Supplementary Material

to

Performance Metrics for Evaluating Liquid Chromatography-Tandem Mass Spectrometry Systems in Shotgun Proteomics

Rudnick et al.

A. SOP for the preparation of the yeast protein lysate

B. Digestion and characterization of the yeast protein lysate

C. SOPs for CPTAC Network Study 5

D. SOPs for CPTAC Network Study 6

A. Preparation of the yeast protein lysate

Reagents:

- YPD (Bio101 system, Cat# 4001-032, Lot# 4001-032-101401)
- Kanamycin Sulfate (Shelton Scientific Inc, Cat# IBU2120, Lot# 6I1150)
- Trichloroacetic Acid (TCA) (VWR (100% W/V), Cat# 3372-2, Lot# 7022)
- Acetone (Burdick & Jackson, Inc, Cat# AH010-4, Lot# 10071741)
- Yeast stock BY4741 (MATa leu2D0 met15D0 ura3D0 his3D1) (Open Biosystems, Cat# YSC1048-645440)

Protocol

All steps of this protocol were completed by Boston Biochem (Cambridge, MA), the commercial concern that produced the lysate.

- Yeast from glycerol stock were struck out onto a YPD plate and incubated overnight at 30˚C. The plate contained colonies of uniform shape, size and color.
- Inoculated 500 ml of YPD (supplemented with 25 µg/ml Kanamycin) with yeast from patch of cells (not an individual colony) and grown overnight at 30˚C with shaking.
- Diluted 100 ml of overnight culture into 10 L of YPDKan in a fermentor and grown at 30˚C. Initial OD600 = 0.21. Growth was done with aeration at 10K CC/min, agitation at 30 rpm.
- The OD600 was monitored every 30 minutes using a spectrophotometer.
- When the culture reached OD600 = 0.93 (~ 5 hours), the yeast was harvested using a Sharples continuous-flow centrifuge (over 30K rpm).
- Total pellet wet weight obtained = 5.4 g.
- The cell pellet was resuspended and washed 3 times in 150 ml ice-cold sterile water to remove residual media. The washes were monitored at OD600 until absorbance was at baseline.
- The washed cell pellet was then resuspended in 160 ml of ice-cold sterile water. The suspension was split into two sterile tubes and 80 ml of ice-cold 20% TCA was added to each (final TCA concentration is 10%).
- Each tube was incubated with agitation at 4˚C for 1 hour to facilitate cell lysis.
- The precipitate was collected by centrifugation at 18.3K rpm (50,000g) for 30 minutes.
- Each pellet was washed with 160 ml ice-cold 90% acetone. The pellet was recovered by centrifugation a at 18.3K rpm (50,000g) for 30 minutes.
- The acetone wash and centrifugation were repeated and all liquid removed.
- The pellet was flash-frozen in liquid nitrogen, transferred to a sterile 50 ml conical tube, and lyophilized.
- The lyophilized pellet was stored at -80˚C or dry ice (as required).

C. Digestion and characterization of the yeast protein lysate

To prepare the material for LC-MS analysis, the solubilized lysate was digested with trypsin using the procedure of Piening *et al.*, (J. Proteome. Res. 2006, 5, 1527). In brief, the yeast protein (approximately 1 mg/ml in 50 mM ammonium bicarbonate, 8 M urea at pH 8.0), was reduced and denatured at 60 °C for 1 h following the addition of dithiothreitol at a concentration of 15 mM. Next, the proteins were alkylated by adding iodoacetamide to a concentration of 25 mM. After quenching the reaction by the addition of dithiothreitol (DTT) to a concentration of 50 mM, the solution was diluted 8-fold with 50 mM aqueous ammonium bicarbonate. Digestion with trypsin was carried out at 37 °C for approximately 18 h. The digestion was halted by the addition of acetic acid to a concentration of 1% and the sample was stored at -80 °C prior to analysis. All reagents used, including trypsin, were from Sigma and used without further purification.

For LC-MS analysis, varying amounts of the trypsin digest, corresponding to approximately 5, 25 and 50 pmol of total yeast protein, were injected onto an Atlantis dC18 column (3 µm particle size, 300 µm x 150 mm; Waters, Milford, MA). Elution was accomplished with a 2 h gradient from 3 % to 40 % acetonitrile in an aqueous solution of 0.1 % formic acid using a flow rate of 15 µl/min. For the LC-MS analysis, a Waters nanoAcquity LC was coupled to a Waters QTof Premier mass spectrometer. Mass spectra were obtained from m/z 400 to m/z 1600 with a scan time of 1 s. A 300 µM solution of glu-fibrinopeptide in a 50 % acetonitrile, 50 % water was sprayed at 2 µl/min, orthogonally to the LC effluent spray, for lock-mass calibration.

Prior to data analysis, the Waters RAW file was converted to mzXML format using *massWolf (http://tools.proteomecenter.org/MassWolf.php)*. For feature detection/analysis of the LC-MS TIC, *msInspect* (build # 4603, http://proteomics.fhcrc.org/CPL/msinspect.html) was used. To count the total number of features, the output file generated by *msInspect* was imported into Excel and the total number of entries was used to determine the total number of features detected.

LC-MS/MS analysis of the digestion was performed on a Thermo LTQ using the original 95 min gradient as described in the SOP optimization. The results were searched using the MyriMatch algorithm and the results run through the IDPicker algorithm to determine the total confidently identified spectra, peptides, proteins and most parsimonious protein groups.

