CORE RESEARCH
FACILITIES

Proteomics

Bioanalytical MS has multiple workflows that may be customized en route to faithfully characterize complex matrices

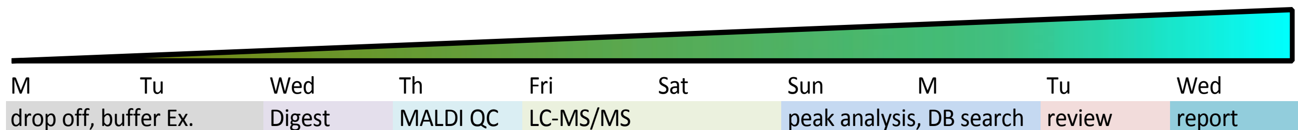
COSTS:	**Protein Identification**	**Biomarker Discovery**	**Absolute Quantification**	**Post Translational Modifications**	**Rapid Bacterial Biotyping**	**User Rates, Training and FAQ**
This link will provide the cost structure of proteomics applications organized by objective.	MS/MS is an unbiased measure of identity that relies upon real-time verification of peptide sequence.	Several MS approaches can reveal the *fold-change* of individual proteins between distinct phenotypes.	With specificity exceeding sandwich ELISA, MRM can quantify dozens of proteins in multiplexed assays.	Sub-stoichiometric and difficult to ionize, custom techniques are often required.	Identify cultured pathogens with a simple MALDI preparation procedure and a few seconds of acquisition.	After training researchers can schedule personal use of the instruments. Woe to those who screw up.

<u>BackGround:</u> The identification (and quantification) of proteins relies upon mass spectrometry (MS) measurements, most often coupled to capillary LC separations.

- These procedures require several days of very clean preparations and quality checks to avoid cross contamination and ensure accurate results with high-fidelity.
- Certain points in each workflow often need customization and routine procedures used by other experimental protocols often have to be significantly altered and optimized for use with MS.
- The output reports from a typical mass spectrometry run can be very complex, representing the sum of (tens of) thousands of spectra.

We work closely with researchers to help them understand and apply the information to achieve optimal relevancy in their ongoing work. To view most reports you will need the latest Scaffold viewer from PoteomeSoftware

<u>Time line</u> for *routine* protein identification (~7-10 days)



We accept samples on Monday or Tuesday, digest Tues. (sometimes Wed.) then samples require a day to desalt, re-concentrate and check by MALDI-TOF. If QC are met, samples are prepped for LC-MS/MS on Thursday. On Friday, we interrogate initial results and evaluate which samples have sufficient coverage based upon the stated objective, the chromatographic profile and the complexity of the original gel band. In this exercise, we attempt to categorize 3 outcomes:

1. The chromatogram is what we would expect given the gel band.
2. The chromatogram looks under sampled. By checking against the MALDI QC and the system suitability chromatograms, a decision is made to either rerun the sample, w/ same instrument or another instrument, repeat the sample prep w/ an new aliquot or discontinue the analysis.
3. The chromatogram is great but it's too complex. Possibly rerun with a longer gradient or recommend prior fractionation.

Those decisions determine what is complete or prepped a second time to run over the weekend. On Monday, data transfers begin and searches are set up. The results of two independent search engines are reviewed on Tuesday, merged into Scaffold and reviewed a second time. If objectives are met, the final report is sent out on Wednesday.

Below, "dynamic range" refers to the protein complexity as:				(gray)	Fixed component	(green)	Optional
Modest < 30	Typical < 50	Wide < 70	Extreme > 90	(yellow)	Alternatives; select one	(pink)	Optional Follow-Up

Protein ID from a gel band: As low as $80/band for 5 bands

			Optional analysis route. Uses MALDI QC spectra w/o LC			Choose one of the following for data collection				Optional Add-ons
Protein ID from Gels: Gel documentation, excision, Reduction, Alkylation, proteolysis, prep for MS, MALDI confirm, QA run @ 20 fmole, data processing, 2 independent search engines, compilation and final report in Scaffold	Band Extraction and Trypsinization	MALDI QC run	MALDI mass fingerprint searching from the QC spectra.	Combined report of MALDI fingerprint data matching	Sample logistics, 2 independent search engines, compilation and final report in Scaffold	Structural confirmation with MALDI TOF/TOF	LC-MS/MS "Modest" dynamic rage (30 min gradient)	LC-MS/MS "Typica" dynamic rage (60 min gradient)	LC-MS/MS "Wide" dynamic rage (90 min gradient)	Iterative exclusion list
First Sample	$30	$30	$30/hr	$30	$45	$25	$40	$65	$90	$25
Subsequent SAMPLES	$30	$30				$25	$40	$65	$90	$25

