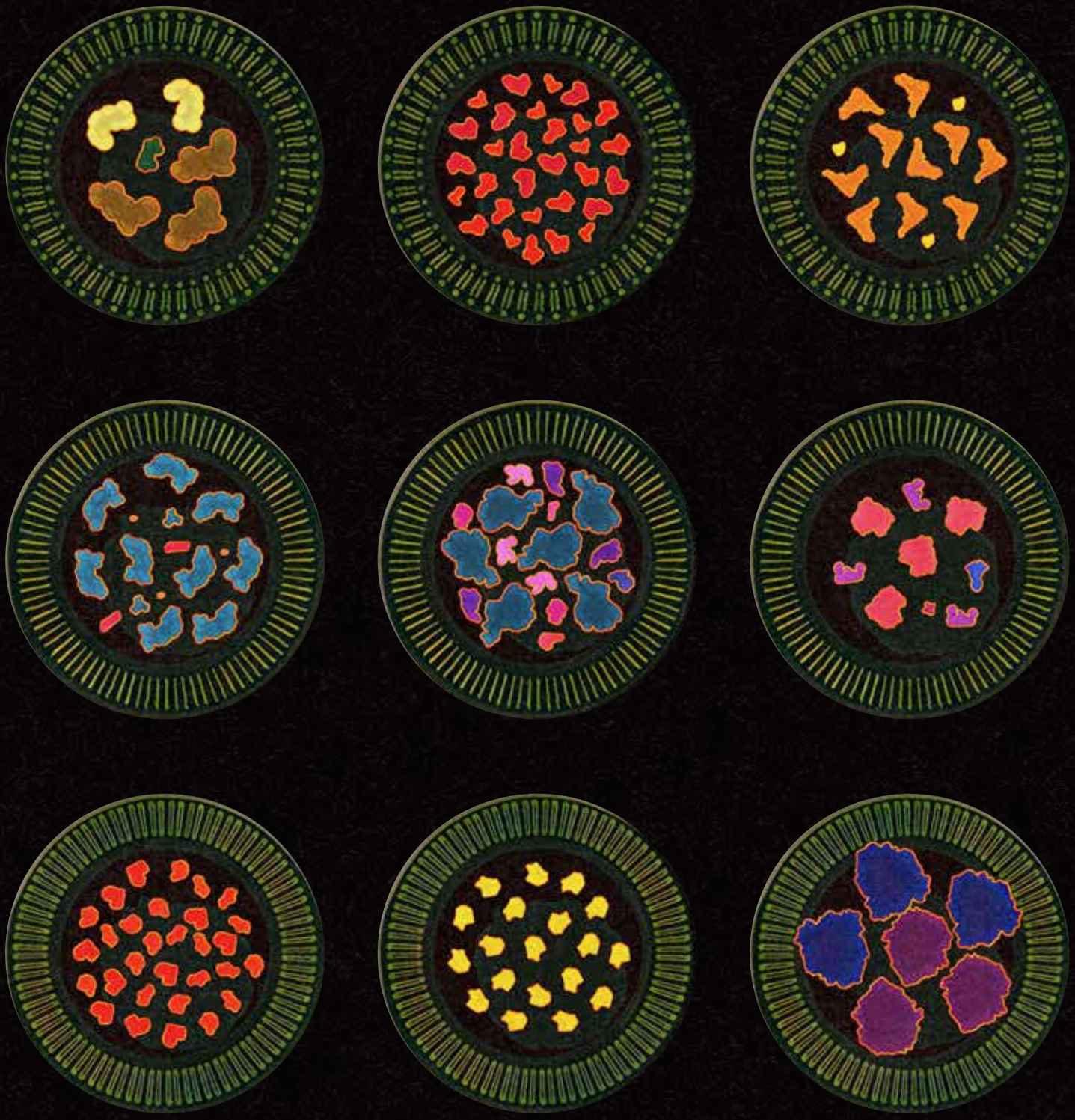
NUCLEUS | PURE



BOOK OF PROTOCOLS

Nucleus PURE Workshop Protocols

Overview

This book contains modified versions of the open source Nucleus protocols that are needed to make your own OnePot PURE including Small Molecule Mix, Protein Mix, tRNAs, and Ribosomes. Complete versions of these protocols are available at https://nucleus.bnext.bio/pure-protocols. These protocols are living documents and are updated and expanded upon approximately every three months.

