Nucleus Protocols

Make tRNAs

Hazardous Materials

- ☐ **Acid Phenol**
  - ☐ Corrosive, toxic, rapidly absorbed through skin, & respiratory irritant
  - ☐ Use in fume hood, wear neoprene gloves, lab coat, and goggles.
- ☐ **Acetic Acid**
  - ☐ Corrosive to skin and eyes
  - ☐ Use appropriate PPE and handle under fume-hood
- ☐ **Ethanol**
  - ☐ Highly flammable, toxic, and irritant
  - ☐ Wear PPE, use in well-ventilated areas, and keep away from open flames

1. Prepare Stock Buffers

☐ Make the following stock solutions from solid. Use ultrapure water (18.2 MΩ, e.g., Milli-Q) and keep everything RNase-free.

Stocks	Final Concentration (mM)	MW (g/mol)	Mass to add (g)	Final Vol (mL)	Storage (°C)
Sodium Acetate	1000	136.08	136.08	1000	room temp
Magnesium Acetate	1000	214.46	214.46	1000	room temp
NaCl (5 M)	5000	58.44	292.2	1000	room temp
NaCl (1 M)	1000	58.44	11.688	200	room temp

2. Prepare Buffers

☐ **Extraction Buffer** — used to resuspend biomass for acid-phenol extraction.

Reagent	Final Concentration (mM)	Stock Concentration (mM)	Volume to Add (mL)
Sodium Acetate	50	1000	50
Magnesium Acetate	10	1000	10
Acetic Acid (glacial)	—	—	to pH 5.0
Ultrapure water	—	—	to 1000
Total			**1000**

☐ **NaOAc (300 mM, pH 5.0)** — used to dissolve the nucleic-acid pellet before DNA removal.

Reagent	Final Concentration (mM)	Stock Concentration (mM)	Volume to Add (mL)
Sodium Acetate	300	1000	30
Acetic Acid (glacial)	—	—	to pH 5.0
Ultrapure water	—	—	to 100

Reagent	Final Concentration (mM)	Stock Concentration (mM)	Volume to Add (mL)
Total			**100**

3. Prepare Working Buffers

- ☐ **Ethanol (70% v/v)** — used to wash nucleic-acid pellets during precipitation.

Reagent	Final Concentration	Stock Concentration	Volume to Add (mL)
Ethanol	70% (v/v)	100% (v/v)	700
Ultrapure water	—	—	300
Total			**1000**

4. Cell culture

- ☐ Add 3 mL Luria Broth (LB) under sterile conditions to three (3) 14 mL culture tubes. Two (2) tubes will be used to prepare 6 mL of overnight culture and one (1) tube will be used as a negative control. 6 mL of overnight culture is enough to inoculate 4 x 450 mL of bulk outgrowths.
- ☐ Label two tubes “(+)” and seed with an A19 stock (colony or glycerol stock; see note above).
- ☐ Label the other tube “(-)”. This will be your negative control, used to test your sterile technique.
- ☐ Incubate all tubes overnight at 37 °C / (225 - 250) rpm / (10 - 16) h.
- ☐ Check if (-) has growth. If not, continue.
- ☐ Seed 4x 450 mL fresh media in 4x 2L baffled Erlenmeyer flasks with 1:500 overnight culture (e.g., 900 µL overnight into each flask with 450 mL media).
- ☐ Incubate back diluted cultures at 37 °C / (225-250) rpm to mid-log phase (OD_{600} between 0.6 and 0.8). This took us ∼3 h.
- ☐ Fill 1 L centrifuge bottles with culture. Balance centrifuge bottles against each other and pellet cultures at 16 000 rcf / 4 °C / 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 in 500 mL cold (4 °C) NaCl (0.9%) then pelleting again at 16 000 rcf / 4 °C / 10 min.
- ☐ Transfer pellets by spatula into a tared bag weigh and record the mass.
- ☐ Flash freeze pellet in liquid nitrogen and store at –80 °C.

5. Nucleus acid extraction by precipitation

- ☐ Set centrifuge to 4 °C and set shaking incubator to 37 °C.
- ☐ Resuspend 2 g of biomass into 18 mL of Extraction Buffer: NaOAc (50 mM), Mg(OAc)2 (10 mM), pH 5.0 in a 50 mL centrifuge tube by vortexing.
- ☐ In a fume hood and wearing the appropriate PPE, add 18 mL of Acid Phenol (pH 4.5) using a glass serological pipette.
- ☐ Cap the 50 mL centrifuge tube and seal with parafilm to prevent your sample spilling.
- ☐ Incubate at 37 °C / 225 rpm / 30 min in a shaking incubator. Tape tubes against the bottom plate of the shaking incubator horizontally so that samples are shaking laterally.