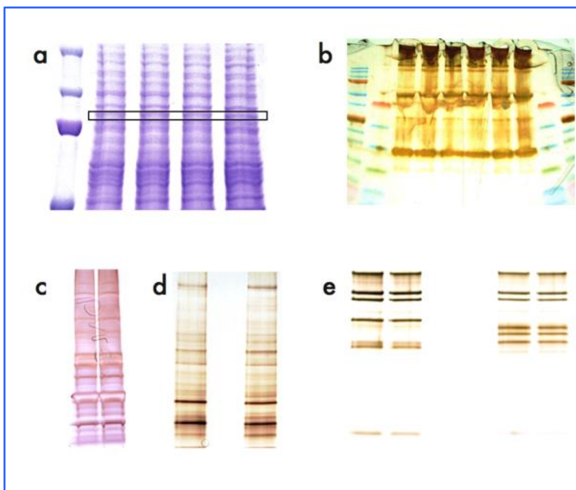
Protein ID from a Mixture in Solution or a Crowded Gel

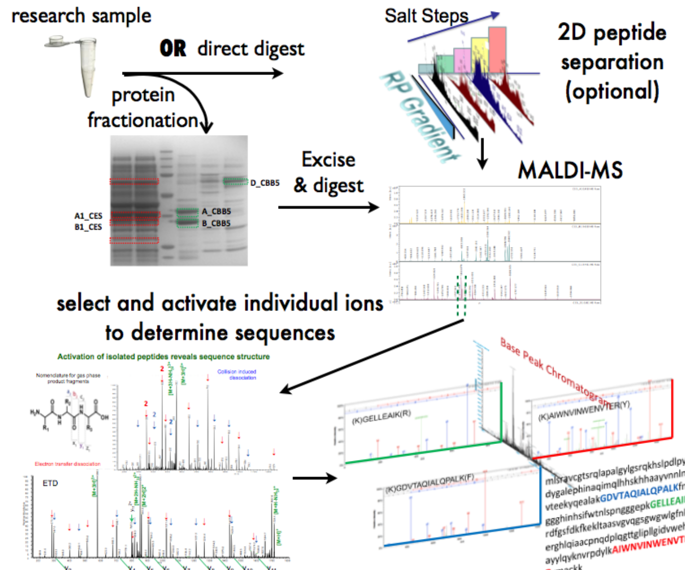
Uses 2 Chromatography steps for maximal peak capacity and sensitivity.

				Choose one of the following for data collection				Optional add-ons	
Protein ID from Solution: Buffer Exchange, Reduction, Alkylation, proteolysis, prep for MS, MALDI confirm, QA run @ 200 fmole, data processing, 2 independent search engines, compilation and final report in Scaffold.	Detergent removal, reduction, aklylation, proteolysis and cleanup prior to off-line LC	MALDI QC run	Data search. Data controls. Data report.	Structural confirmatin with MALDI TOF/TOF	LC-MS/MS "Typical" dynamic range (60 min gradient)	LC-MS/MS "wide" dynamic range (90 min gradient)	LC-MS/MS "Extreme" dynamic range (120 min gradient)	Iterative exclusion list	IEX fractionation includes multiple C18 clean-up
Umbrella Cost	$50		$45						$200
Analysis for each SCX fraction		$20		$25	$65	$90	$120	$25	

Researchers, especially those new to proteomics, should consider commercial PAGE mini gels to separate their proteins of interest. With PAGE, it's easy to check the purity and complexity of the sample and the gels can assist in enriching for particular post-translational modifications. In the Core, we refer to gel images to evaluate feasibility, status and next steps for most projects. In general, PAGE is a great way to prepare your samples and removes contaminating metabolites, detergents, and small peptides. Commercial gels, as well as fresh staining reagents and glassware is highly advised since routine lab equipment is typically very contaminated with proteins and other substances that will interfere with subsequent mass-spectrometry analysis.

