Icahn School of Medicine at Mount Sinai LINCS Center for Drug Toxicity Signatures

Standard Operating Procedure:
Protein Spectra Acquisition by Data Dependent Acquisition (DDA) on LC/MS/MS

DToxS SOP Index: A-8.0
Last Revision: 1/25/16
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Quality Assurance/Control (QA/QC) steps are indicated with green highlight.

Metadata recording is highlighted with yellow highlight and superscript indices.
Literature references are indicated by numbers in square brackets.

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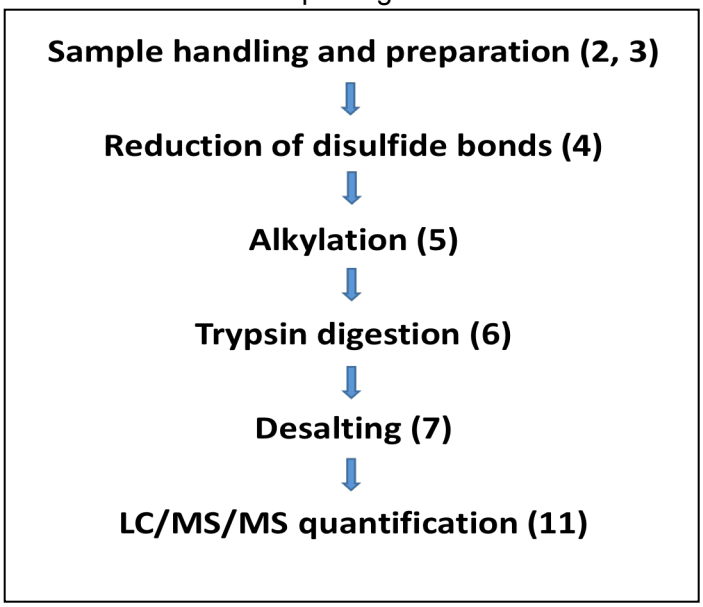
1) **DDA LC/MS/MS Proteomics Workflow.** This schematic depicts the overall steps of the proteomics workflow. Details of each step are given below.



2) **Sample handling.** Place proteins isolated by Mt. Sinai (SOP Index A-2.0—received on dry ice) in a -80ºC freezer upon arrival.

3) **Sample preparation**.

a. Thaw a batch of 8 samples on ice.
b. Vortex briefly and then sonicate at room temperature for 30 sec.
    i. We use a Transsonic 460 sonicator.
c. Mix 55 µL of the protein solution from each sample with 18.5 µL of 4x Laemmli Sample loading buffer (BIO-RAD, Cat. No 161-0747) and 3.5 µL of 2-Mercaptoethanol (BIO-RAD, 161-0710)
d. Load samples into a 1.5 mm thick, 12% BioRad mini SDS-PAGE gel casted in the laboratory according to the manufacturer's directions.
    i. Perform electrophoresis according to the manufacturer's instructions. We use100 volts for 65 min on the Bio-Rad PAC 1000 apparatus. The dye front should be approximately halfway down the gel.
e. Estimate sample protein concentration via Coomassie blue staining.
    i. We include a HeLa cell protein extract of known protein amont (25 - 100 ug) in the same SDS-PAGE for calibration. The amount of HeLa protein is chosen to be similar to the estimated amount of the sampleThe density of each sample lane is measured using Image Lab software (Bio-Rad). The protein concentration is estimated using the following equation:
    Protein concentration = [Hela cell protein amount × (Sample density/Hela cell density)]/volume of samples.
f. Cut the entire gel lane for each sample and dissect into ~1×1 mm gel blocks.
g. Place the gel blocks from each sample into a 15 mL centrifuge tube (CELLTREAT SCIENTIFIC PRODUCTS, product code 229411).
h. Wash the gel blocks from each sample with 10 mL of wash buffer (30% ACN[1] and 70% 100 mM NH_4HCO_3[1] in H_2O) for 3 h. Aspirate the buffer and repeat the wash 3 more times.
i. Remove the wash buffer and dehydrate the gel blocks with 1 mL of 100% ACN[1] for 30 min, and then remove the ACN completely.