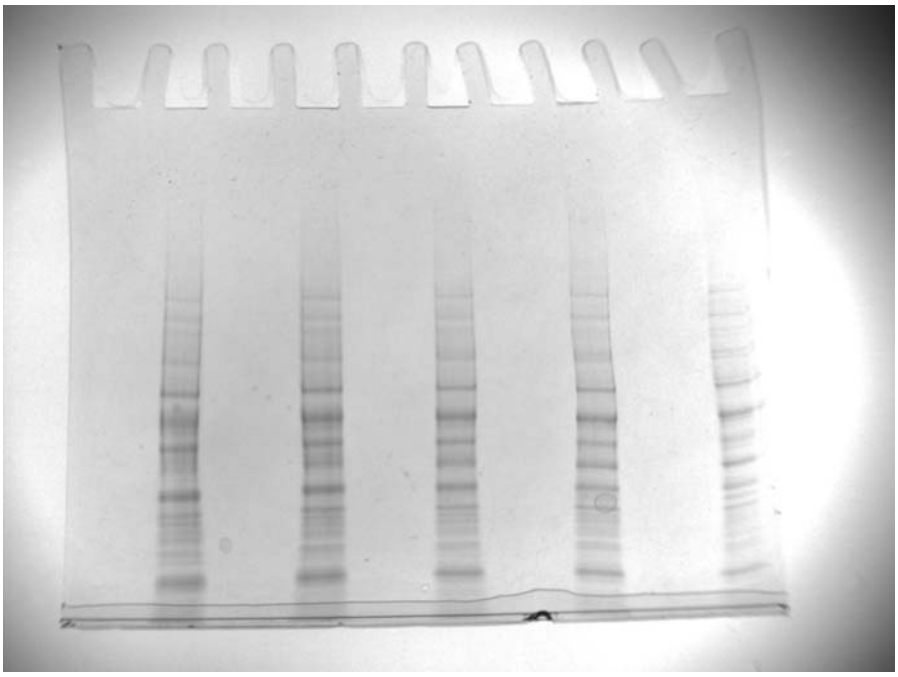

Figure 11D-PAGE results from the analysis of the yeast lysate. The sample loading for the 5 lanes, from left to right, was approximately 12.5 µg, 10 µg, 7.5 µg, 5 µg, and 2.5 µg total protein

Table 1 Total number of LC-MS featured detected at various sample loads of the trypsin digest of the yeast lysate. The number of features was determined using *msInspect.*

Total Protein (pre-digestion) on Column	Number of Features Detected
5 pmol	303
25 pmol	3588
50 pmol	7924

D. SOPs for CPTAC Network Study 5

The CPTAC Unbiased Discovery Working Group was tasked to design a renewable, standardized set of reference samples for benchmarking LC-MS/MS platforms with respect to performance characteristics relevant to shotgun proteomics-based biomarker discovery. The first CPTAC-wide experiment of this working group was to test a yeast lysate spiked with a human protein mix. Due to complications with the human protein mix that we were to use, we have modified this experiment to include only a single protein, bovine serum albumin (BSA). This initial spiked sample will provide a means for comparing performance of LC/MS/MS platforms amongst the CPTAC participants. In the future, our goal is to develop a performance standard that will provide a means for comparing performance of LC/MS/MS platforms i) as a quality control over time, ii) after the addition of new technologies to evaluate their effectiveness compared to historic data, or iii) between laboratories to inform optimization and troubleshooting.

The experiment has been designed to meet two primary goals. First, the study will provide an assessment of run-to-run and lab-to-lab variability for a complex mixture of proteins. Also, the study will provide a measure of the sensitivity of a mass-spec platform in a yeast lysate. This will be achieved by calculating the power required to detect a two-fold difference for the overall sensitivity of the platform for a given decade of protein abundance.

This proposed standard operating procedure (SOP) was developed after initial testing of several yeast lysate samples at Vanderbilt University. After an initial "dress rehearsal" run, the SOP was re-evaluated and refined to achieve better compliance as well as standardize new parameters that may have led to variance in the initial dress rehearsal runs.

Study Samples:

The yeast lysate and BSA was digested using RapiGest as denaturant and iodoacetamide as alkylating reagent. A 20 h digest was performed at 37 °C with proteomics-grade trypsin. After digestion, both samples were lyophilized and resuspended in 0.1% formic acid in water. The yeast sample has been diluted to a total protein concentration of 60 ng/µL. Therefore, the yeast samples are ready to be analyzed without additional dilution. Both yeast samples (Sample 3-A and 3-B) contains approximately 100 µL.

Table 1 contains descriptive and concentration information on the study samples:

Table 2 NCI CPTAC Study 5 samples

Sample	Description	[Total Yeast Protein]	[BSA]
Sample 3-A	unspiked digested yeast	60 ng/µL	0
Sample 3-B	BSA digest in digested yeast	60 ng/µL	10 fmol/µL
Sample 1-B	digested NCI-20 protein mix	0	0

For Study 5, 1 vial of digested, unspiked yeast (Sample 3-A) and 1 vial of digested yeast spiked with a BSA digest (Sample 3-B) was sent to study participants along with 1 vial of digested NCI-20 (Sample 1B), which will be used as a QC sample for the study. Prior to analysis, **Sample 1B should be diluted 150-fold with 0.1% formic acid in water**.

