Supplementary Information

Repeatability and Reproducibility in Proteomic Identifications by Liquid Chromatography—Tandem Mass Spectrometry

October 16, 2009

Contents

Figure 1: CPTAC Sample Relationships

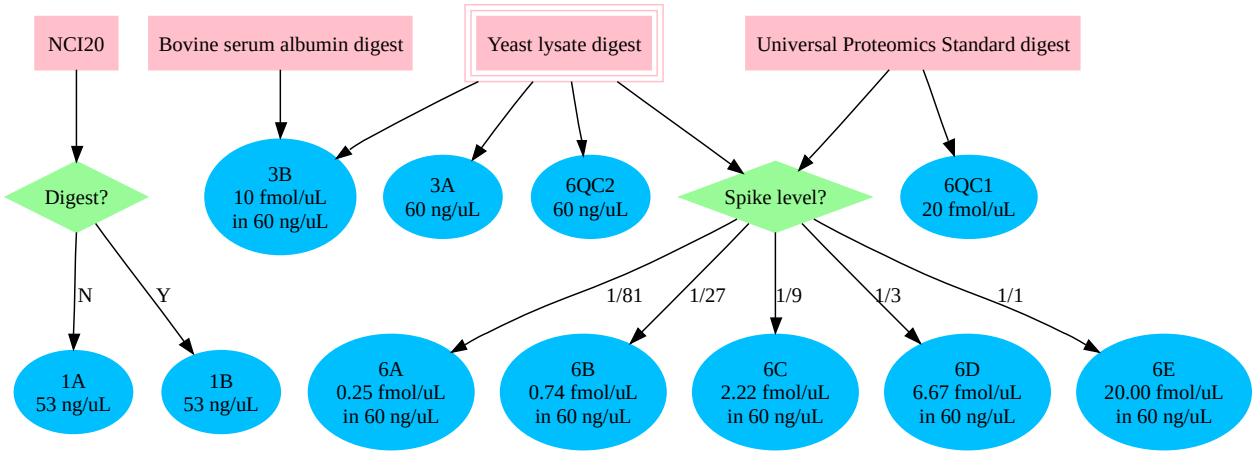


Table 1: NCI-20 Sample Composition

Protein	NCI-20 Target Concentration	Biological Source	Swiss-Prot Number	Swiss-Prot Abbrev	IPI ver. 3.48
Albumin	5 g/L	*Pichia pastoris*	P02768	ALBU_HUMAN	IPI00745872 IPI00384697
Fibrinogen, α subunit	2 g/L*	human plasma	P02671	FIBA_HUMAN	IPI00021885 IPI00029717
Fibrinogen, β subunit			P02675	FIBB_HUMAN	IPI00298497
Fibrinogen, γ subunit			P02679	FIBG_HUMAN	IPI00021891 IPI00219713
Transferrin	1 g/L	human plasma	P02787	TRFE_HUMAN	IPI00022463
Beta-2-microglobulin	2 mg/L	*E.Coli*	P61769	B2MG_HUMAN	IPI00004656
C-reactive protein	1 mg/L	*E. Coli*	P02741	CRP_HUMAN	IPI00022389 IPI00218876
Gastrin-17[a]	0.5 mg/L	peptide synthesis	P01350	GAST_HUMAN	IPI00001624
Transferrin receptor	500 µg/L	human placental tissue	P02786	TFR1_HUMAN	IPI00022462
VEGF	50 µg/L	Sf21 clone of *Spodoptera frugiperda*	P15692	VEGFA_HUMAN	IPI00852889 IPI00216555 IPI00872936 IPI00607893
IGF-1	100 µg/L	*E. Coli*	P01343	IGF1A_HUMAN	IPI00001610
Prolactin	20 µg/L	*E. Coli*	P01236	PRL_HUMAN	IPI00873653
Alpha fetoprotein	15 µg/L	human AFP cell line	P02771	FETA_HUMAN	IPI00022443
CEA	10 µg/L	Sf9 clone of *Spodoptera frugiperda*	P06731	CEAM5_HUMAN	IPI00027486
Calcitonin[b]	10 µg/L	peptide synthesis	P01258	CALC_HUMAN	IPI00000914 IPI00215863
PSA	5 µg/L*	human seminal fluid	P07288	KLK3_HUMAN	IPI00010858
ACT		human plasma	P01011	AACT_HUMAN	IPI00847635 IPI00607870
Thyroglobulin	1 µg/L	human thyroid	P01266	THYG_HUMAN	IPI00306129 IPI00549199
GM-CSF	50 ng/L	*E. Coli*	P04141	CSF2_HUMAN	IPI00022270
Tumor necrosis factor-α	25 ng/L	*E. Coli*	P01375	TNFA_HUMAN	IPI00001671
Interleukin-2	50 ng/L	*E. Coli*	P60568	IL2_HUMAN	IPI00003099
Interleukin-6	5 ng/L	*E. Coli*	P05231	IL6_HUMAN	IPI00007793
Erythropoietin	5 IU/L	Chinese Hamster Ovary cells	P01588	EPO_HUMAN	IPI00307226

* Total mass concentration for the protein complex.

[a] Gastrin-17: 17 amino acid subsequence, 76-92, ("little gastrin" or simply gastrin) of GAST_HUMAN, QGPWLEEEEEAYGWMDF

[b] Calcitonin: 32 amino acid subsequence, 85-116, of CALC_HUMAN, CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP

Table 2: SOP Comparison Chart

	Study 2 SOP v1.0	*Study 3 SOP v2.0*	*Study 5 SOP v2.1*	*Study 6 SOP v2.2*
Source/APstack parameters				
Capillary temperature	NS[a]	NS	150 ± 25°C	150 ± 25°C
Capillary voltage	NS	NS	48 ± 20 V	48 ± 20 V
Tube Lens	NS	NS	100 ± 25 V	100 ± 25 V
Source voltage	NS	NS	2.0 ± 0.5 kV	2.0 ± 0.5 kV
Tune File Parameters				
FTMS Full Microscans (Orbitrap only)	1	1	1	1
Ion Trap MSn Microscans	2	1	1	1
FTMS Full Max Ion Time (Orbitrap only)	1000	1000	1000	1000
Ion Trap Full Max Ion Time (LTQ only)	100	100	100	100
Ion Trap MSn Max Ion Time	100	100	100	100
FTMS Full AGC Target (Orbitrap only)	1,000,000	1,000,000	1,000,000	1,000,000
Ion Trap MSn AGC Target (Orbitrap only)	30,000	10,000	10,000	10,000
Ion Trap MSn AGC Target (LTQ only)	10,000	10,000	10,000	10,000
MS detector				
Acquire time (min)	135	184	184	184
Start delay (min)	0	15	15	15
Segments	1	1	1	1
Scan Events	9	9	9	9
Scan Range for Event 1	m/z 300-2000	m/z 300-2000	m/z 300-2000	m/z 300-2000
Mass range for all events	Normal	Normal	Normal	Normal
Scan Rate for all events	Normal	Normal	Normal	Normal
Polarity for all events	Positive	Positive	Positive	Positive
Data type for MS scan (Orbitrap)	Profile	Profile	Profile	Profile
Data type for MS scan (LTQ)	Centroid	Centroid	Centroid	Centroid
Data Type for MSn Scan	Centroid	Centroid	Centroid	Centroid
Additional microscans	0	0	0	0
Resolution of MS scan (Orbitrap only)	60,000	60,000	60,000	60,000
Lock mass is NOT enabled (Orbitrap only)	NS	NS	T[b]	T
Dynamic Exclusion				
Repeat Count	1	1	1	1
Repeat duration (s)	0	0	0	0
Exclusion list size	500	150	150	150
Exclusion duration (s)	30	60	60	60
Exclusion mass width	By mass	By mass	By mass	By mass
Exclusion mass width (low) (Orbitrap only)	- 15 ppm	0.6	0.6	0.6
Exclusion mass width (high) (Orbitrap only)	+ 20ppm	0.6	0.6	0.6
Exclusion mass width (low) (LTQ only)	1	1	1	1
Exclusion mass width (high) (LTQ only)	3.5	3.5	3.5	3.5
Early expiration is NOT used	T	T	T	T