Some examples of gels suitable for protein I.D. are shown below. **(a)** is a Coomassie-stained gel and **b-e**, are silver-stained with MS-compatible reagents. Silver-staining is 50-fold more sensitive than Coomassie. The **(e)** gel is a far simpler mixture of proteins than **(a)**. Indeed, in the indicated band of gel **(a)**, we Identified >120 proteins. This level of





Hundreds of proteins may be identified by combining dynamic sequencing with RPLC separations

sample complexity can hinder the ability to identify particular post-translational modifications of a given protein.

PAGE is a cheap and efficient protein pre-fractionation step but it's not the only option. In fact, some objectives, such as high-throughput PTM identification and quantification of sera or tissue samples are not compatible with PAGE. For these reasons, we also offer protocols that bypass gels.

Researchers may opt for direct Trypsin digestion of their sample in solution followed by one or two distinct chromatographic separation methods prior to MS analysis. Spreading a complex sample across several chromatographic fractions, each that are less complex, boosts the ability to detect and quantify individual peptides—especially important if one is looking for proteins at low relative abundance.

All digest fractions are verified by MALDI for quality before proceeding with further steps. Sometimes, a protein ID can be made using this MALDI step alone, particularly if the sample is not complex. However, more often, the sample will go on to an LC-MS/MS analysis where peptides are separated on a C18 chromatography nano-column and subjected to MS analysis of the peptides (MS1) and fragments of the peptides (MS2). As indicated in the price list, users can specify what type of C18 elution profile they would like to use for their sample. For less complex samples, a 30 min gradient typically suffices. However, if the band or sample still has many components, then spreading the peptides out more with a longer C18 chromatography profile is desirable. The right choice can be made by analyzing the MALDI profile of the sample, the original gel, and factoring the overall goal of the investigator.

Quantitative Comparisons using Differential Labeling Strategies: Biomarker ID

Culture samples labeled metabolically (SILAC) may be fractionated in gels or analyzed with direct digestion.

				Choose one of the following for data collection			Optional add-ons	
SILAC: Use natural metabolism in cell culture to incorporate stable isotpic forms of AA into the entire proteome. Compare two or more phenotypes by pooling them in the same expermen. Typicall run **3 replicates** and expect substantial analysis time'	Brand Extraction and Trypsinization	MALDI QC run	Data search, Data controls, Data report. Per five multiplexed bands.	3 Tech reps LC-MS/MS "Typical" dynamic range (60 min gradient)	3 Tech reps LC-MS/MS "Wide" dynamic range (90 min gradient)	3 Tech reps LC-MS/MS "Extreme" dynamic range (120 min gradient)	IEF Off-gel with clean-up. May follow with direct digest or 1D PAGE	Additional work such as further reporting, renormalizing, de-novo sequencing, graphical layout, etc.
Mixed labeled bands in gel	$40	$20	$45	$180	$240	$360	$200	$50/hr

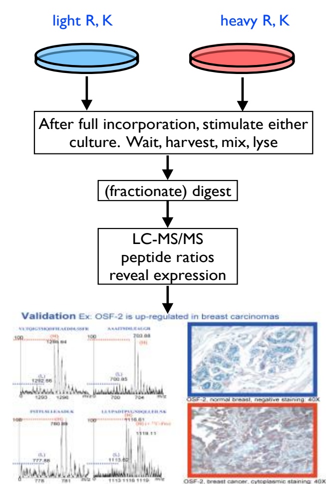
Differential Peptide Labeling and analysis from gel bands or complex mixtures.

					Choose one of the following for data collection				Optional add-ons	
Chemical tagging: The fidelity of isotopic labeling is best when performed directly after digestion. "Global" analyses often require separate steps of peptide seperation on unique solid phase supports. Typically run **forward and corssover repilcates** and extract substantial analysis time.	Detergent removal, reduction, aklylation, proteolysis and cleanup prior to off-line LC	Desalt, tag each state with unique isotope, recombine. Price per component state.	MALDI QC check of each fraction proceeding to nanoRPLC	Data search, Data controls, Data report. Per five multiplexed samples.	Each LC-MALDI-MS/MS (60 min gradient)	Forward and X-over gradients LC-MS/MS "Typical" dynamic range (60 min gradient)	Forward and X-over gradients LC-MS/MS "Wide" dynamic range (90 min gradient)	Forward and X-over gradients LC-MS/MS "Extreme" dynamic range (120 min gradient)	IEX (includes multiple C18 clean-up). May not be required for very simple samples, such as TAP.	Additional work such as further reporting, renormalizing, de-novo sequencing, graphical layout
Umbrella cost	$50	$40		$45					$200	$50/hr
Analysis for each SCX fraction			$20		$90	$130	$180	$240		

Quantification of protein abundance. The Proteomics facility supports relative quantification using isotopic tags. This approach achieves high precision with fewer replicates and is very useful for comparing one sample vs another—for instance an immunoprecipitation with antibodies to different proteins or w/ and w/o kinase inhibitor.

