**Supplementary file 1**

Quick Start™ Bradford Protein Assay

**Reagent used:** 1x Bradford Reagent

**Introduction:** The Bradford assay is a method to estimate the protein concentration in a sample. It involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976). Under acidic conditions, the dye exists in a doubly protonated red cationic form (Amax = 470 nm). When it binds to the protein, it is converted to a stable unprotonated blue form (Amax = 595 nm). This blue form (protein-dye complex) can be detected at 595 nm using a spectrophotometer.

**Standard preparation:** Prepare the stock solution of BSA (Bovine serum albumin) with 2 mg/ml concentration by weighing 2 mg of BSA and dissolving it in 1 ml Mili Q water. Prepare the standards of varying concentration by serially diluting the stock solution of BSA.

**Protocol:**

1. Add 20 µl of each standard and unknown sample into clean tubes.

2. Add 1 ml of 1x Bradford reagent to each tube and vortex it.

3. Incubate the tubes at room temperature for 5 min. and measure the absorbance at 595 nm after the blank correction.

4. Prepare a standard curve by plotting the absorbance of standards versus their concentration in μg/µl.

5. Use the standard curve to determine the protein concentration in each sample.

**Supplementary file 2**

**LC-MS parameters for Label-free quantitation (LFQ) and iTRAQ experiments**

1. **Sample pickup and loading:** Sample pickup and loading parameters for the experiments are mentioned below. It summarizes the sample volume, flow and pressure of the column.

|  |
| --- |
| Sample pickup: |
| Volume (μl) | 2.00 μl for LFQ, 3.00 μl for iTRAQ |
| Flow (μl/min) | 5.00 |
| Sample loading: |
| Volume (μl) | 10.00 μl for LFQ, 12.00 μl for iTRAQ |
| Flow (μl/min) | Unspecified |
| Max. pressure (Bar) | 800.00 μl for LFQ, 750.00 μl for iTRAQ |

1. **Chromatography gradients:** Below is the liquid chromatography gradient used for the experiments. Tables contain the time duration for solvent B at different intervals of time with constant flow rate of 300 nl/min.
2. **For label-free quantitation (LFQ):**

|  |  |  |  |
| --- | --- | --- | --- |
| Time [mm:ss] | Duration [mm:ss] | Flow [nl/min] | Mixture [%B] |
| 00:00 | 00:00 | 300 | 0 |
| 05:00 | 05:00 | 300 | 5 |
| 80:00 | 75:00 | 300 | 30 |
| 110:00 | 30:00 | 300 | 60 |
| 115:00 | 05:00 | 300 | 90 |
| 120:00 | 05:00 | 300 | 90 |

1. **For iTRAQ experiment:**

|  |  |  |  |
| --- | --- | --- | --- |
| Time [mm:ss] | Duration [mm:ss] | Flow [nl/min] | Mixture [%B] |
| 00:00 | 00:00 | 300 | 0 |
| 70:00 | 70:00 | 300 | 35 |
| 77:00 | 07:00 | 300 | 95 |
| 90:00 | 13:00 | 300 | 95 |

1. **Column equilibration:** 0.1% (v/v) FA was used to equilibrate the column with below mentioned parameters for LFQ and iTRAQ experiments.

|  |
| --- |
| Pre-column equilibration: |
| 1. | Volume (μl) | 10.00 μl for LFQ, 20.00 μl for iTRAQ |
| 2. | Flow (μl/min) | Unspecified |
| 3. | Max. pressure (Bar) | 750.00 |
| Analytical column equilibration: |
| 1. | Volume (μl) | 8.00 μl for LFQ, 3.00 μl for iTRAQ |
| 2. | Flow (μl/min) | Unspecified for LFQ, 0.30 μl for iTRAQ |
| 3. | Max. pressure (Bar) | 750.00 for LFQ, unspecified for iTRAQ |
| Autosampler wash: |
| 1. | Flush volume (μl) | 100 |

1. **OT-HCD-OT MS/MS Method:** MS parameters used for the experiments are mentioned below:

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| --- |
| Global Settings: |
| 1. | Method Duration (min) | 120 min for LFQ and 90 min for iTRAQ experiment |
| 2. | Application Mode | Peptide  |
| 3. | Default Charge State | 1  |
| 4. | Internal mass calibration | 445.12003 m/z |
| 5. | Experiment  | 1  |
| 6. | Experiment Name | MS  |
| 7. | Start Time (min) | 0  |
| 8. | End Time (min) | 120  |
| 9. | Cycle Time (sec) | 3  |

