INSTRUCTIONS

Pierce™ Mass Spec Sample Prep Kit for Cultured Cells

84840 2522.1

Number	Description
84840	**Pierce Mass Spec Sample Prep Kit for Cultured Cells**, sufficient reagents for processing 20 samples of 100µg of cell lysate protein for MS analysis

Kit Contents:

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.

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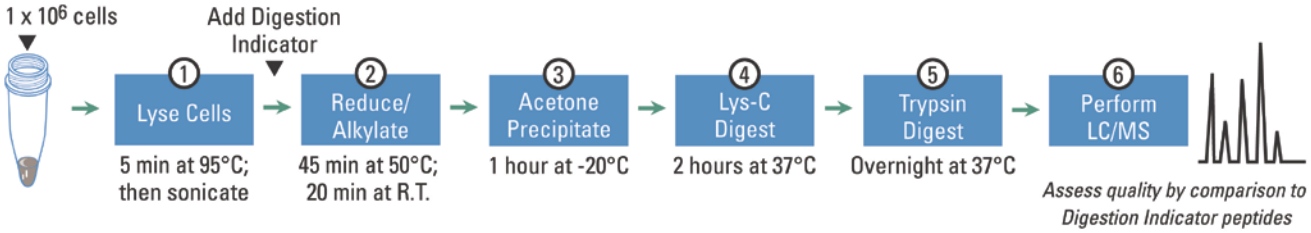
Introduction

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 and an optimized protocol to generate MS-compatible peptide samples from whole-cell lysates. Consistent sample preparation is vital, but difficult, for robust MS analysis. Although there are numerous MS sample preparation methods, most are highly variable and time consuming, which results in excessive peptide modification (e.g., oxidation, carbamylation, over-alkylation) and subsequent inefficient data analysis and interpretation. Enzymatic digestion reproducibility is another source of sample prep variability and is especially critical to monitor for quantitative MS-proteomic analysis. The kit uses a double-digestion protocol with Lys-C and trypsin to more completely proteolize complex protein samples and generates peptides with < 10% miscleavages. Additionally, the kit contains Thermo Scientific™ Pierce™ Digestion Indicator, which is used as a standard protein digestion indicator to assess the digestion reproducibility for multiple samples.

Procedure Summary



Important Product Information

- Warm the Cell Lysis Buffer and Digestion Buffers to room temperature before use. Store buffers at 4°C.
- Pierce Digestion Indicator contains 10µg of protein per vial in a suitable buffer. The actual concentration is printed on the bottle label. Digestion efficiency may be verified by determining the sequence coverage of the Pierce Digestion Indicator (see the Additional Information Section).

Additional Materials Required

- Microcentrifuge tubes
- Microtip probe sonicator or nuclease (e.g., Thermo Scientific™ Pierce™ Universal Nuclease for Cell Lysis, Product No. 88700)
- Heating block
- Chilled (-20°C) 100% acetone and 90% acetone
- Trifluoroacetic acid (TFA)
- Phosphate-buffered saline (PBS)
- Protein assay kit (e.g., Thermo Scientific™ BCA Protein Assay Kit, Product No. 23227)
- 75-300µm capillary C18 reversed-phase column
- Mass spectrometer with online or off-line nano-flow liquid chromatography (LC) system
- Vacuum concentrator (e.g., Thermo Scientific™ SpeedVac™ Vacuum Concentrator)
- Data analysis software (e.g., Thermo Scientific™ Proteome Discoverer™ Software)

Procedure for Preparation of Peptides from Cultured Cells for MS Analysis

A. Material Preparation

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

B. Cell Lysis

1. Culture cells to harvest at least 100µg of protein. For best results, culture a minimum of 1×10^6 cells.

   Note: Rinse cell pellets 2-3 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.
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 (Product No. 88700) to enzymatically digest DNA and RNA. If using nuclease, add 25 units of nuclease to 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 (Product No. 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 10-200µg of cell lysate protein with an appropriate amount of reagents (DTT, IAA, Lys-C and trypsin). When using 10µg of cell lysate, a protein concentration of 0.2-1mg/mL may be used.

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.

   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 microtubes to the pouch containing the desiccant pack.
5. Add 2.1µL of 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.
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 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 460µL (4 volumes) of pre-chilled (-20°C) 100% acetone to sample. Vortex tube and incubate at -20°C for one 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 and resuspend by gently pipetting up and down to break the pellet.