<u>SILAC (Stable isotope labeling by amino acids in cell culture):</u> Investigators working with cultured cells can use simple metabolic growth to incorporate isotopic tags with very high fidelity. Commercial media kits provide dialyzed serum and culture depleted of arginine and lysine. Customers add back either the standard amino acids or versions labeled with ^{13}C to create media with "light" or "heavy" Arg/Lys. These heavy or light amino acids are then incorporated into proteins thus differentially labeling proteins from two populations of cells. After that, researchers may apply any stimulus of interest (eg. kinase, proteosome, or phosphotase inhibitors, UV or mechanical perturbation). After pooling the cells and lysing, core personnel are able to identify peptides and measure the ratio of the same peptide from the heavy and light cells. (Mann. M., (2006) Nature Rev. **7**, 952-958)

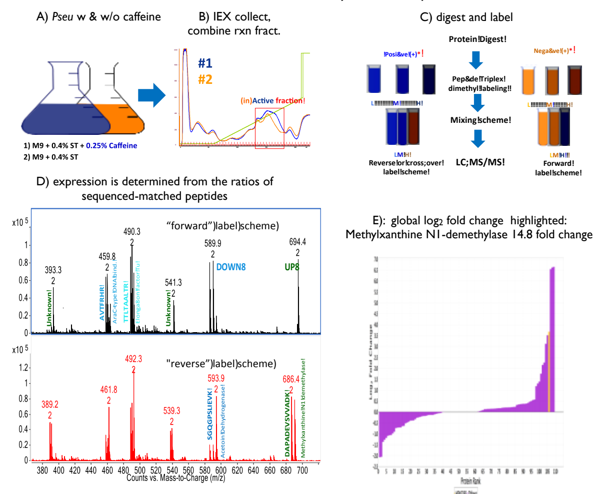
SILAC Tags Metabolically



<u>Triplex Labels</u> : If the isotope tags can not be incorporated metabolically, quantification approaches must be modified. Core personnel have validated global application of isotopic chemical tags in peptide digests. These tags are very selective for free amine residues and confer a mass difference between phenotypes of 4 Da per primary amine. Reagent costs are much lower than other schemes and tagging reaches completion with few side effects. This is an important acheivement for translational studies because we can monitor expression changes in tissue and sera. (Mohammed, S. et al. (2012) Anal Bioanal Chem **404**, 991–1009)

Triplex labeling for studying tissues and sera

Ex. metabolism induced by caffeine exposure

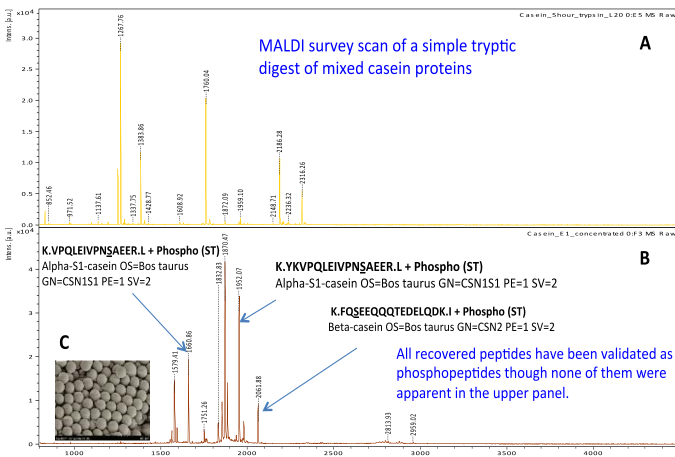


Identification of Phosphorylation Sites

					Choose one of the following for data collection				Optional add-ons		
Targeted Phosphoprotein Analysis: All of the above as well as preperation, QC (construction & validation) of custom phosphopeptide enrichment columns. **Prerequisite:** demonstrate 60% coverage of the protein of interest	Band Extraction and Trypsinization	MALDI-TOF before and after enrichment	Automated final report in Mascot, Spectrum Mill or Scaffold	Enrichment on custom phsopho - affinity column	Confirm structure with MALDI TOF/TOF	LC-MS/MS "Modest" dynamic range (30 min gradient)	LC-MS/MS "Typical" dynamic range (60 min gradient)	LC-MS/MS "Wide" dynamic range (90 min gradient)	Iterative exclusion list	IEX (includes multiple C18 clean-up)	Differential chemical tagging of peptide pools
First Sample	$30	$25	$45	$125	$25	$40	$65	$90	$25	$200	$40
Subsequent Samples	$30	$25		$95	$25	$40	$65	$90	$25		

