Protein Purification by Column Binding (Cytiva HisTrap HP, 1 mL, IMAC)

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Created by: Cecilia

Origin: provided by Cytiva, adapted by last and this year's iGEM team, finalised to this version. Protocol = HisTrap HP, 1 mL (manual no. 71502768 AL): https://geneseesci.asset.akeneo.cloud/Technical_Documents/media/HisTrapHP1mland5mlInstructions.pdf

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Background

Immobilized Metal Affinity Chromatography (IMAC) purifies proteins by their affinity for metal ions. HisTrap columns are pre-charged with **nickel (Ni^{2+}) ions** immobilized on the resin. Proteins engineered with a **$(histidine)_6$-tag** bind strongly to the nickel via their histidine side chains. When a protein mixture is passed through the column: His-tagged proteins bind to the nickel, non-tagged proteins flow through, and the bound His-tagged protein is then eluted using an imidazole-containing buffer that competes with the His-tag for nickel binding — giving relatively specific and efficient purification.

Column Information

- **Column**: Cytiva HisTrap HP, 1 mL **column volume (CV)**
- **Recommended flow rate:** 1 mL/min – Maximum: 4 mL/min
- **Storage**: 20% ethanol, room temperature (or 4°C BUT in fire fridge)
- **Binding capacity:** at least 40 mg His6-tagged protein / mL medium

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Materials / Reagents

- **Clarified cell lysate** (supernatant after sonication + centrifugation), kept cold; filtered (1.2 → 0.8 → 0.45 → 0.2 µm)
    - Cytiva does not do this multi-step filter cascade in their own protocol — adapted by us (via Camille) to reduce column clogging.
- Cytiva pre-packed affinity (IMAC) column; HisTrap HP, 1 mL column volume (CV)
- Peristaltic pump with tubing, set up for gravity/pump-driven flow
- Vivaspin centrifugal concentrator (MWCO below your protein's size — 30 kDa cutoff used previously)
- 50% Glycerol (optional, for long-term storage of the final eluate)
- 20% Ethanol and distilled water (column storage / cleaning)

Buffer Preparation

Made from already-prepared stock solutions: 3 M NaCl, 1 M sodium phosphate buffer (pH 7.4), 1 M imidazole. All buffers used in the column must stay within a pH range of 3–12 for normal operation, and 3–14 for cleaning.

- Imidazole stock (1 M): 68 g per 1 L MQ water (34 g for 500 mL)
- **Cell Wash Buffer** (used before lysis, and for Vivaspin washes): 0.5 M NaCl, 20 mM sodium phosphate
- **Binding Buffer:** 500 mM NaCl, 20 mM sodium phosphate, 30 mM imidazole; pH 7.4
    - Imidazole can be adjusted between 20–40 mM depending on the aim: higher imidazole → higher purity; lower imidazole → higher yield
- **Elution Buffer:** 500 mM NaCl, 20 mM sodium phosphate, 500 mM imidazole; pH 7.4
- **Buffer-exchange buffer**: 100 mM potassium phosphate, pH 6.5
    - For 500 mL: 4.509 g potassium phosphate monobasic (136.09 g/mol) + 2.937 g potassium phosphate dibasic (174.18 g/mol), topped up to 500 mL with MQ water
    - Check pH (target 6.5) before use; store at room temperature

Reagent	Cell Wash Buffer (500 mL)	Binding Buffer (500 mL)	Elution Buffer (250 mL)
3M NaCl (aq)	83 mL	83 mL	42 mL
1M Sodium Phosphate Buffer (pH 7.4)	10 mL	10 mL	5 mL
1M Imidazole (aq)	-	15 mL	125 mL
Milli Q Water	up to 500 mL	up to 500 mL	up to 250 mL

Step-by-Step Protocol

NOTE: columns from Cytiva arrive already charged with Ni^{2+} (packed with Ni Sepharose High Performance). Recharging is only needed after roughly 5–7 purifications, or if the column needed fixing (e.g. trapped air bubbles). Full recharge procedure is under “Stripping & Recharging” below.

1. **Calibrate the pump** (every time, before use)
    a. Set the flow rate between 1–4 mL/min (**1.5 mL/min** used routinely; 1 mL/min for a slower, more controlled run).
    b. Check the transit time: with the pump running, time how long liquid takes to travel from the start of the inlet tubing to the outlet. This is the delay to wait before switching collection flasks in every step below (≈3 min at 1.5 mL/min).