	Study 2 SOP v1.0	*Study 3 SOP v2.0*	*Study 5 SOP v2.1*	*Study 6 SOP v2.2*
Global Data-Dependent Settings				
Charge state screening enabled (Orbitrap only)	T	T	T	T
Monoisotopic precursor selection enabled (Orbitrap only)	T	T	T	T
Non-peptide monoisotopic recognition enabled (Orbitrap only)	NS	T	T	T
Charge state +1 rejected (Orbitrap only)	NS	T	T	T
Unassigned charge state rejected (Orbitrap only)	NS	T	T	T
Scan Event				
Minimum MS signal Threshold for MS2 trigger	500	500	500	500
Mass determined from scan event	1	1	1	1
Activation type	CID	CID	CID	CID
Default charge state	4	4	4	4
Isolation width (m/z)	2	2	2	2
Normalized collision energy	28	28	28	28
Activation Q	0.25	0.25	0.25	0.25
Activation time (s)	30	30	30	30
Mass lists and global mass lists NOT used	T	T	T	T
Wideband Activation NOT used	Specified for LTQ only	Specified for LTQ only	NS	T
Zoom scans NOT used (LTQ only)	T	T	NS	NS
For each Current Scan Event, the Nth most intense ion is set at 8-1 for scan events 2-9, respectively	T	T	T	T
HPLC Parameters and chromatography				
Autosampler temperature	10°C	10°C	10°C	10°C
Column dimensions	NS[c]	11-13 cm x 100μm[d]	11-13 cm x 100μm[e]	11-13 cm x 100μm[e]
Precolumn dimensions	NS	2-4 cm x 100 μm[e]	2-4 cm x 100 μm[f]	2-4 cm x 100 μm[f]
Column packing material	C18 reversed phase[g]	Jupiter C18 [g]	Jupiter C18 [g]	Jupiter C18 [g]
Column temperature	35 °C (if possible)	Room temperature	Room temperature	Room temperature
Mobile phase A	0.1% (v/v) formic acid in water	0.1% (v/v) formic acid in water	0.1% (v/v) formic acid in water	0.1% (v/v) formic acid in water
Mobile phase B	0.1% (v/v) formic acid in acetonitrile	0.1% (v/v) formic acid in acetonitrile	0.1% (v/v) formic acid in acetonitrile	0.1% (v/v) formic acid in acetonitrile
Injection volume	2.0 μL[h]	2.0 μL	2.0 μL	2.0 μL
Injection loop (or needle) volume	NS	NS	8.0 μL[i]	8.0 μL
Flow rate for gradient	As per individual lab protocol	600 +/- 200 nL/min at column tip	600 +/- 200 nL/min at column tip	600 nL/min at column tip
Flow rate for loading phase	N/A[j]	As per individual lab protocol	As per individual lab protocol	1.2 μL/min
Gradient used for blanks	Gradient 1	Gradient 2	Gradient 3	Gradient 3
Gradient used for NCI20	Gradient 1	Gradient 2	Gradient 3	Gradient 3
Gradient used for yeast samples	N/A	Gradient 2	Gradient 2	Gradient 2
Specified column packing procedure?	no	yes[k]	yes	yes

[a]NS = Not specified

[b]T = True

[c]Column dimensions were not specified in Study 2, but the concentration to be injected was to adjusted according to the size of chromatography column that was used.

[d]The following comment was added with respect to this parameter in the SOP for Study 2: "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 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."

[e]The following comment was added with respect to this parameter in the SOP for Studies 5 & 6: "Column length may vary depending upon the individual nanospray set up, but inner diameter should remain the same."

[f]The following comment was added with respect to this parameter in the SOP for Studies 5 & 6: "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."

[g]The exact type of C18 resin was not specified in Study 2. For Studies 3, 5 & 6, all labs were sent the resin from the same lot of Phenomenex Jupiter C18 resin (5 micron, 300 angstrom).

[h]Concentration of injected volume for Study 2 was adjusted according to diameter of chromatographic column.

[i]Since the design of the Agilent autosampler that was used by several groups in these studies has an 8µL needle for injection that was not interchangable, other autosamplers attempted to conform by standardizing the injection loop size to 8 µL.

[j]N/A = Not applicable

[k]Column packing procedures were specified in the SOP for Studies 3, 5, &6 and the following comment was added: "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."

Gradient 1

Mobile phase A = 0.1 % (v/v) formic acid in water

Mobile phase B = 0.1 % (v/v) formic acid in acetonitrile

HPLC Gradient:

Time, min	% A	% B
0	97	3
5	97	3
100	60	40
105	5	95
108	5	95
113	97	3
135	97	3

Gradient 2

Mobile phase A = 0.1 % (v/v) formic acid in water

Mobile phase B = 0.1 % (v/v) formic acid in acetonitrile

HPLC Gradient:

Time	%A	%B	Flow
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
135	60	40	To column
150	10	90	To column
155	10	90	To column
159	10	90	To column
164	95	5	To column
169	100	0	To column
184	100	0	To column

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

Gradient 3

Mobile phase A = 0.1 % (v/v) formic acid in water

Mobile phase B = 0.1 % (v/v) formic acid in acetonitrile

HPLC Gradient:

Time	%A	%B	Flow
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
50	75	25	To column
65	10	90	To column
70	10	90	To column
74	10	90	To column
75	95	5	To column
80	100	0	To column
95	100	0	To column

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

Study 1 Experimental Plan

Introduction

This document describes the initial to be conducted by Clinical Proteomics Technology Assessment for Cancer (CPTAC) teams. The overall goals of this experiment are to benchmark and begin to evaluate the capabilities of three major components of proteomic measurement methods (sample preparation, instrumentation, and data analysis), to identify of variability, and to identify peptides and proteins in a protein mixture.

