Protein Capture from Cell Lysate using Agarose Resin

Introduction

Many commercial protein enrichment kits provide great results, but are often expensive and provide fixed amounts of reagents, which limits optimized or off-protocol use of these kits. “Home-made” kits are a viable alternative to commercially available kits, in particular when all of the components are widely available from a number of suppliers. The amounts of reagents and the click reaction conditions are very similar between many commercial kits. Using the provided protocols, a researcher will be able to assemble an azide or alkyne labeled protein enrichment assay to covalently capture labeled proteins. Once covalently attached to the resin via copper catalyzed click chemistry, the resin can be washed with the highest stringency, virtually eliminating any non-specifically bound proteins to yield a highly enriched population of nascent molecules.
Captured proteins can be protease-digested to yield a highly pure peptide pool that is ideal for mass spectrometry (e.g., LC MS/MS) based analysis, or they can be released from beads under appropriate cleavage conditions for downstream analysis.
This protocol is compatible with non-cleavable agarose azide/alkyne, as well as Dde, PC, and disulfide agarose resin.

Materials Required

- Alkyne or azide agarose resin
- THPTA
- Copper (II) Sulfate pentahydrate
- Sodium ascorbate
- Azide or alkyne probe for protein labeling
- Protease inhibitor
- Tris
- SDS
- Urea
- DTT
- Iodoacetamide
- Acetonitrile
- Trypsin (mass spectrometry grade)
- TFA
- C18 desalting columns

Important: Any solution directly involved in the click reaction should not contain copper chelators (EDTA, EGTA, etc.) or reducing agents (DTT, 2-mercaptoethanol, TCEP, etc.).

Material Preparation

Lysis Buffer	200 mM Tris pH 8.0, 4% CHAPS, 1 M NaCl, 8 M Urea. Store refrigerated for up to 1 week or at -20°C for 1 year to avoid decomposition of urea Note: Add Protease Inhibitor Cocktail to Lysis buffer at twice the amount recommended by manufacturer. 1 mL of lysis buffer is sufficient for 50-200 million cells or 5-20 mg tissue extract .
Agarose SDS Wash Buffer	100 mM Tris, 1% SDS, 250 mM NaCl, 5 mM EDTA, pH 8.0
Sodium Ascorbate	Add 2 mL of deionized water to 400 mg of **Sodium Ascorbate** and vortex until fully dissolved. After use, store remaining stock solution at -20°C for up to 1 year

Protein Enrichment Protocol

1. Preparation of Agarose Resin

1. Mix the 50% resin slurry until the resin is completely resuspended
2. Before the resin settles, transfer 200 µL of well-mixed resin with a 1 mL pipette into a clean microcentrifuge tube
3. Wash the resin with 1.3 mL of deionized water
4. Pellet the resin by centrifugation at 1000 x g for 2 min
5. Carefully aspirate the supernatant leaving settled resin at the bottom of the tube. Take care not to aspirate settled resin

2. Lysate Preparation

1. Add 1 mL of **Lysis Buffer** containing protease inhibitor to each alkyne- or azide-containing cell or tissue pellet in a 2 mL microcentrifuge tube
2. Incubate the lysis mixture on ice for 5-10 minutes
   Note: The lysate will become very viscous. While on ice, add Benzonase® Nuclease to the lysate or sonicate using a probe sonicator by applying two 3-second pulses, until the lysate is no longer viscous .
3. Centrifuge the lysate at 16,000 x g for 5 min
4. Place lysate back on ice until ready for the click reaction

3. Preparation of 2X Copper Catalyst Solution

1. Prepare 1 mL of 2X Copper Catalyst Solution per enrichment reaction as follows:

860 µL	Deionized water
100 µL	100 mM THPTA
20 µL	100 mM Copper (II) Sulfate Solution
20 µL	Sodium Ascorbate

2. Vortex 2X Copper Catalyst Solution to mix

4. Lysate/Agarose Click Reaction

1. Assemble the click reaction in a 2 mL microcentrifuge tube as follows

200 µL	Washed Agarose resin (Step 1.5)
800 µL	Cell or tissue lysate (Step 2.4)
1000 µL	2X Copper Catalyst Solution (Step 3.2)

