Abstract

The developments of electrospray ionization (ESI) and modern LC/MS techniques have greatly expanded the mass spectrometrist's capabilities for analyzing complex biomolecular samples. Traditionally, processing and interpretation of the mass spectral data from biomolecular samples has been laborious and time consuming. The development of new data processing strategies, involving new robust algorithms for reliable and automated charge deconvolution, has opened up new application areas where ESI/MS can play a vital role. In this presentation, automated analytical strategies are described that demonstrate both high-throughput (e.g, 3000 samples/day) and detailed LC/MS analysis applications of oligonucleotides. The strategies presented encompass all aspects from sample handling and injection to data processing and reporting.

Overview

Purpose

To develop strategies enabling both high-throughput and detailed automated mass spectrometric analysis of oligonucleotides for manufacturing QC as well as therapeutic (drug development applications)

Methods

- Analytical methods utilize negative ion electrospray ionization (ESI) liquid chromatography/mass spectrometry (LC/MS) with buffers optimized for chromatography and high-sensitivity detection.
- High-speed injection systems are used with HPLC employing column switching capability.
- Automated ESI charge deconvolution is used to confirm synthesized oligos as well as identify common impurities, degradants, and metabolites.
- High-throughput (HT) methods are mainly used to confirm expected masses and provide limited information about sample purity and the identification of major components present. HT methods incorporate on-line desalting but no real chromatographic separation. HT methods are amenable to oligos up to ~130 bases. The upper limit of HT methods is mainly limited by sample purity and NOT the ability of the sample to be ionized.
- Detailed LC/MS with gradient HPLC separation is used when more information is desired, e.g., to determine the levels and identities of impurities. LC/MS separation is also useful when purity is low, as is often the case with unpurified long oligos, particularly above 100 bases.

Results

- Two analytical systems were developed to address the different analysis goals of HT vs. detail.
- For modest throughput applications where flexibility is desired, a single-arm autosampler can achieve a throughput of ~1500 samples/24 hrs and is automatically switchable from HT to detailed LC/MS analysis modes (**Figure 1**).
- When maximum throughput is required, a staggered injection, dual-arm autosampler is used that achieves an injection rate of ~3000 samples/24 hrs (**Figure 2**).
- A detailed LC/MS application is presented which demonstrates the use of the system for metabolic stability and metabolite i.d. of oligonucleotides from serum samples.

