SureQuant™ AKT Pathway IP and MS Sample Prep Module

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

Multiplex immunoprecipitation to mass spectrometry (IP-MS) kits from Thermo Fisher Scientific are developed for simultaneous enrichment and quantitation of multiple total and phosphorylated proteins in the AKT Pathway Signaling Pathway. The AKT Pathway multiplex panel for absolute or relative quantitation contains two modules:

- IP and MS Sample Prep Module includes all the reagents necessary to immunoenrich AKT-mTOR pathway proteins and perform in-solution MS sample preparation method
- Absolute or Relative Quantitation Module includes system suitability standard and AQUA Ultimate Heavy and/or Light Peptide mixtures.

The immuno-enriched and digested samples spiked with internal standards can be processed using the discovery MS method (nanoLC-MS/MS) and targeted MS method (nanoLC-PRM/MS) analysis.

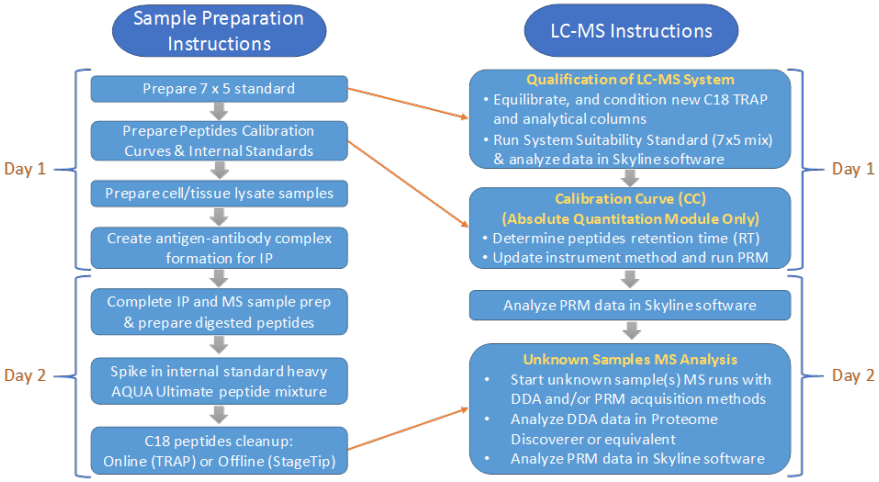
The SureQuant™ AKT Pathway IP and MS Sample Preparation Module enables highly effective antigens for immunoprecipitation (IP) and co-immunoprecipitation (co-IP) for mass spectrometry (MS) analysis. The high-capacity streptavidin magnetic beads maximize the recovery of low-abundant targets while their low non-specific binding minimizes background protein identifications. Cells are first lysed in a non-ionic, detergent-containing buffer and incubated with an IP-MS verified, specific biotinylated antibody mixture to form an immune complex for each AKT-mTOR pathway target protein. The bound complexes are thoroughly washed with detergent-free buffers to greatly reduce nonspecific binding and to remove any residual detergent. A trypsin enzyme elution method is used to dissociate the bound immune complexes from the streptavidin beads, which can then be digested directly in-solution and followed up by MS analysis on a same day; no SDS-PAGE purification is required. The high affinity biotin-streptavidin interaction allows the antigen to be eluted with significantly less antibody contamination compared to other antibody capture ligands such as Protein A, G, or A/G.

Contents

Components	Storage
Positive Control Lysate - A549 (+hIGF-1) at 1 mg/mL, 2 × 0.5 mL (Shipped separately)	-80°C
Trypsin Protease, 20 µg Trypsin Storage Solution, 250 µL	-20°C
AKT Pathway Biotinylated Antibody Mix, 0.2 mL Multiplex[1] Pierce™ Streptavidin Magnetic Beads, 1 mL IP-MS Cell Lysis Buffer, 100 mL IP-MS Wash Buffer A, 75 mL IP-MS Wash Buffer B, 40 mL IP Elution and MS Sample Prep Buffer, 10 mL Chloroacetamide, 2 mg/vial Low Protein-Binding Collection Tubes (1.5 mL), 50 each Bond-Breaker™ TCEP Solution, Neutral pH, 0.5 mL 10% Pierce™ Trifluoroacetic Acid (TFA), Sequencing Grade, 0.5 mL	4°C

[1] Antibody mixture is powered by Cell Signaling Technology™ (CST™) antibodies.