QA/QC1 for Step 3:

a. The protein samples are thawed and sonicated immediately prior to loading onto the SDS-PAGE gels to preserve protein stabilities.
b. Protein samples and the known amounts of the HeLa cell extracts must be loaded onto the same gels for estimation of protein amounts.

4) Reduction of the disulfide bonds [1]

a. Freshly prepare 25 mM DTT[1] solution (dissolve 46.2 mg DTT[1] in 12 mL of 100 mM NH_4HCO_3[1] in H_2O).
b. Add 1 mL of the 25 mM DTT solution to each tube of the dehydrated gel blocks from Step 3.
c. Incubate at 55°C for 30 min.
d. Add 9 mL of 100% ACN[1] and incubate for 10 min at RT.
e. Completely aspirate supernatant with a pipette.

5) Alkylation [1]

a. Add 1 mL of 50 mM iodoacetamide (IAM)[1] solution (111 mg IAM[1] in12 mL 100 mM NH_4HCO_3[1] in H_2O) to the gel blocks from each sample in the 15 mL centrifuge tubes from Step 4.
b. Incubate at 37°C in the dark for 30 min.

c. Add 9 mL of 100% ACN[1] for 10 min at RT.
d. Completely aspirate supernatant with a pipette.

6) Trypsin digestion and tryptic peptide extraction [1].

a. Prepare the trypsin solution by dissolving 60 µg of trypsin[1] into 12 mL of 50 mM NH_4HCO_3.
b. Add1 mL of trypsin solution per 20 µg of total protein to each sample (estimated from Step 3)..
c. Incubate at 37°C for 16 hrs.
d. Add 600 µL of peptide extraction buffer (4 mL 100 mM NH_4HCO_3[1], 8 mL 100%ACN[1], 200 µL of formic acid[1]) to each sample tube.
    i. This will extract peptides from the gel blocks.
e. Vortex the centrifuge tubes for 30 sec, centrifuge them at 1,000 g for 1 min, and incubate the samples at 37 ºC for 30 min.
f. Transfer 1.2 mL from each sample tube to a fresh 1.5 mL Eppendorf tube (Fisher Brand, Premium, Cat No.05-408-129).
    i. The gel pieces absorb the solution so volume loss is expected.
g. Add 600 µL 100% ACN to each sample tube, vortex for 30 sec, centrifuge at 1,000 g for 1 min, and incubate at 37 ºC for 30 min. Transfer the extract to a fresh 1.5 ml Eppendorf tube.
    i. You should have two 1.5 mL tubes for each sample.
    ii. The gel blocks are left behind.
h. Place 1.5 mL tubes in a SpeedVac (Vacufuge ™, Eppendorf) until ~250 µL per tube remains. (~3 hours)
i. Combine the contents of the 2 tubes for each sample.
j. Check pH to make sure the pH < 2.0 .Take 0.5ul from each sample and test it on pH stripe (pH-Fix 0.0-6.0, Macherey-NaGEL, REF 92115) if necessary, add 10% TFA[1] in H_2O (v/v) to adjust the pH to < 2.0.

7) Desalting using the Pierce™ C_{18} spin columns (Thermo Scientific, Cat No. 89870) **[1].**