Below are checklists that outline the HPLC and mass spectrometry parameters of this SOP for both a Thermo LTQ and Thermo Orbitrap. The run order of samples follow the checklists. Participants will adhere as closely to these guidelines as possible with the understanding that not all labs are able to utilize a precolumn and,

depending upon their nanospray set up, these chromatographic dimensions (length only) may change somewhat. Derivations from the protocol in this regard will be recorded. All participants should use the Jupiter C18 resin sent to their respective labs.

If possible, participants should pack their columns at 1000 PSI in methanol and then finish packing with the HPLC at 50:50 0.1% formic:acetonitrile on the HPLC for 30 min prior to cutting back the columns to their final dimensions. Please note any discrepancies from this procedure. The column should also be conditioned prior to use with an injection of a concentrated protein digest (such as a BSA digest) followed by several injections of a less concentrated digest. Several blank runs should be performed after conditioning to eliminate carryover of the conditioning digest into the first blank of the study.

It is recommended that each lab run a preliminary test run with the SOP methods and check the parameters recorded in the data file against the check lists provided. This will help to find errors in tune file parameters that can sometimes be tricky to spot.

Study 5 LTQ SOP Checklist

Source/APstack parameters				Notes
	☐	Capillary temperature	150 ± 25°C	
	☐	Capillary voltage	48 ± 20 V	
	☐	Tube Lens	100 ± 25 V	
	☐	Source voltage	2.0 ± 0.5 kV	
Tune File Parameters				Notes
	☐	Ion Trap Full Microscans	1	
	☐	Ion Trap MSn Microscans	1	
	☐	Ion Trap Full Max Ion Time	100	
	☐	Ion Trap MSn Max Ion Time	100	
	☐	Ion Trap Full AGC Target	30,000	
	☐	Ion Trap MSn AGC Target	10,000	
MS detector				Notes
	☐	Acquire time (min)	184	
	☐	Start delay (min)	15	
	☐	Segments	1	
	☐	Scan Events	9	
	☐	Scan Range for Event 1	m/z 300-2000	
	☐	Mass range for all events	Normal	
	☐	Scan Rate for all events	Normal	
	☐	Polarity for all events	Positive	
	☐	Data type for MS scan	Centroid	
	☐	Data Type for MSn Scan	Centroid	
	☐	Additional microscans	0	
Dynamic Exclusion				Notes
	☐	Repeat Count	1	
	☐	Repeat duration (s)	0	
	☐	Exclusion list size	150	
	☐	Exclusion duration (s)	60	
	☐	Exclusion mass width	By mass	
	☐	Exclusion mass width (low)	1.0	
	☐	Exclusion mass width (high)	3.5	
	☐	Early expiration is NOT used		
Scan Event				Notes
	☐	Charge state screening and Rejection is NOT enabled		
	☐	Minimum MS signal Threshold for MS2 trigger	500	
	☐	Mass determined from scan event	1	

	☐	Activation type	CID	
	☐	Default charge state	4	
	☐	Isolation width (m/z)	2.0	
	☐	Normalized collision energy	28	
	☐	Activation Q	0.250	
	☐	Activation time (s)	30.0	
	☐	Mass lists and global mass lists NOT used		
	☐	For each Current Scan Event, the Nth most intense ion is set at 8-1 for scan events 2-9, respectively		
HPLC Parameters and chromatography				Notes
	☐	Autosampler temperature	10C	
	☐	Column dimensions	11-13 cm x 100μm*	
	☐	Precolumn dimensions	2-4 cm x 100 μm**	
	☐	Column packing material	Jupiter C18	
	☐	Column temperature	Room temperature	
	☐	Mobile phase A	0.1% (v/v) formic acid in water	
	☐	Mobile phase B	0.1% (v/v) formic acid in acetonitrile	
	☐	Injection volume	2.0 μL	
	☐	Injection loop (or needle) volume	8.0 μL***	
	☐	Flow rate for gradient	600 +/- 200 nL/min at column tip	
	☐	Flow rate for loading phase	As per individual lab protocol	
	☐	184 min HPLC gradient for yeast samples	As per attached table	
	☐	95 min HPLC gradient for NCI20 and blanks samples	As per attached table	

* Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same.

** Precolumn is optional depending upon individual laboratory expertise, but should be used if at all possible. Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same.

*** If not possible to achieve, use same set up as dress rehearsal and make note of change.

HPLC Gradient with “vented” column

Time	%A	%B	Flow	Notes
0	100	0	To Waste*	
0.01	100	0	To Waste	
10	100	0	To Waste	
15	98	2	To Waste	
15.01	98	2	To column (source)	
135	60	40	To column (source)	
150	10	90	To column (source)	
155	10	90	To column (source)	
159	10	90	To column (source)	
164	95	5	To column (source)	
169	100	0	To column (source)	
184	100	0	To column (source)	

* If using a precolumn, the flow should be directed to waste if possible.

95 min HPLC Gradient with “vented” column for blanks and NCI-20

Time	%A	%B	Flow	Notes
0	100	0	To Waste*	
0.01	100	0	To Waste	
10	100	0	To Waste	
15	98	2	To Waste	
15.01	98	2	To column (source)	
50	75	25	To column (source)	
65	10	90	To column (source)	
70	10	90	To column (source)	
74	10	90	To column (source)	
75	95	5	To column (source)	
80	100	0	To column (source)	
95	100	0	To column (source)	

*If using a precolumn, the flow should be directed to waste.