Experimental

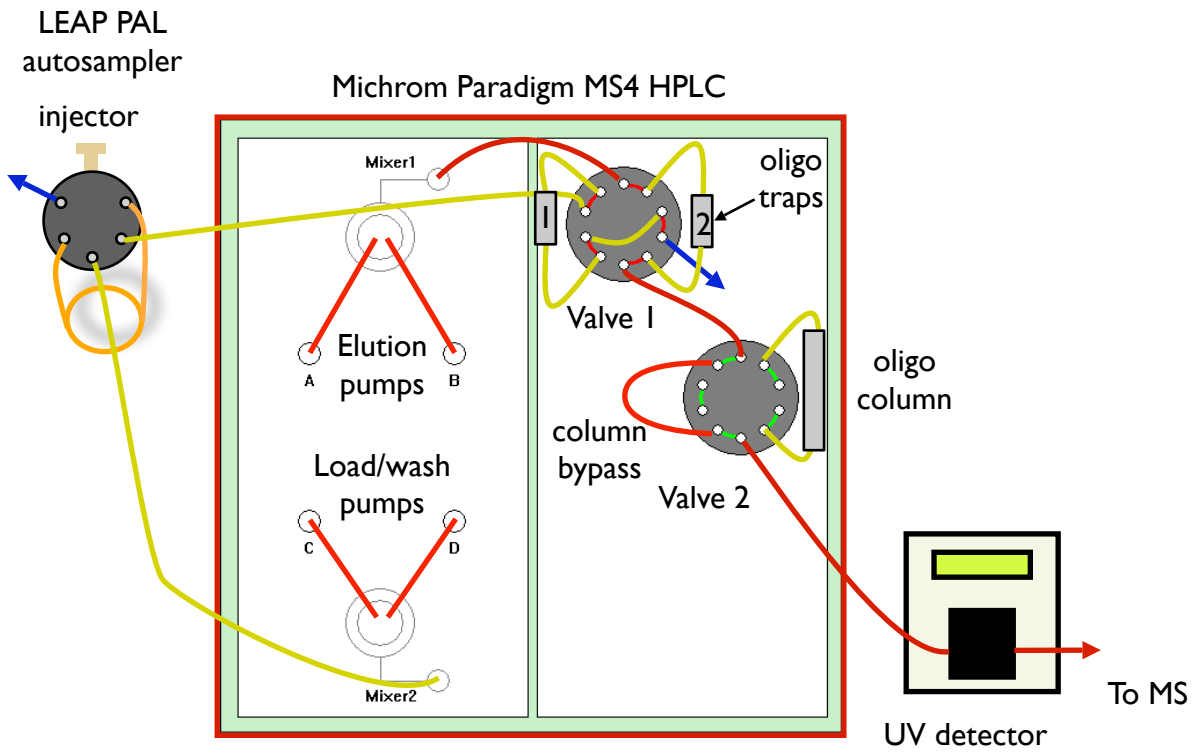
Mass Spectrometry

- LCQ series or LTQ mass spectrometers (Thermo Scientific) running Xcalibur 1.4 or 2.0 and Novatia ProMass Deconvolution software, (also available from Thermo Scientific)
- Full scan, negative ion mode detection

Chromatography

- LEAP HTS PAL or Twin HTS-PAL autosamplers with macros written in Cycle Composer
- Michrom Paradigm MS4 HPLC and UV detector
- Mobile Phases: A = H_2O/0.0375% DIEA/0.075% HFIPA/10 uM EDTA (pH ~8.5), B = same components as A except containing 65-90% MeOH (DIEA = diisopropylethylamine, HFIPA = hexafluoroisopropanol). Mobile phase A also serves as wash solvent for HT methods. Gradients are typically 5-40% MeOH.
- Trap Columns: Oligo HTCS trap columns, 10 µM 300A C18, 1 x 10 mm (Novatia)
- Analytical column for metabolite i.d. work: PLRP-300A (Polymer Labs) operated at 40C

