**Preparing Whole Cell Protein Extracts**

**for Differential Protein Expression Analysis in the Proteomics and Metabolomics Core (PMC)**

**David Kakhniashvili, Ph.D., Director**

**Before you start your project!!!**

* All users must contact Dr. Kakhniashvili and discuss specific project details before submitting samples to the PMC.
* Read the Sample Preparation Basics SOP for the PMC.

**Important Information for Sample Preparation**

***Differential Protein Expression Analysis*** determines the relative abundances of identical proteins (the molar ratios) in two or more samples representing different conditions (groups) - e.g. control vs patient, wild type vs mutant, treated vs untreated, individual time points, etc. An optimal number of biological and/or technical replicates must be analyzed per condition (group) for statistical validation of results. **The investigator is expected to define the study conditions (groups) and to then determine (in collaboration with a statistician) an optimal/required number of replicates per condition. Please consult with Dr. Daniel Johnson in the Molecular Bioinformatics (mBIO) core (x8-3743) if you are unsure about statistical requirements for an experiment.**

***Cell Lysis and Protein Extraction***

The main objective of this procedure is:

a. to efficiently lyse cells and extract proteins

 b. to preserve proteins from degradation and other uncontrolled modifications

There is no absolute single “best way” to lyse cells and extract proteins. A variety of homemade (published) and commercial buffers have been optimized for different cell (or sample) types. Conditions optimal for a specific sample should be selected.

***Quality and Amount of Protein Extract Required***

Processing/preparation of protein extracts for LC/MS analysis include trypsin digestion and labeling of the generated peptides with either iTRAQ or TMT reagents. Before trypsin digestion, protein extracts must be essentially free of a) protease inhibitors, denaturing agents, detergents, etc. that inhibit trypsin digestion, b) compounds with primary amino groups and free thiols competing with peptides in labeling reaction, and c) protein stabilizers – glycerol, PEG, which severely interfere with MS analysis. Protein extracts can be separated from these low MW components by filtration using centrifugal filter devices of a low MWCO (e.g. FASP columns) or by acetone precipitation. Purified protein extracts are then dissolved and trypsin digested in an appropriate buffer. The required amount of digested protein in submitted samples is 25-100 µg per sample (per replicate).

***Protein Extracts Containing Extremely Abundant Proteins***

Analysis of medium and low abundant proteins is extremely difficult (or impossible) in the presence of highly abundant proteins (e.g. hemoglobin in red blood cells, albumin in blood plasma). Selective depletion of abundant proteins from protein extracts (to at least average abundance level) is required to facilitate analysis of less abundant proteins of interest.

**Preparing Whole Cell Protein Extracts – Basic Protocol**

**Introduction**

The Thermo Scientific™ Pierce™ Mass Spec Sample Prep Kit for Cultured Cells enables reproducible processing of cultured mammalian cells for proteomic mass spectrometry (MS) analysis. The kit contains all of the necessary buffers, reagents, MS-grade enzymes; an optimized protocol generates MS-compatible peptide samples from whole-cell lysates. Sample preparation can be performed in 2 alternative ways using

• **Acetone precipitation** (refer to **appendix A**)

or

• **FASP processing** (refer to **appendix B**)

**Appendix A**

**Preparing Whole Cell Protein Extracts via Acetone Precipitation**

**Materials Required**

1. **Pierce™ Mass Spec Sample Prep Kit for Cultured Cells, P/N 84840**

**Kit Contents** (sufficient for processing 20 samples of 100μg of cell lysate protein):

• Cell Lysis Buffer, 5ml

• Digestion Buffer, 5ml

• No-Weigh™ DTT, 24 microtubes, each containing 7.7mg of dithiothreitol (DTT)

• Iodoacetamide, Single-Use, 24 microtubes, each containing 9.3mg of iodoacetamide (IAA)

• Trypsin Storage Solution, 250μl

• Pierce Digestion Indicator, 10μg

• Lys-C Protease, MS Grade, 20μg

• Pierce™ Trypsin Protease, MS Grade**,** 2 × 20μg

**Storage:** Upon receipt, remove Insert A (containing Pierce Digestion Indicator, Lys-C Protease and Pierce Trypsin Protease, MS Grade) and store at -20°C. Store the remaining components at 4°C. Product is shipped on dry ice.