Run order:

Run number	Sample	Gradient
1	**Sample 1-B-NCI20**	95 min gradient
2	Wash*	
3	Blank	95 min gradient
4	**Sample 3-A-Yeast**	184 min gradient
5	Wash*	
6	Blank	95 min gradient
7	**Sample 3-A-Yeast**	184 min gradient
8	Wash*	
9	Blank	95 min gradient
10	**Sample 3-A-Yeast**	184 min gradient
11	Wash*	
12	Blank	95 min gradient
13	**Sample 1-B-NCI20**	95 min gradient
14	Wash*	
15	Blank	95 min gradient
16	**Sample 3-B-Spiked Yeast**	184 min gradient
17	Wash*	
18	Blank	95 min gradient
19	**Sample 3-B-Spiked Yeast**	184 min gradient
20	Wash*	
21	Blank	95 min gradient
22	**Sample 3-B-Spiked Yeast**	184 min gradient
23	Wash*	
24	Blank	95 min gradient
25	**Sample 1-B-NCI20**	95 min gradient
26	Wash*	
27	Blank	95 min gradient
28	**Sample 3-A-Yeast**	184 min gradient
29	Wash*	
30	Blank	95 min gradient
31	**Sample 3-A-Yeast**	184 min gradient
32	Wash*	
33	Blank	95 min gradient
34	**Sample 3-A-Yeast**	184 min gradient
35	Wash*	
36	Blank	95 min gradient
37	**Sample 1-B-NCI20**	95 min gradient
38	Wash*	
39	Blank	95 min gradient
40	**Sample 3-B-Spiked Yeast**	184 min gradient
41	Wash*	
42	Blank	95 min gradient
43	**Sample 3-B-Spiked Yeast**	184 min gradient
44	Wash*	
45	Blank	95 min gradient
46	**Sample 3-B-Spiked Yeast**	184 min gradient
47	Wash*	
48	Blank	95 min gradient
49	**Sample 1-B-NCI20**	95 min gradient

* As per individual laboratory protocol

Study 5 Orbitrap SOP Checklist

Source/APstack parameters				Notes
	☐	Capillary temperature	150 ± 25°C	
	☐	Capillary voltage	48 ± 20 V	
	☐	Tube Lens	100 ± 25 V	
	☐	Source voltage	2.0 ± 0.5 kV	
Tune File Parameters				Notes
	☐	FTMS Full Microscans	1	
	☐	Ion Trap MSn Microscans	1	
	☐	FTMS Full Max Ion Time	1000	
	☐	Ion Trap MSn Max Ion Time	100	
	☐	FTMS Full AGC Target	1,000,000	
	☐	Ion Trap MSn AGC Target	10,000	
MS detector				Notes
	☐	Acquire time (min)	184	
	☐	Start delay (min)	15	
	☐	Segments	1	
	☐	Scan Events	9	
	☐	Scan Range for Event 1	m/z 300-2000	
	☐	Mass range for all events	Normal	
	☐	Scan Rate for all events	Normal	
	☐	Polarity for all events	Positive	
	☐	Data type for MS scan	Profile	
	☐	Data Type for MSn Scan	Centroid	
	☐	Additional microscans	0	
	☐	Resolution of MS scan	60,000	
	☐	Lock mass is NOT enabled		
Dynamic Exclusion				Notes
	☐	Repeat Count	1	
	☐	Repeat duration (s)	0	
	☐	Exclusion list size	150	
	☐	Exclusion duration (s)	60	
	☐	Exclusion mass width	By mass	
	☐	Exclusion mass width (low)	0.6	
	☐	Exclusion mass width (high)	0.6	
	☐	Early expiration is NOT used		
Global Data-Depentent Settings				Notes
	☐	Charge state screening enabled		
	☐	Monoisotopic precurson selection enabled		

	☐	Non-peptide monoisotopic recognition enabled		
	☐	Charge state +1 rejected		
	☐	Unassigned charge state rejected		
Scan Event				Notes
	☐	Minimum MS signal Threshold for MS2 trigger	500	
	☐	Mass determined from scan event	1	
	☐	Activation type	CID	
	☐	Default charge state	4	
	☐	Isolation width (m/z)	2.0	
	☐	Normalized collision energy	28	
	☐	Activation Q	0.250	
	☐	Activation time (s)	30.0	
	☐	Mass lists and global mass lists NOT used		
	☐	For each Current Scan Event, the Nth most intense ion is set at 8-1 for scan events 2-9, respectively		
HPLC Parameters and chromatography				Notes
	☐	Autosampler temperature	10C	
	☐	Column dimensions	11-13 cm x 100μm*	
	☐	Precolumn dimensions	2-4 cm x 100 μm**	
	☐	Column packing material	Jupiter C18	
	☐	Column temperature	Room temperature	
	☐	Mobile phase A	0.1% (v/v) formic acid in water	
	☐	Mobile phase B	0.1% (v/v) formic acid in acetonitrile	
	☐	Injection volume	2.0 μL	
	☐	Injection loop (or needle) volume	8.0 μL***	
	☐	Flow rate for gradient	600 +/- 200 nL/min at column tip	
	☐	Flow rate for loading phase	As per individual lab protocol	
	☐	184 min HPLC gradient for yeast samples	As per attached table	
	☐	95 min HPLC gradient for NCI20 and blanks samples	As per attached table	

* Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same.