Procedure summary



Additional information

- Do not centrifuge, dry or freeze the magnetic beads, because this can cause the beads to aggregate and lose binding activity.
- A small amount of antibody may co-elute with the immunoprecipitated antigen. MS will identify some heavy and light chain peptides; however, the target identification will not be affected.
- IP-MS Cell Lysis Buffer has been tested on representative cell types including, but not limited to: MCF7, HeLa, Jurkat, A431, A549, MOPC, NIH/3T3, HEK293, HCT116, BT-549, and U2OS. Typically, 10^6 HeLa cells yield ~10 mg of cell pellet and ~3 µg/µL (or 300 µg) of protein when lysed with 100 µL of buffer.
- To minimize protein degradation, include protease inhibitors (e.g., Thermo Scientific™ Halt™ Protease Inhibitor Cocktail, EDTA-free (100X), Product No. 78425) in preparation of cell lysates.
- The IP-MS Cell Lysis Buffer is compatible with the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Product No. 23225), Thermo Scientific™ Pierce™ Detergent Compatible Bradford Assay Kit (Product No. 23246) and Thermo Scientific™ Pierce™ 660nm Protein Assay Kit (Product No. 22662).

Materials required but not provided

- Phosphate-buffered saline (PBS, 100mM sodium phosphate, 100mM NaCl; pH 7.2; Product No. 28372)
- Antigen sample (cell lysate or tissue lysate)
- Vacuum concentrator (e.g., Thermo Scientific™ SpeedVac™ Vacuum Concentrator Kit, Product No. SPD121P or equivalent)
- Thermomixer, heat block or incubator
- Magnetic stand (e.g., Thermo Scientific™ DynaMag™–2 Magnet, Product No. 12321D)
- Platform shaker or rotating mixer
- Tissue strainer (Product No. 87791)

Procedure

Lyse mammalian cells

Lyse cell monolayer (adherent) cultures

1. Carefully remove culture medium from cells.
2. Wash the cells 3 times with PBS or HBSS.
3. Add ice-cold IP-MS Cell Lysis Buffer (with added protease and phosphatase inhibitor) to the cells and scrape the cells with a squeegee or other scraping device to lyse.

Table 1 Suggested volume of IP-MS Cell Lysis Buffer to use for different standard culture plates.

Plate Size/Surface Area	Volume of IP-MS Cell Lysis Buffer
100 × 150 mm	400-800 µL
100 × 100 mm	300-500 µL
100 × 60 mm	100-300 µL
6-well plate	200-400 µL/well
24-well plate	100-200 µL/well

4. Collect cells into a common polypropylene conical tube or equivalent.
5. Incubate on ice for 10 minutes with periodic mixing.
6. Transfer the lysate to microcentrifuge tubes and centrifuge at ~16,000 × *g* for 10 minutes to pellet the cell debris.
7. Transfer supernatant to a new tube for protein concentration determination and further analysis.

Lyse cell suspension cultures

1. Centrifuge the cell suspension at 1000 × *g* for 5 minutes to pellet the cells. Discard the supernatant.
2. Wash cells 3 times by suspending the cell pellet in PBS or HBSS. Centrifuge at 1,000 × *g* for 5 minutes to pellet cells.
3. Add ice-cold IP-MS Cell Lysis Buffer (with added protease and phosphatase inhibitor) to the cell pellet. Use 500 µL of IP-MS Cell Lysis Buffer per 50 mg of wet cell pellet (i.e., 10:1 v/w). If using a large amount of cells, first add 10% of the final volume of IP-MS Cell Lysis Buffer to the pellet and pipette the mixture up and down to mix. Add the remaining volume of IP-MS Cell Lysis Buffer to the cell suspension.
4. Incubate lysate on ice for 10 minutes with periodic mixing. Remove cell debris by centrifugation at ~13,000 × *g* for 10 minutes.
5. Transfer supernatant to a new tube for protein concentration determination and further analysis.