|  |
| --- |
| Scan Master Scan: |
| 1. | MSn Level | 1  |
| 2. | Use Wide Quad Isolation | True  |
| 3. | Detector Type | Orbitrap |
| 4. | Orbitrap Resolution | 60K |
| 5. | Mass Range | Normal |
| 6. | Scan Range (m/z) | 375-1700 |
| 7. | Maximum Injection Time (ms) | 50 |
| 8. | AGC Target | 400000 |
| 9. | Microscans | 1 |
| 10. | RF Lens (%) | 60 |
| 11. | Use ETD Internal Calibration | False |
| 12. | Data Type | Profile |
| 13. | Polarity | Positive |
| 14. | Source Fragmentation | False |
| 15. | Scan Description | Filter MIPS |
| 16. | MIPS Mode | Peptide |

|  |
| --- |
| Filter Charge State: |
| 1. | Include charge state (s) | 2-6 |
| 2. | Include undetermined charge states | False |
| 3. | Include charge states 25 and higher | False |
| Filter Dynamic Exclusion: |
| 1. | Exclude after n times | 1 |
| 2. | Exclusion duration (s) | 40 |
| 3. | Mass Tolerance | ppm |
| 4. | Mass tolerance low | 10 |
| 5. | Mass tolerance high | 10 |
| 6. | Exclude isotopes | True |
| 7. | Perform dependent scan on single charge state per precursor only | False |
| Filter Intensity Threshold: |
| 1. | Maximum Intensity | 1E+20 |
| 2. | Minimum Intensity | 5000 for LFQ and 20000 for iTRAQ experiment |
| 3. | Relative Intensity Threshold | 0 |
| 4. | Intensity Filter Type | Intensity Threshold |
| Data Dependent Properties: |
| 1. | Data Dependent Mode | Cycle Time |
| 2. | Scan Event | 1 |

|  |
| --- |
| Scan ddMSn Scan: |
| 1. | MSn Level | 2 |
| 2. | Isolation Mode | Quadrupole |
| 3. | Isolation Offset | Off |
| 4. | Isolation Window | 2 for LFQ and 1.2 for iTRAQ experiment |
| 5. | Reported Mass | Original Mass |
| 6. | Multi-notch Isolation | False |
| 7. | Scan Range Mode | Auto Normal |
| 8. | First Mass | 100 |
| 9. | Scan Priority | 1 |
| 10. | Activation Type | HCD |
| 11. | Collision Energy Mode | Fixed |
| 12. | Collision Energy (%) | 30 % for LFQ and 35% for iTRAQ experiment |
| 13. | Detector Type | Orbitrap |
| 14. | Orbitrap Resolution | 15K |
| 15. | Maximum Injection Time (ms) | 30 |
| 16. | AGC Target | 10000 |
| 17. | Inject ions for available parallelizable time | True |
| 18. | Microscans | 1 |
| 19. | Use ETD Internal Calibration | False |
| 20. | Data Type | Centroid |
| 21. | Polarity | Positive |
| 22. | Source Fragmentation | False |

**Supplementary file 3**

**Experimental plan for iTRAQ experiment:** Following plan was followed while performing iTRAQ experiment.

**Figure 1.** Tissue sample labeling plan for iTRAQ experiment



**Table 1.** Strategy of iTRAQ labeling

|  |  |
| --- | --- |
|  | **iTRAQ Reagents** |
| **Reaction** | **114** | **115** | **116** | **117** |
| **I** | Tissue 1 | Tissue 2 | Tissue 3 | Tissue 4 |
| **II** | Tissue 2 | Tissue 3 | Tissue 4 | Tissue 1 |
| **III** | Tissue 3 | Tissue 4 | Tissue 1 | Tissue 2 |
| **IV** | Tissue 4 | Tissue 1 | Tissue 2 | Tissue 3 |

**Supplementary file 4**

**Parameters for data analysis**

**A. Processing workflow:**

**Spectrum Files RC.**

1. Search Settings:

Protein Database: Human\_Proteome\_22082019.fasta

Enzyme Name: Trypsin (Full)

Precursor Mass Tolerance: 20 ppm

Fragment Mass Tolerance: 0.5 Da

Static Modification: Carbamidomethyl / +57.021 Da (C)

**Mascot**

1. Input Data:

Instrument: Default

Protein Database: Human\_22082019

Enzyme Name: Trypsin

Maximum Missed Cleavage Sites: 2

Taxonomy: All entries

2. Tolerances:

Fragment Mass Tolerance: 0.05 Da

Precursor Mass Tolerance: 10 ppm

Use Average Precursor Mass: False

4. Dynamic Modifications:

Show All Modifications: False

1. Dynamic Modification: Oxidation (M)

2. Dynamic Modification: Phospho (ST.)

3. Dynamic Modification: Phospho (Y)

4. Dynamic Modification: Acetyl (Protein N-term)

5. Static Modifications:

Static Modification: Carbamidomethyl (C)