The strength of this experiment resides in the team-oriented approach underlying the structure of the study. Every sample will be analyzed on many different platforms and by different laboratories. Data will be compared across labs to ensure repeatability and reproducibility. Consequently, complete and accurate reporting using commonly accepted formatting and terminology of all the requested data and metadata is imperative for the success of this study.

Given their experience in metrology and measurement quality assessment materials, the National Cancer Institute (NCI) is partnering with the National Institute of Standards and Technology (NIST) in overseeing this experiment. NIST is developing the materials and will store and analyze the data resulting from the interlab studies.

Also partnering with the NCI is Texas A&M University (TAMU). TAMU brings expertise in statistical design of experiments, which complements that of each group. Both TAMU and NIST have been working with the NCI to develop an experimental design for the initial experiment. Future experiments will also involve input from the five CPTAC teams.

Experimental Timeline

STUDY I: "Benchmark Study of Defined Mixtures"

Experiment

Week 1: November 6, 2006

- NCI CPTAC Sample Type 1A and Sample Type 1B distributed to the CPTAC teams by NIST
- Raw data shall be submitted to NIST no later than November 13, 2006
- Processed data shall be submitted to NIST no later than November 20, 2006

Week 2: November 13, 2006

- NCI CPTAC Sample Type 1A and Sample Type 1B distributed to CPTAC teams by NIST
- Raw data shall be submitted to NIST no later than November 20, 2006
- Processed data shall be submitted to NIST no later than December 1, 2006

Data Analysis

December 4–15, 2006

- NCI, NIST, Texas A&M, and CPTAC teams conduct data analysis to identify variations within and between laboratories and instruments

December 18, 2006

- CPTAC teleconference to present data and discuss results

Procedural Instructions

STUDY IA: "Benchmark Study of Protein Mixtures"

Goals

- to benchmark the starting performances of proteomic measurement methods
- to provide results to aid in the planning of subsequent studies
- to establish working protocols for transferring data between CPTAC teams and NCI/NIST/TAMU, including data format issues
- to detect platforms that have some difficulties attaining repeatability
- to find platforms that have similar performance characteristics
- to establish stable protein materials that may be later used as standards and to establish material and data transfer protocols

Description

- Teams may use their chosen methods to discern the composition of an unknown protein mixture (NCI CPTAC Sample Type 1A). These results will be used to identify the variables involved in identifying proteins in a complex mixture and will provide valuable information to the Program Coordinating Committee. The mixture is simple enough that a 1-D LC separation of the peptides derived from proteolysis of the protein mixture should provide sufficient resolution.
- NCI CPTAC Sample Type 1A consists of a mixture of proteins, supplied as a solution in an aqueous buffer. The concentrations of the proteins in a Sample Type 1A span the range of proteins found in human plasma, and the total protein concentration is approximately 8 g/L. The mixture was prepared in 25 mM phosphate buffered saline, pH 7.4, containing 5 mM sodium azide.
- Samples will be shipped frozen, in screw-capped 0.5 mL polypropylene vials, filled with approximately 0.1 mL of each sample.

Instructions for Week 1 (November 6 - 13, 2006)

Sample prep and measurement

1. On November 7, 2006, receive three aliquots of NCI CPTAC Sample Type 1A per proteomic platform to be used in these studies.
2. Prepare each aliquot according to the individual laboratory's specified operating procedures, as reported in the survey (see 5 below if changes are necessary).
3. Analyze each aliquot on each proteomic platform described in the questionnaire (see 5 below is differences are necessary).
4. Analyze data to identify proteins in NCI CPTAC Sample Type 1A.
5. Record any deviations from standard operating procedures, and include any information needed to interpret results.
6. If Step 2 does not include trypsin digestion, repeat Steps 1–5 with trypsin digestion.

Data analysis and reporting

1. The name of each data file must contain the identification number of the aliquot from which it was generated.
2. By Monday, November 13, 2006, send raw MS data files for each aliquot to NIST.
3. By Monday, November 20, 2006, send to NIST MS peak lists, search engine results, and a summary report for each aliquot, including output data files created by library search software.
4. In the summary report, please include:

a. Proteins (including isoforms) and peptide ions identified, PTMs, and their numbers as well as confidence measures.
b. Methods for separating true and false positives.

Instructions for Weeks 2 (November 13-20, 2006)

Instructions for Week 2 are identical to those for Week 1 except for the dates.

As much as possible, staff involved in the sample preparation and analysis should remain the same through both weeks.

Sample prep and measurement

1. All three aliquots should be run within 24 hours of receipt.

Data analysis and reporting

1. All three raw MS data files should be sent to NIST by Monday, November 20, 2006.
2. All processed data files should be sent to NIST by December 1, 2006

STUDY IB: "Benchmark Study of Digested Protein Mixtures"

Goals (in addition to those from IA)

- Benchmark performance of proteomic methods with a digested sample
- Determine the extent of variability arising from instrumentation
- Determine the extent of variability arising from data analysis

Description

- This study is designed to control for differences in sample preparation. Each team will receive aliquots of a protein mixture (NCI CPTAC Sample Type 1B) that have already been digested by trypsin. The aliquots will be run and analyzed as they were in Study IA, except for the sample preparation steps. This is the first step toward isolating the source of variability in proteomics experiments.
- NCI CPTAC Sample Type 1B will be peptides resulting from the reduction, cysteine-alkylation, and trypsin digestion of the proteins in Sample Type 1A by NIST. The reduction, alkylation, and trypsin digestion of Sample Type 1A will involve minimal dilution and, therefore, the molar concentration of the peptides in Sample Type 1B will be similar to the molar concentration of the proteins in Sample Type 1A.
- Samples will be shipped frozen, in screw-capped 0.5 mL polypropylene vials, filled with approximately 0.1 mL of each sample.

Instructions for Week 1 (November 6-13, 2006)

Sample prep and measurement

1. On November 7, 2006, receive three aliquots of NCI CPTAC Sample Type 1B per proteomic platform in laboratory.
2. Analysis each aliquot using the proteomic platform.
3. Process and analyze data to identify proteins in NCI CPTAC Sample Type 1B.
4. Record any deviations from laboratory standard operating procedure

Data analysis and reporting

1. The name of each data file must contain the identification number of the aliquot from which it was generated.
2. By Monday, November 13, 2006, send raw MS data files for each aliquot to NIST.
3. By Monday, November 20, 2006, send to NIST MS peak lists, search engine results, and a final protein report for each aliquot.
4. In the protein report, please include:

a. Proteins (including isoforms) and peptide ions identified, PTMs, and their numbers as well as confidence measures.
b. Methods for separating true and false positives.

Instructions for Week 2 (November 13–20, 2006)

Instructions for Week 2 are identical to those for Week 1 except for the dates. Below are the relevant instructions to keep up with the CPTAC timeline.