** Precolumn is optional depending upon individual laboratory expertise, but should be used if at all possible. Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same.

*** If not possible, use same set up as dress rehearsal and make note of change.

184 min HPLC Gradient with "vented" column for yeast samples				
Time	%A	%B	Flow	Notes
0	100	0	To Waste*	
0.01	100	0	To Waste	
10	100	0	To Waste	
15	98	2	To Waste	
15.01	98	2	To column (source)	
135	60	40	To column (source)	
150	10	90	To column (source)	
155	10	90	To column (source)	
159	10	90	To column (source)	
164	95	5	To column (source)	
169	100	0	To column (source)	
184	100	0	To column (source)	

*If using a precolumn, the flow should be directed to waste.

95 min HPLC Gradient with "vented" column for blanks and NCI-20				
Time	%A	%B	Flow	Notes
0	100	0	To Waste*	
0.01	100	0	To Waste	
10	100	0	To Waste	
15	98	2	To Waste	
15.01	98	2	To column (source)	
50	75	25	To column (source)	
65	10	90	To column (source)	
70	10	90	To column (source)	
74	10	90	To column (source)	
75	95	5	To column (source)	
80	100	0	To column (source)	
95	100	0	To column (source)	

*If using a precolumn, the flow should be directed to waste.

Run order:

Run Number	Sample	Gradient
1	**Sample 1-B-NCI20**	95 min gradient
2	Wash*	
3	Blank	95 min gradient
4	**Sample 3-A-Yeast**	184 min gradient
5	Wash*	
6	Blank	95 min gradient
7	**Sample 3-A-Yeast**	184 min gradient
8	Wash*	
9	Blank	95 min gradient
10	**Sample 3-A-Yeast**	184 min gradient
11	Wash*	
12	Blank	95 min gradient
13	**Sample 1-B-NCI20**	95 min gradient
14	Wash*	
15	Blank	95 min gradient
16	**Sample 3-B-Spiked Yeast**	184 min gradient
17	Wash*	
18	Blank	95 min gradient
19	**Sample 3-B-Spiked Yeast**	184 min gradient
20	Wash*	
21	Blank	95 min gradient
22	**Sample 3-B-Spiked Yeast**	184 min gradient
23	Wash*	
24	Blank	95 min gradient
25	**Sample 1-B-NCI20**	95 min gradient
26	Wash*	
27	Blank	95 min gradient
28	**Sample 3-A-Yeast**	184 min gradient
29	Wash*	
30	Blank	95 min gradient
31	**Sample 3-A-Yeast**	184 min gradient
32	Wash*	
33	Blank	95 min gradient
34	**Sample 3-A-Yeast**	184 min gradient
35	Wash*	
36	Blank	95 min gradient
37	**Sample 1-B-NCI20**	95 min gradient
38	Wash*	
39	Blank	95 min gradient
40	**Sample 3-B-Spiked Yeast**	184 min gradient
41	Wash*	
42	Blank	95 min gradient
43	**Sample 3-B-Spiked Yeast**	184 min gradient
44	Wash*	
45	Blank	95 min gradient
46	**Sample 3-B-Spiked Yeast**	184 min gradient
47	Wash*	
48	Blank	95 min gradient
49	**Sample 1-B-NCI20**	95 min gradient

* As per individual laboratory protocol

Upload Instructions for Study5: Unbiased Discovery, Yeast + BSA Experiment

1.) Browse to https://chemdata.nist.gov/cptac/ (ignore the security warning and click OK. A signed third party certificate is in the process of being purchased to prevent these warnings in the future).
2.) Enter your 'log-in' name and password. (Sent to each contact in a separate e-mail.)
3.) Select '**Study5**' from the selector at the top of the navigation bar. (Do not use the Study3 section for these data files).
4.) Find your site and the instrument for which you are uploading data on the navigation bar. Click on 'Yeast + BSA Experiment' under that instrument.
5.) Follow the instructions in the box for Step 3. All data files and any annotation forms should be first copied to a **single directory** for upload. Please include the completed SOP checklist (above) in that directory. Uploading multiple directories or replicate experiments is allowed.
6.) Enter the name of your SOP contact in the box at Step 4. Click 'Sign.' The 'Upload' hyperlink at Step 5 is now active.
7.) Click on 'Upload' to launch Tranche. This requires Java JRE to be installed. Browse to the directory you wish to upload to begin the data transfer.
8.) Once you see a box displaying "*Upload Complete. Please check NIST website*" your upload has completed. You do not have to leave you web browser open for the upload to continue. You can view all uploads by clicking 'Download Data' from the navigation bar.

Timeline for Study 5

Task	Duration	Start Date	End Date
NIST sends out samples for full study	2 days	10/4/2007	10/5/2007
Labs perform full study	14 days	10/8/2007	10/24/2007
Labs upload data to Tranche	1 day	10/25/2007	10/25/2007
Yeast data is analyzed	30 days	10/29/2007	12/7/2007
Interim results reported to PCC	1 day	10/23/2007	10/23/2007
Yeast results reported to PCC	1 day	12/18/2007	12/18/2007