Lyse tissue

1. If frozen, thaw on ice, then, using a balance, carefully section into approximate 500 mg pieces.
2. Wash each piece 3 times with 5 mL of cold PBS or HBSS.
3. In cold PBS, mince the tissue to very small pieces (smallest pieces produce optimal extraction) with scissors or razor blades .
4. Centrifuge at 500 × *g* for 5 minutes at 4°C.
5. After centrifugation, remove the supernatant. If removal of supernatant is difficult, centrifugation at higher speed is recommended.
6. Add ice-cold IP-MS Cell Lysis Buffer (with added protease and phosphatase inhibitor) to the tissue lysate at a 1:20 w/v ratio. For example, if minced tissue weighs 100 mg, add 2 mL of IP-MS Cell Lysis Buffer.
7. Homogenize tissue on ice with electronic Polytron Handheld Tissue Tearer or equivalent.
8. Incubate on ice for at least 10 minutes.
9. For optimal removal of tissue debris, use a tissue strainer with 2 mL capacity (Product No. 87791). Centrifuge sample at 1,000 × *g* for 10 minutes at 4°C.

Keep the supernatant and determine the protein concentration as described above.

Note: Alternatively, centrifuge at 5,000 × *g* for at least 5 minutes if the tissue strainer is not used. Keep the supernatant and determine protein concentration as described above.

Prepare immune complex

1. Thaw positive control lysates and/or unknown cell/tissue lysates on ice.
2. For positive control, combine 0.5 to 1 mL of lysate with 20 µL biotinylated antibody mix per sample in a low protein-binding collection tube. For sample lysate, use 0.5 to 1 mg of protein in a volume not to exceed 1 mL.
3. Ensure tubes are completely sealed and wrap each with paraffin film.
4. Incubate lysate and biotinylated antibody mix at 4°C overnight with gentle end-over-end rotation on a Thermomixer or platform shaker to form immune complex.

Manually perform immunoprecipitation and prepare MS samples

Note: To ensure streptavidin magnetic bead homogeneity, mix the vial thoroughly by repeated inversion, vortexing, or using a rotating platform.