2. Rotate end-over-end on sample rotator for 16-20 hours

5. Reduction & Alkylation of Resin Bound Proteins

1. Centrifuge agarose resin (Step 4.2) at 1000 x g for 2 min. Aspirate the supernatant to waste, taking care not to aspirate the resin
2. Add 1.8 mL of deionized water to the resin, centrifuge at 1000 x g for 2 min, aspirate the supernatant to waste taking care not to aspirate resin. This water wash step prevents clumping of the resin caused by interaction of residual Lysis Buffer with the SDS in the Agarose Wash Buffer
3. Add 1 mL of SDS Agarose Wash Buffer and 10 µL of 1M DTT to the resin. Vortex briefly to resuspend the resin
4. Heat the resin at 70°C on a heat block for 15 min, then cool to room temperature for 15-30 min
5. Centrifuge the resin at 1000 x g for 5 min, aspirate the supernatant to waste taking care not to aspirate the resin
6. Prepare 1 mL of a 40 mM iodoacetamide solution per enrichment reaction by dissolving 7.4 mg of iodoacetamide into 1 mL of SDS Agarose Wash Buffer
7. Add 1 mL of 40 mM iodoacetamide solution to the resin, vortex to resuspend the resin, incubate the reaction in the dark for 30 minutes at room temperature

6. Resin Wash

Note: All washes can be performed using empty spin columns (e.g., Bio-Rad). SDS Agarose Wash Buffer is used for stringent removal of non-specifically bound proteins. After this wash, it is critical to remove residual SDS by washing the resin exhaustively with 8 M urea and 20% acetonitrile prior to mass spectrometry analysis .

1. Centrifuge the resin from step 5.7 at 1000 x g for 2 min
2. Wash the resin with H_2O, centrifuge at 1000 x g for 2 min
3. Wash the resin 5 times with SDS Agarose Wash Buffer, centrifuging at 1000 x g for 2 min after each wash
4. Wash the resin 5-10 times with 8 M urea/100 mM Tris pH 8.0, centrifuging at 1000 x g for 2 min after each wash
5. Wash the resin 5-10 times with 20% acetonitrile, centrifuging at 1000 x g for 2 min after each wash

7. Protease Digestion of Resin-Bound Proteins

1. After step 6.5, wash the resin twice with digestion buffer (100 mM Tris pH 8.0, 2 mM $CaCl_2$, 10% acetonitrile), centrifuging at 1000 x g for 2 min after each wash
2. Aspirate the supernatant to waste, resuspend the resin in 200 µL of digestion buffer
3. Add 10 µL of 0.1 µg/µL Trypsin to the resin slurry, gently mix the slurry, incubate at 37°C for 6 hours to overnight

8. Preparation of Digest for Mass Spectrometry Analysis

1. Pellet the resin from Step 7.3 by centrifugation at 1000 x g for 5 min, then carefully transfer the digested supernatant to a clean tube
2. Add 500 µL of deionized water to the resin. Vortex briefly to mix, then pellet the resin by centrifugation at 1000 x g for 5 min
3. Transfer the rinse supernatant over the resin to the digest supernatant from Step 8.1
4. Add additional deionized water to the digest to a final volume of 1 mL
   Note: this dilutes the acetonitrile concentration to 2% .
5. Acidify the diluted digest by adding 2 µL of TFA
6. Desalt the digest on a C-18 cartridge using vacuum or gravity flow, allowing each solution to completely flow through the cartridge before adding the next solution
   a. Add 1 mL of 50% acetonitrile/0.1% TFA to the cartridge and discard the effluent

b. Add 1 mL of 0.1% TFA to the cartridge and discard the effluent. Repeat one more time

c. Add the acidified, diluted digest to the cartridge and discard the effluent

d. Add 1 mL of 0.1% TFA to the cartridge and discard the effluent. Repeat one more time

e. Place a clean 1.5 mL tube below the C-18 cartridge outlet

f. Elute the peptides into a clean 1.5 mL tube by adding 700 µL of 50% acetonitrile/0.1% TFA to the C-18 cartridge

7. Dry the eluate containing the desalted peptide digest in a vacuum concentrator. Store at -20°C until ready for MS analysis

Troubleshooting

Problem	Possible Cause	Solution
Low yield of enriched proteins	Inefficient protein capture or low abundance of alkyne- or azide-tagged proteins	Increase lysate concentration (use more cells) or pre-enrich the proteins (e.g., soluble lysate, membrane lysate, lectin enrichment, etc.)
		Confirm peptide recovery by measuring A280 after
	Inefficient digestion of resin-bound proteins	Use high quality trypsin
High background with unlabeled control cells	Insufficient washing of resin	Increase column washes. Use only high purity reagents. Prepare filtered buffers fresh. Ensure proper preparation of copper catalyst solution
Signal suppression during MS analysis	SDS contamination in digest	Wash the resin thoroughly after the SDS Agarose Wash Buffer with another buffer such as 8M urea and 20% acetonitrile to remove all traces of SDS detergent