Sample prep and measurement

1. All three aliquots should be run within 24 hours of receipt.

Data analysis and reporting

1. All three raw MS data files should be sent to NIST by Monday, November 20, 2006.
2. All processed data files should be sent to NIST by December 1, 2006

Study 2 Experimental Plan: SOP v1.0

SOP for CPTAC Study 2

This standard operating procedure (SOP) is intended to provide a more uniform LC-MS/MS measurement of a peptide mixture (Sample 1B) between different labs all using a Thermo LTQ or LTQ-Orbitrap. This SOP is intended to provide a common and reasonable set of experimental parameters for this inter-laboratory study, so is not necessarily optimal for each laboratory.

Because different LC-MS/MS instrument configurations will be used in this study, the SOP is intended to be flexible enough to accommodate this variety. Therefore, please note that LC column dimensions, flow rates, and the ESI source type and source parameters are not given. These are to be decided by the individual labs.

The analysis of data will be done in the same way as done for Study 1, using the NIST peptide identification pipeline described earlier. A password-protected web-based data entry form will be provided to permit filling in experimental details not fixed by the SOP. In this form, locations for copying the resulting raw data files will also be provided. As before, uploading will be to a NIST sFTP site.

Sample Handling:

a. NCI Sample 1B (two sets of 3 vials) should be stored at -80 °C
b. Prior to analysis, thaw samples to room temperature for 1-2 h.
c. Mix samples thoroughly by vortexing
d. Spin samples down briefly by centrifuging (2 min at 5000 rpm)
e. If necessary (see table below), dilute each of the 3 samples with HPLC- or MS-grade water, mix thoroughly and transfer an aliquot to an autosampler vial. Use only one aliquot from each vial of Sample 1B and not multiple aliquots from the same vial.

HPLC column diameter to be used…	Dilution of Sample 1B performed…
2.1 mm	**none (undiluted)**
1 mm	**3-fold**
320 µm	**30-fold**
150 µm	**150-fold**

f. Prepare a blank by adding HPLC- or MS-grade water to an additional autosampler vial

HPLC Parameters:

Autosampler temperature= 10 ^{o}C

Column type = C18 reversed phase column

Column temperature = 35 °C **when possible, otherwise 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**

HPLC Gradient:

Time, min	**% A**	**% B**
0	**97**	**3**
5	**97**	**3**
100	60	40
105	5	95
108	5	95
113	**97**	**3**
135	**97**	**3**

MS Parameters:

- The MS parameters below reflect those of a Thermo LTQ with the following software versions: Xcalibur 2.0 SR2 and LTQ MS 2.2
- Electrospray source parameters will be dictated by the LC flowrate and type of source used and the value for source parameters are left to the operator to decide what is appropriate
- The LTQ parameters below reflect a data dependent MS/MS experiment where one MS scan is followed by 8 MS/MS scans on the most intense ions, **in the order of least intense to most intense**
- User defined settings are summarized in the tables below, but default values should be used when a value is not specified

Tune Plus

	LTQ	**LTQ-Orbitrap**
Full microscans	1	1
MS^n microscans	2	2
Full Max Ion Time (ms)	100	**1000**
MSn Max Ion Time (ms)	100	100
Full AGC Target	30,000	**1,000,000**
MS^n AGC Target	10,000	**30,000**

MS Detector

	LTQ	**LTQ-Orbitrap**
Acquire time (min)	135	135
Start delay (min)	0	0
Segments	1	1
Scan Events	9	9
Scan Ranges for Event 1	m/z 300-2000	m/z 300-2000
Mass Range for all Events	Normal	Normal
Scan Rate for all Events	Normal	Normal
Scan Type for Event 1	Full	Full
Polarity for all Events	Positive	Positive
Data type for MS Scan	Centroid	Profile
Data type for MS^n Scan	Centroid	Centroid
Additional microscans	0	0
Resolution of MS scan	—	60,000

- Zoom scans and wideband activation are not used for LTQ runs

For Scan Events 2-9, the Dependent Scan box is checked and the following settings are used:

Dynamic Exclusion

	LTQ	**LTQ-Orbitrap**
Repeat count	1	1
Repeat duration (s)	0	0
Exclusion list size	500	500
Exclusion duration (s)	30	30
Exclusion mass width	by mass	by mass
Exclusion mass width (low)	1.0	**- 15 ppm**
Exclusion mass width (high)	3.5	**+ 20 ppm**

For the LTQ-Orbitrap:

- **Charge State Screening is enabled**
- **Find C12 is turned on**

For the LTQ:

- **Charge State Screening and Rejection is not enabled**

Scan Event

	LTQ	**LTQ-Orbitrap**
MS2 Minimum signal threshold	500	500
Mass determined from scan event	1	1
Activation type	CID	CID
Default charge state	4	4
Isolation width (m/z)	2.0	2.0
Normalized collision energy	**28**	**28**
Activation Q	0.250	0.250
Activation time (ms)	30.0	30.0

- For each Current Scan Event, the N^{th} most intense ion is set at 8-1 for scan events 2-9, respectively
- Mass lists and global mass lists are not used
- Early expiration is not used

LC-MS/MS Analysis:

a. Sample should be analyzed in the following order:
    1. blank
    2. Sample 1B, vial 1
    3. **blank**
    4. Sample 1B, vial 2
    5. **blank**
    6. Sample 1B, vial 3

b. The second set of 3 vials of Sample 1B should be analyzed approximately one week after the first set, preferably after the instrument has been used for other analyses.

Tracking Annotation:

The purpose of the SOP is that all samples be run identically on each of the team's instruments. In the event of a deviation from the SOP, or to record parameters not explicitly listed in the SOP, NIST has set up a user-friendly website to record such deviations. This site will also serve to centralize and standardize collection of CPTAC sample analysis metadata with respect to the SOP. Metadata from all Study 2 runs shall be submitted to this site. Ideally, metadata should be submitted on the same day the sample is analyzed.

All teams are required to run three samples in Week 1 and three samples in Week 2. The week in which the sample was run is to be recorded for each sample on the website. One or more operating procedures (OP) must also be created for each team. This record stores the values actually used when the sample was run, and must be assigned to each group of samples on the day of analysis. This process constitutes annotation for this SOP based study, and the values will be used during analysis of results.

The site is located at: http://chemdata.nist.gov/cptac/

Each team was provided with a username and password for this site.

Instructions for using the site are linked on the site. Please direct questions or feedback to Dr. Paul Rudnick (paul.rudnick@nist.gov).

Background Information:

A primary goal of NCI CPTAC Study 2 is to assess between-lab comparability on comparable instrument, specifically the Thermo LTQ or LTQ-Orbitrap. However, while the MS instrumentation might be comparable between labs, the LC inlets used are not, with probably the most critical differences being the LC column dimensions and the flow rates used.