2. **Assemble Column**



   a. Remove the outlet and the stopper
   b. Fill the open end of the column with distilled water and carefully close it while liquid is still dropping (to prevent air bubbles in the column)
3. **Equilibrate Column with**
   a. Equilibrate with 10 CV Binding Buffer before loading any sample.
4. **Load the sample (Flowthrough)**
   a. Place the inlet tube into the clarified, filtered lysate/supernatant.
   b. After the **calibrated transit time**, move the outlet tube from Waste into the Flowthrough flask.
   c. Run the entire sample volume through the column.
5. **Wash**
   a. Switch the inlet tube into the Binding Buffer.
   b. After the calibrated transit time, move the outlet tube from Flowthrough into the Washing flask.
   c. Run **≈10 CV** of Binding Buffer through the column (e.g. 10 mL for a 1 mL CV column).
6. **Elute**
   a. Switch the inlet tube into Elution Buffer.
   b. After the calibrated transit time, move the outlet tube from Washing into the Elution flask.
   c. Run **≈5 CV** of Elution Buffer through the column (e.g. 5 mL for a 1 mL CV column).
7. **Clean the column**
   a. Between different samples on the same column: wash with Binding Buffer for 10 CV.
   b. After the final use: wash sequentially with Binding Buffer (10 CV) → distilled water (10 CV) → 20% Ethanol (10 CV).
   c. Turn off the pump and close the outlet of the column. Next, detach the inlet connector, turn on the pump again, fully fill the column opening with the 20% ethanol and then close it with the column stopper, making sure no air bubbles are trapped inside.
   d. Store at room temperature (or, if not used often, at 4 °C in the fire fridge)
   e. Fully empty the tubing.

8. **Concentrate Eluate (Vivaspin)**
    a. Load 4 mL (1 mL for SDS) of the eluate into a Vivaspin centrifugal concentrator (MWCO 30 kDa) and add 4 mL of exchange buffer (e.g. 100 mM potassium phosphate, pH 6.5). Pipette up and down.
    b. Centrifuge at 4,000 × g for 30 minutes.
    c. Add exchange buffer again so the total volume is around 5 mL, pipette up and down (especially close to the membrane, to prevent fouling) & centrifuge again to dilute out the imidazole.
    d. Repeat this wash-and-spin step until the volume is concentrated at ~ 2 mL.
9. **Storage of Eluate**
    a. Add glycerol to the final concentrated eluate in a 1:1 ratio for long-term storage.
    b. If glycerol is added, wash it back out via Vivaspin (at least ~4 wash-and-spin cycles) before using the sample for further analysis.

Stripping & Recharging

Not needed after every purification — only after roughly 5–7 uses of the same column, or if the column needed fixing.

Stripping buffer
Final: 20 mM sodium phosphate, 0.5 M NaCl, 50 mM EDTA, pH 7.4

- Add ~70% of the final volume of dH_2O to a clean flask or beaker.
- Weigh out the required powders (see table below) and add to the water.
- Stir/mix until fully dissolved.
- Check pH with litmus paper or a calibrated pH meter and adjust if necessary.
- Transfer to a graduated cylinder or volumetric flask and top up to final volume with dH_2O.
- Sterilize by filtration through a 0.22 µm filter.

Total volume	***di-Sodium hydrogen phosphate dihydrate***	***Sodium dihydrogen phosphate dihydrate***	***NaCl***	***EDTA***
100 ml	0.218 g	0.121 g	2.992 g	1.461g
50 ml	0.109 g	0.061 g	1.496 g	0.731 g
25 ml	0.055 g	0.030 g	0.748 g	0.365 g

Stripping

1. Wash with 5 – 10 mL stripping buffer
2. Wash with 5 – 10 mL binding buffer
3. Wash with 5 – 10 mL distilled water
4. *(If not immediately recharged, load with 20 % ethanol - but then wash with binding buffer and distilled water again!)*

Recharging

1. Load 0.5 mL of 0.1 M $NiCl_2$ (or $NiSO_4$ as stated in the original protocol). Nickel waste needs to be separately collected and discarded in the appropriate waste canister (nickel waste in fume hood).
2. Wash with 5 mL distilled water
3. Wash with 5 mL binding buffer
4. Store in 20% ethanol.

Cleaning in Place (CIP)

Clean when back pressure increases. Always strip Ni^{2+} first (procedure above), then clean using the relevant row below, then wash with 5 CV of 20% ethanol and store in 20% ethanol.

Problem Type	***Cleaning Solution***	***Notes***
Ionically bound proteins	Several CV of 1.5 M NaCl	Follow with approximately 10 CV of distilled water
Precipitated protein, hydrophobically bound proteins and lipoproteins	1 M NaOH, contact time 1–2 h	Follow with 1 ml binding buffer and 5-10 ml of distilled water
Lipids/lipoproteins	30% isopropanol, 15–20 min	Follow with 10 mL of distilled water

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SDS-PAGE Samples for Quality Control

Possible samples to collect for later quality control via SDS-PAGE:

Sample	What it checks
Lysate (pre-filtration)	Total protein before any processing
Lysate (post-filtration)	Whether filtration itself caused target protein loss
Flow-through	Leakage / weakly-bound protein not captured by the column (adjust imidazole depending on yield and concentration wanted)
Wash	No target protein
Eluate	Main product; this shows purity and yield
Eluate, post Vivaspin	Target protein as well as unspecific proteins
Flowthrough of Vivaspin	Check what proteins got lost
Supernatant of Vivaspin	Final concentrated product

Troubleshooting

For troubleshooting help on inclusion bodies or other issues, check the Cytiva manual: https://geneseesci.asset.akeneo.cloud/Technical_Documents/media/HisTrapHP1mland5mlInstructions.pdf