In this workshop, we will make the four components described above, perform component unit tests, optimize formulation, and use Nucleus Hub to analyze and share the results.

Credits

Many people have worked with b.next to help create these protocols.

Nucleus OnePot PURE Development Workshop:

- Charlie Newell (University College London)
- Evan Kalb (University of Minnesota)
- Matas Deveikis (Imperial College London)

With important contributions from:

- Drew Endy (Stanford University, FreeGenes)
- Elizabeth Strychalski (National Institute of Standards and Technology)
- Jenny Molloy (University of Cambridge, Open Bioeconomy Lab)
- Kate Adamala (University of Minnesota)

- Michael Booth (University College London)
- Miki Yun (Caltech)
- Richard Murray (Caltech)
- Paul Freemont (Imperial College London)
- Yan Zhang (Caltech)
- Yoshihiro Shimizu (RIKEN)
- Zoila Jurado (Caltech)

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- Astera Institute
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- Schmidt Sciences

OnePot PURE was originally developed by Barbora Lavickova in the Maerkl Lab at EPFL:

- Lavickova, B. & Maerkl, S. J. A Simple, Robust, and Low-Cost Method To Produce the PURE Cell-Free System. ACS Synth. Biol. 8, 455–462 (2019).

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Day 1

1. Protein Mix - Grow and Induce Expression Strains
2. Energy Mix - Make Amino Acid Mix
3. Energy Mix - Make Small Molecule Mix
4. Mesaure - Small Molecule Mix

Protein Mix - Grow and Induce Expression Strains

Overview

This protocol has been established to grow and induce expression strains for producing OnePot PURE proteins following Lavickova's OnePot PURE protocol [Grasemann, 2021] with a few deviations.

At your bench

Item	Quantity	Temperature	Location	Notes
Deep well plate with starter culture (35 strains -EF-Tu)	1	37C	Bench	This is prepared the night before and corresponds to the first step of the protocol below
culture tube with EF-Tu	1	37C	Bench	Removed from incubator immediately before use
Reagent reservoir	1	-	Bench	
2 L baffle flask	1	-	Bench	
Terrific broth	500 mL	RT	Bench	Pre autoclaved
1 L centrifuge bottle	1	-	Bench	

Item	Quantity	Temperature	Location	Notes
Resealable plastic pouch	1	-	Bench	
Cuvette	5	-	Bench	
IPTG	1	4C	Shared 4C	Stored at -20C but thawed for use during the day
Biohazard waste	1	RT	Shared bench	

Protocol

- [ ] **Prepare starter cultures (night before)**
  - [ ] Working under flame, Prepare the media by mixing 20 mL LB with 20 uL of Kan (50 mg/mL).
  - [ ] In a deep-well plate, add 300 uL of media into 35 wells and inoculate each well with the 35 PURE proteins (omitting EF-Tu). Seal the plate with a breathable membrane and incubate at 37C / 260 rpm / overnight.
  - [ ] For EF-Tu, inoculate 3 mL of LB + Kan media in a 14 mL cell culture tube and incubate at 37C/ 260 rpm/ (12 - 16) hrs.
- [ ] **Inoculate Co-culture**
  - [ ] (Optional) Confirm all strains have grown by measuring the OD600 on a 96-well plate with 10x dilutions or by visually confirming turbidity.
  - [ ] Transfer 500 mL LB + Kan into baffled 2L Erlenmeyer flask and inoculate with the starter cultures:
    - [ ] 5 mL total inoculation volume (~140x back-dilution).
      - [ ] 2.33 mL of EF-Tu culture (~300x back-dilution).
      - [ ] 75 uL of each culture from deep-well plate into reagent reservoir.
- [ ] **Outgrowth & Harvest**
  - [ ] Thaw IPTG at 4C.