**Additional Materials Required**

• Microcentrifuge polypropylene tubes

• Microtip probe sonicator or nuclease (e.g., Thermo Scientific™ Pierce™ Universal Nuclease

 for Cell Lysis, P/N. 88700)

• Protein assay kit (e.g., Thermo Scientific™ BCA Protein Assay Kit, P/N 23227)

• Pierce Quantitative Colorimetric peptide Assay (P/N 23275)

• Heating block

• Chilled (-20°C) 100% acetone and 90% acetone

• Trifluoroacetic acid (TFA)

• Phosphate-buffered saline (PBS)

• Vacuum concentrator (e.g., Thermo Scientific™ SpeedVac™ Vacuum Concentrator)

**Procedure for Preparation of Peptides from Cultured Cells**

**A. Material Preparation**

1. Pre-chilled 90% acetone: Prepare 90% acetone in ultrapure water (e.g mix 45mL of

 100% acetone with 5mL of ultrapure water) and store at -20°C.

2. Pre-chilled 100% acetone: Store 100% acetone at -20°C.

3. Warm the Cell Lysis Buffer and Digestion Buffers provided with Pierce kit to room

 temperature before use. Store buffers at 4°C.

**B. Cell Lysis**

1. Culture cells to harvest at least 100μg of protein. For best results, culture a minimum of 2 ×

 106 cells.

**Note:** Rinse cell pellets 3 times with 1X PBS to remove cell culture media. Pellet cells using low-speed centrifugation (i.e., < 1000 × *g*) to prevent premature cell lysis.

2. Lyse the cells by adding five cell-pellet volumes of Cell Lysis Buffer (i.e., 100μl of Cell

 Lysis Buffer for a 20μl cell pellet). Pipette sample up and down to break up the cell clumps

 and gently vortex sample to mix.

3. Incubate the lysate at 95°C for 5 minutes.

4. Cool the lysate on ice for 5 minutes, spin down.

5. Sonicate lysate on ice using a microtip probe sonicator to reduce the sample viscosity by

 shearing DNA. Alternatively, use Pierce Universal Nuclease for Cell Lysis (P/N 88700) to

 enzymatically digest DNA and RNA. If using nuclease, add 25 units of nuclease per 1ml of

 cell lysate and incubate at room temperature for 15 minutes.

6. Centrifuge lysate at 16,000 × *g* for 10 minutes at 4°C.

7. Carefully separate the supernatant and transfer into a new tube.

8. Determine the protein concentration of the supernatant using established methods such as

 the BCA Protein Assay Kit (e.g., Thermo Scientific™ BCA Protein Assay Kit, P/N 23227).

**C. Reduction, Alkylation and Acetone Precipitation**

**Note:** This procedure is optimized for 100μg of cell lysate protein at 1mg/mL concentration; however, the procedure may be used for 50-100μg of cell lysate protein with an appropriate (proportional) amount of reagents (DTT, IAA, Pierce Digestion Indicator, Lys-C and trypsin).

1. Warm and equilibrate the Pierce Digestion Indicator to room temperature.

2. Add 100μg of lysate protein to a polypropylene microcentrifuge tube and adjust the sample

 volume to 100μL using Cell Lysis Buffer to a final concentration of 1mg/ml.

3. Add 0.5μg (0.5% w/w) of Pierce Digestion Indicator to the sample (i.e. 0.005μg of Pierce

 Digestion Indicator per μg of sample protein).

**Note:** The actual concentration is printed on the bottle label. Refer to the label to determine the required volume.

4. Immediately before use, puncture the foil covering of the Thermo Scientific™ No-Weigh™

 DTT tube with an empty pipette tip. Add 100μl of ultrapure water to the tube and gently

 pipette up and down to dissolve the contents of the tube. The final concentration of DTT is

 ~500mM.

**Note:** To preserve DTT stability between uses, return unused micro-tubes to the pouch containing the desiccant pack.

 5. Add 2.1μl of 500mM DTT solution to the sample (final DTT concentration is ~10mM). Mix

 and incubate at 50°C for 45 minutes. Discard any unused DTT solution.

 6. Cool the sample to room temperature for 10 minutes, spin down.

 7. Immediately before use, puncture the foil covering of the Single-Use Iodoacetamide tube

 with an empty pipette tip. Add 100μl of Cell Lysis Buffer to the tube and gently pipette up

 and down to dissolve the contents of the tube. The final concentration of IAA is ~500mM.