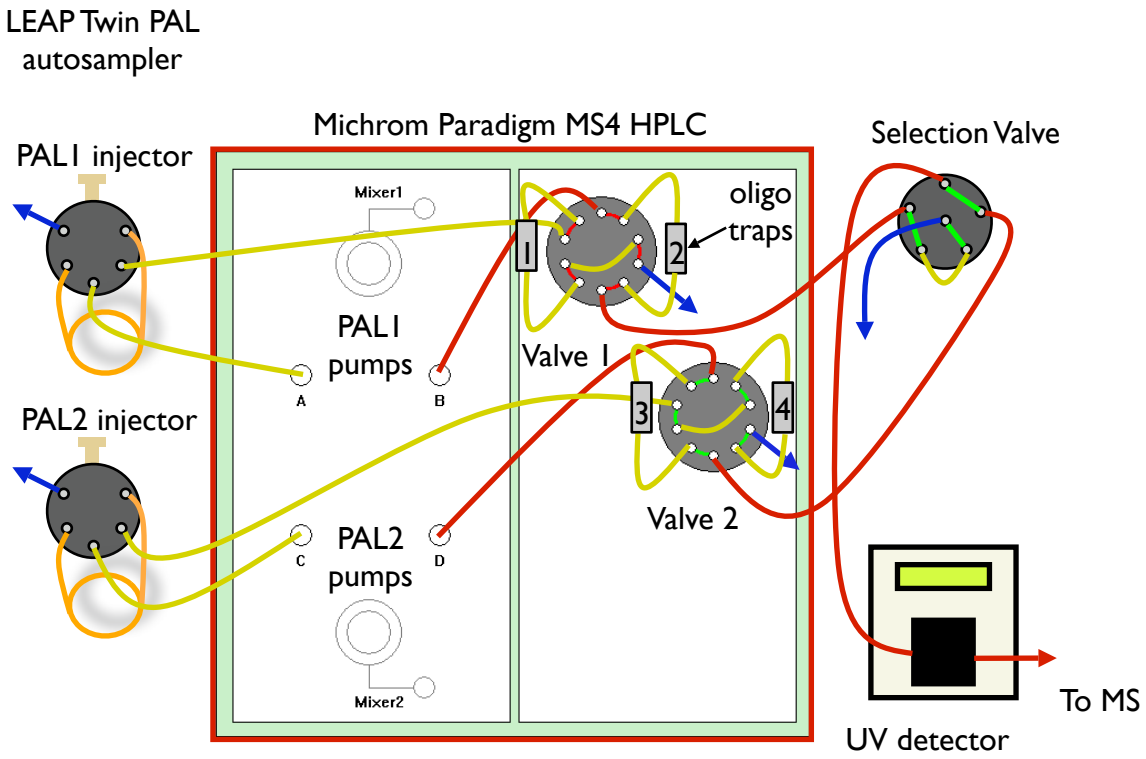
Figure 1. Instrument configuration allowing for ~1500 samples/24 hrs HT MS analysis and automated switching to detailed LC/MS.



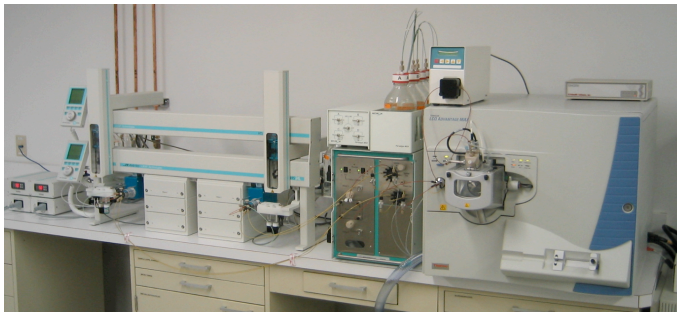
Description

- Sample is flushed from the autosampler to the oligo trap columns using wash solvent from pump C at 1mL/min.
- After 20 sec rinse, Valve 1 is toggled.
- In HT mode, Valve 2 is in column bypass and sample is eluted directly into MS using 65%B (no gradient), resulting in ~1 min analysis time, or ~1500 samples/24 hrs.
- In detailed LC/MS mode, oligo column on Valve 2 is switched in line and a gradient is run thru trap and oligo column.
- Solvent from pump D is used to clean flow path and traps between runs.

FIGURE 2. Instrument configuration allowing for ~3000 samples/24 hrs dedicated HT MS analysis.

