The Click Chemistry Capture Kit provides all necessary reagents to covalently capture specific sub-classes of proteins by a Cu(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC). The proteins of interest need to be metabolically, enzymatically or chemically azido- or alkyne-tagged. Subsequently, the resin containing the covalently attached proteins can be washed with high stringency, virtually eliminating any non-specifically bound proteins. Upon protease digestion, this yields a highly pure peptide pool that is ideal for mass spectroscopy (e.g. LC MS/MS) based analysis.

**Content:**
- 7 ml Lysis buffer
  - store at 4 °C
- 4.8 g Urea
  - store at ambient temperature
- 1.5 ml Additive 1
  - store at 4 °C
- 0.5 ml Copper (II) Sulfate (100 mM)
  - store at ambient temperature
- 400 mg Additive 2
  - store at ambient temperature
- 200 ml Agarose wash buffer
  - store at 4 °C
- 10 Empty spin columns
  - store at ambient temperature

**Materials required but not provided for capturing of Azide-tagged proteins**
- 5-20 mg azido- or alkyne-tagged cell or tissue extract
- Alkyne or Azide agarose resin
- Unlabeled negative control cells or tissue
- Sample rotator
- Table top centrifuge
- Protease inhibitor
- 2 ml microcentrifuge tubes
- aqua bidest.
- Probe sonicator

**Materials required but not provided for on-resin digestion with protease**
- DTT
- Iodoacetamide
- 8 M Urea, 100 mM Tris, pH 8
- Acetonitrile
- Mass-spec grade trypsin
- 0.1 % TFA
- C-18 desalting cartridges
- Digestion Buffer (100 mM Tris, 2 mM CaCl₂, 10 % acetonitrile)
- Heat block

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Click Chemistry Capture Kit

• Vacuum concentrator

Preparation of stock solutions

Lysis Buffer (200 mM Tris, 4 % CHAPS, 1 M NaCl, 8 M Urea, pH 8.0)
• Add the solid urea (4.8 g) to the Lysis Buffer (7 ml) provided.
• Mix the solution on a rotator at room temperature until the urea is completely dissolved (1-2 hours). Store at -4 °C (for up to 1 week) or at -20 °C for 1 year to avoid decomposition of urea.
• Note: 30 min before starting the enrichment protocol, add 20 µl Protease Inhibitor Cocktail (e.g. Sigma 8340) per ml of Lysis Buffer (sufficient for 50-200 million cells or 5-20 mg tissue extract).

Additive 2
• Add 2 ml of aqua bidest. to Additive 2 and vortex until fully dissolved.
• After use, store remaining stock solution at -20°C for up to 1 year.

Protein Enrichment Protocol (per enrichment)

Step 1: Preparation of Alkyne- or Azide-Agarose Resin
• Mix the 50 % resin slurry until the resin is completely resuspended.
• Before the resin settles, transfer 200 µl of well-mixed resin with a 1 ml pipette into a clean 2 ml microfuge tube.
• Add 1.3 ml aqua bidest. to the resin.
• Pellet the resin by centrifugation for 2 min at 1000 x g.
• Carefully discard the supernatant leaving approximately 200 µl of settled resin at the bottom of the tube. Take care not to aspirate settled resin.

Step 2: Lysate Preparation
• Add 1 ml Lysis Buffer including Protease Inhibitor (see Preparation of stock solutions) to each azide- or alkyn-containing cell or tissue extract (5-20 mg protein) in a 2 ml microfuge tube.
• Incubate the lysis mixture on ice for 5-10 min.
• While on ice, sonicate the mixture using a probe sonicator by applying two 3 second pulses. Take care not to overheat the sample.
• Repeat the sonication on ice until the lysate is no longer viscous (e.g. viscosity of water).
• Centrifuge the lysate at 10,000 x g for 5 min.
• Place the lysate back on ice until performing the click reaction.

Step 3: Preparation of 2x Copper Catalyst Solution
• Prepare 1 ml of 2x Copper Catalyst Solution per enrichment reaction as follows:
  - 860 µl Aqua bidest.
  - 100 µl Additive 1
  - 20 µl Copper (II) Sulfate Solution
  - 20 µl Additive 2
• Voretex the solution

Step 4: Lysate/Agarose Click Reaction
• Assemble the click reaction in a 2 ml microfuge tube as follows:
  - 200 µl washed Alkyne or Azide Agarose resin (from step 1)
  - 800 µl cell or tissue lysate (from step 2)
  - 1000 µl 2x Copper Catalyst Solution (from step 3)
• Rotate end-over-end on sample rotator for 16-20 hours