- [ ] Incubate culture at 37C/ 260 rpm/ ~2.5 hours or until the OD600 reaches 0.6-0.8.
    - [ ] At +2 hours, measure OD600 from 1 mL sample in a cuvette. Repeat every 20 minutes until OD600 reaches target.
    - [ ] Mark inoculation time.
- [ ] Induce protein expression with 500 uL of 0.5 M IPTG and incubate at 37C / 260 rpm / 2.5 hours. Mark induction time.
- [ ] Harvest the cells via centrifugation at 16,000 x g / 4C / 15 minutes.
    - [ ] Tare plastic bag on a coarse scale.
    - [ ] Decant supernatant into biohazardous waste.
    - [ ] Scoop cell pellet (”cellet”) into pre weighed ziploc bag. Weigh and record mass on bag.
    - [ ] (Optional) flash freeze plastic bag in liquid nitrogen.
- [ ] **Storage**
    - [ ] **Store the cellet at -80C.**

Notes

Use this page to record any important details and reflections

Energy Mix - Make Amino Acid Mix

Overview

You want to make PURE, which will need amino acids. You will need to make Amino Acid Mix. Here, we make stock solutions for each amino acid, plus an equimolar (3.25 mM) master mix of amino acids for use in downstream protocols.

Resource

Nucleus_v0.3.0_AA_worksheet.xlsx

At your bench

Item	Quantity	Temperature	Location	Notes
19x amino acid stocks (-tyrosine)	1	-20C	Shared -20C	These are prepared before hand
0.22 um syringe filter	1	-	Bench	
10 mL plastic syringe	1	-	Bench	

Protocol

- [ ] **Plan experiment with Amino Acid Mix Worksheet**

- [ ] Using the included Amino Acid Mix (”AA Mix”) worksheet at the top of this page, determine the minimum mass you will need for each amino acid given how much amino acid mix you want to make.

- [ ] **Weight each amino acid except tyrosine**
  - [ ] For each amino acid (except Tyrosine):
    - [ ] tare a microcentrifuge tube (1.5 mL) on an analytical balance.
    - [ ] add at least the minimal mass you determined previously.
    - [ ] input the actual mass weighed in the worksheet.

- [ ] **Determine resuspension volumes**
  - [ ] For each amino acid (except Tyrosine), resuspend in Ultrapure water (18.2 MOhm, e.g., Milli-Q) with "calculated resuspension volume" in the worksheet.

▼ **Tips: vortex, heat, and/or sonciate stocks to aid resuspension**

Some amino acids are less soluble than others. Don’t fret! Use a vortexer to resuspend these insoluble amino acids. Additionally, you can use moderate heat (e.g., 65C) to aid in resuspension.

- [ ] **For Tyrosine**
  - [ ] Tare a small weigh boat on an analytical balance.
  - [ ] Add at least the minimal mass you determined previously.
  - [ ] input the actual mass weighed. Note how much of each amino acid stock (except for Tyrosine) that you will require given how much Tyrosine you have weighed and confirm that you have enough.
  - [ ] Transfer Tyrosine to a 15 mL conical tube.

▼ **Note: Tyrosine is not very soluble in water.**

Tyrosine (Tyr) is the least soluble amino acid, and therefore, a larger volume is required to solubilize tyrosine. To avoid making a concentrated Tyrosine stock, we dissolve Tyrosine directly AA Mix -Tyr to its target concentration.

  - [ ] Add Ultrapure water to amino acid mix to reach the target final volume. Resuspend Tyrosine, with heat and/or vortexing as needed.

- [ ] **Filter the final mixture**
    - [ ] Filter AA Mix with a syringe filter (0.22 um).
    - [ ] Aliquot AA Mix into 1.5 mL microcentrifuge tubes (between 200 uL and 500 uL per aliquot).
- [ ] **Storage**
    - [ ] Return amino acid stock solutions to -20C.
    - [ ] Store 1 mL AA Mix aliquots at -20C.

Notes

Use this page to record any important details and reflections

Energy Mix - Make Small Molecule Mix

Overview

You want to make PURE. You'll need Energy Mix, the combination thirty-one (31) small molecules (*e.g.*, rNTPs, amino acids, buffering salts, etc.) and tRNAs (from *E. coli*) needed to fuel transcription and translation. We refer to Energy Mix that does not contain tRNAs as Small Molecule Mix.