a. Activate the C_{18} spin column: Add 200 µL of 50% ACN[1] in H_2O (v/v) onto the column, spin it at 1500 × g for 1 min; discard the flow-through; repeat this step once more.
b. Equilibrate the C_{18} spin column: Add 200 µLof 5% ACN-0.1%TFA[1] in H_2O (v/v) onto the column, spin it at 1,600 × g for 1 min; discard the flow through; repeat this step twice more.
c. Load the peptides: Add the entire digested peptide solution from each sample from Step 6 onto one C_{18} spin column, and spin it at 1,600 × g for 1 min.
    i. Each spin column is used for only one unique sample.
d. Re-load the flow-through back onto the column and spin it again as in step c.
e. Repeat step d. There are 3 spin steps in total.
    i. Keep the flow-through temporarily until the peptide yields can be confirmed by the later steps (The flow -through can be discarded after confirmation in Step 11).
f. Wash the C_{18} spin column: Add 200 µL of 5% ACN-0.1% TFA[1] in H_2O (v/v) onto the column, spin it at 1,600 × g for 1 min.
    i. Keep the flow through temporarily as above.
g. Repeat step f 2 more times.
h. Elute the tryptic peptides: Add 20 µL of 30% ACN[1] in H_2O (v/v) onto the column, spin at 1,600g, and add eluate to a fresh 1.5 mL tube.
i. Add 20 µL of 70 % ACN[1] in H_2O (v/v) onto the column, spin at 1,600g, and combine eluate with that from step h.
j. Repeat step i twice more.

k. Use a SpeedVac (Vacufuge ™, Eppendorf) to dry the peptide eluents at 30°C to less than 5 µL without completely drying (~30 min)
l. Resuspend the peptides with Solvent A (ACN[1]: H_2O: FA[1]=2 %: 97.9 %: 0.1 %, v/v/v) to a final concentration of peptides equivalent to 500 ng/µL (based on the protein concentration determined for the sample).

8) **QA/QC2 for Steps 4-7. Evaluate digestion efficiency.** We use a Thermo Scientific™ Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Velos-MS) with the instrument conditions outlined below before a formal LC/MS/MS quantification on a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Step 11). A Dionex Nano LC system is connected to the Velos-MS as a sample inlet. We use 4 µL of each sample from above.

a. Nano LC separation conditions[2]

i. **Instrument:** Dionex Ultimate® 3000 RSLCnano LC System
ii. **Column**: 75 µm x 15 cm, 3 µm, 100 Å, C_{18} column (CMP Scientific P/N c-75-002-015)
iii. **Flow rate:** 250 nL/min
iv. **Solvent A:** ACN[1]: H_2O: FA[1]=2%: 97.9%: 0.1%, v/v/v.
v. **Solvent B:** ACN[1]: H_2O: FA[1]=85%: 14.9%: 0.1%, v/v/v.
vi. **LC gradient**:

Time (minutes)	B%
0	1.0
6.1	1.0
60	30
65	50
70	95
74	95
75	1.0
95	1.0

b. Mass spectrometry Analysis [2]

i. **Instrument:** Velos-MS
Method duration: 85 min
Polarity: positive
Default charge state: 2
Number of Scan Events: 11
ii. **Full scan MS**
Analyzer: FTMS
Resolution: 60,000
Scan range: 300 to 1650 m/z
iii. **Data dependent (dd)-MS²**
Activation Type: CID
Min. Signal Required: 5000.0
Isolation Width: 3.00m/z
Normalized Coll. Energy: 30.0
Default Charge State: 2
Activation Q: 0.250
Activation Time: 30.000
iv. **dd settings**
Use separate polarity settings disabled

Parent Mass List:	(none)
Reject Mass List:	(none)
Neutral Loss Mass List:	(none)
Product Mass List:	(none)
Neutral loss in top:	3
Product in top:	3
Most intense if no parent masses found	not enabled
Add/subtract mass	not enabled
FT master scan preview mode	enabled
Charge state screening	enabled
Charge state dependent ETD time	not enabled
Monoisotopic precursor selection	enabled
Charge state rejection	enabled
Unassigned charge states:	rejected
Charge state 1:	rejected
Charge state 2:	not rejected
Charge state 3:	not rejected
Charge state 4:	not rejected

c. QA/QC acceptance criteria:

The samples will be considered passing QA/QC and proceed to Step 11 only when the highest ion chromatogram intensity from Velos-MS run reaches > 3.0E8 and rich MS/MS spectra with at least 6 fragment ions with signal to noise ≥ 5 per 1000 peptide mass units.