Because MS is a concentration dependent analytical technique, column diameter and LC flow rate will have a pronounced effect on measurement sensitivity because, for a given amount of sample injected on-column, lower flowrates and smaller column dimensions result in a lower elution volume and higher elution concentration. It is believed that for a given amount of sample on-column, the MS measurement sensitivity relates to the LC column diameter through a $\approx 1/r^2$ factor, where r is the radius of the column.

Therefore, in order to provide the most comparable results from different LC set-ups, we propose varying the amount injected on-column based on the dimensions of the LC column used through a $1/r^2$ scaling factor.

First, the calculations are based on the approximate total protein concentration used to prepare Sample B = 4 µg/µL ≈ 60 pmol/µL (using the molecular mass of HSA, the most abundant protein in Sample B, as a representative protein mass).

The starting point for the calculations in the table below was the goal to perform a 2 µL injection of 750 fmol on-column when using a column with an inside diameter of 150 µm or smaller. On-column injection amounts were scaled up approximately, relative to the $1/r^2$ factor, for columns of differing dimension. The dilution factor for NCI-20 Sample B was calculated based on keeping the injection volume for all column dimensions fixed at 2 µL

Column ID	**≈ $1/r^2$**	**Amount injected on-column**	**Concentration of Sample for 2 µL injection**	**NCI-20 Sample B Dilution**
2.1 mm	1	150,000 fmol	75,000 fmol/µL	undiluted
1 mm	4	37,500 fmol	18,750 fmol/µL	3-fold
320 µm	40	3,750 fmol	1,875 fmol/µL	30-fold
150 µm + below	200	**750 fmol**	375 fmol/µL	150-fold

Study 3 Experimental Plan: SOP v2.0

[Note: Study 3 was originally intended as a "dress rehearsal" for Study 3. The group later renamed this test experiment to be Study 3.]

Proposed SOP for Dress Rehearsal of CPTAC Study 3 (Yeast)

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 will be to test a yeast lysate spiked with a human protein mix. If successful, the spiked sample 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 twofold 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. Three major areas of potential optimization were tested, 1) digestion conditions, 2) chromatography and 3) mass spectrometric parameters. The development of this SOP involved the use of either a Thermo LTQ or a Thermo LTQ-Orbitrap instrument. In contrast to procedures for prior CPTAC Studies, this SOP has been optimized for high sensitivity. We have prioritized peptide diversity and high protein counts over the reproducible identification of a core set of peptides or proteins.

This study will be carried out in two phases. The first phase—the "dress rehearsal"—will be carried out with a digested, unspiked yeast lysate sample and be used to fine tune this SOP and sort through any issues of data handling and analysis. The second phase will be the full study with four digested yeast samples, each spiked with varying amounts of digested Sigma-48 proteins.

This is the SOP for the dress rehearsal. It describes the samples, specifies settings for sample preparation and analysis, and provides instructions for submitting and annotating the data. Finally, the sample scheme and schedule for the dress rehearsal conclude the document.

Study Samples:

The yeast lysate and Sigma-48 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 sample is ready to be analyzed without additional dilution. The yeast sample (Sample 3-A) contains approximately 100 µL.

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

Table 1 NCI CPTAC Study 3 dress rehearsal samples

Sample	Description	[Total Yeast Protein]	[Individual Sigma-48]
Sample 3-A	unspiked digested yeast	60 ng/µL	0
Sample 1-B	digested NCI-20 protein mix	0	0

For the "dress rehearsal" of Study 3, 1 vial of digested, unspiked yeast (Sample 3-A) 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**.

HPLC Parameters:

Autosampler temperature= 10 ^{o}C

Column type = 11 cm x 100 µm Jupiter C18 reversed phase column with a 2 cm x 100 um "vented" precolumn with same packing material

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**

Flow-rate = 600 +/- 200 nL/min at column tip

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 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 30min 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.

Recommended HPLC gradient with "vented" column if possible (first 15 minutes diverted to waste if utilizing a "vented" column).

Time	A%	B%
0	100	0
0.01	100	0
10	100	0
15	98	2
15.01	98	2
135	60	40
150	10	90
155	10	90
159	10	90
164	95	5
169	100	0
184	100	0

MS Parameters:

- Parameters in **bold** are those that deviate from the SOP from the CPTAC Study 2
- The MS parameters below reflect those with the following software versions for the Thermo LTQ and LTQ-Orbitrap respectively: **Xcalibur 2.0 SR2, LTQ MS 2.2 and Xcalibur 2.0 SR2, FT programs 2.0.2 0614, LTQ Orbitrap MS 2.2**
- Electrospray source parameters will be dictated by the LC flowrate and type of source used and the value for source parameters are left to the operator to decide what is appropriate
- The LTQ parameters below reflect a data dependent MS/MS experiment where one MS scan is followed by 8 MS/MS scans on the most intense ions, in the order of least intense to most intense
- User defined settings are summarized in the tables below, but default values were used when a value is not specified

Tune Plus

	LTQ	**LTQ-Orbitrap**
Full microscans	1	1
MS^n microscans	**1**	**1**
Full Max Ion Time (ms)	100	1000
MSn Max Ion Time (ms)	**100**	**100**
Full AGC Target	30,000	1,000,000
MS^n AGC Target	10,000	**10,000**

MS Detector

	LTQ	**LTQ-Orbitrap**
Acquire time (min)	**184**	**184**
Start delay (min)	**15**	**15**
Segments	1	1
Scan Events	9	9
Scan Ranges for Event 1	m/z 300-2000	m/z 300-2000
Mass Range for all Events	Normal	Normal
Scan Rate for all Events	Normal	Normal
Scan Type for Event 1	Full	Full
Polarity for all Events	Positive	Positive
Data type for MS Scan	Centroid	Profile
Data type for MS^n Scan	Centroid	Centroid
Additional microscans	0	0
Resolution of MS scan	—	60,000

- Zoom scans and wideband activation are not used for LTQ runs

For Scan Events 2-9, the Dependent Scan box is checked and the following settings are used:

Dynamic Exclusion

	LTQ	**LTQ-Orbitrap**
Repeat count	1	1
Repeat duration (s)	0	0
Exclusion list size	**150**	**150**
Exclusion duration (s)	**60**	**60**
Exclusion mass width	by mass	by mass
Exclusion mass width (low)	1.0	**0.6**
Exclusion mass width (high)	3.5	**0.6**

For the LTQ-Orbitrap:

- **Charge State Screening is enabled**
- **Monoisotopic precursor selection enabled**
- **Non-peptide monoisotopic recognition enabled**
- **Charge state +1 rejected**
- **Unassigned charge state rejected**

For the LTQ:

- **Charge State Screening and Rejection is not enabled**

Scan Event

	LTQ	LTQ-Orbitrap
MS2 Minimum signal threshold	500	500
Mass determined from scan event	1	1
Activation type	CID	CID
Default charge state	4	4
Isolation width (m/z)	2.0	2.0
Normalized collision energy	28	28
Activation Q	0.250	0.250
Activation time (ms)	30.0	30.0