Here, we describe the components of SM Mix, how to make it, and how to store it properly. This SM Mix is magnesium depleted, alloing you to fine tune the concentration against your other PURE components made in later steps.

At your bench

Item	Quantity	Temperature	Location	Notes
HEPES-KOH (1 M, pH 7.6)	1× 50-100 uL aliquot	-20C	Shared -20C	Prepared before hand
Potassium glutamate (2.5 M)	1× 50-100 uL aliquot	-20C	Shared -20C	Prepared before hand
Magnesium acetate (1 M)	1× 50-100 uL aliquot	-20C	Shared -20C	Prepared before hand
rNTP mix	1× 50-100 uL aliquot	-20C	Shared -20C	Prepared before hand; contains rATP, rGTP at 100 mM; rCTP, rUTP at 50 mM)

Item	Quantity	Temperature	Location	Notes
Creatine phosphate (1 M)	1× 50-100 uL aliquot	-20C	Shared -20C	
TCEP-HCl (500 mM, pH 7)	1× 50-100 uL aliquot	RT	Bench	Prepared before hand
Folinic acid (5 mM)	1× 50-100 uL aliquot	-20C	Shared -20C	Prepared before hand
Spermidine (200 mM)	1× 50-100 uL aliquot	-20C	Shared -20C	Prepared before hand
Ultrapure water	1× 50 mL aliquot	RT	Bench	

Protocol

☐ Prepare Folinic Acid Stock (5 mM)

- For more information please see our developer note: An Unexpected Enzyme in PURE: Why Folinic Acid Needs Extra Help

☐ Weigh 12.5 mg folinic acid.

☐ Dissolve to a final volume of 4.89 mL.

☐ Aliquot and freeze at -20C.

☐ Combine SM Mix components

Component	Stock concentration (mM)	Concentration of components in reaction (mM)	Concentration in small molecule solution (mM)	Final volume to add (µL)
HEPES	1000	50	166.67	33.3
Potassium glutamate	2500	100	333.33	26.7
Magnesium acetate	1000	6	20.00	4.0

Component	Stock concentration (mM)	Concentration of components in reaction (mM)	Concentration in small molecule solution (mM)	Final volume to add (µL)
rATP	100	2	6.67	13.3
rGTP	100	2	6.67	
rCTP	50	1	3.33	
rUTP	50	1	3.33	
Creatine phosphate	1000	20	66.67	13.3
TCEP	500	1	3.33	1.3
Folinic acid	5	0.02	0.07	2.7
Spermidine	200	2	6.67	6.7
Amino Acid solution	3.25	0.3	1.00	61.5
tRNA	40	3.5	11.67	**omitted**
Water				37.1
Total Volume				**200**

- [ ] **Storage**
  - [ ] Aliquot SM Mix into 4× 50 uL volumes in 1.5 mL microfuge tubes and store at -80C.

Notes

Use this page to record any important details and reflections

Measure - Small Molecule Mix

Overview

The goal of this experiment is to quickly check if your SM Mix is functional. We are intentionally adding less Magnesium (as Mg(OAc)2, 40 mM) now so that we can optimize it's concentration in a later experiment for your particular preparations of PURE components (we recommend this practice generally for each new component prep). Today, we will add a Magnesium supplement to ensure that your PURE reaction works.

PURE reaction setup

Component	Experiment (uL)	NEB SM control (uL)	NEB +DNA (uL)	NEB -DNA (uL)
NEB Sol A -AA -tRNA		5		
NEB AA		2.5		
NEB tRNA	2.5	2.5		
Workshop SMS	7.5			
Mg(OAc)2 supplement (40 mM)	2.5			
NEB Sol A			10	10
NEB Sol B	7.5	7.5	7.5	7.5
RNase Inhibitor	1.25	1.25	1.25	1.25
pT7-plamGFP plasmid (120 ng/uL)	1.25	1.25	1.25	0
Ultrapure	2.5	5	5	6.25
Total	**25**	**25**	**25**	**25**

