

Diazo Biotin Probes

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The following is a general protocol for enrichment of proteins labeled with Diazo Biotin Probes from cell lysates. The Diazo Biotin-tagged proteins captured onto streptavidin agarose resin can be either released for downstream analysis or digested on beads followed by the release of peptides bound agarose resin. The investigators may wish to determine optimal resuspension/binding buffer(s) as well as on-beads digestion conditions and peptides preparation for downstream analysis.

The protocol provided is optimized for 1 mL soluble proteins at 1 mg/mL concentration using our high capacity streptavidin agarose. This protocol can be scaled up or down, depending on the amount of biotin labeled protein to be captured on the resin.

Binding Biotinylated Proteins to Streptavidin Agarose Resin.

- 1.1 Resuspend air-dried protein pellets (after click reaction and precipitation) by bath sonication in at 1 mg/mL in resuspension buffer (50 mM Tris, 150 mM NaCl and 1% SDS). Other denaturing pull down buffers (for example 6 M urea, 2 M thiourea, and 10 mM HEPEs) are also compatible with downstream analysis.
- 1.2 Mix 50% Streptavidin Agarose slurry until the resin is completely resuspended.
- 1.3 Transfer 100 μ L of 50% slurry (50 μ L of settled resin) to a microfuge tube. Use a 200 μ L pipet with 4-5 mm cut tip to ensure that the resin is transferred properly. Wash streptavidin agarose resin two times with 1 mL of PBS and one time with 1 mL of resuspension buffer.
- 1.4 Resuspend streptavidin agarose resin in 200 μ L of resuspension buffer, and add the slurry to the solubilized proteins.
- 1.5 Incubate samples on a rotator for 2 hours.
- 1.6 Wash beads two times with resuspension buffer (1 mL), two times with 1% SDS in PBS (1 mL), and two times with PBS buffer (1 mL).

Stringency of washing may be increased (e.g., 1% SDS in 8 M urea washes) if high protein background is observed by MS or Western blot.
- 1.7 The resin now contains bound biotin labeled protein and is ready for cleavage/elution or on-beads digestion.

Cleavage and Recovery of Captured Proteins

- 2.1 Resuspend the beads in 100 μ L of freshly prepared sodium dithionite solution (1% SDS, 25 mM sodium dithionite)
- 2.2 Incubate for 30 minutes at room temperature.
- 2.3 Centrifuge beads for 2 minutes at 2,000 g, and collect the eluent.
- 2.4 Repeat steps 2.1 - 2.3, and combine the eluents.
- 2.5 Precipitate proteins in ice cold methanol (1 mL) 2 hours to overnight at -20°C . Collect protein was by centrifugation (10 minutes, 10,000 g, 4°C), the pellet was allowed to air-dry for 5 minutes.

Note: this step can be replaced by desalting

On Beads Trypsin Digestion of Enriched Proteins

- 3.1 Resuspend beads from Step 3.6 in 500 μ L of 10 mM DTT in PBS, vortex briefly.
- 3.2 Heat to 70°C on a heat block for 15 minutes, and then cool to room temperature for 15-30 minutes.
- 3.3 Centrifuge resin for 5 minutes at 2,000 g, aspirate the supernatant to waste taking care not to aspirate the resin.
- 3.4 Add 1 mL 40 mM iodoacetamide solution to the resin, vortex to resuspend the resin, incubate the reaction in the dark for 30 minutes at room temperature.
- 3.5 Pellet the beads by centrifugation (2,000 g, 3 minutes) and wash once with PBS (1 mL).
- 3.6 Pellet the beads by centrifugation (2,000 g, 3 minutes).
- 3.7 Add 300 μ L of digestion buffer (100 mM Tris, 2 mM CaCl_2 , 0.5 M urea) to the resin.
- 3.8 Add 3 μ g trypsin from a stock solution to the resin slurry, gently mix the slurry, then incubate at 37°C for 6-12 hours with over-the-end rotation.

Note: Alternative proteases and trypsin digestion procedures are also compatible with this protocol and may be used to increase peptide coverage.
- 3.9 Pellet the beads by centrifugation (2,000 g, 3 minutes), and collect the supernatant digest.

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- 3.10 Wash the beads with PBS (200 μ L) and water ($2 \times 200 \mu$ L).
- 3.11 Combine the washes with the supernatant digest to form the “trypsin fraction”.
- 3.12 Concentrate the “trypsin fraction” to dryness using a speedvac set to 40°C.
- 3.13 The “trypsin fraction” may be stored for at least 12 months at -80°C.

Cleavage and Recovery of Peptides

- 4.1 Resuspend the beads in 200 μ L of freshly prepared sodium dithionite solution (25 mM sodium dithionite)
- 4.2 Incubate for 30 minutes at room temperature.
- 4.3 Centrifuge beads for 2 minutes at 2,000 g, and collect the eluent. Repeat the elution step, and combine the eluents.
- 4.4 Resuspend the beads in 200 μ L of 50% acetonitrile-water, centrifuge beads for 2 minutes, and collect the eluent. Combine all eluents.
- 4.5 Concentrate the “cleavage fraction” to dryness using a Speedvac heated to 40°C. Store at -20°C until desalting and MS analysis.
- 4.6 For long term storage, the “cleavage fraction” may be stored for at least 12 months at -80°C.

Preparation of Digest for Mass Spectrometry Analysis

The following protocol is provided for desalting using ZipTip C18 P10. Sample recovery for typical peptides is > 85%, but could be as low as 35% for hydrophilic peptides. Other protocols for sample preparations for MS also can be used.

- 5.1 Resuspend the dried “trypsin fraction” and “cleavage fraction” in 1% FTA in water (25.0 μ L). Gently vortex the sample and briefly centrifuge to collect all liquid at the bottom
- 5.2 Wet the tip by aspirating 10 μ L of 50% ACN in water and then discarding solvent. Repeat once.
- 5.3 Equilibrate tip by aspirating 10 μ L of 0.1% TFA and discarding solvent. Repeat twice.
- 5.4 Load the peptides by pipetting the sample 10–15 times. Carefully pipette the sample such that air bubbles do not pass through the C18 tip. Air bubbles decrease loading capacity.
- 5.5 Wash the pipette tip with 1% TFA in water (50 μ L) and discard the eluent.
- 5.6 Slowly aspirate 25 μ L of 50% ACN:Water with 1% TFA. Repeat twice.
- 5.7 Concentrate the sample using a speedvac heated to 40°C. Store at -20°C until analysis by LC/MS. For long term storage, the desalted samples may be stored for at least 12 months at -80°C.

References

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