E. SOPs for CPTAC Study 6

NCI CPTAC Study 5 samples

Sample	Description	[Total Yeast Protein]	[Sigma48]
Sample 6-QC1	Sigma48	0	20 fmol/uL
Sample 6-QC2	unspiked digested yeast	60 ng/µL	0
Sample 1-B	digested NCI-20 protein mix		
Sample 6A	0.25 fmol/uL Sigma48 spiked into yeast digest	60 ng/uL	0.25 fmol/uL
Sample 6B	0.74 fmol/uL Sigma48 spiked into yeast digest	60 ng/uL	0.74 fmol/uL
Sample 6C	2.2 fmol/uL Sigma48 spiked into yeast digest	60 ng/uL	2.2 fmol/uL
Sample 6D	6.7 fmol/uL Sigma48 spiked into yeast digest	60 ng/uL	6.7 fmol/uL
Sample 6E	20 fmol/uL Sigma48 spiked into yeast digest	60 ng/uL	20 fmol/uL

An equimolar mixture of 48 human proteins (Sigma UPS1) was prepared for Study 6 as follows:

TFE-assisted Digestion of Sigma 48

1. Resuspend contents of a single Sigma 48 vial in:
50 uL 100 mM ammonium bicarbonate pH 8
50 uL TFE (Acros Organics)

Incubate at 60 ̊C for 45 minutes and vortex repeatedly. You'll see solid material "stuck" at the bottom of the vial so I simply vortex to try to get as much of this into solution.

2. Reduce (using 1/10 volume) with 10 uL of 50 mM DTT (prepared in water) and incubate at 55-66^{o}C for 20 minutes.

3. Cool to room temperature and alkylate with 10 uL of 100 mM IAM (also prepared in water) and incubate in the dark for 20 minutes.

4. Dilute with enough volume of Ambic pH 8 (360 uL) so the final concentration of TFE is about ≤10 % prior to adding the trypsin.

5. Add trypsin (Promega) in 1:50 ratio. Our trypsin concentration is 0.01 ug/uL in 100 mM Ambic, and based on the average protein concentration of a Sigma 48 vial (around 6.5 ug) I added 20 uL of the 0.01 ug/uL trypsin stock.

Digest at 37̊ C overnight, dry down and reconstitute in 0.1 % formic acid for LC/MS/MS analysis. I reconstituted in 50 uL = 100 fmol/uL

(Note: DTT and IAM were from Sigma.)

For Study 6, 1 vial of digested Sigma48 (Sample 6-QC1), 1 vial of digested, unspiked yeast (Sample 6-QC2), 1 vial of digested NCI 20 protein mix (Sample 1B) and 5 vials with varying concentrations of digested Sigma48

spiked into digested yeast (Samples 6A-6E) will be sent to each laboratory. Prior to analysis, **Sample 1B should be diluted 150-fold with 0.1% formic acid in water**. All other samples do not need be diluted.

Below are checklists that outline the HPLC and mass spectrometry parameters of this SOP for both a Thermo LTQ and Thermo Orbitrap. The run order for the samples follow the checklists.

If possible, participants should pack their columns at 1000 PSI in methanol and then finish packing with the HPLC at 50:50 0.1% formic:acetonitrile on the HPLC for 30 min prior to cutting back the columns to their final dimensions. The column should also be conditioned prior to use with an injection of a concentrated protein digest (such as a BSA digest) followed by several injections of a less concentrated digest. Several blank runs should be performed after conditioning to eliminate carryover of the conditioning digest into the first blank of the study.

Study 6 LTQ SOP Checklist

Source/APstack parameters				Notes
	☐	Capillary temperature	150 ± 25°C	
	☐	Capillary voltage	48 ± 20 V	
	☐	Tube Lens	100 ± 25 V	
	☐	Source voltage	2.0 ± 0.5 kV	
Tune File Parameters				Notes
	☐	Ion Trap Full Microscans	1	
	☐	Ion Trap MSn Microscans	1	
	☐	Ion Trap Full Max Ion Time	100	
	☐	Ion Trap MSn Max Ion Time	100	
	☐	Ion Trap Full AGC Target	30,000	
	☐	Ion Trap MSn AGC Target	10,000	
MS detector				Notes
	☐	Acquire time (min)	184	
	☐	Start delay (min)	15	
	☐	Segments	1	
	☐	Scan Events	9	
	☐	Scan Range for Event 1	m/z 300-2000	
	☐	Mass range for all events	Normal	
	☐	Scan Rate for all events	Normal	
	☐	Polarity for all events	Positive	
	☐	Data type for MS scan	Centroid	
	☐	Data Type for MSn Scan	Centroid	
	☐	Additional microscans	0	
Dynamic Exclusion				Notes
	☐	Repeat Count	1	
	☐	Repeat duration (s)	0	
	☐	Exclusion list size	150	
	☐	Exclusion duration (s)	60	
	☐	Exclusion mass width	By mass	
	☐	Exclusion mass width (low)	1.0	
	☐	Exclusion mass width (high)	3.5	
	☐	Early expiration is NOT used		
Scan Event				Notes
	☐	Charge state screening and Rejection is NOT enabled		
	☐	Minimum MS signal Threshold for MS2 trigger	500	
	☐	Mass determined from scan event	1	