Post Translational Modifications (PTM): Most PTMs involve a mass shift of both the precursor peptide and peptide fragments that carry the chemical adduct. Hence, MS/MS can detect PTM and assign the specific residue affected. For each modification there are unique barriers to success. For instance, detecting phosphorylation can be difficult

MALDI analysis (A) of a tryptic digest of mixed casein proteins and (B) enrichment of embedded pPeptides using home-brew Ti-phosphonate resin (C)



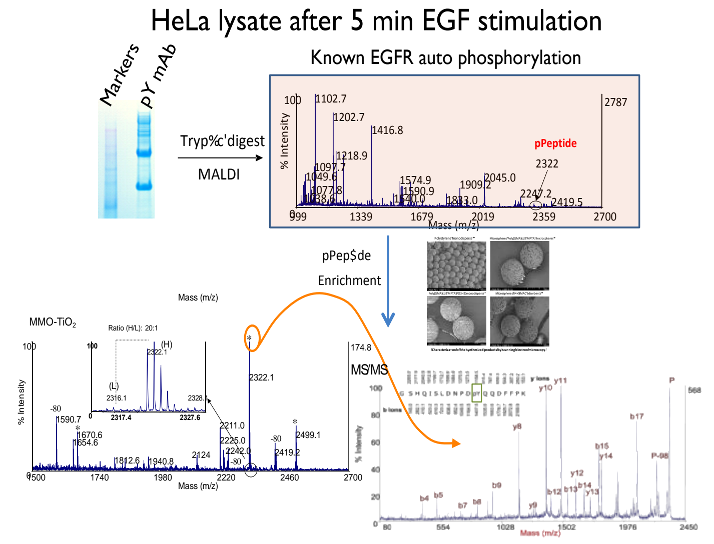
because its presence is substoichiometric, reversible, and suppresses peptide ionization. Moreover, Ser or Thr phospho-residues kinetically favor neutral loss of HPO_2, so that MS2 peptide fragment sequence ions are attenuated. These characteristics mandate special preparation and scanning procedures.

Phosphorylation: Due to the very low stoichiometry and transient nature of phosphorylation, phosphorylated peptides are often undetectable in single-stage MS scans. To find and validate phosphorylated peptides, custom enrichment steps and specialized expertise in ion fragmentation are usually required. Further, phosphorylation changes are often orchestrated in a networked response to stimulus. Tracking such changes globally is an extreme challenge that engages the top research laboratories in the country.

Enrichment: We use immobilized metal affinity chromatography (IMAC) to enrich peptides that are phosphorylated. This is done with customized beads, coated with a hydrophilic micellar foam that supports phosphonate ligands via a 9 carbon linkage. The ligands are titrated with $TiCl_2$ to create dative bonding sites with low steric hindrance. (a. Yu et al., (2009) Analytica Chi. Acta, 636, 34-41. b. Zhou et al. (2013) Nature Protocols. **8,** 461-480.)

Unlike negatively charged amino acid sidechains, the phosphate moiety on phosphopeptides is not neutralized in 0.1% TFA. This allows phosphopeptides to bind IMAC columns and be separated from non-phosporylated peptides.

Global Studies: What may be achieved with a combination of labeling and enrichment is illustrated in the figure below.



Anti pTyr enrichment of HeLa cell lysate following 5 min EGF stimulation of the heavy-labeled cell population is shown; control is light labeled. The very small abundance of the phosphopeptide at m/z 2322 in the digest of the EGFR band typifies the rather low ionization efficiency observed for pPeptides. The spectrum shown at the middle left illustrates a dramatic enhancement in the spectral signal observed for pPeptides with specific enrichment on our customized surface. The lower right spectrum illustrates the fragmentation pattern of m/z 2322.1. The sequence fragments of this auto-phosphorylation site are also confirm with ESI MS/MS.

