Protein purification via Sepharose beads (Camille's protocol)

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Immobilized Metal Affinity Chromatography (IMAC) is a method used to purify proteins based on their affinity for metal ions. Here, proteins are purified using Sepharose beads charged with **nickel (Ni^{2+})** ions, in 2 mL Eppendorf tubes, **to test batches of different conditions.**

Proteins engineered with a **histidine tag (His-tag)** bind strongly to nickel ions due to the interaction between the histidine side chains and the metal. When a protein mixture is passed through the column:

1. **His-tagged proteins** bind to the nickel on the column.
2. **Non-tagged proteins** flow through (FT fraction)
3. Wash buffer containing lower imidazole concentration **washes away** the **miscellaneous proteins** that have **unspecific** interactions, improving final purity.
4. The bound His-tagged proteins are then **eluted** using a buffer containing **imidazole**, which competes with the His-tag for binding to the nickel.

This allows for relatively specific and efficient purification of the His-tagged protein.

Materials:

- Lysis buffer
    - For ci2350: 100 mM sodium phosphate, 200 mM NaCl, 5 mM imidazole
    - For AKR1D1: 20 mM Tris-HCl pH 7.9 plus 5 mM imidazole in milliQ water
    - For atcABC: 20 mM Tris-HCl pH 7.8
        - OR same as ci2350, might not have a difference, inquire Camille
        - Consider adding reducing agents, 5 mM DTT or BME? ← sensitive to oxygen
        - Purified AtcABC has a pH optimum of 6.5
- Wash buffer A (same as Lysis buffer, but 20 mM imidazole)
- Wash buffer B (same as Lysis buffer, but 50 mM imidazole)*
- Elution buffer (same as Lysis, but 500 mM imidazole)
    - For atcABC: Elution was performed using buffer (20 mM Tris–HCl and 500 mM NaCl, pH 7.8) using a gradient 0-100% ← though I believe it can be the same as ci and AKR1D1 (with imidazole), since we can't do a gradient
- Ice
- Microcentrifuge (RT)
- MQ water
- Ethanol

- **Ni^{2+}** ion solution
- 2 mL Eppendorf tubes

* NOTE: Wash buffers wash away other proteins that bind unspecifically. You can test which wash buffer concentration works best (we tested with 20, 50, 100 mM Imidazole). When tested, can prepare only one that works best, and switch to Cytiva's protein purification protocol.

Steps:

1. **Charge Sepharose beads with Ni^{2+}**
   1.1. **…** inquire Camille how she did it

2. **Removal of Ethanol:**

Sepharose beads are stored by suspending them in 20% ethanol, since this increases their stability. This ethanol needs to be removed before using them:

2.1. If beads are floating around, centrifuge the tube at 6000 RPM (~3300 g), 1 min. (Or 8000 RPM (~3400 g) in the mini microcentrifuge on our table). If beads are settled, proceed to b).
2.2. Discard the supernatant (ethanol). Beads are easily disturbed, if they are accidentally resuspended, centrifuge again.
2.3. Add 1 mL of MQ H_2O.
2.4. Resuspend by inverting the tube 10 times. NEVER VORTEX.
2.5. Centrifuge (6000 RPM (~3300 g), 1 min).
2.6. Discard the supernatant (water)
2.7. Repeat 2.3-2.6 three times.

NOTE: Assuming the beads were stored in a single tube, you will need to aliquot the beads (250 µL of beads for one purification batch). You can either take 250 µL of beads using 1 mL pipette after you've discarded the ethanol or, you could resuspend the beads (either with the same ethanol or during c) step), and then aliquot them equally into separate 2 mL tubes.

3. **Equilibrating the Sepharose beads**

The beads need to have the same buffer that is used for cell lysis. To do so:

3.1. Add 1 mL Lysis buffer solution.
3.2. Resuspend by inverting the tube 10 times.
3.3. Centrifuge (6000 RPM (~3300 g), 1 min).
3.4. Discard the supernatant.
3.5. Repeat 3.1-3.5 three times.

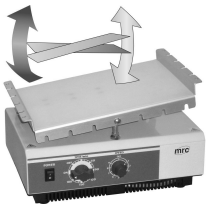
NOTE: discard the pipettes and tubes containing any buffer with imidazole to a separate plastic bag, then throw that away in chemical waste (blue bag near electrophoresis station).
Liquids with imidazole go into the imidazole waste bottle in the wastes' fume hood.

4. **Loading the protein:**

4.1. Add 1-1.5 mL of Lysed cell SN (supernatant (the soluble protein fraction))
4.2. Resuspend by inverting the tube 10 times.

4.3. Let it incubate for 20 min, while shaking in the tilting shaker
4.4. Centrifuge (6000 RPM (~3300 g), 1 min).
4.5. KEEP the supernatant (FT - Flowthrough fraction).

Note: If you want to load more protein, repeat 4.1-4.5 (e.g. if you want to load 2 mL, first add 1 mL, do 4.1-4.5, then add the remaining 1 mL).



Note 2: ⬆ used the old orange one near the heating block, secured the tube rack with tape, then put the Eppendorf tubes in it.

5. Washing away miscellaneous proteins:

5.1. Add 1 mL Wash buffer (A OR B)
5.2. Resuspend by inverting the tube 10 times.
5.3. Centrifuge (6000 RPM (~3300 g), 1 min).
5.4. Collect the supernatant (Wash fraction).

Note: can use either one wash buffer for each protein batch. Check different wash buffers and use SDS-PAGE and Bradford assay to compare which one works best for you.

6. Elution of target protein:

6.1. Add 1 mL Elution buffer.
6.2. Resuspend by inverting the tube 10 times.
6.3. Let it incubate for 20 min, while shaking in the tilting shaker.
6.4. Centrifuge (6000 RPM (~3300 g), 1 min).
6.5. Collect the supernatant (Elution fraction)

Elution fraction (along with others) can be used for total protein quantification using Bradford assay (will need ~400 µL)
For SDS-PAGE, aliquot 15 µL of each fraction you want to test.

NOTE: **DO NOT FREEZE THE ELUTION FRACTION** (and the Wash fractions), if you're not using a desalting column to filter away the imidazole (or doing a buffer exchange), store them in the fridge (+4C). High concentrations of imidazole destabilizes proteins during the freezing and thawing process, leading to aggregation and precipitation. It is also not recommended to keep them in the fridge for a long time either due to imidazole (a few days should be fine).

7. Recovery of the sepharose beads:

7.1. Wash them with 1 mL MQ H_2O, 3 times (repeat 2.3-2.6).
7.2. Wash them with 1 mL Ethanol (20%), 3 times (same procedure)
7.3. Pool the beads together into one tube, add 1 mL Ethanol (20%), leave in fridge.

Sepharose beads can be used 5-6 times this way, after which they will need to be restripped, then charged with Ni^{2+} once more.