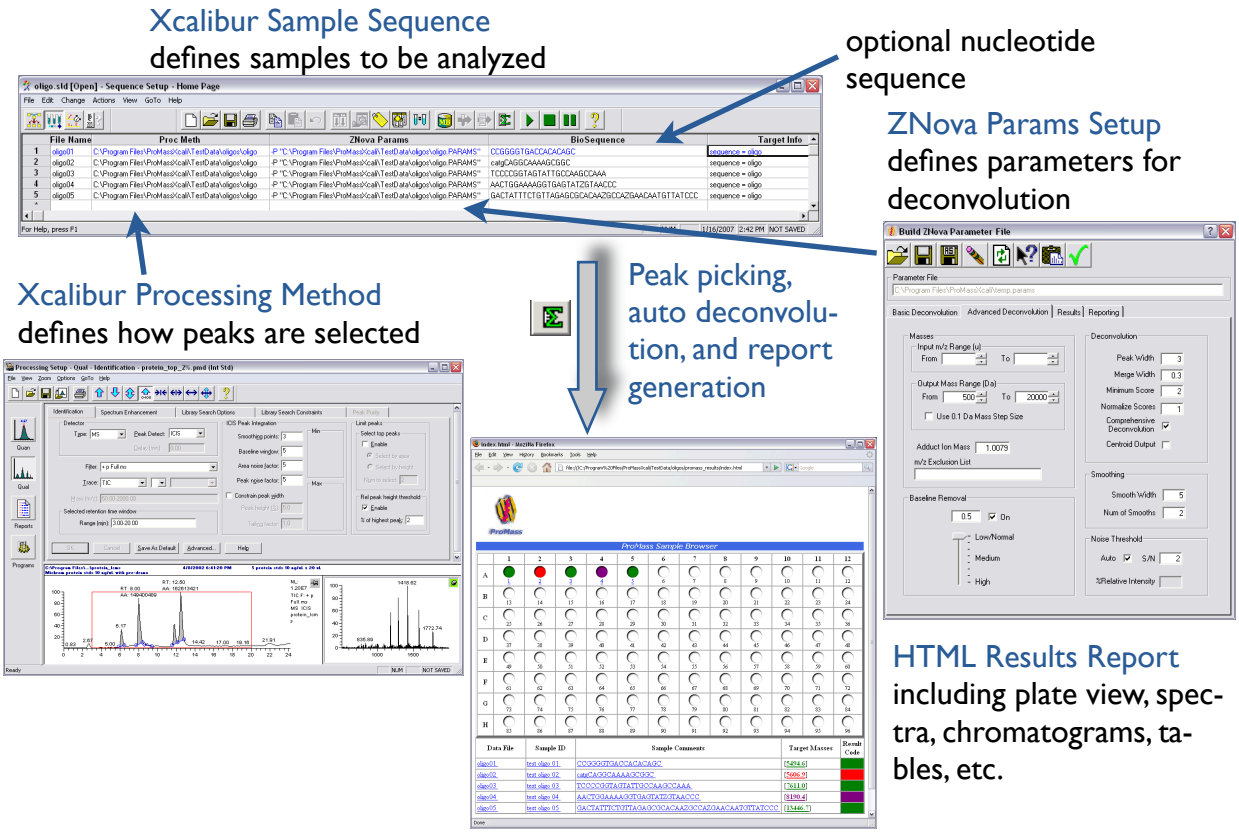


DESCRIPTION



- Staggered injections are performed by a special injection macro on the Twin PAL autosampler. PAL1 injects while PAL2 cleans its syringe.
- Pumps A and C load and wash 2 sets of oligo trap columns at 1mL/min with 20 sec load/wash time.
- Pumps B and D deliver pre-mixed eluent (buffer containing 65% MeOH) at 300 uL/min.
- Valves 1 and 2 are toggled at the appropriate time after their respective wash cycles are complete.
- A selection valve determines which trap is eluted into the MS system.
- Resulting analysis time is ~29 sec or ~3000 samples/24 hrs.

FIGURE 3. Data processing work flow for automated ESI/LC/MS analysis of oligonucleotides.



DESCRIPTION

- Data processing work flow is driven from the Xcalibur sample list.
- Parameters for chromatogram peak selection and charge deconvolution are specified.
- Target masses and optional oligo sequences are specified.
- At the end of the sample sequence acquisition, data processing is triggered, as shown by the example in Figure 4.
- ProMass deconvolutes all selected peaks and produces a web-based color-coded summary indicating the presence of expected target masses in the deconvoluted spectra. The color-coded sample wells in the ProMass browser are hyper-linked to detailed reports for each sample allowing the raw and deconvoluted spectra to be viewed interactively.

