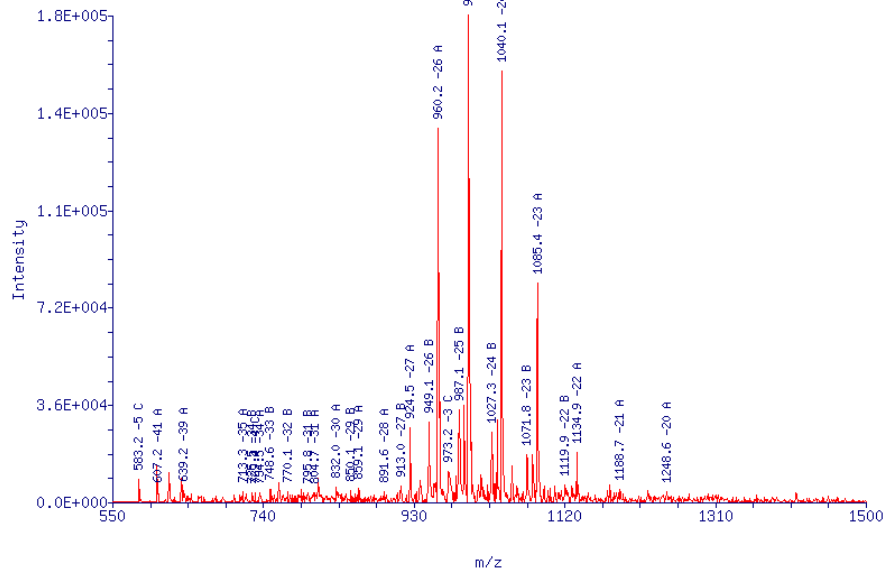
Jupiter C18 300A 2x100 mm, 5-40%B in 20 min