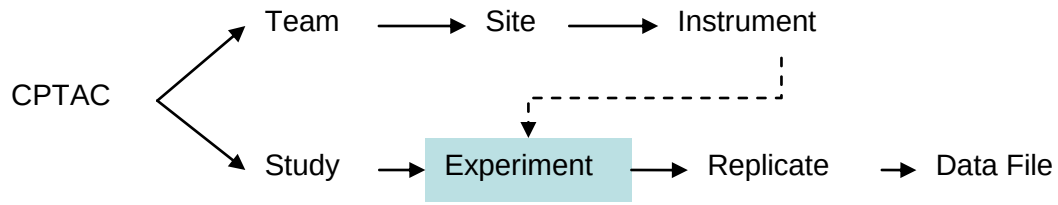
- For each Current Scan Event, the N^{th} most intense ion is set at 8-1 for scan events 2-9, respectively
- Mass lists and global mass lists are not used
- Early expiration is not used

Annotation / Data Transfer for Study3 "Dress Rehearsal"

A new CPTAC website is being developed for Study 3 that uses Tranche (http://www.proteomecommons.org/dev/dfs/) to store data files; annotation will be recorded using spreadsheets. Access to CPTAC data files will be restricted by passphrases stored in a database accessible by the website. The goal of this site is to collect valuable experimental information and connect it to the data. It also provides a means to record 'lab-specific' information not specified by the SOP along with any necessary deviations from the SOP so that this information may be systematically evaluated during the analysis phase of the study.

The CPTAC annotation website now provides the following services to participating teams: (1) secure team login and access to pre-loaded, lab/site-specific information, (2) access to annotation forms (xls), (3) a field to track SOP compliance, (4) direct upload links to Tranche, and (5) study-wide single and batch Tranche download access.

The following simplified illustration shows the current organization of data elements in the study and is based on a model developed by the 'Data Analysis, Storage, and Dissemination Working Group' (all depicted entity relationships are '1-to-many'):



The database is designed to store multiple CPTAC studies, but only one study at a time is active. This phase of the Study will be loaded as an Experiment under each Instrument for each participating Team account. The current "dress rehearsal" phase will be run as a single replicate consisting of 13 runs. **Note:** The functions and layout of the site are subject to change during the course of the Study.

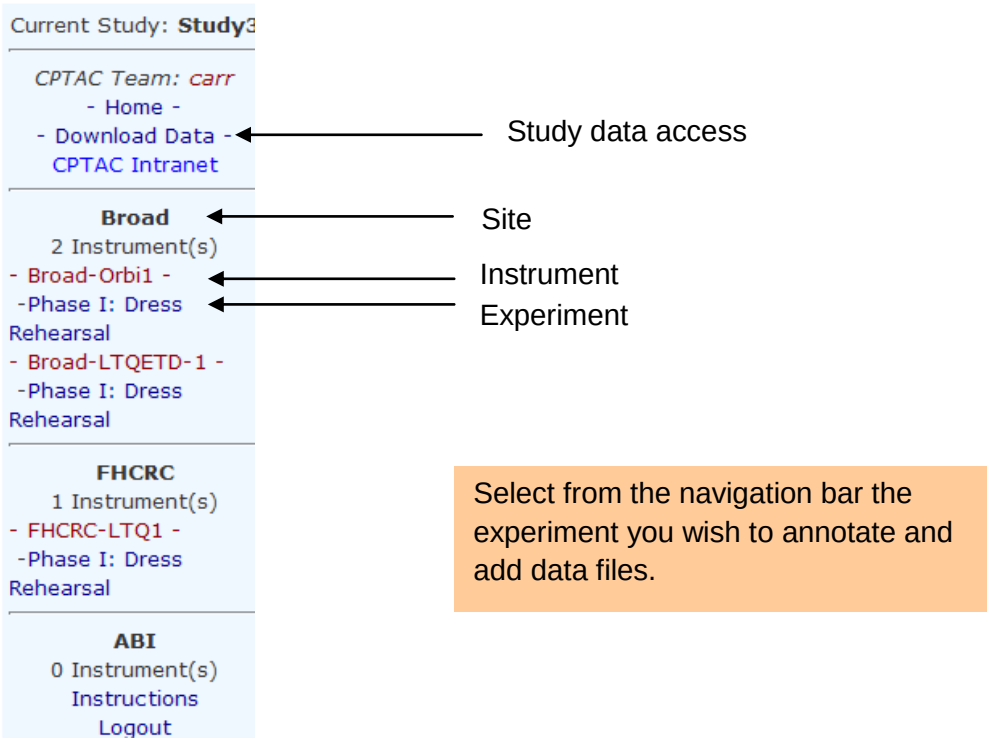
Access and Annotation/Data Upload

The **new** URL for the site is:

https://chemdata.nist.gov/cptac/

[**Note:** This is a secure 'https' website. Although traffic on the site is encrypted, its certificate is not signed by a third party outside of NIST. This will prompt a security warning on most browsers and users should click 'allow this site' or similar message to proceed. The process of installing a security certificate is underway to eliminate these warnings.]

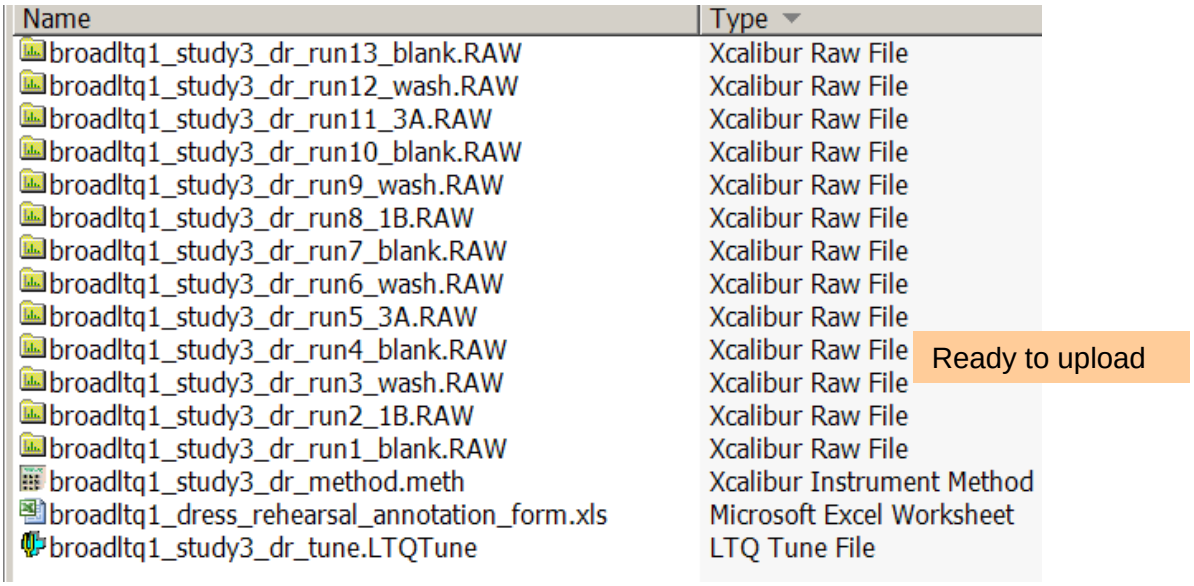
Users login using the name of the team lead in all lower case (i.e. **'carr', 'fisher', 'liebler', 'regnier'** or **'tempst'**). Passwords will be e-mailed to data contacts and team leads at the beginning of the dress rehearsal. There is only one login password per team.