1. Place 70 µL of Pierce™ Streptavidin Magnetic Beads into a 1.5 mL low protein-binding microcentrifuge tube.
2. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.
3. Add 140 µL of IP-MS Cell Lysis Buffer to the beads and gently vortex to mix. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant. Repeat this step once.
4. Quick spin antigen sample/antibody mixture (from "Prepare immune complex" section) by centrifugation and add to the tube containing pre-washed magnetic beads. Incubate at room temperature for 1 hour with end-over-end rotation on a Thermomixer or platform shaker.
5. Quick spin by centrifugation. Collect the beads with a magnetic stand and completely remove supernatant so that no liquid remains.
6. Add 500 µL of IP-MS Wash Buffer A to the tube and mix by vortexing for 10-15 seconds. Collect the beads and carefully remove the supernatant. Perform this wash a total of 3 times.
7. Add 500 µL of IP-MS Wash Buffer B to the tube and mix by vortexing for 10-15 seconds. Collect the beads and carefully remove the supernatant. Perform this wash a total of 2 times. Prior to last wash, quick spin by centrifugation to avoid bead loss.
8. Prepare 0.2 µg/µL trypsin by adding 100 µL of trypsin storage solution to 20 µg trypsin in a vial. Pipette solution 5-10 times to solubilize the trypsin and keep the enzyme solution on ice until use.
9. Per each sample, prepare trypsin elution by adding 5 µL of 0.2 µg/µL trypsin to 95 µL of IP Elution and MS Sample Prep Buffer and add to beads. Incubate at 37°C for 1 hour using a thermomixer or equivalent set to 1,400 to 1,500 rpm. For optimal results, the mixing speed is critical.
10. Quick spin by centrifugation and collect the beads by placing on a magnet. Transfer 90 µL of the supernatant containing the target antigens to a new 1.5 mL low protein-binding collection tube. Do not remove remaining 10 µL of supernatant.
11. Prepare 5X single-pot reduction/alkylation mixture as follows:
    a. Add 12 µL of 0.5 M TCEP to 228 µL of IP Elution and MS Sample Prep Buffer.
    b. Transfer 214 µL of diluted 25 mM TCEP solution into 1 tube of no-weigh chloroacetamide.
    c. Vortex for 60 seconds. Use immediately and discard any unused solution.
12. Add 25 µL of 5X single-pot reduction/alkylation mixture to each sample and incubate at 95°C for 5 minutes.
13. After reduction/alkylation, place samples at 4°C for 1 minute.
14. Add 7 µL of IP Elution and MS Sample Prep Buffer and 3 µL of 0.2 µg/µL trypsin to each sample. Store remaining trypsin in 5 to 10 µL aliquots at -20°C.
15. Vortex briefly and incubate for 2 hours at 37°C using a thermomixer set to 500 rpm.
16. After trypsin digestion, remove the samples and add 10 µL of 10% TFA and vortex briefly to stop digestion.
17. Centrifuge sample for 2 minutes at 15,000 × *g* to remove any remaining particulates.
18. Remove 125 µL of supernatant and transfer to a new low protein-binding collection tube.
19. Dry the supernatant in a speed vacuum concentrator.

MS quantitation

1. Prepare internal standard AQUA Ultimate HeavyPeptide internal standard spiked-in solution (see instructions in the AKT Pathway Absolute or Relative Quantitation Kit Module).
2. Add 20 µL of internal standard HeavyPeptide mixture to each dried sample.
3. Vortex for at least 60 seconds and quick centrifuge.
4. Clean up spiked-in and digested samples before LC-MS analysis using one of the following methods:
   a. For C18 **on-line** trap column (Product No. 164564) sample clean up, transfer each sample to a labeled autosampler vial for LC-MS/MS analysis or store at -20°C for later analysis.
   b. For C18 **off-line** clean up, process digested and internal standard spike-in samples with Pierce™ C18 Spin Tips (Product No. 84850) using recommended protocol before MS analysis.

Troubleshooting

Observation	Possible cause	Recommended action
Antigen(s) did not immunoprecipitate or low amount of recovered protein.	Sample did not contain sufficient antigen to detect.	Verify protein expression and/or lysis efficiency by SDS-PAGE or Western blot. Use more lysate for IP if required.
	Protein degraded.	Add protease and phosphatase inhibitors.
	Insufficient magnetic beads used.	Ensure magnetic beads are evenly suspended before use.
	Short liquid-chromatography (LC) gradient and slow-scanning mass spectrometer were used.	Use nano LC with longer gradient (60 minutes) and fast-scanning mass spectrometer (e.g., Thermo Scientific™ Q Exactive™/Orbitrap™ Fusion Mass Spectrometer).
	Low recovery of peptides after in-solution digestion.	Use low protein-binding microcentrifuge tubes (provided in kit) for maximum recovery of target peptides .
High non-specific background proteins identified.	Non-specific proteins bound to the magnetic beads.	Pre-clear sample by incubating with Pierce™ Streptavidin Magnetic Beads without antibody before forming the immune complex.
Magnetic beads aggregated.	Magnetic beads were frozen or centrifuged.	Handle the beads as directed in the instructions.

Limited product warranty

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