 Protect solution from light.

 8. Add 11.5μl of 500mM IAA solution to the sample (final IAA concentration is ~50mM). Mix

 and incubate at room temperature for 20 minutes protected from light. Discard any

 unused IAA solution.

 9. After alkylation with IAA, immediately add 690μl (6 volumes) of pre-chilled (-20°C) 100%

 acetone to sample. Vortex tube and incubate at -20°C for four hour to overnight to

 precipitate proteins.

10. Centrifuge at 16,000 × *g* for 10 minutes at 4°C. Carefully remove acetone without

 dislodging the protein pellet.

11. Add 50μl of pre-chilled (-20°C) 90% acetone, vortex to mix and centrifuge at 16,000 × *g*

for 5 minutes at 4°C.

12. Carefully remove acetone without dislodging the protein pellet. Allow the pellet to dry for

 2-3 minutes and immediately proceed to Section D. Enzymatic Protein Digestion.

**Note:** Do not dry the acetone-precipitated protein pellet for more than 2-3 minutes; excess drying will make the pellet difficult to re-suspend in the Digestion Buffer.

**D. Enzymatic Protein Digestion**

1. Add 100μl of Digestion Buffer to the acetone-precipitated protein pellet (final protein

 concentration is 1mg/ml). and resuspend by gentle pipetting up and down to break the

 pellet**. It is important to dissolve as much protein as possible**; water bath sonication

 may facilitate the process.

**Note:** An acetone-precipitated protein pellet may not completely dissolve; however, after proteolysis at 37°C, all the protein will be solubilized.

2. Immediately before use, add 40μL of **ultrapure water** to the bottom of the vial containing

 20μg Lys-C and incubate at room temperature for 5 minutes. Gently pipette up and down

 to dissolve. Store any remaining Lys-C solution in single-use volumes at -80°C.

3. Add 2μl of Lys-C (1μg, enzyme-to-substrate ratio = 1:100) to the sample. Mix and

 incubate at 37°C for 2 hours.

4. Immediately before use, add 40μl of **Trypsin Storage Solution** to the bottom of the vial

 Containing 20μg trypsin and incubate at room temperature for 5 minutes. Gently pipette up

 and down to dissolve. Store any remaining trypsin solution in single-use volumes at -80°C

 for long-term storage.

5. Add 4μl of trypsin (2μg, enzyme-to-substrate ratio = 1:50) to the sample. Mix and incubate

 overnight at 37°C.

6. Acidify the sample with TFA (to 0.1%) to stop digestion, spin down.

7. Speed vac the sample (106μl) for at least 2 hr. to remove the (volatile) Digestion Buffer.

8. Resuspend the sample containing 100μg of digested proteins in 100μl of 10% acetonitrile.

9. Determine the peptide concentration in the samples using Pierce Quantitative Colorimetric

 Peptide Assay (P/N 23275) according to the manufacturer’s protocol.

10. Transfer at least 25μg of the digested protein sample into a new tube. Transfer equal

 amount of each sample into corresponding new tubes; **record the transferred amount**.

11. Speed vac the samples to dryness. The samples are ready to be submitted to the facility

 for further processing.

**Appendix B**

**Preparing Whole Cell Protein Extracts via FASP Processing**

**Materials Required**

1. **Pierce™ Mass Spec Sample Prep Kit for Cultured Cells, P/N 84840**

**Kit Contents** (sufficient for processing 20 samples of 100μg of cell lysate protein):

• Cell Lysis Buffer, 5ml

• Digestion Buffer, 5ml

• No-Weigh™ DTT, 24 micro-tubes, each containing 7.7mg of dithiothreitol (DTT)

• Iodoacetamide, Single-Use, 24 microtubes, each containing 9.3mg of iodoacetamide (IAA)

• Trypsin Storage Solution, 250μl

• Pierce Digestion Indicator, 10μg

• Lys-C Protease, MS Grade, 20μg

• Pierce™ Trypsin Protease, MS Grade**,** 2 × 20μg

**Storage:** Upon receipt, remove Insert A (containing Pierce Digestion Indicator, Lys-C Protease and Pierce Trypsin Protease, MS Grade) and store at -20°C. Store the remaining components at 4°C. Product is shipped on dry ice.