Protocol

- [ ] **Assemble PURE reactions**
  - [ ] Gather reagents from the table.
  - [ ] Thaw reagents on ice.
  - [ ] Prepare a PCR strip in a strip holder, on ice, to assemble reactions into.
  - [ ] For each reaction, assemble by adding the volume of reagents from the table in the order listed:
    - [ ] Vortex Solution A aggressively (≥ 1 min, 10s on/10s on ice). Spin down, pipette mix, and add to the reaction tubes.
    - [ ] Briefly spin down Solution B, *gently* pipette mix, and add to the reaction tubes. **Do not vortex.**
    - [ ] Add RNase Inhibitor to the reaction tubes.
    - [ ] Vortext and spin Mg(OAc)2 supplement (40 mM) and add to the reaction tube.
    - [ ] Vortex and spin `pT7-plamGFP` plasmid (120 ng / uL), pipette mix, and add to the reaction tubes.
  - [ ] Close lids on the PCR tubes and briefly spin down to eliminate bubbles.
  - [ ] Hold assembled reactions on ice until ready for measurement.
  - [ ] Array onto black 384-well optical plate and take note of your plate map.
  - [ ] Measure.
- [ ] **Return reagents to their appropriate storage locations.**
  - [ ] Add a black dot to the lid of each of PURE Solution A and B. The number of dots indicates freeze–thaw cycles.

Notes

Use this page to record any important details and reflections

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Day 2

1. Protein Mix - Lyse Bacteria and Purify Proteins

2. Mesaure - Protein Mix

Protein Mix - Lyse Bacteria and Purify Proteins

Overview

This protocol has been established to lyse bacteria and purify the PURE proteins following Lavickova's OnePot PURE protocol [Grasemann, 2021] with a few deviations.

At your bench

Item	Quantity	Temperature	Location	Notes
Lysis Buffer	30 mL	RT	Bench	Prepared beforehand
Wash Buffer	50 mL	RT	Bench	Prepared beforehand
Elution Buffer	7.5 mL	RT	Bench	Prepared beforehand
Dialysis Buffer	50 mL	RT	Bench	Prepared beforehand
Storage Supplement Buffer (60% glycerol)	10 mL	RT	Bench	Prepared beforehand
TCEP-HCl (500 mM, pH 7)	400 uL	RT	Bench	
Protease inhibitor tablet	1/4	4C	Shared 4C	
PD-10 gravity column	1		Bench	

Item	Quantity	Temperature	Location	Notes
3 kDa MWCO, 15 mL centrifugal filter	1		Bench	
3 kDa MWCO, 4 mL centrifugal filter	1		Bench	
3 kDa MWCO, 0.5 mL centrifugal filter	1		Bench	
10 mL syringe	2		Bench	
0.22 um syringe filter	2		Bench	
Flowthrough catch	1		Bench	
Protein low bind microfuge tubes			Shared bench	
Temperature probe			Shared bench	

Protocol

- [ ] **Finish Buffers**
  - [ ] Add TCEP (0.5M, 500x) to finish Lysis Buffer, Wash Buffer, Elution Buffer, Dialysis Buffer, and Storage Supplement Buffer. E.g., 50 mL Wash Buffer requires 100 uL TCEP.
  - [ ] Resuspend one protease inhibitor tablet per four protein preps in 4 mL Lysis Buffer in a 15 mL conical tube. Resuspend by vortexing vigorously. Add one quarter of the resuspended protease inhibitor mixture (1 mL) to your tube of Lysis Buffer and mix by inversion or vortexing.
- [ ] **Cell lysis**

- [ ] Resuspend cellet in its plastic bag with 10 mL Protein Wash Buffer. Massage cellet until suspended.
- [ ] Transfer resuspended to 15 mL conical tube and keep on ice.
- [ ] Lyse cells by sonication using a 130-watt probe sonicator (6 mm diameter probe). Set sonicator to 25% amplitude/ 15 seconds on/30 seconds off. Sonicate samples on ice for 2 minutes on-time. It may take 2-4 cycles to sufficiently lyse cells. Do NOT let the sample heat up. If so, allow the sample to cool on ice before continuing sonication.
- [ ] Clarify lysate by centrifugation at 16,000 x g/ 4C/ 20 min.
- [ ] Use a 10 mL syringe with 0.22 um syringe filter to filter the supernatant into a fresh 15 mL tube.
- [ ] Discard pellet in biohazardous waste.