- ☐ Centrifuge at 4000 rcf / 4 °C / 15 min. You should observe three (3) layers: the aqueous (top) fraction, the organic (lower) fraction, and a middle fraction of cell debris separating them.
- ☐ Carefully collect the aqueous fraction by serological pipette, without disturbing the cell debris fraction, and transfer to a fresh 50 mL centrifuge tube.
- ☐ Add 14 mL of Extraction Buffer to Acid Phenol, seal the 50 mL centrifuge tube with parafilm, and incubate at 37 °C / 225 rpm / 15 min.
- ☐ Centrifuge at 4000 rcf / 4 °C / 15 min.
- ☐ Collect the aqueous fraction and combine with the first nucleic acid extraction (total volume between 30 mL and 32 mL).
- ☐ Set centrifuge to 25 °C.
- ☐ Add NaCl (5 M) to the aqueous phase to a final concentration of 0.2 M (∼1.5 mL). Mix by inversion and split evenly into 2x 50 mL centrifuge tubes.
- ☐ Precipitate nucleic acids by adding one volume of isopropanol (∼17 mL) to each tube and incubate at room temperature for 10 min. The mixture should turn visibly cloudy.
- ☐ Pellet nucleic acid precipitate via centrifugation at 14 500 rcf / 25 °C / 15 min.
- ☐ Wash the pellet with EtOH (70%):
    - ☐ Decant supernatant and wash nucleic acid pellet with 10 mL cold (-20 °C) EtOH (70%).
    - ☐ Re-pellet nucleic acid pellet by centrifugation at 14 500 rcf / 25 °C / 5 min.
    - ☐ Decant the supernatant and allow the pellet to air dry for 10 min.
- ☐ Resuspend each pellet into 15 mL of cold (4 °C) NaCl (1 M) by vortexing or pipetting. Ensure the pellet is fully dissolved. Allow NaCl (1 M) solution to hydrate pellet for (10 - 15) min at room temperature to help the pellet dissolve.
- ☐ Precipitate rRNA by centrifugation at 9500 rcf / 4 °C / 20 min.
- ☐ Decant the supernatant to a new 50 mL centrifuge tube.
- ☐ Add 2 volumes (approximately 30 mL) of cold (-20 °C) EtOH (100%) to the supernatant and incubate at –20 °C / >30 min to precipitate remaining nucleic acids. You can perform this step overnight.
- ☐ Centrifuge at 14 500 rcf / 4 °C / 5 min.
- ☐ Wash the pellet with EtOH (70%) as above.
- ☐ Set centrifuge to 25 °C.
- ☐ Dissolve the pellet in 6 mL of NaOAc (300 mM, pH 5.0). As needed to ensure the pellet is fully dissolved, heat samples up to 60 °C, pipette mix, and/or vortex. If the pellet is visibly small, you can dissolve each pellet in 3 mL of NaOAc (0.3 M, pH 5.0) and pool them together, totaling 6 mL.
- ☐ Add 0.56 volumes of isopropanol (∼3.4 mL) to each nucleic acid solution and incubate at room temperature for 10 min.
- ☐ Centrifuge at 14 500 rcf / 25 °C / 5 min. Decant the supernatant to a 15 mL centrifuge tube.
- ☐ Set the centrifuge to 4 °C.
- ☐ Add 2.3 mL of isopropanol to the supernatant (supernatant:isopropanol is 100:95) and incubate at –20 °C / >30 min. This step can be performed overnight.
- ☐ Centrifuge the suspension at 14 500 rcf / 4 °C / 15 min.
- ☐ Wash the pellet with EtOH (70%) as above.
- ☐ Resuspend the tRNA pellet in 1.5 mL of nuclease-free water and keep on ice.

6. Dialysis

- ☐ Hydrate the dialysis membrane: Remove the cassette from its protective pouch and immerse in nuclease-free water for 2 min.
- ☐ Add Sample:

- ☐ Open the cassette by twisting the cap counter-clockwise.
- ☐ Carefully pipette 1.5 mL of resuspended tRNAs into the cassette. Avoid puncturing the membrane!
- ☐ Remove the excess air in the cassette by simultaneously pressing the membrane gently on both sides and inserting the cap and locking it into place.

☐ Dialyze Sample:

- ☐ Float cassette in 500 mL nuclease-free water in a large (>600 mL) beaker and gently stir at 4 °C / 2 h. We do this by putting our beaker in a bucket of ice on a stir plate.
- ☐ Change the dialysis buffer and continue dialyzing overnight.

7. Concentrate

☐ Pipette dialyzed tRNAs to the upper chamber of an Amicon® Ultra-0.5 mL Centrifugal Filter, 3 kDa MWCO.
☐ Centrifuge at 14 000 rcf / 10 min and check the remaining volume in the upper chamber. Repeat until you hit your target volume.

8. Quality control

☐ Prepare a 1:1000 dilution of your tRNAs in water.
☐ Measure absorbance at 260 nm, 280 nm, and 230 nm.
☐ Estimate your yield by A_{260} ([tRNA] = A_{260} * 40 mg/mL). Typical yield of tRNA is typically between (4-20) mg per gram of cell mass.
☐ Estimate your purity by A_{260}/A_{230} and A_{260}/A_{280} (both should be $\geq$1.8).
☐ (optional) Prepare TBE-Urea 10% Gel

- ☐ Load gel into gel dock with running buffer.
- ☐ Pre-run gels at 100 V / 30 min.
- ☐ Wash wells with running buffer by syringe. You should be able to see urea displaced from the well by change in refractive index.

☐ Prepare tRNA samples:

- ☐ Dilute tRNAs to 40 ng/µL tRNA in nuclase-free water.
- ☐ Prepare 20 ng/µL sample by adding 10 µL of 40 ng/µL tRNA to 10 µL 2x TBE-Urea sample buffer.
- ☐ Prepare an ssRNA ladder. We use the NEB low range ssRNA (2 µL ladder + 2 µL 2x sample buffer).
- ☐ Incubate sample and and ladder at 65 °C / 3 min arrow.r 4 °C / hold using a thermal cycler.

☐ Load 200 ng of tRNA (10 µL at 20 ng/µL) onto the TBE-Urea gel and run at 125V / 2.5 h.
☐ Meanwhile, prepare SYBR-Green stain (4 µL in 40 mL water) to stain gel.
☐ Soak gel in SYBR-Green stain and visualize gel using UV or blue-light transilluminator. You should see multiple distinct bands around 75-90 nt.

9. Formulation

☐ Dilute your tRNA stocks in nuclease-free water to 35 µg/µL, which is 10x working concentration for cytosol reactions.

10. Storage

- ☐ Aliquot your tRNAs to reduce freeze / thaw cycles and store at –80 °C.