1. **FASP Protein Digestion Kit, Expedeon P/N 44250, Thermo Fisher P/N EX44250**

**Kit Contents** (sufficient for processing 8 samples):

• Spin Columns, 8

• Collection Tubes, 16

• Tris-HCl solution,100mM, pH 8.5, 20ml

• Urea, single-use, 8 micro-tubes, each containing 0.75g of urea

• NaCl solution, 500mM, 20ml

• Iodoacetamide (IAA), single-use, 8 micro-tubes - **Do not use for this protocol**

• Ammonium Bicarbonate Solution, 50mM, 20ml – **Do not use for this protocol**

**Additional Materials Required**

• Microtip probe sonicator or nuclease (e.g., Thermo Scientific™ Pierce™ Universal Nuclease

 for Cell Lysis, P/N. 88700)

• Protein assay kit (e.g., Thermo Scientific™ BCA Protein Assay Kit, P/N 23227)

• Pierce Quantitative Colorimetric peptide Assay (P/N 23275)

• Micro-centrifuge polypropylene tubes

• Heating block

• Vortex

• Trifluoroacetic acid (TFA)

• Phosphate-buffered saline (PBS)

 Triethyl-ammonium bicarbonate (TEAB) solution, 1M (Sigma, P/N T7408-100ml)

• Vacuum concentrator (e.g., Thermo Scientific™ SpeedVac™ Vacuum Concentrator)

**Procedure for Preparation of Peptides from Cultured Cells**

**A. Material Preparation**

1. Warm the Cell Lysis Buffer and Digestion Buffer provided with Pierce kit to room

 temperature before use. Store buffers at 4°C.

2. Urea Sample Solution: Add 1 mL Tris Hydrochloride Solution provided with the FASP Kit to

 one tube of Urea, also provided with the FASP Kit. Vortex the tube until all the powder

 dissolves.

3. TEAB Solution, 50mM: e.g. add 1ml of 1M TEAB to 19ml of ultrapure water, mix.

**B. Cell Lysis**

1. Culture cells to harvest at least 100μg of protein. For best results, culture a minimum of 2 ×

 106 cells.

**Note:** Rinse cell pellets 3 times with 1X PBS to remove cell culture media. Pellet cells using low-speed centrifugation (i.e., < 1000 × *g*) to prevent premature cell lysis.

2. Lyse the cells by adding five cell-pellet volumes of Cell Lysis Buffer (i.e., 100μl of Cell

 Lysis Buffer for a 20μl cell pellet). Pipette sample up and down to break up the cell clumps

 and gently vortex sample to mix.

3. Incubate the lysate at 95°C for 5 minutes.

4. Cool the lysate on ice for 5 minutes, spin down..

5. Sonicate lysate on ice using a microtip probe sonicator to reduce the sample viscosity by

 shearing DNA. Alternatively, use Pierce Universal Nuclease for Cell Lysis (P/N 88700) to

 enzymatically digest DNA and RNA. If using nuclease, add 25 units of nuclease per 1ml of

 cell lysate and incubate at room temperature for 15 minutes.

6. Centrifuge lysate at 16,000 × *g* for 10 minutes at 4°C.

7. Carefully separate the supernatant and transfer into a new tube.

8. Determine the protein concentration of the supernatant using established methods such as

 the BCA Protein Assay Kit (e.g., Thermo Scientific™ BCA Protein Assay Kit, P/N 23227)

**C. Reduction and Alkylation**

**Note:** This procedure is optimized for 100μg of cell lysate protein at 1mg/mL concentration; however, the procedure may be used for 50-100μg of cell lysate protein with an appropriate (proportional) amount of reagents (DTT, IAA, Pierce Digestion Indicator, Lys-C and trypsin).

1. Warm and equilibrate the Pierce Digestion Indicator to room temperature.

2. Add 100μg of lysate protein to a polypropylene microcentrifuge tube and adjust the sample

 volume to 100μL using Cell Lysis Buffer to a final concentration of 1mg/ml.

3. Add 0.5μg (0.5% w/w) of Pierce Digestion Indicator to the sample (i.e. 0.005μg of Pierce

 Digestion Indicator per μg of sample protein).

**Note:** The actual concentration is printed on the bottle label. Refer to the label to determine the required volume.

4. Immediately before use, puncture the foil covering of the Thermo Scientific™ No-Weigh™

 DTT tube with an empty pipette tip. Add 100μl of ultrapure water to the tube and gently

 pipette up and down to dissolve the contents of the tube. The final concentration of DTT is

 ~500mM.