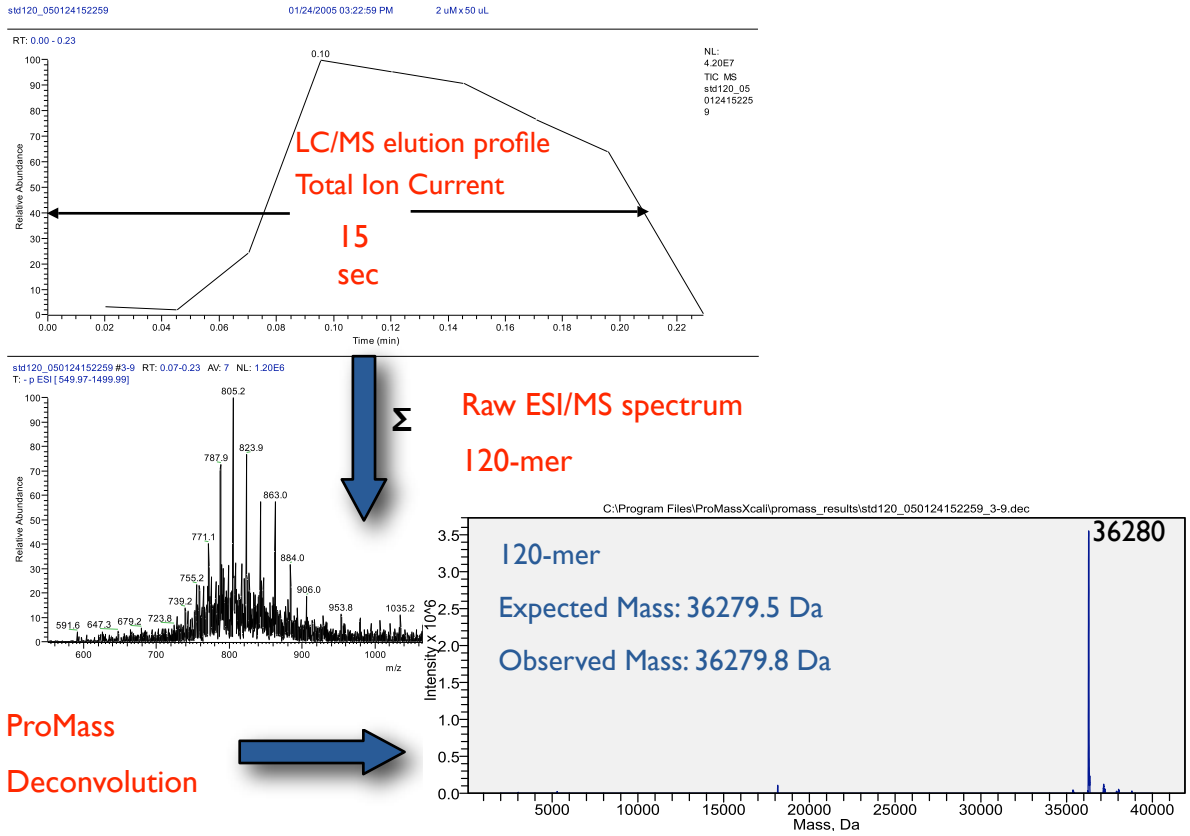
Target mass found as major component

Target mass found with other significant components or low spectral quality

Target mass found but not as major peak

Target mass not found

FIGURE 4. Automated ESI/MS analysis of a 120-mer oligo using HT systems shown in Figure 2. Spectra are automatically averaged over LC/MS peak profile by the Xcalibur processing method. Data is then exported to ProMass for deconvolution.



Detailed LC/MS Application

Metabolic Stability and Metabolite Identification of Oligos in Serum Extracts

Overview

- The potential use of oligos in therapeutic applications is increasing.
- Stability of oligo-based drug candidates in serum over time needs to be measured.
- Potential metabolites need to be identified.
- Stability and metabolite i.d. information is fed back into process for drug candidate optimization.