9) QA/QC3 for Step11 using BSA digest:

a. LC condition QA/QC: Run two injections of the BSA trypsin digest (Waters, MassPREP BSA Digestion Standard Cat No. 186002329, 100 fmol) in a parallel dual HPLC configuration as a quality control to evaluate the LC instrument performance,

i. For the BSA QC test, the nano LC will use the following gradient: [3]

Gradient 1 (peptide separation):

Time (minutes)	B%
0	1.0
1.5	5
50	30
60	50
65	95
70	95

Gradient 2 (column washing, equilibration and sample loading):

Time (minutes)	B%
0	95
5	95
40	1
70	1

ii. Analysis Logistics:

Both gradient 1 (for peptide separation) and gradient 2 (for column washing and equilibration) are 70 mins in lengths. In the first 70 mins of gradient, 100 fmol of the BSA digest is injected into column 1. The peptides are separated using gradient 1 on column

1. In the meantime, column 2 is run using gradient 2, in which the column is washed, equilibrated and loaded with the second sample at 55 min of gradient 2 for a subsequent LC/MS/MS analysis. Once the first 70 min gradient is finished, column 1 is switched from gradient 1 to gradient 2 for column washing, equilibration and the loading of a third sample at 55 min, while column 2 with the 2nd sample already loaded is switched from gradient 2 to gradient 1 for peptide separation.

iii. QA/QC acceptance criteria:

We will monitor the retention time and average peak width. The retention time shifts for 4 select BSA peaks (m/z 417.212, m/z 509.298, m/z 512.255, and m/z 722.816) should be less than 5 min compare to the historic runs. The average peak width for these 4 selected peaks should be less than 0.2 min. Otherwise the quality control is considered as failed and the causes of the increase in dead volumes need to be checked.

b. MS condition QA/QC:

Run two injections of BSA trypsin digest (Waters, MassPREP BSA Digestion Standard Cat No. 186002329,100 fmol) in the parallel HPLC configuration as the quality control to evaluate the MS instrument performance.

Detailed MS method is as following: [5]

i.	**Instrument:**	QE-MS
	Method duration:	70 min
	Polarity:	positive
	Default charge state:	3
	Inclusion:	-
	Exclusion:	-
	Tags:	-
ii.	**Full MS**	
	Resolution:	140,000
	AGC target:	3E6
	Maximum IT:	100ms
	Scan range:	400 to 1750 m/z
iii.	**dd-MS^2**	
	Resolution:	17,500
	AGC:	5E4
	Loop Count:	15
	Isolation window:	2.0m/z
	Fixed first mass:	150m/z
	NCE:	27.0
iv.	**dd settings**	
	underfill ratio:	1.0 %
	Apex trigger:	-
	Charge exclusion:	unassigned, 1, 6-8, >8
	Peptide match:	preferred
	Exclude isotopes:	on
	Dynamic exclusion:	20.0 s

c. QA/QC acceptance criteria:

BSA sequence coverage should be >65% and the number of identified peptides should be >50.

10) QA/QC4 for Step11 using HeLa cell digest

a. LC condition QA/QC: Run two injections of 200 ng of HeLa cell digests (Pierce, Prod# 88329) in parallel configuration 1 as the quality control to ensure good LC/MS/MS overall performance.

i. For the HeLa cell test, the nano LC will use the following gradient: [4]

Gradient 1 (peptide separation):

Time (minutes)	B%
0	1.0
1.5	5
195	30
215	50
230	95
240	95

Gradient 2 (column washing, equilibration and sample loading):

Time (minutes)	B%
0	95
40	95
200	1
240	1

ii. Analysis logistics:

Both gradient 1 (for peptide separation) and gradient 2 (for column washing and equilibration) are 240 mins in length. In the first 240 mins of gradient, 200 ng of HeLa cell digest is injected into column 1. The peptides are separated using gradient 1 on column 1. In the meantime, column 2 is run using gradient 2, in which the column is washed, equilibrated and loaded with the second sample at 225 min of gradient 2 for a subsequent analysis. Once the first 240 min gradient is finished, column 1 is switched from gradient 1 to gradient 2 for column washing, equilibration and loading of a third sample at 225 min, while column 2 is switched from gradient 2 to gradient 1 for peptide separation.

iii. QA/QC acceptance criteria:

We monitor the retention time and average peak width for selected peptide ions. The retention time shifts between runs should be less than 5 min compared to historic runs. The average peak width should be less than 0.2 min. If these criteria are not met, quality control has failed, and the machines need maintenance or cleaning.

b. Protein ID QA/QC: Run two injections of 200ng of HeLa cell digests (Pierce™ HeLa Protein Digest Standard, Cat No. 88329) in parallel configuration as quality control to ensure LC-MS/MS overall performance for complex protein ID.

Detailed MS method is as following: [5]

i. **Instrument:** QE-MS [6]

Method duration:	240 min
Polarity:	positive
Default charge state:	3
Inclusion:	-
Exclusion:	-
Tags:	-

ii. **Full MS**

Resolution:	140,000
AGC target:	3E6
Maximum IT:	100ms
Scan range:	400 to 1750 m/z

iii. **dd-MS^2**

Resolution:	17,500
AGC:	5E4
Loop Count:	15
Isolation window:	2.0m/z
Fixed first mass:	150m/z
NCE:	27.0

iv. **dd settings**

underfill ratio:	1.0 %
Apex trigger:	-
Charge exclusion:	unassigned, 1, 6-8, >8
Peptide match:	preferred
Exclude isotopes:	on
Dynamic exclusion:	45.0 s

c. QA/QC acceptance criteria:

We monitor the number of identified protein which should be > 3,000 and identified peptides which should be > 14,000. If these criteria are not met, quality control has failed, and the machine needs maintenance or cleaning.

11) LC/MS/MS analysis for protein identification and quantification [2; 3; 4]

Peptides derived from 2 µg of each sample obtained from step 7l is subjected to LC/MS/MS analysis with the following conditions.

a. Nano LC separation conditions for the analysis of 2 separate samples [7]

i. **Instrument:** Dionex Ultimate@ 3000 RSLCnano Duo LC System
ii. **Column**: 75 µm x 50 cm, 3 µm, 100 Å, C_{18} column (Thermo, P/N 164768)
iii. **Flow rate:** 250 nL/min
iv. **Solvent A:** ACN[1]: H2O: FA[1]=2%: 97.9%: 0.1%, v/v/v.
v. **Solvent B:** ACN[1]: H2O: FA[1]=85%: 14.9%: 0.1%, v/v/v.
vi. **Column oven temperature**: 40 °C
vii. **Injection volume:** 4 µl (the sample was resuspended into Solvent A to the final concentration of peptides equivalent to 500 ng/µl.)
viii. **LC Instrument configuration:** parallel configuration 1
ix. **LC gradient:**

Gradient 1 (peptide separation):

Time (minutes)	B%
0	1.0
1.5	5
195	30
215	50
230	95
240	95

Gradient 2 (column washing, equilibration and sample loading):

Time (minutes)	B%
0	95

40	95
200	1
240	1

b. **Analysis logistics**:

Both gradient 1 (for peptide separation) and gradient 2 (for column washing and equilibration) are 240 min. In the first 240 min of gradient, 2 µg equivalent peptides from the first sample is injected into column 1. The peptides are separated using gradient 1 on column 1. In the meantime, column 2 is run using gradient 2, in which the column is washed, equilibrated and loaded with the second sample at 225 min of gradient 2 for a subsequent LC/MS/MS analysis. Once the first 240 min gradient is finished, column 1 is switched from gradient 1 to gradient 2 for column washing, equilibration and loading the third sample at 225 min, while column 2 is switched from gradient 2 to gradient 1 for peptide separation. Once the second 240 gradient is finished, column 1 is switched back from gradient 2 to gradient 1 for peptide separation while column 2 is switched back from gradient 1 to gradient 2 for column washing, equilibration and loading the fourth sample at 225 min. In this way, odd numbered samples are separated by column 1 while even numbered samples are separated by column 2. All samples are loaded at 225 min of gradient 2 via autosampler with flow rate of 5 µL/min and separated on the analytical columns by gradient 1 with a flow rate of 250 nL/min. Raw data spectra are submitted to ISMMS, and used for protein identification and quantitation (see SOP Index CO-1.0)

c. Mass spectrometry data acquisition conditions:[7]

i. **Instrument:** QE-MS

Method duration:	240 min
Polarity:	positive
Default charge state:	3
Inclusion:	-
Exclusion:	-
Tags:	-

ii. **Full MS**

Resolution:	140,000
AGC target:	3E6
Maximum IT:	100ms
Scan range:	400 to 1750 m/z

iii. **dd-MS^2**

Resolution:	17,500
AGC:	5E4
Loop Count:	15
Isolation window:	2.0m/z
Fixed first mass:	150m/z
NCE:	27.0

iv. dd settings

underfill ratio:	1.0 %
Apex trigger:	-
Charge exclusion:	unassigned, 1, 6-8, >8
Peptide match:	preferred
Exclude isotopes:	on
Dynamic exclusion:	45.0 s

References

1. PRIME-XS Protocol NPC in Solution Enzymatic Digestion, http://www.primexs.eu/protocols/func-startdown/66/
2. Bantscheff M[1], Schirle M, Sweetman G, Rick J, Kuster B, Quantitative mass spectrometry in proteomics: a critical review. *Anal Bioanal Chem.* 2007 Oct; 389(4):1017-31. doi: 10.1007/s00216-007-1486-6
3. Neilson KA, Ali NA, Muralidharan S, Mirzaei M, Mariani M, Assadourian G, Lee A, van Sluyter SC, Haynes PA. Less label, more free: approaches in label-free quantitative mass spectrometry, *Proteomics.* 2011 Feb; 11(4):535-53. doi: 10.1002/pmic.201000553.
4. Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR, Resing KA, Ahn NG. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol Cell Proteomics.* 2012; 893:321-41. doi: 10.1007/978-1-61779-885-6_20.

Metadata

1) Reagents for use in reduction, alkylation, digestion, and LC/MS carriers

Reagent name	Abbreviation	Companies	Cat. No	Lot No	Grade
Acetonitrile	ACN	Fisher Scientific	A998-4	13I026891	HPLC grade
Ammonium Bicarbonate	NH_4HCO_3	Fisher Scientific	A643-500	144046	Certified
Dithiothreitol	DTT	Bio-Rad	161-0611	210009645	Electrophoresis
Iodoacetamide	IAM	Bio-Rad	163-2109	0907B517	99.4%
Water	H_2O	J.T. Baker	4218-03	0000093738	HPLC grade
Formic acid	FA	Sigma-Aldrich	94318	BCBF8669V	For mass spec
Trifluoroacetic acid	TFA	Sigma-Aldrich	302031	SHBC1388V	HPLC grade
Trypsin (modified)		Promega	V5113	0000112439	Sequencing grade

2) Procedure 8. Conditions for quality evaluation of the protein digest samples on the Velos-MS

a. Nano LC separation conditions

i. **Instrument:** Dionex Ultimate@ 3000 RSLCnano LC System

ii. **Column**: 75 μm x 15 cm, 3 μm, 100 Å, C_{18} column (CMP Scientific P/N c-75-002-015)

iii. **Flow rate:** 250 nL/min

iv. **Solvent A:** ACN: H_2O: FA=2%: 97.9%: 0.1%, v/v/v.

