Nucleus Protocols

Make OnePot Protein Mix

1. Starter Plate Preparation

- ☐ Prepare a OnePot PURE starter plate containing each of the 36 strains, stored as 25% glycerol stocks at an OD_{600} = 0.5. Each well of the starter glycerol stock plate should contain 40 µL of the corresponding strain at OD_{600} = 0.5 and be stored at –80 °C.

2. Buffer Preparation

2.1. 1M HEPES (250 mL)

- ☐ Dissolve 59.6 g HEPES (MW 238.3 g/mol) in 200 mL ddH_2O.
- ☐ Adjust to a final volume of 250 mL.
- ☐ Sterilize by autoclaving or filter sterilization.
- ☐ Store at 4 °C with the bottle wrapped in foil to protect from light. Avoid storing HEPES for longer than one week; prepare only the amount required for the intended number of preparations.

2.2. 1M Magnesium Chloride (250 mL)

- ☐ Dissolve 50.8 g $MgCl_2$ (MW 203.3 g/mol) in 200 mL ddH_2O.
- ☐ Adjust to a final volume of 250 mL.
- ☐ Sterilize by autoclaving or filter sterilization.
- ☐ Store at 4 °C for up to 6 mo.

2.3. 2M Potassium Chloride (250 mL)

- ☐ Dissolve 37.3 g KCl (MW 74.55 g/mol) in 200 mL ddH_2O.
- ☐ Adjust to a final volume of 250 mL.
- ☐ Sterilize by autoclaving or filter sterilization.
- ☐ Store at 4 °C for up to 6 mo.

2.4. Buffer A (1 L)

- ☐ Add 53.5 g NH_4Cl to a 1 L bottle and dissolve in 500 mL ddH_2O.
- ☐ Add 50 mL of 1 M HEPES.
- ☐ Add 10 mL of 1 M $MgCl_2$.
- ☐ Bring the volume up to 1 L with ddH_2O.
- ☐ Adjust pH to ∼7.6 using KOH pellets.
- ☐ Filter sterilize.
- ☐ Store at 4 °C, wrapped in foil to protect from light; suitable for multiple preparations.

2.5. Buffer B (500 mL)

- ☐ Add 17 g Imidazole to a 500 mL Duran bottle and dissolve in 300 mL ddH_2O.
- ☐ Add 25 mL of 1 M HEPES.
- ☐ Add 5 mL of 1 M $MgCl_2$.
- ☐ Add 25 mL of 2 M KCl.
- ☐ Bring the volume to 490 mL with ddH_2O.
- ☐ Adjust pH to ∼7.6 using HCl.
- ☐ Bring the final volume to 500 mL with ddH_2O.
- ☐ Filter sterilize.
- ☐ Store at 4 °C, wrapped in foil to protect from light; suitable for multiple preparations.

2.6. Buffer HT (2 L)

- ☐ Use a sterile 2 L bottle and sterile (filtered) HEPES, $MgCl_2$, KCl, and water to avoid the need for filter sterilization.
- ☐ Add 100 mL of 1 M HEPES.
- ☐ Add 20 mL of 1 M $MgCl_2$.
- ☐ Add 100 mL of 2 M KCl.
- ☐ Add 1780 mL sterile ddH_2O.
- ☐ Store at 4 °C, wrapped in foil to protect from light; suitable for multiple preparations.

2.7. Stock 60 (100 mL)

- ☐ Add 5 mL of 1 M HEPES (filtered) to a sterile 100 mL Duran bottle.
- ☐ Add 1 mL of 1 M $MgCl_2$ (filtered).
- ☐ Add 5 mL of 2 M KCl (filtered).
- ☐ Add 60 mL of 100% sterile glycerol.
- ☐ Add 29 mL sterile ddH_2O.
- ☐ Store at 4 °C, wrapped in foil to protect from light; suitable for multiple preparations.
- ☐ Prepare Stock 30 using Stock 60 before adding any reducing agent to Stock 60.

2.8. Stock 30 (50 mL)

- ☐ Add 25 mL of filtered Buffer HT (without reducing agent) to a sterile 100 mL Duran bottle.
- ☐ Add 25 mL of filtered Stock 60 (without reducing agent).
- ☐ Store at 4 °C, wrapped in foil to protect from light; suitable for multiple preparations.