Approach

- Incubate oligo with serum over a suitable range of time points (~10 uM oligo in serum).
- Digest samples with proteinase K to "chew up" serum proteins.
- Centrifuge samples and inject on LC/MS system (**Figure 1**) using LTQ mass spectrometer in detailed LC/MS mode.
- Measure disappearance of oligo by measuring LC/MS peaks areas of extracted ion traces relative to t(0) (see **Figure 6**).
- A linear fit of natural log of % oligo remaining vs. time is used to determine half-life.
- Use ProMass deconvolution software to "fish out" potential metabolites.
- Comparison of data with extracted serum blanks allows false positive matches from serum background to be eliminated.

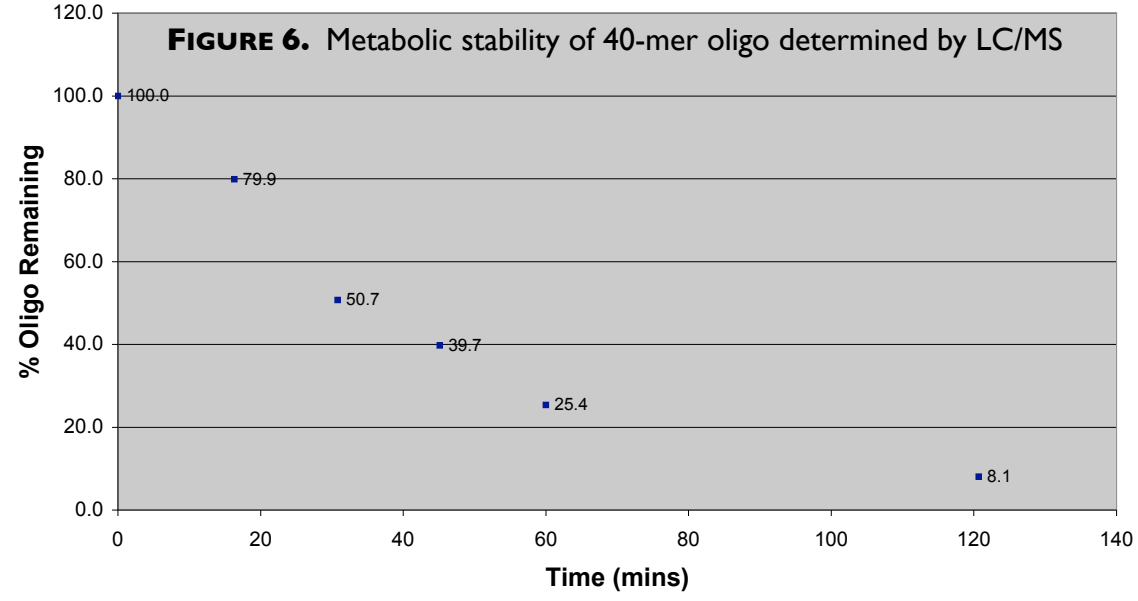
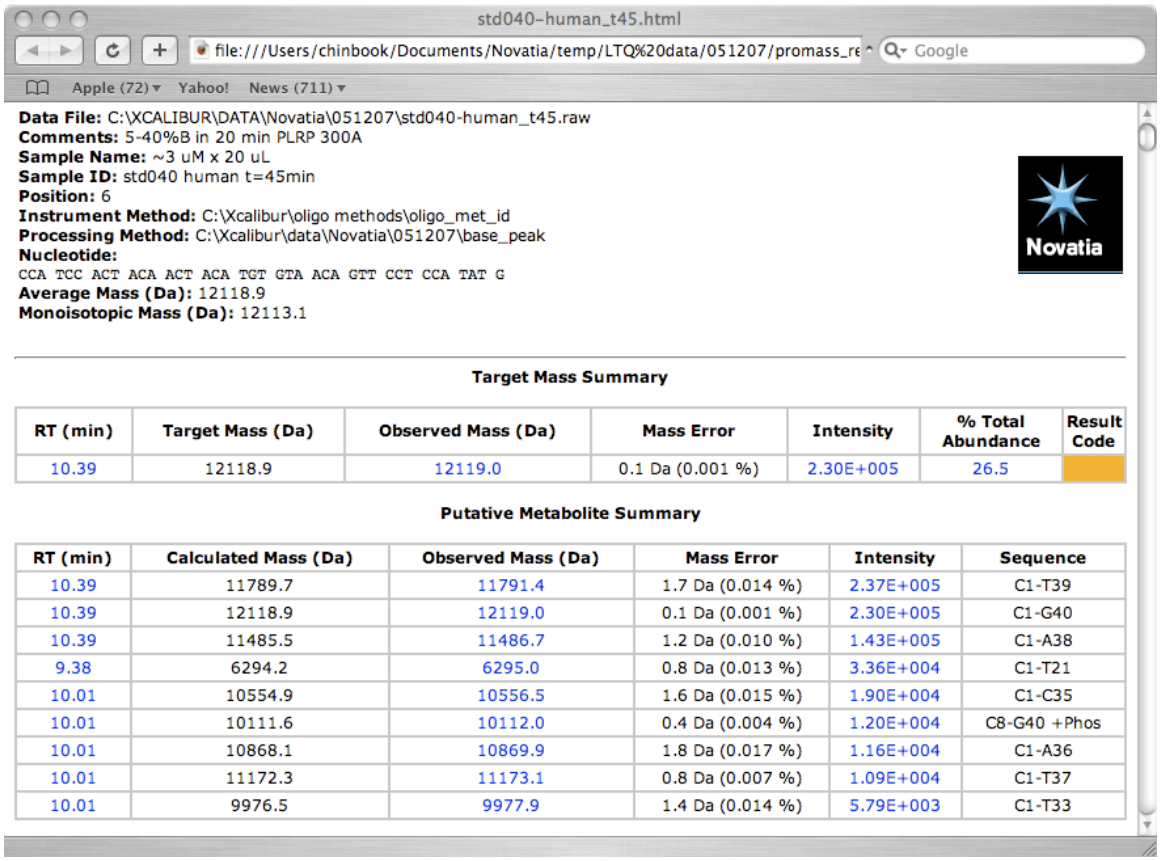