v. **Solvent B:** ACN: H_2O: FA=85%: 14.9%: 0.1%, v/v/v.

vi. **LC gradient**:

Time (minutes)	B%
0	1.0
6.1	1.0
60	30
65	50
70	95
74	95
75	1.0
95	1.0

b. Mass spectrometry Analysis

i. **Instrument:** Velos-MS
Method duration: 85 min
Polarity: positive
Default charge state: 2
Number of Scan Events: 11

ii. **Full scan MS**
Analyzer: FTMS
Resolution: 60,000
Scan range: 300 to 1650 m/z

iii. **Data dependent (dd)-MS^2**
Activation Type: CID

Min. Signal Required:	5000.0
Isolation Width:	3.00m/z
Normalized Coll. Energy:	30.0
Default Charge State:	2
Activation Q:	0.250
Activation Time:	30.000

iv. **dd settings**

Use separate polarity settings disabled	
Parent Mass List:	(none)
Reject Mass List:	(none)
Neutral Loss Mass List:	(none)
Product Mass List:	(none)
Neutral loss in top:	3
Product in top:	3
Most intense if no parent masses found	not enabled
Add/subtract mass	not enabled
FT master scan preview mode	enabled
Charge state screening	enabled
Charge state dependent ETD time	not enabled
Monoisotopic precursor selection	enabled
Charge state rejection enabled	
Unassigned charge states:	rejected
Charge state 1:	rejected
Charge state 2:	not rejected
Charge state 3:	not rejected
Charge state 4:	not rejected

3) Procedure 9. QA/QC using BSA digest

a. nano LC will use the following gradient:

Gradient 1 (peptide separation):

Time (minutes)	B%
0	1.0
1.5	5
50	30
60	50
65	95
70	95

Gradient 2 (column washing, equilibration and sample loading):

Time (minutes)	B%
0	95
5	95
40	1
70	1

b. detailed MS method is as follows:

i. **Instrument:** QE-MS

Method duration: 70min

Polarity:	positive
Default charge state:	3
Inclusion:	-
Exclusion:	-
Tags:	-

ii. **Full MS**

Resolution:	140,000
AGC target:	3E6
Maximum IT:	100ms
Scan range:	400 to 1750 m/z

iii. **dd-MS^2**

Resolution:	17,500
AGC:	5E4
Loop Count:	15
Isolation window:	2.0m/z
Fixed first mass:	150m/z
NCE:	27.0

iv. **dd settings**

underfill ratio:	1.0 %
Apex trigger:	-
Charge exclusion:	unassigned, 1, 6-8, >8
Peptide match:	preferred
Exclude isotopes:	on
Dynamic exclusion:	20.0 s

4) Procedure 10. QA/QC using Hela cell digest

a. nano LC will use the following gradient:

Gradient 1 (peptide separation):

Time (minutes)	B%
0	1.0
1.5	5
195	30
215	50
230	95
240	95

Gradient 2 (column washing, equilibration and sample loading):

Time (minutes)	B%
0	95
40	95
200	1
240	1

b. detailed MS method is as follows:

i. **Instrument:** QE-MS

Method duration:	240min
Polarity:	positive
Default charge state:	3
Inclusion:	-
Exclusion:	-
Tags:	-

ii. Full MS

Resolution:	140,000
AGC target:	3E6
Maximum IT:	100ms
Scan range:	400 to 1750 m/z

iii dd-MS^2

Resolution:	17,500
AGC:	5E4
Loop Count:	15
Isolation window:	2.0m/z
Fixed first mass:	150m/z
NCE:	27.0

iv dd settings

underfill ratio:	1.0 %
Apex trigger:	-
Charge exclusion:	unassigned, 1, 6-8, >8
Peptide match:	preferred
Exclude isotopes:	on
Dynamic exclusion:	45.0 s