3. Procedure

3.1. Day 1: Starter Culture

- ☐ Starter culture incubation needs to begin at 6 pm. As such begin subsequent steps at 5 pm to allow sufficient time to start incubation at 6 pm.
- ☐ Thaw a PURE Starter Plate on ice. This starter plate contains each of the 36 strains frozen in 25% glycerol at OD_{600} 0.5.
- ☐ Add 50 µL 1000x Kanamycin (50 mg/mL) to 50 mL of sterile LB in a sterile falcon tube.
- ☐ Label 1.5 mL sterile tubes from 1 to 36 (except number 25) and add 1 mL of LB + Kan to each tube.
- ☐ Add 10 µL of each of the 36 glycerol stocks (except number 25) to the corresponding labeled tubes and mix well by vortexing.
- ☐ Add 300 µL of inoculated culture from each tube into the corresponding well of a sterile 96 deep well plate. Seal the plate using a Breath-easy sealing membrane.
- ☐ Inoculate 3 mL of LB + Kan in 15 mL falcon tubes with 10 µL of EF-Tu glycerol stock. Do this in duplicate.
- ☐ Incubate deep well culture plate and 15 mL falcon tubes at 260 rpm / 37 °C / 16 h.
- ☐ Start incubation at 6 pm. Check OD_{600} at 10 am the next day (16 h incubation).
- ☐ Place 1.5 L of sterile LB in 37 °C static incubator to prewarm overnight.

3.2. Day 2: Main Growth

- ☐ The next day, measure the OD_{600} of the starter plate using a 96 well plate by adding 30 µL of each starter to the bottom of each well and 270 µL of LB + Kan on top (10x dilution). Measure EF-Tu culture density at this time too. Expected OD_{600} with 10x dilution is 0.2 - 0.3.

- ☐ After 16 h of incubation, all strains should be at OD_{600} = 2-3. If a strain is growing very slowly, remove the starter volume from the 96-deep well plate and place it into a 2 mL sterile tube, and shake at 260 rpm / 37 °C independently until desired OD is reached. The remaining starters can be left in the deep well plate on the bench. If a starter has overgrown above OD_{600}, dilute the starters to OD_{600} = 3 using LB + Kan in a sterile tube.
- ☐ Once all starter strains have been equilibrated to OD_{600} 2-3, proceed to main growth by adding 500 mL of prewarmed LB + Kan into a 2.5 L baffled flask (2x flasks).
- ☐ Into a sterile 5 mL tube add 55 µL of each starter culture (excluding EF-Tu), and 1675 µL of EF-Tu starter culture. Mix well by vortexing and add entire content to 500 mL of LB + Kan in 2.5 L baffled flask. Repeat for the second culture flask.
- ☐ Incubate the cultures at 260 rpm / 37 °C / (1.5 - 2) h (until OD_{600} reaches 0.2 - 0.3). Check OD_{600} after 1 h as baffled flask may cause cells to grow faster.
- ☐ Once OD_{600} of 0.2- 0.3 is reached, inoculate each culture flask with 500 µL of 100 mM IPTG to achieve a final induction concentration of 0.1 mM IPTG.
- ☐ Incubate cultures for a further at 260 rpm / 37 °C / 3 h.
- ☐ During incubation periods of main growth, prepare protein purification buffers as described below and store at 4 °C until use. Don't add TCEP at this point.

Buffer Type	Buffer A (mL)	Buffer B (mL)	Total (mL)	0.5 M TCEP (µL)
Resuspension/Equilibration Buffer	200	0	200	400
Wash Buffer	99	1	100	200
Elution Buffer	2	18	20	40

- ☐ 15 min before the end of the incubation, cool table top centrifuge to 4 °C and prepare an ice bucket and cool centrifuge bottles.
- ☐ At the end of the 3 h incubation, place baffled flask into ice bucket and remove samples for OD_{600} measurement. Final expected OD_{600} is 2-3.
- ☐ Fill each centrifuge bottle with 500 mL of culture from each flask and spin at 5000 g / 4 °C / 15 min and discard supernatant.
- ☐ Add 20 mL of sterile LB into each bottle and resuspend the cell pellet thoroughly and move resuspension into labeled sterile 50 mL falcon tubes.
- ☐ Centrifuge the Falcon tubes at 2000 g / 4 °C / 8 min, remove the supernatant by decanting.
- ☐ Centrifuge Falcon tubes again at 2000 g / 4 °C / 2 min, remove residual supernatant by pipetting.
- ☐ The pellets can be flash frozen in liquid nitrogen and stored at –80 °C for up to 3 d until protein purification.