▼ **Note: Visually inspect pellet for lysis**

White material in pellet indicates lysed cell mass. A sufficiently lysed pellet should appear smaller than the original pellet, lighter in color, and have a translucent ring around the pellet. Note if you see black material at the base of the pellet. This indicates oversonication.

- [ ] **Column Prep**
  - [ ] Assemble columns.
    - [ ] Cut tips off of columns off using a razor blade.
    - [ ] Pack a filter into the bottom of the column (we use the back end of a cell spreader).

▼ **Notes: columns have ridges!**

The filter needs to be pushed across two circular ridges on the inside of the column, one ~2 cm from the opening of the column and another ~5 cm further down the column

    - [ ] Assemble buffer reservoir to column.
  - [ ] Wash empty columns.

- [ ] Put column assembly on your column holder over your flowthrough catch.
- [ ] Wash column, buffer reservoir, and filter with ≥ 10CV (20 mL) Ultrapure water. Discard flowthrough.

▼ **Notes: “CV” = Column Volume**

One “Column Volume” is equal to the volume of affinity resin in your column. For example, “wash with 5 CV of Wash Buffer” means that for 2 mL of resin, wash with 10 mL of Wash Buffer. Specifying buffer volumes by CVs allows practitioners to increase or decrease the scale of the purification and still use the same protocol.

- [ ] Load and Equilibrate Ni2+ affinity Resin into columns.
  - [ ] Resuspend Ni2+ resin (50% v/v suspension) by shaking.
  - [ ] Add 2 mL of resuspended resin by pipette to the column.
  - [ ] Wash column with ≥ 10 CV (20 mL) mL Ultrapure water. Discard flowthrough.
  - [ ] Equilibrate columns with ≥ 10 CV (20 mL) of Wash Buffer (4C). Discard flowthrough.
- [ ] **(*Optional*)** store Columns.
  - [ ] Seal each column with a cap and bung.
  - [ ] Store columns at 4C for up to 48 hrs.

▼ **Notes: store columns long term in EtOH (20%)**

Packed and loaded columns can harbor bacterial growth, even at 4C. If you are not going to use your columns within 48 hours of preparation, or if you want to save your used columns for later reuse, store them in EtOH (20%). Wash the columns as described above in Wash Buffer, then wash with 10 CV Ultrapure water to remove salts. Fill the gravity column with EtOH (20%), seal bung and lid with parafilm, and store at 4C until ready for use.

- [ ] **Gravity Column Ni-resin Purification**
  - [ ] Add bung to gravity column to prevent flowthrough.

- [ ] Add clarified lysate (supernatant) to the equilibrated column.
- [ ] Seal top of column with cap.
- [ ] Incubate at 4C / 1hr with rotation.
- [ ] Mount column on a stand with a beaker below to capture flow through.
- [ ] Remove bung and let the flow through elute off the column.
- [ ] Wash column with 12.5 CV (25 mL) of Wash buffer and discard flow though.
- [ ] Elute sample with 2.5 CV (5 mL) Elution Buffer and capture eluent in 15 mL tube. This is your sample.

- [ ] **Buffer Exchange by diafiltration**
  - [ ] Dilute your eluent (5 mL) with 10 mL Dialysis Buffer (3x dilution) and transfer to a 15 mL centrifugal filter.
  - [ ] Spin samples at 4000 g/ 4C / 1 hr. Check your samples frequently (~20 min) and note the volume. Target ≤ 500 uL.
  - [ ] Dilute your sample with 15 mL fresh Dialysis Buffer (≥90x dilution) and spin down again to ≤ 500 uL.
  - [ ] (Optional) dilute and spin your samples again with 15 mL Dialysis Buffer (≥270x dilution).

- [ ] **Concentrate buffer exchanges proteins**
  - [ ] Pipette mix the sample in the spin filter to resuspend any proteins that have collected on the filter.
  - [ ] Check the concentration of your Protein Mix by A280. Calculate your estimated final volume to target (25 - 30) ug / uL.

    ▼ **Note: A280 overestimates protein concentration**

    PURE uses Protein Mix at 20 ug / uL. We will use Pierce660, a robust and accurate colormetric assay, to do precise quantitation of your protein concentration at a later step. Here, we just want a quick estimate of the concentration at this step.