The illustration above shows one team's navigation bar following login. After selecting the appropriate "Phase I: Dress Rehearsal", the annotation page will be displayed listing the steps for annotation and upload. All annotation and data files will be uploaded as a **single directory** to Tranche. Individual download of the same directory using Tranche will allow selection of individual files from a directory listing.

Follow these steps to annotate and upload study data. These (or similar) steps will appear on the website with links:

1. Download the annotation form.
2. Complete the annotation form. All required fields are highlighted. Use the 'site' and 'instrument name' provided on the website's navigation bar to fill-in the top section. The middle section is specific to the entire "dress rehearsal" and information entered here is expected to stay consistent for all runs. The bottom section tracks individual run/file information. The sample name from the tube you were shipped must be entered for each study sample along with the date and raw file name. Comments specific to a single run can also be entered here.
3. Copy raw data file(s) from **each step** of the SOP (13 total sample/blank/wash runs) to a single directory. If you are using an instrument that generates multiple raw files per sample, please create a single zip file for each step.
4. If running an LTQ, copy your 'tune' and 'method' files used for the experiment into the same directory.
5. Copy your completed annotation form to this directory. The contents of your directory should look something like this prior to upload:



6. Verify that all parameters have been checked according to the SOP **or** that deviations have been otherwise recorded. Have the designated SOP authority at your site "sign" the web form by entering his/her name in the box. Should there be any deviations from the SOP or other questions regarding your lab's operation of the experiment, the SOP authority will serve as the primary contact.
7. Click on the 'Upload Data' link provided on the website. This will launch the CPTAC/Tranche upload tool. Select the directory to upload; the progress indicator will display the time left for the transfer; your browser does not have to remain open during this time. Your Tranche hash and passphrase will be recorded on the website after the upload has completed. These values will be used to later access your data.

Data Downloading

After uploads have completed, all of the "dress rehearsal" data can be downloaded from the 'Download Data' link on the navigation bar. From this page, click on a single link or use the check boxes to generate a multiple download 'csv' file. This file can be used as input for a 'multi-download' used in combination with the latest Tranche Tool. More information on this feature can be found here, http://www.proteomecommons.org/dev/dfs/examples/nci-cptac/.

Note: A 'multi-download' csv file contains your Tranche hashes and passphrases and can be used by anyone to access your data files. You may keep this file as a "back-up" but **do not share** it with parties outside of the CPTAC program until this level of data sharing has been authorized.

Sampling Scheme:

Samples should be analyzed in the following order:

Dress rehearsal:

1. blank (full gradient, injection of 0.1% formic acid in H_2O(mobile phase A))
2. Sample 1B (QC sample, full gradient)
3. autosampler and column wash (per individual lab's protocol)
4. blank (full gradient, injection of 0.1% formic acid in H_2O(mobile phase A))
5. Sample 3-A (digested, unspiked yeast)
6. autosampler and column wash (per individual lab's protocol)
7. blank (full gradient, injection of 0.1% formic acid in H_2O(mobile phase A))
8. Sample 1B (QC sample, full gradient)
9. autosampler and column wash (per individual lab's protocol)
10. blank (full gradient, injection of 0.1% formic acid in H_2O(mobile phase A))
11. Sample 3-A (digested, unspiked yeast)
12. autosampler and column wash (per individual lab's protocol)
13. blank (full gradient, injection of 0.1% formic acid in H_2O(mobile phase A))

Key Dates for Dress Rehearsal

	Time allotted	Finish date
SOP and analysis scheme understood and approved by participating labs	4d	7/10/2007
Labs perform dress rehearsal experiments	10d	7/20/2007
Labs upload data to Tranche	4d	7/24/2007
Dress rehearsal data analyzed and issues with individual lab's data are sorted out	20d	8/13/2007
Troubleshoot dress rehearsal data and protocol	18d	8/31/2007
Begin experiments on spiked yeast samples		9/4/2007

Study 5 Experimental Plan: SOP v2.1

CPTAC Unbiased Discovery Working Group Study 5 SOP

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.

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

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

Study 6 Experimental Plan: SOP v2.2

CPTAC Unbiased Discovery Working Group Study 6 SOP

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 performed a preliminary experiment to include only a single protein, bovine serum albumin (BSA). This initial spiked sample provided a means for comparing performance of LC/MS/MS platforms amongst the CPTAC participants and helped to further refine this SOP. As we have since sorted out issues with the digestion of Sigma's Universal Protein Standard (Sigma48), we are moving forward with our goal to assess the limits of detection for biomarker discovery in our model standard yeast. In addition, we are developing 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 difference for the overall sensitivity of the platform for a given protein abundance.

This proposed standard operating procedure (SOP) was developed after initial testing of several yeast lysate and Sigma48 samples at Vanderbilt University. After an initial Study 3 and Study 5 results, 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 studies.

Study Samples:

The yeast lysate was digested using RapiGest as denaturant and iodoacetamide as alkylating reagent at NIST. The Sigma48 sample was digested at Vanderbilt using a protocol involving TFE solubilization and also using iodoacetamide as the alkylating reagent. The digested Sigma48 was spiked in at 5 different concentrations from 20 fmol/uL to 0.25 fmol/uL. The yeast digest alone, the Sigma48 digest alone and the NCI 20 will all be used as QC standards for these experiments. Given the limited quantity of Sigma48, these will be sent in **25uL aliquots**, so plan experiments carefully.

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

Table 3 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

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. 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. This updated SOP has been changed slightly and these changes are highlighted in the respective SOP's. **The flow rate for these experiments has now been standardized too 600 nL/min and should not be deviated from. In addition, it has been specified that wideband activation is NOT to be used.** Any derivations from the protocol must 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.