5) Procedure 11. LC/MS/MS quantification

a. Nano LC separation conditions for the analysis of 2 separate samples

v. **Instrument:** Dionex Ultimate@ 3000 RSLCnano Duo LC System
vi. **Column**: 75 μm x 50 cm, 3 μm, 100 Å, C_{18} column (Thermo, P/N 164768)
vii. **Flow rate:** 250 nL/min
viii. **Solvent A:** ACN: H2O: FA=2%: 97.9%: 0.1%, v/v/v.
ix. **Solvent B:** ACN: H2O: FA=85%: 14.9%: 0.1%, v/v/v.
x. **Column oven temperature**: 40 °C
xi. **Injection volume:** 4 µl (the sample was resuspended into Solvent A to the final concentration of peptides equivalent to 500 ng/µl.)
xii. **LC Instrument configuration:** parallel configuration 1
xiii. **LC gradient:**

Gradient 1 (peptide separation):

Time (minutes)	B%
0	1.0
1.5	5
195	30
215	50
230	95
240	95

Gradient 2 (column washing, equilibration and sample loading):

Time (minutes)	B%
0	95
40	95
200	1
240	1

b. Mass spectrometry data acquisition conditions:

i. **Instrument:** QE-MS

	Method duration:	240min
	Polarity:	positive
	Default charge state:	3
	Inclusion:	-
	Exclusion:	-
	Tags:	-
ii.	**Full MS**	
	Resolution:	140,000
	AGC target:	3E6
	Maximum IT:	100ms
	Scan range:	400 to 1750 m/z
iii.	**dd-MS^2**	
	Resolution:	17,500
	AGC:	5E4
	Loop Count:	15
	Isolation window:	2.0m/z
	Fixed first mass:	150m/z
	NCE:	27.0
iv.	**dd settings**	
	underfill ratio:	1.0 %
	Apex trigger:	-
	Charge exclusion:	unassigned, 1, 6-8, >8
	Peptide match:	preferred
	Exclude isotopes:	on
	Dynamic exclusion:	45.0 s

Quality Assurance/Control Steps (QA/QC)

QA/QC1 for Step 3:

a. The protein samples are thawed and sonicated immediately prior to loading onto the SDS-PAGE gels to preserve protein stabilities.
b. Protein samples and the known amounts of the HeLa cell extracts must be loaded onto the same gels for the protein amount estimations.

QA/QC2 for Steps 4-7:

The digestion efficiency is evaluated with Thermo Scientific™ Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Velos-MS) with the instrument conditions outlined in SOP Steps 8

QA/QC3 for Step 11 using BSA digest:

a. Run two injections of BSA trypsin digest (Waters, MassPREP BSA Digestion Standard Cat No. 186002329, 100 fmol) in parallel configuration 1 as quality control to evaluate the MS instrument performance. We will monitor the retention time of 4 select BSA peaks (m/z 417.212, m/z 512.255, m/z 722.816 and m/z 509.298) (retention time shift between runs should be less than 5 min) and their average peak width (should be less than 0.2min). The condition of LC and MS are outlined in SOP Step 9a.

b. Run two injections of BSA trypsin digest (Waters, MassPREP BSA Digestion Standard Cat No. 186002329, 100 fmol) in parallel configuration as quality control to evaluate the MS instrument performance. BSA sequence coverage should be >65% and the number of identified peptides should be >50. The detail MS method is listed in SOP Step 9b.

QA/QC4 for Step 11 using HeLa digest:

a. Run two injections of 200 ng of HeLa cell digests (Pierce, Prod# 88329) in parallel configuration 1 as quality control to ensure LC-MS/MS overall performance. We will monitor the retention time (retention time shift should be less than 5 min) and peak width (should be less than 0.2 min). The condition of LC and MS are outline in SOP Step 10a.

b. Run two injections of 200ng of HeLa cell digests (Pierce™ HeLa Protein Digest Standard, Cat No. 88329) in parallel configuration as quality control to ensure LC-MS/MS overall performance. The number of identified proteins should be >3000 and identified peptides >14,000. The detail MS method is listed in SOP Step 10b.