Troubleshooting Failed Experiment

IDeA National Resource for Proteomics
2020 Workshop for Faculty and Students
Outline

- Experimental Design
- General rules for sample processing
- Troubleshooting for sample preparation
- Troubleshooting for Instrumentation
- Troubleshooting for data analysis
Experimental Design (Before cell culturing)

- Well communication between users and core staffs
  - Clear background and goal from users
  - Different approaches, cost and availability in the core
  - Ask questions and talk about your concerns at the beginning
  - Biostatistician and Bioinformatician consultants
  - Biological replicates
  - Available sequence database
  - Use iLab

- Common issues
  - Statistical power: too small sample size for a too big goal
  - Potency vs physiological dose (preliminary experiment)
  - Too many variables (control in the experiment or statistical analysis)
General rules for sample processing

- Technical consistancy
- Start with higher amount of protein (100 ug)
- Preserve back up sample in each step
- Avoid time delay across samples
  - Randomized or reversed sequence
  - Manifold and repeater
- Well preserved and fresh prepared reagents
  - DTT, IAA, Urea, AmBic..
  - TMT label reagent
- Document everything
Sample preparation

- Reliance on the core:
  - Protein quantification (how many ways can you do?)
  - Buffer blank
  - SDS PAGE and gel cutting

- Incompatible lysis buffer:
  - Report the correct buffer formula (SDS, detergent, salt)
  - Amine containing buffer

- Protein preservation:
  - On ice at all time and dry ice with shipping
  - Protease inhibitors and phosphatase inhibitors
  - Don’t dry samples at protein level
Common issues in sample prep

- **Wrong Buffer!**
  - ✓ We can’t handle detergen or higher % of SDS (4%)
  - ✓ Solution 1: Buffer exchange (dialysis, diafiltration..)
  - ✓ Solution 2: Use ideal lysis buffer provided by the core

- **Wrong total protein quantification**
  - ✓ No buffer blank
  - ✓ Limit of Detection

- **Poor SDS gel or cutting**
Recommendations for Sample Preparation for Gel Band-based

- Use commercially available 1.0 mm SDS-PAGE and skip lanes between samples
- Commassie Staining instead of silver staining
- Stain the gel in a “clean” unautoclaved container (don’t use Western blot container) and destain the gel with milliQ water
- Wear gloves the entire time of handling the samples
- Take a digital image of the gel indicating the band(s) to cut. Provide a print copy with the gel submission and a digital file to the director via email
- Make an appointment to bring the gel over in a covered container with milliQ H2O. For long distance shipping, gels can be sent in well-sealed plastic foils with ice pack
- If the core users want to run the gel, have them provide protocols
- If the core user want to cut the gel band, have them follow our protocol and store the gel piece(s) in Eppendorf tube(s) at 4°C

Modified from HICCC Proteomics Shared Resource, Columbia University Medical Center
Sample preparation issue: Cell or tissue lysis and protein solubility

- Pellets can’t be completely lysed and resolubilized in 2% SDS lysis buffer
  - Solution 1: Use 4% SDS with 95°C incubation
  - Solution 2: By pass protein quant and Normalize sample at peptide level (e.g. human serum). Use cell count instead of BCA. Therefore, add 8M urea and TCEP directly and perform FASP digestion.
  - Solution 3: Only use supernatant and have other extraction methods for the precipitate.

- Particularly for membrane protein and fat containing tissues
  - Solution: Use separate protocol to extract proteins (e.g. liquid liquid extraction or acetone precipitation)
Sample preparation issue: FASP protocol

- Centrifugation seems like taking forever
  - Solution 1: Make sure no precipitate before adding urea
  - Solution 2: Start with lower amount (25 µg for TMT)
  - Solution 3: Extra procedure for fat-containing samples

- FASP filter O-ring comes off
  - Solution: Quickly check after every spinning. Snap it back to the unit. Reload the filtrate to the filter
The impact of the FASP O-ring in a TMT study
Sample preparation issue: TMT Labeling

- How to troubleshoot a long FASP-TMT study?
  - Solution 1: 3 QCs (protein BCA, peptide assay and ratio check)
Filter Aided Sample Preparation (FASP) and TMT Labeling

Sample protein amount (2 mg):
- Cell Pellet: 50 μl (15 cm dish)
- Tissue: 25 mg minced; Freeze/thaw; Pestle; Cryo Bead Homogenizer
- Serum: Abundant Protein Depletion
- Protease /phosphatase inhibitor

Lysate Preparation:
- 100 mM DTT
- 2% SDS
- Heat (95°C) 3 min

DNA Shredding:
- Sonication
- QIAshredder

Protein Quant (100 μg)
- Bradford or BCA
- Qubit™ 3.0 NGS

FASP processing: 30K filter unit
- Denaturation: 8M Urea
- Alkylation: 0.05M IAA
- Washing: Urea and AMBIC
- Overnight Digestion: Trypsin
- Stop reagent: Formic acid

Digestion clean up:
- Sep-Pak Vac C18 (50 mg)
- High throughput: manifold

Peptide Quant

TMT Labeling

Clean up

Fusion Lumos

Combine Samples

Ratio Check (MS3)
Sample preparation issue: TMT Labeling

➢ How to troubleshoot a long FASP-TMT study?
  ✓ Solution 1: 3 QCs (protein BCA, peptide assay and ratio check)
  ✓ Solution 2: Make room for failure
Make room for failure

300 µg proteins in homogenate

Use 100 µg of proteins for FASP digestion

50 µg of peptides

Use 25 µg of peptides for 10plex TMT labeling

Combine 12.5 µg of labeled peptides from each channel (125 µg total)

Use 60 µg of mixture and make 12 super fractions

5 µg per fraction

Prepare 5 µg for the UHPLC/MS/MS

Inject 1 µg
Sample preparation issue: TMT Labeling

- How to troubleshoot a long FASP-TMT study?
  - Solution 1: 3 QCs (protein BCA, peptide assay and ratio check)
  - Solution 2: Make room for failure

- Do we need that much labeling material? No
  - Solution 1: Coordinate different studies at the dry peptide level and label all studies with one kit at the same time
  - Solution 2: Preserve small aliquot?
  - Solution 3: Test other conditions or QC
TMT labeling efficiency

1st Biological replicate

2nd Biological replicate

3rd Biological replicate (TMT$^5$ and TMT$^{10}$)
Common instrument issue and troubleshooting

- Time to change column
- Time to tune/calibrate LC and MS instruments
- When introduce new LC column or LC-MS/MS method
  - Analysis of a known sample to assess system performance (BSA digest)
  - System suitability
    - MS response, sensitivity
    - Mass accuracy
    - Precision
    - Retention time
    - Peak shape, FWHM
    - Chromatographic resolution
- Sequence of injection for large study
  - Blank injection in between samples (not superfractions)
  - Add pooled samples
  - Block by biological replicates
  - Randomize the sequence
Data collection and analysis

QC your data before interpret the result

- Principal component analysis: outlier and data clustering
- Venn diagram: missing value
- Heatmap and volcano plot: the multiple correction cut-off and numbers of differentially expressed proteins
- Think about any confounder in your samples or procedures
  - Solutions: stratification, multivariate models, logistic regression, linear regression, analysis of covariance

Back up your data: we can’t save your data forever!