**Note:** To preserve DTT stability between uses, return unused micro-tubes to the pouch containing the desiccant pack.

 5. Add 2.1μl of 500mM DTT solution to the sample (final DTT concentration is ~10mM). Mix

 and incubate at 50°C for 45 minutes. Discard any unused DTT solution.

 6. Cool the sample to room temperature for 10 minutes, spin down.

 7. Immediately before use, puncture the foil covering of the Single-Use Iodoacetamide tube

 with an empty pipette tip. Add 100μl of Cell Lysis Buffer to the tube and gently pipette up

 and down to dissolve the contents of the tube. The final concentration of IAA is ~500mM.

 Protect solution from light.

 8. Add 11.5μl of 500mM IAA solution to the sample (final IAA concentration is ~50mM). Mix

 and incubate at room temperature for 20 minutes protected from light. Discard any

 unused IAA solution.

 9. After alkylation with IAA, immediately add 100μl of Urea Sample Solution and proceed to

 Section D, FASP Protein digestion

**D. FASP Protein Digestion**

1. Add 200μl of Urea Sample Solution to a Spin Filter and centrifuge at 14,000 x *g* for 5 min.

**Note:** The centrifugation times may need adjustment – keep it short but long enough to let all solvent flow through the filter to the collection tube.

2. Transfer the alkylated protein sample (step C9) into the Spin Filter. Centrifuge at

 14,000 x *g* for 15 min. Discard the flow-through from the collection tube

3. Add 200μL of Urea Sample Solution to the Spin Filter, cap the filter, vortex and centrifuge

 at 14,000 x *g* for 12 min. Discard the flow-through from the collection tube. Repeat this

 step once.

4. Add 100μl of 50 mM TEAB Solution to the Spin Filter, cap the filter, vortex and centrifuge

 at 14,000 x *g* for 10 min. Discard the flow-through from the collection tube. Repeat this

 step twice.

5. Add 100μl of Digestion Buffer provided with Pierce kit

6. Immediately before use, add 40μL of **ultrapure water** to the bottom of the vial containing

 20μg Lys-C and incubate at room temperature for 5 minutes. Gently pipette up and down

 to dissolve. Store any remaining Lys-C solution in single-use volumes at -80°C.

7. Add 2μl of Lys-C (1μg, enzyme-to-substrate ratio = 1:100) to the sample, cap the filter,

 **vortex 1 min**, and incubate at 37°C for 2 hours.

8. Immediately before use, add 40μl of **Trypsin Storage Solution** to the bottom of the vial

 Containing 20μg trypsin and incubate at room temperature for 5 minutes. Gently pipette up

 and down to dissolve. Store any remaining trypsin solution in single-use volumes at -80°C.

9. Add 4μl of trypsin (2μg, enzyme-to-substrate ratio = 1:50) to the sample, cap the filter,

 vortex, and Incubate overnight at 37°C. Wrap the tops of the tubes with Parafilm to

 minimize the effects from evaporation.

10. Transfer the Spin Filter to **a new collection tube** and centrifuge at 14,000 x *g* for 10 min.

 **Do not discard the filtrate.**

11. Add 50μl of 50 mM TEAB Solution to the Spin Filter and centrifuge at 14,000 x *g* for 10

 min. **Do not discard the combined filtrate.**

12. Add 50μl 0.5 M Sodium Chloride Solution provided with the FASP Kit and centrifuge the

 Spin Filter at 14,000 x *g* for 10 min. **Save the combined (206μl) filtrate.**

11. Speed vac the sample (206μl, containing ~ 100μg of digested proteins) to ~20-50μl and

 desalt using C18 ZipTips (or equivalent) of appropriate capacity according to the

 manufacturer’s protocol.

11. Speed vac the desalted sample to dryness.

12. Resuspend the sample in 100μl of 10% acetonitrile.

13. Determine the peptide concentration in the samples using Pierce Quantitative

 Colorimetric Peptide Assay (P/N 23275) according to the manufacturer’s protocol.

14. Transfer at least 25μg of the digested protein sample into a new tube. Transfer equal

 amount of each sample into corresponding new tubes; **record the transferred amount**.

15. Speed vac the samples to dryness. The samples are ready to be submitted to the facility

 for further processing.