# ESI Mass Spectra of DMT-on Oligo

80-mer from LC/MS run, RT = 16.5 min

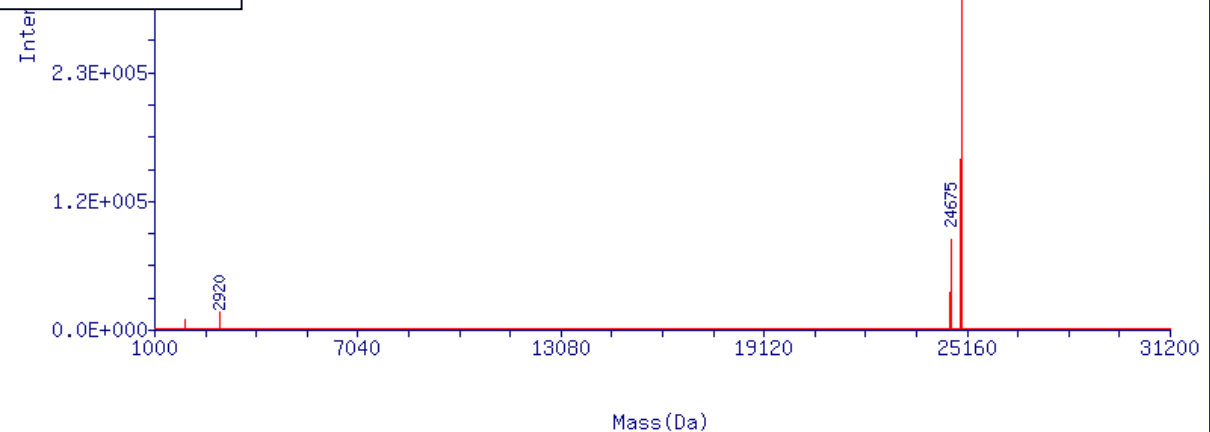
**ESI/MS**



**ProMass Deconvolution**

Expected Mass: 24988.4 Da

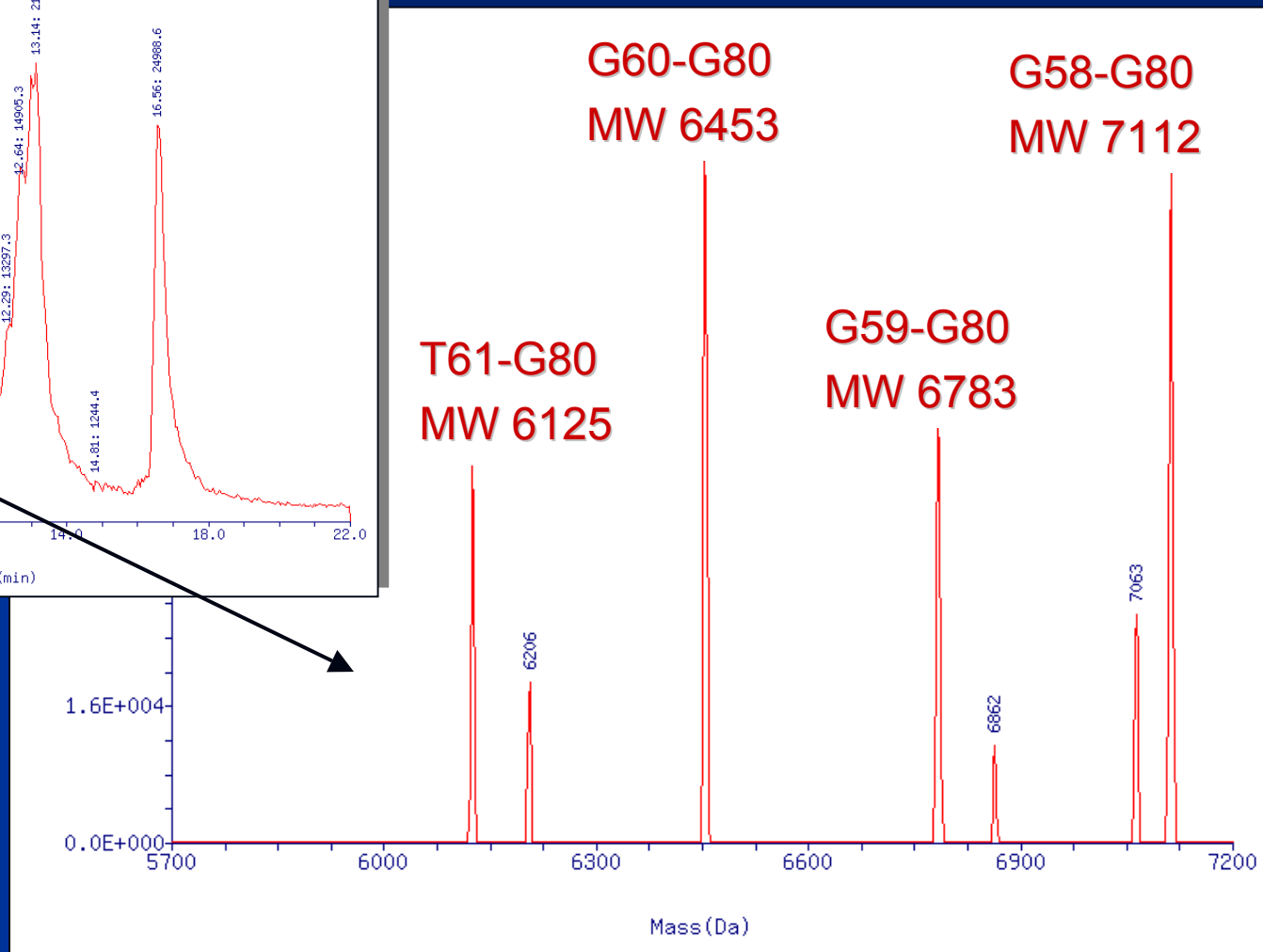
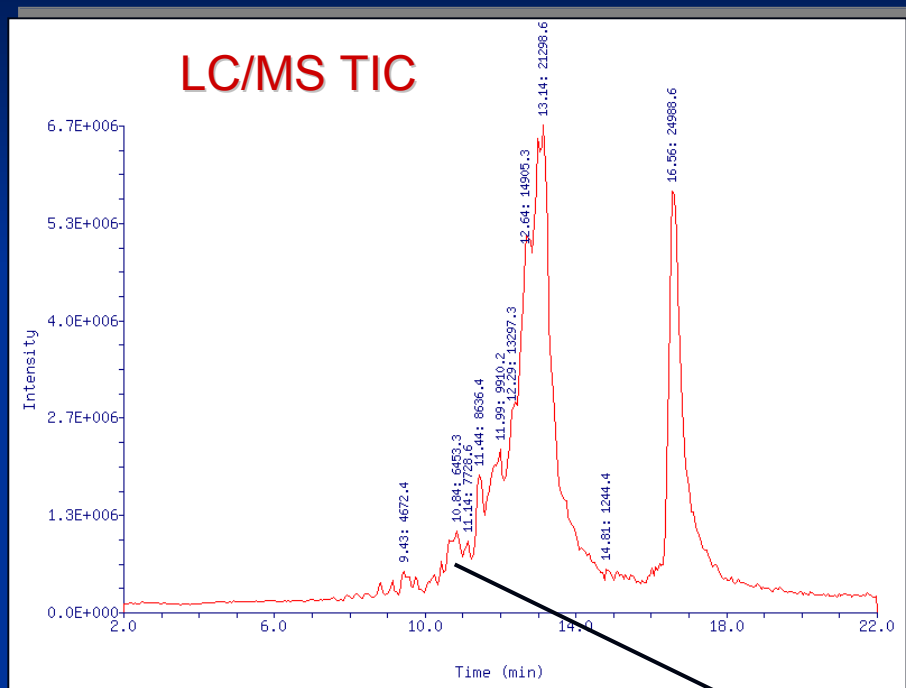
Observed Mass: 24988.6 Da



# Detailed LC/MS Analysis of Failure Products

## LC/MS of 80-mer

5'-TAATACGACTCACTATAGGGTAATACGACTCACTATAGGGTAATACGACTCACTATAGGG-3'



# Conclusions

- A fully-automated system for HT oligo MS analysis was described
  - -ve ion detection with optimized mobile phase
  - High-speed on-line desalting
  - Automated ESI deconvolution and web-based reporting
  - Sample throughput of >1000 samples/day in HT MW confirmation mode
- MALDI-ToF and ESI-MS offer comparable mass accuracy below ~40-mers
- Better resolution with quadrupole ESI-MS vs. linear MALDI-ToF across entire mass range
- ESI-LCMS is the technique of choice for analysis of long and/or fragile oligonucleotides
  - “Softer” ionization
  - No degradation of mass resolution with increasing mass
  - Mass accuracy (< 100 ppm) across entire mass range
- Detailed LC/MS profiling capability on the same system via automatic valve switching

# Acknowledgements

- Brian Elliott – IDT
- Co-workers – Jeff Whitney, David Detlefsen, Kathleen Anderson
- Kerry Nugent – Michrom BioResources