Percolator

1. Input Data:

Maximum Delta Cn: 0.05

Maximum Rank: 0

2. Decoy Database Search:

Target FDR (Strict): 0.01

Target FDR (Relaxed): 0.05

Validation based on: q-Value

**Sequest HT**

1. Input Data:

Protein Database: Human\_Proteome\_22082019.fasta

Enzyme Name: Trypsin (Full)

Max. Missed Cleavage Sites: 2

Min. Peptide Length: 6 for LFQ and 7 for iTRAQ

Max. Peptide Length: 144

Max. Number of Peptides Reported: 10

2. Tolerances:

Precursor Mass Tolerance: 10 ppm

Fragment Mass Tolerance: 0.05 Da

Use Average Precursor Mass: False

Use Average Fragment Mass: False

3. Dynamic Modifications:

Max. Equal Modifications Per Peptide: 3

Max. Dynamic Modifications Per Peptide: 4

i. Dynamic Modification: Oxidation / +15.995 Da (M)

ii. Dynamic Modification: Phospho / +79.966 Da (S, T, Y)

iii. Dynamic Modification (only for iTRAQ study): iTRAQ 4-plex / +144.102 Da (K)

4. Dynamic Modifications (protein terminus):

N-Terminal Modification: Acetyl / +42.011 Da (N-Terminus)

5. Dynamic Modifications (peptide terminus):

N-Terminal Modification (only for iTRAQ study): iTRAQ 4-plex / +144.102 Da (N-307Terminus)

6. Static Modifications:

Static Modification: Carbamidomethyl / +57.021 Da (C)

**Minora Feature Detector**

1. Peak & Feature Detection:

Min. Trace Length: 513

Min. # Isotopes: 2 Peaks

Max. ΔRT of Isotope Pattern Multiplets [min]: 0.2

2. Feature to ID. Linking:

PSM Confidence At Least: High

**B. Consensus workflow:**

**MSF Files**

1. Storage Settings:

Spectra to Store: Identified or Quantified

Feature Traces to Store: All

2. Merging of Identified Peptide and Proteins:

Merge Mode: Globally by Search Engine Type

File Limit for Automatic Merge.: 10

3. FASTA Title Line Display:

Reported FASTA Title Lines: Best match

Title Line Rule: standard

4. PSM Filters:

Maximum Delta Cn: 0.05

Maximum Rank: 0

Maximum Delta Mass: 0 ppm

**PSM Grouper**

1. Peptide Group Modifications: Site Probability Threshold: 75

**Peptide Validator**

1. General Validation Settings:

Validation Mode: Only PSM level FDR Calculation based on score

Target FDR (Strict) for PSMs: 0.01

Target FDR (Relaxed) for PSMs: 0.05

Target FDR (Strict) for Peptides: 0.01

Target FDR (Relaxed) for Peptides: 0.0514

2. Specific Validator Settings:

Validation Based on: q-Value

Use Concatenated FDR Calculation for PSM Level FDR Calculation Based on Score:

True

Reset Confidences for Nodes without Decoy Search (Fixed score thresholds): False

**Processing node 3: Peptide and Protein Filter**

1. Peptide Filters:

Peptide Confidence At Least: High

Keep Lower Confident PSMs: False

Minimum Peptide Length: 6

Remove Peptides Without Protein Reference: False

2. Protein Filters:

Minimum Number of Peptide Sequences: 1

Count Only Rank 1 Peptides: False

Count Peptides Only for Top Scored Protein: False

**Protein FDR Validator**

1. Confidence Thresholds:

Target FDR (Strict): 0.01

Target FDR (Relaxed): 0.05

**Protein Marker**

1. Contaminant Database:

Protein Database: contaminants\_26042018.fasta

2. Annotate Species:

As Species Map: False

As Species Names: False

**Feature Mapper**

1. Chromatographic Alignment:

Perform RT Alignment: True

Maximum RT Shift [min]: 5

Mass Tolerance: 10 ppm

Parameter Tuning: Coarse

2. Feature Linking & Mapping:

RT Tolerance [min]: 0

Mass Tolerance: 0 ppm

Min. S/N Threshold: 5

**Precursor Ions Quantifier**

1. General Quantification Settings:

Peptides to Use: Unique + Razor

Consider Protein Groups for Peptide Uniqueness: True

Reject Quan Results with Missing Channels: False

2. Precursor Quantification

Precursor Abundance Based On: Area

Min. # Replicate Features [%]: 0

3. Normalization and Scaling:

Normalization Mode: Total Peptide Amount

Scaling Mode: On All Average

**Display Settings**

1. General:

Filter Set: Filter Set Master Protein Filter

Row Filter for Target Protein: Master is equal to Master

Layout Definition: (not specified)