Rapid Biotyping of Microbial Pathogens

MALDI Bacterial Biotyping: Spectral analysis of simple protein preparations from culture.	Customer Prep	Search, ID, and Report	Instrument Calibration per run	Per sample < 50 samples	Per sample > 50 samples
		$30	$8	$1.35	$1

Biotyping Rationale: Currently, biochemical tests form the basis of most techniques used to identify microorganisms. Most frequently, reagents, metabolized by specific organisms, are added to cultures so that a characteristic color change may be compared with reference values. Correlating the results of a number of such tests on a culture provides an indication of the organism's identity.

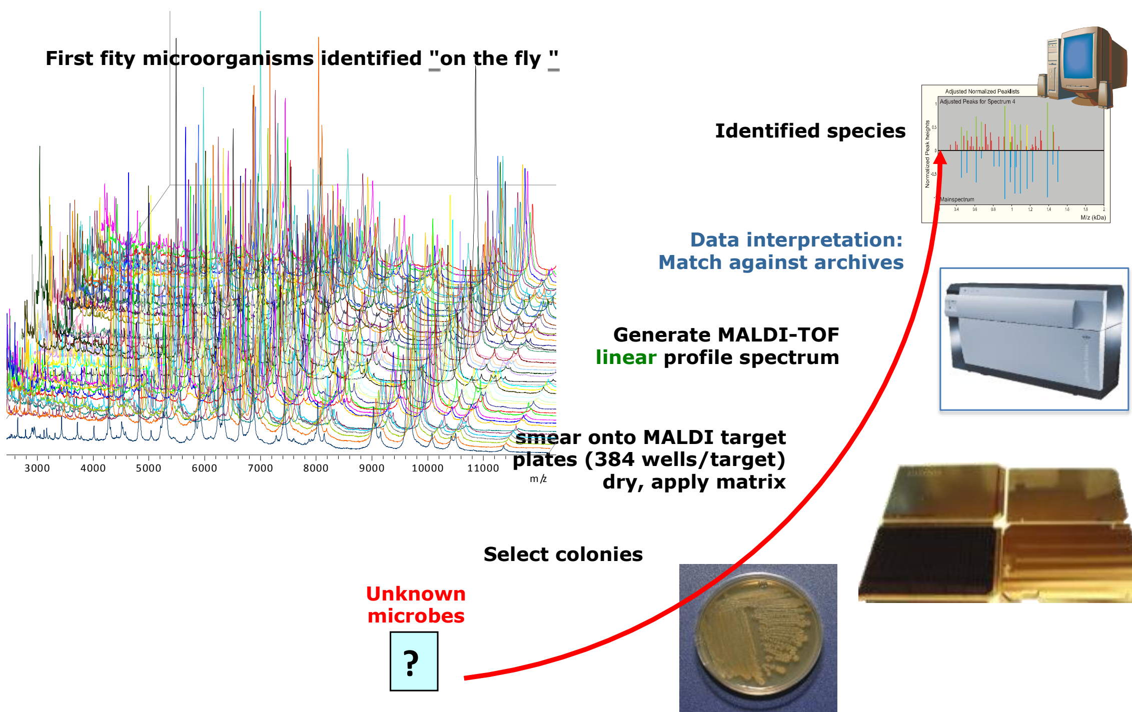
MALDI Biotyping uses a different methodology altogether: it identifies microorganisms by analyzing the expression of their most prolific protein/RNA conjugates using mass spectrometry. This mass spectral pattern of protein expression is compared with reference patterns in a database to identify the pathogen.

The process is cheap and has the sensitivity and specificity to be classified as a clinical diagnostic in Canada and many European countries already. Trials are already taking place in the US which may spur FDA approval. *See* Ryan T. Saffert et al., JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2011, p. 887–892:

Typical results are illustrated below. This method removes the subjectivity from philological classifications and, what is more important, provides a rigorous basis for determining the statistical relevancy of each diagnostic interpretation. This means that future go/no go decisions and their outcomes may be subjected to prospective scrutiny to achieve more universal standards of best practice.

In addition, the research grade analytics we have on hand, our instrument can sequence selected ions, which means we are able to create and validate new metrics used (at least in-house) to improve specificity in speciating microorganisms. Core personnel have access to first-in-class vector machine software to characterize In Source and Post Source Decay (ISD) patterns that may identify sub-species indistinguishable by common metrics. Hence, it is possible to resolve the evolution of pathogenic vectors or conduct (non-GLP) epidemiologic studies of infectivity and efficacious interventions.

