



Nucleus Protocols

Make Ribosomes

1. PROTOCOL

1.1. Cell culture

- **Prepare overnight cultures.**
 - Add 5 mL Luria Broth (LB) under sterile conditions to two (2) 14 mL culture tubes.
 - Label one tube “(+)”. Add 10 uL of A19 glycerol stock to (+).
 - Label the other tube “(-)”. This will be your negative control, testing if your technique is sterile.
 - Incubate both tubes overnight shaking at 37C / (225-250) rpm / (12-16) hr.
- **Perform bulk outgrowth.**
 - Check if (-) has growth. If not, continue.
 - Back dilute overnight 1:250 - 1:1000 into 4x 450 mL fresh LB in 2 L baffled Erlenmeyer flasks (e.g., 1.8 mL overnight into 450 mL LB).
 - Incubate back diluted cultures at 37C / (225-250) rpm to mid-log phase (OD600 between 0.6 and 0.8, typically ~3 hrs).
- **Pellet, wash, and store cells.**
 - Fill 1 L centrifuge bottles with culture. Balance centrifuge bottles against each other and centrifuge cultures at 16 000 rcf / 4C / 10 min.
 - Decant supernatant, add fresh culture, and repeat centrifugation as above, working through the remaining culture. You should end up with large pellets at the bottom of each centrifuge bottle.
 - Wash the pellets by resuspending (4C) NaCl (0.9%) in about 50 mL and transfer the resuspended cells to a single centrifuge bottle. Dilute to ~500 mL and re-pellet at 16 000 rcf / 4C / 10 min.
 - Transfer pellets by spatula into a tared bag weigh and record the mass.
 - Flash freeze pellet in liquid nitrogen and store at -80C.

1.2. Lysis

- Resuspend (2-5) g cell pellet in 25 mL of Ribosome Lysis buffer & lyse cells using 130-watt probe sonicator (probe tip diameter: 6 mm) on ice with following parameters: 50% amplitude, 15s on/ 30s off for 2 minutes on-time. The amount of energy delivered via sonication will vary depending on the amount of cells resuspended.
- Clarify lysate by centrifugation at 16 000 rcf / 4C / 10 min.
- Aspirate supernatant and measure volume. Add an equal volume of Salting Out buffer to adjust the concentration of ammonium sulfate to 1.5 M and mix well. Incubate at 4C / 10 min.
- Remove precipitate by centrifugation at 16 000 rcf / 4C / 10 min.
- Filter supernatant using a 0.22 um syringe filter and keep cold (4C).

1.3. FPLC purification

- **Set-up.**
 - Connect the two Butyl column (5 mL) in tandem, totaling a column volume (CV) of 10 mL.
 - Place A1 in Ribosome Wash Buffer and B1 in Ribosome Elution Buffer. Place sample line in A2. Set the default flow rate to 4 mL / min (except for pump washes: 10 mL/min).

- **Equilibrate HIC column.**
 - Perform a pump wash with Ribosome Wash Buffer (without TCEP) and equilibrate the column with 4 CV of Ribosome Wash Buffer(without TCEP).
 - Once you've equilibrated your columns, add TCEP to Ribosome Wash and Elution Buffer.
 - Load your fraction collector with 15 mL conical tubes and set the fraction volume to 5 mL.
 - If using a sample pump to load samples, place sample line (S1) into sample and load around 90% of sample volume onto the column. Once almost loaded, dilute the sample with Ribosome wash buffer (~5 mL) to load as much sample as possible. DO NOT allow air into the FPLC; make sure the sample line is always submerged.
- **Perform HIC**
 - Wash step 1: wash with 3 CV of Ribosome Wash Buffer to remove unbound components .
 - Wash step 2: wash with 5 CV of 80% Wash Buffer and 20% Ribosome Elution Buffer.
 - Elution: elute the product by applying 3.5 CV (35 mL) of 50% Ribosome Wash Buffer and 50% Ribosome Elution Buffer. Ensure that the fraction collector captures these fractions separately.
 - Wash step 3: Elute all strongly interacting contaminants with 5 CV of 100% Ribosome Elution Buffer.
- **Clean columns**
 - Place inlet into a NaOH (0.5 M) and perform pump wash. Wash the column with 3 CV NaOH (0.5M).
 - Place the inlet into water, perform pump wash, and then wash column in 2 CV filtered Ultrapure water.
 - Place the inlet into AcOH (0.1 M), perform pump wash, and subsequently wash column with 3 CV AcOH (0.1 M).
 - Pump wash with water and wash column with 2 CV filtered MilliQ water.
 - Place all inlets into EtOH (20% v/v). Perform a pump wash, then wash columns with 3 CV EtOH (20% v/v). Store columns at 4C in EtOH (20% v/v) until ready for use.

1.4. Ultracentrifugation

- Gently overlay recovered fractions (should correspond to second peak) onto 35 mL of Cushion Buffer in a polycarbonate ultracentrifuge bottle (70 mL).
- Prepare another polycarbonate ultracentrifuge bottle as a balance. Measure 35 mL of Cushion Buffer, then add Ribosome Buffer until the balance mass is within 0.1 g of the sample bottle mass. **Make sure all bottles are well balanced ($\Delta m \leq 0.1$ g) and have no cracks!**
- Pellet ribosomes by ultracentrifugation at 100 000 rcf / 4C / 16 hrs. A translucent ribosome pellet will be formed at the bottom of the centrifuge bottle. It may be difficult to see.
- Discard the supernatant. Carefully, wash each pellet with 0.5 mL cold ribosome buffer. Repeat this step twice.
- Resuspend the clear pellets in 100 μ L of Ribosome Buffer on ice using a magnetic stir bar (3 mm diameter, 10 mm length) on a magnetic stirrer set at the lowest possible speed. Collect resuspended ribosomes.
- Wash tubes with an additional 50 μ L of Ribosome Buffer to resuspend any remaining ribosomes.

1.5. *Quality Control*

- Determine the ribosome concentration by measuring the absorbance at 260 nm at a 100x dilution in Ribosome Buffer. 10 units of A₂₆₀ from a 100x dilution corresponds to 23 μ M of undiluted solution.
- Dilute to final stock of 10 μ M. To adjust the concentration, dilute the ribosomes with ribosome buffer or concentrate further via centrifugation at 4000 rcf in a 100 kDa centrifugal filter at 4C.
- Protein gel: dilute 10 μ M sample by 4x (Add 2.5 μ L of sample with 7.5 μ L water) and mix with 10 μ L of 4x Laemmli with BME loading buffer. Boil samples at 90C for 10 minutes and load 5 μ L and 2.5 μ L onto 4-20% tris-glycine gel. Run gel at 200 V / 30-45 min or until the loading dye line reaches the bottom of the gel.

1.6. *Storage*

- Aliquot your ribosomes to reduce freeze / thaw cycles and store at -80C.