	☐	Activation type	CID	
	☐	Default charge state	4	
	☐	Isolation width (m/z)	2.0	
	☐	Normalized collision energy	28	
	☐	Activation Q	0.250	
	☐	Activation time (s)	30.0	
	☐	Mass lists and global mass lists NOT used		
	☐	Wideband Activation NOT used		
	☐	For each Current Scan Event, the Nth most intense ion is set at 8-1 for scan events 2-9, respectively		
HPLC Parameters and chromatography				Notes
	☐	Autosampler temperature	10C	
	☐	Column dimensions	11-13 cm x 100μm*	
	☐	Precolumn dimensions	2-4 cm x 100 μm**	
	☐	Column packing material	Jupiter C18	
	☐	Column temperature	Room temperature	
	☐	Mobile phase A	0.1% (v/v) formic acid in water	
	☐	Mobile phase B	0.1% (v/v) formic acid in acetonitrile	
	☐	Injection volume	2.0 μL	
	☐	Injection loop (or needle) volume	8.0 μL***	
	☐	Flow rate for gradient	600 nL/min at column tip	
	☐	Flow rate for loading phase	1.2 uL/min	
	☐	184 min HPLC gradient for yeast samples	As per attached table	
	☐	95 min HPLC gradient for NCI20 and blanks samples	As per attached table	

* Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same.

** Precolumn is optional depending upon individual laboratory expertise, but should be used if at all possible. Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same.

*** If not possible to achieve, use same set up as dress rehearsal and make note of change.

184 min HPLC Gradient with “vented” column for yeast samples					
Time	%A	%B	Flow path	Flow rate	Notes
0	100	0	To Waste*	1.2 uL/min	
0.01	100	0	To Waste	1.2 uL/min	
10	100	0	To Waste	1.2 uL/min	
15	98	2	To Waste	1.2 uL/min	
15.01	98	2	To column (source)	600 nL/min	
135	60	40	To column (source)	600 nL/min	
150	10	90	To column (source)	600 nL/min	
155	10	90	To column (source)	600 nL/min	
159	10	90	To column (source)	600 nL/min	
164	95	5	To column (source)	600 nL/min	
169	100	0	To column (source)	600 nL/min	
184	100	0	To column (source)	600 nL/min	

*If using a precolumn, the flow should be directed to waste.

95 min HPLC Gradient with “vented” column for blanks and NCI-20					
Time	%A	%B	Flow	Flow rate	Notes
0	100	0	To Waste*	1.2 uL/min	
0.01	100	0	To Waste	1.2 uL/min	
10	100	0	To Waste	1.2 uL/min	
15	98	2	To Waste	1.2 uL/min	
15.01	98	2	To column (source)	600 nL/min	
50	75	25	To column (source)	600 nL/min	
65	10	90	To column (source)	600 nL/min	
70	10	90	To column (source)	600 nL/min	
74	10	90	To column (source)	600 nL/min	
75	95	5	To column (source)	600 nL/min	
80	100	0	To column (source)	600 nL/min	
95	100	0	To column (source)	600 nL/min	

*If using a precolumn, the flow should be directed to waste.

Run order:

This series of samples is to be run in **triplicate**. You may run the triplicate runs back-to-back or as three separate replicates with other samples or maintenance in between. If the series is run back-to-back, there is no need to run the Sample 1B (NCI20) two times in a row.

Run number	Sample	Gradient
1	**Sample 1-B-NCI20**	95 min gradient
2	Wash*	
3	Blank	95 min gradient
4	**Sample 6-QC2 –Yeast only**	184 min gradient
5	Wash*	
6	Blank	95 min gradient
7	**Sample 6A - 0.25 fmol/uL Sigma 48 spiked yeast**	184 min gradient
8	Wash*	
9	Blank	95 min gradient
10	**Sample 6B - 0.74 fmol/uL Sigma 48 spiked yeast**	184 min gradient
11	Wash*	
12	Blank	95 min gradient
13	**Sample 6C - 2.2 fmol/uL Sigma 48 spiked yeast**	184 min gradient
14	Wash*	
15	Blank	95 min gradient
16	**Sample 6D - 6.7 fmol/uL Sigma 48 spiked yeast**	184 min gradient
17	Wash*	
18	Blank	95 min gradient
19	**Sample 6E - 20 fmol/uL Sigma 48 spiked yeast**	184 min gradient
20	Wash*	
21	Blank	95 min gradient
22	**Sample 6-QC1 - Sigma 48 only**	184 min gradient
23	Wash*	
24	Blank	95 min gradient
25	**Sample 1-B-NCI20**	95 min gradient

* As per individual laboratory protocol

Study 6 Orbitrap SOP Checklist				
Source/APstack parameters				Notes
	☐	Capillary temperature	150 ± 25°C	
	☐	Capillary voltage	48 ± 20 V	
	☐	Tube Lens	100 ± 25 V	
	☐	Source voltage	2.0 ± 0.5 kV	
Tune File Parameters				Notes
	☐	FTMS Full Microscans	1	
	☐	Ion Trap MSn Microscans	1	
	☐	FTMS Full Max Ion Time	1000	
	☐	Ion Trap MSn Max Ion Time	100	
	☐	FTMS Full AGC Target	1,000,000	
	☐	Ion Trap MSn AGC Target	10,000	
MS detector				Notes
	☐	Acquire time (min)	184	
	☐	Start delay (min)	15	
	☐	Segments	1	
	☐	Scan Events	9	
	☐	Scan Range for Event 1	m/z 300-2000	
	☐	Mass range for all events	Normal	
	☐	Scan Rate for all events	Normal	
	☐	Polarity for all events	Positive	
	☐	Data type for MS scan	Profile	
	☐	Data Type for MSn Scan	Centroid	
	☐	Additional microscans	0	
	☐	Resolution of MS scan	60,000	
	☐	Lock mass is NOT enabled		
Dynamic Exclusion				Notes
	☐	Repeat Count	1	
	☐	Repeat duration (s)	0	
	☐	Exclusion list size	150	
	☐	Exclusion duration (s)	60	
	☐	Exclusion mass width	By mass	
	☐	Exclusion mass width (low)	0.6	
	☐	Exclusion mass width (high)	0.6	
	☐	Early expiration is NOT used		
Global Data-Depentent Settings				Notes
	☐	Charge state screening enabled		
	☐	Monoisotopic precurson		