Biotyping microorganisms with MALDI



Following ionization, the arrival time, abundance and symmetry of protein(/RNA complexes) provides a species-specific ion signature of isolated microbes.

USER Training and User Operation of Core Facility Equipment

	USER RATES after Training. Unassisted. Does not include consumables.			
MALDI TOF/TOF	30	Each 20 min		set-up and QC
LTQ LC-MS/MS	56	Each 1 hr	30	set-up and QC
Q-TOF	96	Each 1 hr	40	set-up and QC
QQQ	56	Each 1 hr	40	set-up and QC

	USER RATES after Training. Partial Assistance Does not include consumables.			
MALDI TOF/TOF	45	Each 20 min		set-up and QC
LTQ LC-MS/MS	84	Each 1 hr	30	set-up and QC
Q-TOF	144	Each 1 hr	40	set-up and QC
QQQ	84	Each 1 hr	40	set-up and QC

Training Sessions: Times and Scope

Users can be trained to use the equipment on their own. This is a great learning experience and if a user has many different samples to run over the course of 1-2 years, they can save some money by learning the instrumentation themselves. However, for most users, the cost-savings will be negligible, but is still a valuable way to understand their experiments better. The most important thing users can do to improve their success in their proteomics project is to work on sample prep. The second step to a better result is to learn how to run and interpret the search programs that find matches between the known proteomes and their samples. Finally, experience with the instruments themselves can be very helpful. For this, we offer periodic classes in which groups of 3-5 people are trained. Training begins on the MALDI and can progress to the LTQ, Q-TOF, or QQQ depending on the needs of the user. Users can also be trained on an individual basis. Contact us for times and availability.

Rules and Responsibilities

1) Sign up ahead of time
2) Leave the instrument in good working order with a run of standards to demonstrate that
3) Document all samples analyzed
4) During training we have agreed on standard QCs and 'fit-for-purpose' standards. Your samples and these QC must by checked by MALDI before they are injected into any LC. Use the MALDI data to determine the suitability of your sample.
5) If a fluid path or instrument becomes blocked, contaminated, or inoperable we will check the data set from (4). If it is poor or missing, you will be billed for the time required to fix the problem.
6) Don’t break anything.
7) If you break something, then call for help immediately. Or—refer to previous rule.

Frequently Asked Questions

Do you have office hours to consult with us about our project?
We are all typically in the lab preparing, running, or analyzing samples. We like it when people drop by, but to sit down with you will best be done by emailing for an appointment of setting one up through our calendar system.

Should I talk to you before I do my project?
We can share some guidelines with you about how you might prepare your samples better and help ensure success. Running a routine immunoprecipitation in a routine lab gel and staining with the lab's common stock of commassie will not typically work. You'll need to customize and we can suggest how to do that.

Do I really need a commercial gel and dedicated equipment to do MS analysis of my sample?
Most Probably. Keratin and other contaminants are everywhere—even on "washed" glassware.

Do I need software to view my results and where can I get that?
Yes, you will want the latest version of Scaffold software found here:
http://www.proteomesoftware.com/products/scaffold/download

How do the prices in the CCOM Proteomics Facility compare with other institutions?
> We are glad you asked. See the chart below.

Price Comparisons

	Protein Extraction, Trypsinization, Sample CleanUp, Sample QC by MALDI, MALDI-TOF/TOF confirmation, Search and Report	Protein Extraction, Trypsinization, Sample CleanUp, Sample QC by MALDI, LC-MS/MS, Search and Report
1 gel band comparison		
UIOWA CCOM proteomics	**100**	**115**
University 1		169
University 2		245
University 3		245
University 4		170
University 5	110	119
University 6	150	
University 7	180	250
University 8 (est)		130
5 gel band comparison		
UIOWA CCOM proteomics	**400**	**550**
University 1		845
University 2		1225
University 3		1225
University 4		850
University 5	550	595
University 6	750	
University 7	900	1250
University 8 (est)		650

	Raw spectra. MALDI-TOF or ESI accurate mass on proteins or peptides. Sample spotting and data collection by facility	MALDI-TOF or ESI accurate mass on proteins or peptides. Alternative sample prep and data collection by facility
Intact Mass (each)		
UIOWA CCOM	**20**	**55**
University 1		
University 2	45	
University 3	70	
University 4		
University 5		42
University 6	20	
University 7	70	
University 8 (est)	15	35