Figure 6. Metabolic stability of 40-mer oligo determined by LC/MS

RESULTS

- Stability of a 40-mer oligonucleotide in human serum was measured by LC/MS, resulting in the graph in **Figure 6**. Half-life for this oligo was determined as ~33 minutes.
- ProMass deconvolution software was used to calculate all potential metabolites containing either the 3' or 5' end of the oligo and report matches found in the experimental data.
- Metabolites are reported in the Putative Metabolite Summary table, as shown below for the t = 45 min time point.
- Most of the metabolites found in this example were the result of cleavage from the 3' terminus.

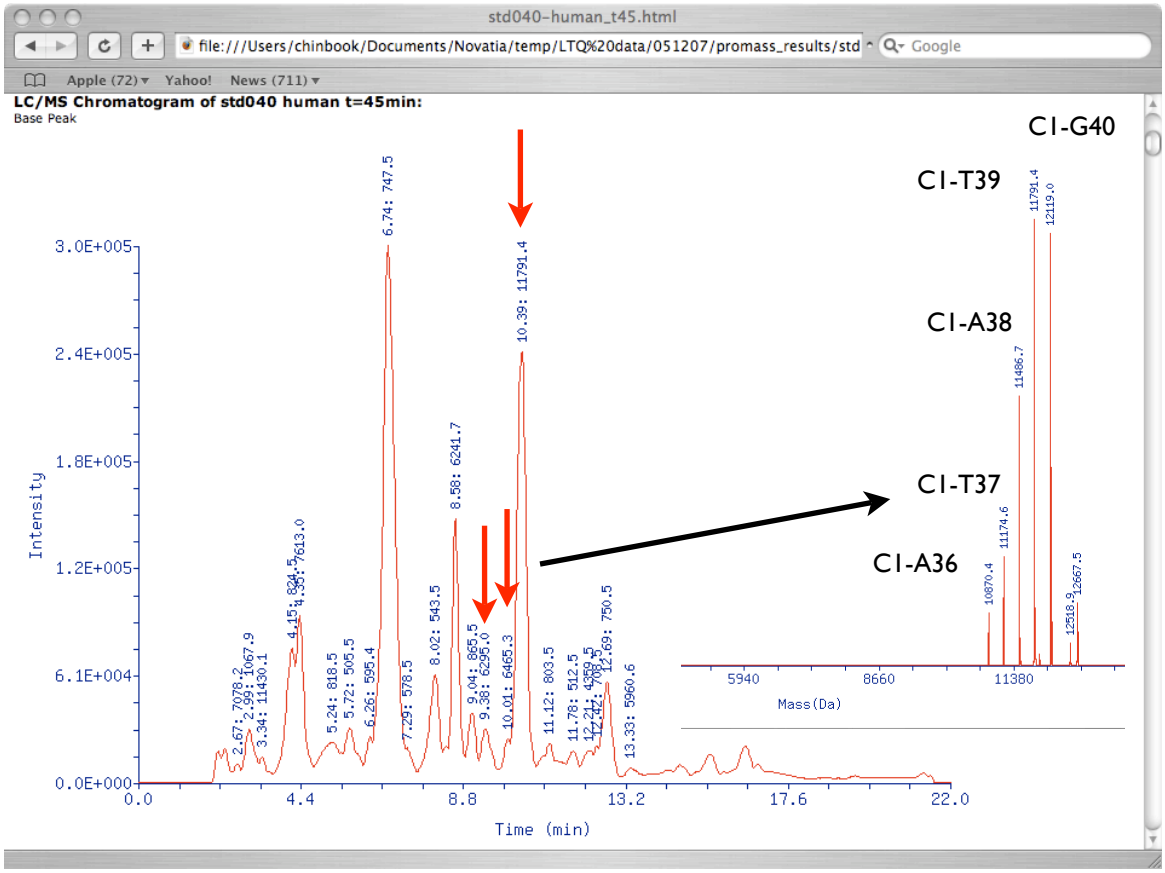


Target Mass Summary

RT (min)	Target Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	% Total Abundance	Result Code
10.39	12118.9	12119.0	0.1 Da (0.001 %)	2.30E+005	26.5	

Putative Metabolite Summary

RT (min)	Calculated Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	Sequence
10.39	11789.7	11791.4	1.7 Da (0.014 %)	2.37E+005	C1-T39
10.39	12118.9	12119.0	0.1 Da (0.001 %)	2.30E+005	C1-G40
10.39	11485.5	11486.7	1.2 Da (0.010 %)	1.43E+005	C1-A38
9.38	6294.2	6295.0	0.8 Da (0.013 %)	3.36E+004	C1-T21
10.01	10554.9	10556.5	1.6 Da (0.015 %)	1.90E+004	C1-C35
10.01	10111.6	10112.0	0.4 Da (0.004 %)	1.20E+004	C8-G40 +Phos
10.01	10868.1	10869.9	1.8 Da (0.017 %)	1.16E+004	C1-A36
10.01	11172.3	11173.1	0.8 Da (0.007 %)	1.09E+004	C1-T37
10.01	9976.5	9977.9	1.4 Da (0.014 %)	5.79E+003	C1-T33



- Red arrows mark the location of metabolites found in the LC/MS chromatogram above.
- All other peaks are due to digested serum background.
- The mass spectrum in the inset figure above shows the deconvoluted mass spectrum from RT = 10.39 min and the identified metabolites.

Acknowledgments

- Thanks are due to Brian Elliott of IDT for providing the oligo standards and for collaboration on the development of the high-throughput MS systems.