Step 5: Reduction & Alkylation of Resin Bound Proteins
• Warm the Agarose wash buffer to room temperature before starting. Ensure that the solution is homogenous and clear before use.
• Centrifuge the agarose resin for 1 min at 1000 x g. Carefully discard the supernatant.
• Add 1.8 ml of aqua bidest. to the resin, centrifuge at 1000 x g and carefully discard the supernatant. This water wash step prevents clumping of the resin caused by interaction of residual Lysis Buffer with the SDS in Agarose wash buffer.
• Add 1 ml Agarose wash buffer and 10 µl of 1 M DTT to the resin. Vortex briefly to resuspend the resin.
• Heat the resin at 70 °C for 15 min on a heat block, then cool to room temperature for 15-30 min.
• Centrifuge resin for 5 min at 1000 x g and carefully discard the supernatant.
• Prepare 1 ml of a 40 mM iodoacetamide solution per enrichment reaction by dissolving 7.4 mg of iodoacetamide in 1 ml of Agarose wash buffer.
• Add 1 ml 40 mM iodoacetamide solution to the resin, vortex to resuspend the resin and incubate the reaction in the dark for 30 min at room temperature.

Step 6: Resin Wash
• Agarose wash buffer is used for stringent removal of non-specifically bound proteins. It is critical to remove residual SDS by exhaustive washing with 8 M urea and 20 % acetonitrile prior to mass spectrometry analysis.
• Twist off the spin column’s bottom closure and remove the cap.
• Use a 1 ml pipette to resuspend the resin, then transfer the resin to a spin column.
• Rinse the resin tube with 0.5 ml H2O and also transfer this
volume to the same spin column.

- Add 2 ml of Agarose wash buffer to the spin column and centrifuge at 1000 x g for 1 min. Repeat this step 5 times.
- Add 2 ml of 8 M Urea/100 mM Tris, pH 8 to the spin column and centrifuge at 1000 x g for 1 min. Repeat this step 5-10 times.
- Add 2 ml of 20 % acetonitrile to the spin column and centrifuge at 1000 x g for 1 min. Repeat this step 5-10 times.

**Protease Digestion of Resin-Bound Proteins**

- Cap the bottom of the spin column and add 500 µl of Digestion Buffer to the resin.
- Use a 1 ml pipette to resuspend the resin in the spin column and transfer the resin to a clean tube.
- Rinse the spin column with 0.5 ml additional Digestion Buffer and add this rinse to the already transferred resin.
- Pellet the resin by centrifugation for 5 min at 1000 x g. Discard the supernatant, but leave approximately 200 µl of Digestion Buffer in the tube above the resin. Take care not to aspirate the resin.
- Add 10 µl of 0.1 µg/µl trypsin to the resin slurry and mix gently. Incubate at 37 °C for 6 hours to overnight.

**Preparation of Digest for Mass Spectrometry Analysis**

- Pellet the resin by centrifugation for 5 min at 1000 x g and carefully transfer the digested supernatant to a clean tube.
- Add 500 µl aqua bidest. to the resin. Vortex briefly and pellet the resin by centrifugation for 5 min at 1000 x g.
- Transfer the supernatant to the already transferred digest supernatant.
- Add additional aqua bidest. to the digest to a final volume of 1 ml (Please note: This dilutes the acetonitrile concentration to 2 %).
- Acidify the diluted digest by adding 2 µl of TFA.
- Desalt the digest on a C-18 cartridge using vacuum or gravity flow. Allow each solution to completely flow through the cartridge before adding the next solution.
- Add 1 ml of 50 % acetonitrile/0.1 % TFA to the cartridge and discard the effluent.
- Add 1 ml of 0.1 % TFA to the cartridge and discard the effluent. Repeat one more time.
- Add the acidified, diluted digest to the cartridge and discard the effluent.
- Add 1 ml of 0.1 % TFA to the cartridge and discard the effluent. Repeat one more time.
- Place a clean 1.5 ml tube below the C-18 cartridge outlet.
- 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.
- Dry the eluate containing the desalted peptide digest in a vacuum concentrator. Store at -20 °C until MS analysis.

**Troubleshooting**

**Problem: Low yield of enriched proteins**

- **Possible reason:** Insufficient protein capture or low abundance of azido- or alkyne-tagged proteins
  - Increase the 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 digestion
- **Possible reason:** Inefficient digestion of resin-bound proteins
  - Use high quality trypsin

**Problem: High background with unlabeled control cells**

- **Possible reason:** Insufficient washing of resin
  - Increase column washes
  - Use only high purity reagents
  - Prepare fresh filtered buffers
  - Ensure proper preparation of copper catalyst solution

**Problem: Signal suppression during MS analysis**

- **Possible reason:** SDS contamination in the digest
  - Wash the resin thoroughly after the Agarose wash buffer wash with another buffer such as 8 M urea and 20 % acetonitrile to remove all traces of SDS detergent (Step 6)