3.3. Day 3: Protein Purification

- ☐ Thaw cell pellets on ice.
- ☐ Add 2 mL of fresh Ni^{2+} affinity resin to chromotography column. Wash column with 30 mL ddH_2O twice to remove ethanol.
- ☐ Add TCEP to Resuspension/Equilibration buffer to a final concentration of 1 mM (see section).
- ☐ Equilibrate column with 30 mL of Resuspension/Equilibration buffer + TCEP and close valve on the column with 5 mL of buffer remaining in the column.
- ☐ Add 7.5 mL of Resuspension/Equilibration buffer + TCEP to each falcon tube containing cell pellet and resuspend thoroughly and store on ice.
- ☐ Lyse cells via sonication at 70% amplitude, 10 s on 10 s off with 2000 J of energy in a ice water bath. Use a clamp stand to hold the falcon tube in place such that the cell suspension is submerged in the ice water bath and place the probe deep enough

into the solution without touching the tube. If a large amount of foam is generated, the energy transfer will be damped. In that case, let the foam settle, lower the probe deeper into the solution, and extend the sonication time. If sonication is successful, the solution will turn darker.

- ☐ Aliquot the sonicated sample in 2 mL tubes (1 mL per tube) and spin at 16 000 g / 4 °C / 20 min.
- ☐ Collate all clarified pellet free supernatant into a fresh 50 mL falcon tube on ice.
- ☐ Resuspend the resin in the remaining 5 mL of Resuspension/Equilibration buffer + TCEP within the column and collate buffer + resin into falcon tube containing supernatant. Seal the lid with parafilm and incubate in a rotating shaker at 4 °C for a minimum of 3 h.
- ☐ After incubation of sample with resin, briefly spin the falcon tubes in a table top centrifuge using pulse mode to collate the resin to the bottom of the tube.
- ☐ Resuspend the sample with resin using a pipette and add the mixture back into the protein purification column.
- ☐ Label three 15 mL falcon tubes as ‘flow through’, ‘wash’ and ‘elution’, respectively. Replicate as required for the number of purifications you are doing.
- ☐ Add TCEP at a final concentration of 1 mM to wash and elution buffers (see section) and store at 4 °C until use.
- ☐ Once the resin has settled into a bed at the bottom of the column, let the buffer run through and collect samples from the middle of the flow through into 15 mL falcon tube labeled ‘flow through’.
- ☐ Wash column with wash buffer + TCEP and collect flow through in 15 mL falcon tube labeled ‘wash’.
- ☐ Add 5 mL elution buffer + TCEP into the column and resuspend the resin a few times with pipette and incubate for 10 min before elution into tube labeled ‘elution’. During this incubation, add 1 L of Buffer HT into a 1L beaker and soak 2 kDa dialysis cassette in buffer with magnetic stir in cold room/fridge.
- ☐ Store eluted protein on ice.
- ☐ Remove dialysis cassette from beaker and add 5 mL of eluted protein into the dialysis cassette. Remove as much air as possible from cassette before putting the lid back on. Dialyze according to protocol stated below to remove imidazole. 5 mL of eluted protein is dialyzed against 1L of buffer HT (without TCEP) at 4 °C for 12 h / overnight. In instances when elutions can be combined, 10 mL of elution can be dialyzed against 2 L of buffer HT.

3.4. Day 4: Protein Concentration

- ☐ After dialysis is complete, remove dialyzed sample from cassette and add to a 3 kDa MWCO Amicon Ultra 15 and top up volume with 10 mL of fresh buffer HT + TCEP.
- ☐ Spin at 3220 g / 4 °C / 60 min. The sample volume will reduce down to 1.5 mL.
- ☐ Remove sample from Amicon tube and split into 2 mL sterile tubes in 500 µL aliquots. Spin to pellet any precipitated proteins at 14 000 g / 4 °C / 10 min.
- ☐ Collate pellet free supernantant into a fresh 1.5 mL tube on ice.
- ☐ Add TCEP to a 1 mL aliquot of Stock-60 to achieve a final concentration of 1 mM.
- ☐ Add equal volume of Stock-60 + TCEP to collated sample. Proceed to determine protein concentration using Pierce660 Assay in triplicate with a calibration curve spanning (0 - 2) mg/mL.
- ☐ Concentrate protein down using 0.5 mL 3 kDa Amicon columns and spin at 14 500 g / 4 °C / 15 min at until desired volume is reached to obtain a final concentration of 15 mg/mL.
- ☐ Spin to pellet any precipitated proteins at 14 000 g / 4 °C / 10 min.

- ☐ Determine final protein concentration using Pierce660 Assay and dilute samples with Stock 30 + TCEP as required to reach correct final concentration of 15 mg/mL.
- ☐ Aliquot 50 µL into PCR tubes, snap freeze using liquid nitrogen, and store at –80 °C.