		selection enabled		
	☐	Non-peptide monoisotopic recognition enabled		
	☐	Charge state +1 rejected		
	☐	Unassigned charge state rejected		
Scan Event				Notes
	☐	Minimum MS signal Threshold for MS2 trigger	500	
	☐	Mass determined from scan event	1	
	☐	Activation type	CID	
	☐	Default charge state	4	
	☐	Isolation width (m/z)	2.0	
	☐	Normalized collision energy	28	
	☐	Activation Q	0.250	
	☐	Activation time (s)	30.0	
	☐	Mass lists and global mass lists NOT used		
	☐	Wideband Activation NOT used		
	☐	For each Current Scan Event, the Nth most intense ion is set at 8-1 for scan events 2-9, respectively		
HPLC Parameters and chromatography				Notes
	☐	Autosampler temperature	10C	
	☐	Column dimensions	11-13 cm x 100μm*	
	☐	Precolumn dimensions	2-4 cm x 100 μm**	
	☐	Column packing material	Jupiter C18	
	☐	Column temperature	Room temperature	
	☐	Mobile phase A	0.1% (v/v) formic acid in water	
	☐	Mobile phase B	0.1% (v/v) formic acid in acetonitrile	
	☐	Injection volume	2.0 μL	
	☐	Injection loop (or needle) volume	8.0 μL***	
	☐	Flow rate for gradient	600 nL/min at column tip	
	☐	Flow rate for loading phase	1.2 uL/min	
	☐	184 min HPLC gradient for yeast samples	As per attached table	
	☐	95 min HPLC gradient for NCI20 and blanks samples	As per attached table	

* Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same.
** Precolumn is optional depending upon individual laboratory expertise, but should be used if at all possible. Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same.

*** If not possible, use same set up as dress rehearsal and make note of change.

184 min HPLC Gradient with "vented" column for yeast samples					
Time	%A	%B	Flow path	Flow rate	Notes
0	100	0	To Waste*	1.2 uL/min	
0.01	100	0	To Waste	1.2 uL/min	
10	100	0	To Waste	1.2 uL/min	
15	98	2	To Waste	1.2 uL/min	
15.01	98	2	To column (source)	600 nL/min	
135	60	40	To column (source)	600 nL/min	
150	10	90	To column (source)	600 nL/min	
155	10	90	To column (source)	600 nL/min	
159	10	90	To column (source)	600 nL/min	
164	95	5	To column (source)	600 nL/min	
169	100	0	To column (source)	600 nL/min	
184	100	0	To column (source)	600 nL/min	

*If using a precolumn, the flow should be directed to waste.

95 min HPLC Gradient with "vented" column for blanks and NCI-20					
Time	%A	%B	Flow	Flow rate	Notes
0	100	0	To Waste*	1.2 uL/min	
0.01	100	0	To Waste	1.2 uL/min	
10	100	0	To Waste	1.2 uL/min	
15	98	2	To Waste	1.2 uL/min	
15.01	98	2	To column (source)	600 nL/min	
50	75	25	To column (source)	600 nL/min	
65	10	90	To column (source)	600 nL/min	
70	10	90	To column (source)	600 nL/min	
74	10	90	To column (source)	600 nL/min	
75	95	5	To column (source)	600 nL/min	
80	100	0	To column (source)	600 nL/min	
95	100	0	To column (source)	600 nL/min	

*If using a precolumn, the flow should be directed to waste.

Run order:

This series of samples is to be run in **triplicate**. You may run the triplicate runs back-to-back or as three separate replicates with other samples or maintenance in between. If the series is run back-to-back, there is no need to run the Sample 1B (NCI20) two times in a row.

Run number	Sample	Gradient
1	**Sample 1-B-NCI20**	95 min gradient
2	Wash*	
3	Blank	95 min gradient
4	**Sample 6-QC2 –Yeast only**	184 min gradient
5	Wash*	
6	Blank	95 min gradient
7	**Sample 6A - 0.25 fmol/uL Sigma 48 spiked yeast**	184 min gradient
8	Wash*	
9	Blank	95 min gradient
10	**Sample 6B - 0.74 fmol/uL Sigma 48 spiked yeast**	184 min gradient
11	Wash*	
12	Blank	95 min gradient
13	**Sample 6C - 2.2 fmol/uL Sigma 48 spiked yeast**	184 min gradient
14	Wash*	
15	Blank	95 min gradient
16	**Sample 6D - 6.7 fmol/uL Sigma 48 spiked yeast**	184 min gradient
17	Wash*	
18	Blank	95 min gradient
19	**Sample 6E - 20 fmol/uL Sigma 48 spiked yeast**	184 min gradient
20	Wash*	
21	Blank	95 min gradient
22	**Sample 6-QC1 - Sigma 48 only**	184 min gradient
23	Wash*	
24	Blank	95 min gradient
25	**Sample 1-B-NCI20**	95 min gradient

* As per individual laboratory protocol