**Nickel Purification of Nanobodies from periplasm**

Use to purify well-behaved nanobodies that do not require protein A to select for well-folded protein

Reagents:

- SET Lysis Buffer:
  - 200 mM Tris pH 8
  - 500 mM Sucrose
  - 0.5 mM EDTA
- Cold $H_2O$
- Benzonase Nuclease
- 3 M $MgCl_2$
- 5 M NaCl
- Wash Buffer 1 (The high salt wash removed non-specific binders from the nickel column)
  - 20 mM Hepes pH 7.5
  - **500 mM NaCl**
  - 20 mM imidazole
- Wash Buffer 2
  - 20 mM Hepes pH 7.5
  - 100 mM NaCl
  - 20 mM imizaole
- Elution Buffer
  - 20 mM Hepes pH 7.5
  - 100 mM NaCl
  - 200 mM imidazole
- 10 kDa cutoff concentrator (Amicon)
- Size Exclusion / Dialysis Buffer
  - 20 mM Hepes pH 7.5
  - 150 mM NaCl
  - 10% Glycerol
- Ni-NTA Resin or column

Protocol:

1. Resuspend cell pellet in 50 mL Room Temperature SET buffer / L of initial culture volume.
2. Once completely resuspended add 2x volume of cold $H_2O$, 5 mM $MgCl_2$, 1 µL benzonase nuclease.
3. Stir 1 hr at room temperature.
4. Spin at 9000-14000 x g for 30 min (For large culture volume, you can use 1 L centrifuge bottles)
5. Add 100 mM NaCl to supernatant. Stir at room temperature for 15 min.
6. Filter supernatant through glass microfibre filter
7. Apply supernatant to Ni-NTA resin equilibrated with wash buffer
    - For gravity purification use ~3-5 mL resin / L culture volume, load at a moderate speed, to increase yield collect flow through run over the column again to ensure full binding
8. Wash with 10 CV of wash buffer 1
9. Wash with 10 CV of wash buffer 2
10. Elute with 3-5 CV of elution buffer
11. Wash resin with 5-10 more CV of elution buffer to clean if you are planning to use resin again
12. Concentrate protein containing fractions in 10 kDa cutoff concentrator. If nanobody precipitates clear by spinning at 15-20,000 x g for 5 minutes at 4°C or filter with 0.22 um filter
    - Some nanobodies that precipitate can be rescued by the addition of extra salt (300-500 mM)
13. Run over superdex S75 or dialyze overnight to buffer exchange.
14. Check purify by SDS-PAGE
    - Occasionally, a second band may be present that is slightly larger than the expected molecular weight of the nanobody. This may be the nanobody with a retained PelB sequence. This can be separated by ion exchange, but will need to be optimized for individual nanobodies (e.g. MonoS column in 20 mM Hepes pH 7.5 with slow gradient between 450-520 mM NaCl for nanobody with a pI of 9.4.)
15. Note: Nanobodies for crystallization can be treated with carboxypeptidases A and B to remove C-terminal His-Tag