   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 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 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. Freeze samples at -80°C to stop digestion. (Optional: stop digestion by acidifying with TFA)
7. Speed vac sample to remove the Digestion Buffer.
8. Resuspend the sample in an appropriate buffer (e.g., 0.1% TFA) and subject for LC-MS analysis.

   Note: Proteolytic digests prepared using this protocol are directly compatible with LC-MS analysis. Clean-up of samples with C18 spin tips or columns (see Related Thermo Scientific Products) is optional.

Troubleshooting

Problem	Possible Cause	Solution
Low protein yield	Insufficient cells	Increase number of cells used for lysis
Difficult to re-suspend the pellet following acetone precipitation	Over-dried protein pellet	Air dry the acetone-precipitated pellet for 2-3 minutes
Incomplete digestion	Inactive enzyme	Store any remaining enzyme solution in single-use volumes at -80°C
Sequence coverage of the Pierce Digestion Indicator is < 50%	Working at the limit of detection of the MS instrument	Increase concentration of the Pierce Digestion Indicator to 1% (w/w)
Over-alkylation	Alkylation was allowed to proceed for too long	Alkylate the reduced protein protected from light for 20 minutes at room temperature and immediately remove the excess reagents by acetone precipitation
		Quench alkylation reactions by adding 10mM DTT (final concentration)

Additional Information

A. MS Data Analysis

Identify proteins by searching the LC-MS/MS data with a database-search software (e.g., Thermo Scientific™ Matrix Science Mascot™ or Proteome Discoverer™ Software) including fixed modification of cysteines (Average mass: 57.0513Da, Monoisotopic mass: 57.021464Da) and variable oxidation of methionines (Average mass: 15.9994Da, Monoisotopic mass: 15.994915Da). To assess digestion efficiency and instrument sensitivity, the protein database should include the Pierce Digestion Indicator protein sequence:

MESDESGLPAMEIECRITGTLNGVEFELVGGGEGTPEQGRMTNKMKSTKGALTFSPYLLSHVMGYGFYHFGTYPSGYENPFLHAI
NNGGYTNTRIEKYEDGGVLHVSFSYRYEAGRVIGDFKVMGTGFPEDSVIFTDKIIRSNATVEHLHPMGDNDLDGSFTRTFSLRDG
GYYSSVVDSHMHFKSAIHPSILQNGGPMFAFRRVEEDHSNTELGIVEYQHAFKTPDADAGEE

The five digestion indicator peptides most commonly detected are underlined above. The Pierce Digestion Indicator peptides are quantified manually with extracted ion chromatograms of the raw LC-MS/MS data or automatically with Thermo Scientific™ Pinpoint™ Software (Table 1).

Table 1. Properties of the five Thermo Scientific Pierce Digestion Indicator peptide sequences.

Digestion Indicator Peptide Sequence	Observed Mass/Charge	Observed Charge	Hydrophobicity Factor
ITGTLNGVEFELVGGGEGTPEQGR	1209.1007	+2	40.59
VMGTGFPEDSVIFTDK	871.9189	+2	40.24
DGGYYSSVVDSHMHFK	610.2701	+3	27.24
SAIHPSILQNGGPMFAFR	648.3367	+3	42.42
VEEDHSNTELGIVEYQHAFK	587.0315	+4	35.13

To assess LC-MS/MS instrument performance and to assist with the quantitative analysis, spike Thermo Scientific™ Pierce™ Retention Time Calibration Mixture (Product No. 88321) into the sample at a 1:100 dilution. This calibration mixture is used to normalize autosampler variability, to verify elution-time consistency and mass calibration, and to provide retention time controls for data analysis using the Pinpoint™ Software.

Related Thermo Scientific Products

84841 **Pierce Digestion Indicator**

23227 **BCA Protein Assay Kit**

88320 **Pierce Peptide Retention Time Calibration Mixture**

28904 **Trifluroacetic acid**

87784 **Pierce C18 Tips, 100µL bed**

89870 **Pierce C18 Spin Columns**

90061 **TMTsixplex™ Isobaric Label Reagent Set**

90051 **Lys-C Protease, MS Grade**

90057 **Pierce Trypsin Protease, MS Grade**

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