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	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:

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 QC-2 –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**	184 min gradient

	Sigma 48 spiked yeast	
20	Wash*	
21	Blank	95 min gradient
22	**Sample QC-1 - 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

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	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:

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 QC-2 –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**	184 min gradient

	Sigma 48 spiked yeast	
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 QC-1 - 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

Upload Instructions for Study6: Unbiased Discovery, Yeast + Sigma48

1.) Browse to https://chemdata.nist.gov/cptac/ (ignore the security warning and click OK.)
2.) Enter your 'log-in' name and password. These are the same as used in earlier studies. If you have forgotten either, send an email to 'paul.rudnick@nist.gov.'
3.) Select '**Study6**' from the selector at the top of the navigation bar.
4.) Click your site on the navigation bar, then the 'Experiment' button, then 'Yeast + Sigma48' to bring up to the transfer page for the dataset you are ready to upload.
5.) Complete the annotation form (Step1), if you have not done so already.
6.) Follow the instructions in box for Step 3. All data files and any annotation forms should be first copied to a **single directory** for upload. Please also include the completed SOP checklist (from this document).
7.) Enter the name of your SOP contact in the box at Step 4. Click 'Sign.' The 'Upload' hyperlink at Step 5 should now be active.
8.) Click on 'Upload' to launch Tranche (Step 5). This requires Java JRE to be installed. Browse to the directory containing your upload begin the data transfer.
9.) 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. An e-mail receipt will be sent to the SOP contact when the upload completes. You can also view all successful uploads by clicking 'Download Data' from the navigation bar.

Timeline for Study 6

Task	Duration	Start Date	End Date
NIST sends out samples for full study	2 days	02/25/2008	02/26/2008
Labs perform full study	30 days	02/27/2008	03/27/2008
Labs upload data to Tranche	1 day	03/27/2008	03/28/2008
Data is analyzed	30 days	03/31/2008	04/25/2008
Results reported to WG	1 day	05/07/2008	05/07/2008
Yeast results reported to PCC	1 day	05/27/2008	05/27/2008

For questions about the timeline, please contact Chris Kinsinger (kinsingc@mail.nih.gov)

CPTAC Network Listing

http://proteomics.cancer.gov/programs/CPTAC/networkmembership/

Broad Institute of MIT and Harvard: Steven A. Carr, Michael Gillette, Karl R. Clauser, Terri Addona, Susan Abbatiello, Ronald K. Blackman, Jacob D. Jaffe, Eric Kuhn, Hasmik Keshishian, Michael Burgess

Buck Institute for Age Research: Bradford W. Gibson, Birgit Schilling, Jason M. Held, Bensheng Li, Christopher C. Benz, Gregg A. Czerwieniec, Michael P. Cusack

California Pacific Medical Center: Dan Moore

Epitomics, Inc: Xiuwen Liu, Guoliang Yu, Youling Zou

Fred Hutchinson Cancer Research Center: Amanda G. Paulovich, Jeffrey R. Whiteaker, Lei Zhao, ChenWei Lin, Regine Schoenherr, Pei Wang, Peggy L. Porter, Constance D. Lehman, Diane Guay, JoAnn Lorenzo, Barbara R. Stein, Marit Featherstone, Lindi Farrell, Stephanie Stafford, Julie Gralow

Hoosier Oncology Group: Linnette Lay, Kristina Kirkpatrick

Indiana University: Randy J. Arnold, Predrag Radivojac, Haixu Tang

Indiana University-Purdue University Indianapolis: Jake Chen, Scott H. Harrison, Harikrishna Nakshatri, Bryan Schneider

Lawrence Berkeley National Laboratory: Joe W. Gray, John Conboy, Anna Lapuk, Paul Spellman, Daojing Wang, Nora Bayani

Massachusetts General Hospital: Steven J. Skates, Trenton C. Pulsipher

Memorial Sloan-Kettering Cancer Center: Paul Tempst, Hans Lilja, Mark Robson, James Eastham, Clifford Hudis, Brett Carver, Josep Villanueva, Kevin Lawlor, Arpi Nazarian, Lisa Balistreri, San San Yi, Alex Lash, John Philip, Yongbiao Li, Andrew Vickers, Adam Olshen, Irina Ostrovnaya, Martin Fleisher

Monarch Life Sciences: Tony J. Tegeler, Mu Wang

National Cancer Institute: Henry Rodriguez, Tara R. Hiltke, Mehdi Mesri, Christopher R. Kinsinger

National Cancer Institute-SAIC-Frederick: Gordon R. Whiteley

National Institute of Standards and Technology – Gaithersburg, MD: Nikša Blonder, Bhaskar Godugu, Yuri Mirokhin, Pedi Neta, Jeri S. Roth, Paul A. Rudnick, Stephen E. Stein, Dmitrii Tchekhovskoi, Eric Yan, Xiaoyu (Sara) Yang, David M. Bunk, Karen W. Phinney, Nathan G. Dodder

National Institute of Standards and Technology – Hollings Marine Lab, Charleston, SC: Lisa E. Kilpatrick

New York University School of Medicine: Thomas A. Neubert, Helene L. Cardasis, David Fenyo, Chongfeng Xu, Sofia Waldemarson, Steven Blais, Åsa Wahlander

Plasma Proteome Institute: N. Leigh Anderson

Purdue University: Fred E. Regnier, Jiri Adamec , Charles Buck, Wonryeon Cho, Kwanyoung Jung, John Springer, Xiang Zhang

Single Organism Software: Jayson A. Falkner

Texas A&M University: Cliff Spiegelman, Lorenzo Vega-Montoto, Asokan Mulayath Variyath

University of Arizona: Dean D. Billheimer

University of British Columbia: Ronald C. Beavis, Dan Evans

University of California at San Francisco: Susan J. Fisher, Michael Alvarado, Mattheus Dahlberg, Penelope M. Drake, Matthew Gormley, Steven C. Hall, Keith D. Jones, Michael Lerch, Michael McMaster, Richard K. Niles, Akraporn Prakobphol, H. Ewa Witkowska

University of Michigan: Philip C. Andrews, Mark Gjukich, Bryan Smith, James Hill

University of North Carolina at Chapel Hill: David F. Ransohoff

University of Texas M.D. Anderson Cancer Center: Gordon B. Mills, Yiling Lu, Jonas Almeida, Katherine Hale, Doris Siwak, Mary Dyer, Webin Liu, Hassan Hall, Natalie Wright

University of Victoria: Terry W. Pearson, Matt Pope, Martin Soste, Morteza Razavi

University of Victoria-Genome BC Proteomics Centre: Angela Jackson, Derek Smith, Christoph Borchers

University of Washington: Michael J. MacCoss, Brendan MacLean

Vanderbilt University School of Medicine: Daniel C. Liebler, Amy-Joan L. Ham, Lisa J. Zimmerman, Robbert J.C. Slebos, David L. Tabb, Carlos L. Arteaga, Ming Li, Melinda E. Sanders, Simona G. Codreanu, Misti A. Martinez, Corbin A. Whitwell, Constantin F. Aliferis, Douglas P. Hardin, Richard M. Caprioli, Dale S. Cornett, Eric I. Purser, Matthew C. Chambers, Scott M. Sobecki, Michael D. Litton, Darla Freehardt, De Lin, Sheryl L. Stamer, Anthony Frazier, Kimberly Johnson, Ze-Qiang Ma, Matthew V. Myers, Haixia Zhang, Alexander Statnikov