A fast A280 measurement can be made with a microvolume spectrophotometer, however the resulting estimate is biased. It is easier to dilute samples than concentrate them, therefore we will aim to overconcentrate our proteins today and dilute them later.

☐ Transfer your samples to a smaller centrifugal filter (4 mL or 0.5 mL, depending on your target concentration) and spin to your target concentration.

☐ Spin your sample down to ~20 ug / uL.

☐ Measure your sample volume by reverse pipetting.

☐ Add an equal volume of Storage Supplement Buffer (60% glycerol) and spin until you reach your target concentration. Verify your concentration by A280.

▼ **Note: Proteins “crash out” at high concentrations**

Concentrating protein samples above 30 ug / uL can cause proteins to aggregate and precipitate out of solution. To make protein stocks in 30% glycerol at 20 ug / uL, you would need to concentrate them first to 60 ug / uL, then add an equal volume of Storage Supplement. This is impractical.

As a result, we add Storage Supplement before reaching our target concentration. However, high viscosity buffers (e.g., 30% glycerol) greatly slow down centrifugal filtration. Thus, we add storage supplement *just* before proteins crash out, and finish concentrating in their final buffer.

☐ **Storage**

☐ Transfer proteins to Protein low bind microfuge tubes.

☐ Store at -80C.

Notes

Use this page to record any important details and reflections

Measure - Protein Mix

Overview

The goal of this experiment is to quickly check if your Protein Mix is functional by running PURE reactions with it. We will compare the activity of your Protein Mix to a control protein mix, supplementing each with Energy Mix and Ribosomes.

PURE reaction setup

Component	Experiment (uL)	NEB Protein control (uL)	NEB +DNA (uL)	NEB -DNA (uL)
NEB Sol B -Ribos		3		
Workshop Protein Mix (___ ug/uL)	3			
NEB Ribosomes	4.5	4.5		
NEB Sol A	10	10	10	10
NEB Sol B			7.5	7.5
RNse Inhibitor	1.25	1.25	1.25	1.25
pT7-plamGFP plasmid (120 ng/uL)	1.25	1.25	1.25	
Ultrapure water	5	5	5	6.25
total	**25**	**25**	**25**	**25**

Protocol

- [ ] **Assemble PURE reactions**
  - [ ] Gather reagents from the table.

- [ ] Thaw reagents on ice.
- [ ] Prepare a PCR strip in a strip holder, on ice, to assemble reactions into.
- [ ] For each reaction, assemble by adding the volume of reagents from the table in the order listed:
    - [ ] Vortex Solution A aggressively (≥ 1 min, 10s on/10s on ice). Spin down, pipette mix, and add to the reaction tubes.
    - [ ] Briefly vortex and spin down Protein Mix and Solution B -Ribos. Add each to the reaction tubes as specified above.
    - [ ] Briefly spin down Ribosomes, *gently* pipette mix, and add to the reaction tubes. **Do not vortex.**
    - [ ] Add RNase Inhibitor to the reaction tubes.
    - [ ] Vortex and spin `pT7-plamGFP` plasmid (120 ng / uL), pipette mix, and add to the reaction tubes.
    - [ ] Add Ultrapure water to the reaction tubes.
- [ ] Close lids on the PCR tubes and briefly spin down to eliminate bubbles.
- [ ] Hold assembled reactions on ice until ready for measurement.
- [ ] Array onto 384 well plate and take note of your plate map.
- [ ] Measure.

- [ ] **Return reagents to their appropriate storage locations.**
    - [ ] Add a black dot to the lid of each of PURE Solution A and B. The number of dots indicates freeze–thaw cycles.

Notes

Use this page to record any important details and reflections

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Day 3

1. Measure- Pierce660 Protein Quantification
2. Measure - Optimize Mg++ Concentration
3. DevNote - Analyze and Draft
4. Ribosomes - Purify and Ultracentrifuge

Measure - Pierce660 Protein Quantification

Overview

Pierce660 is a quick (5 min) colorimetric method for total protein quantitation. Compared to other quantitation assays, we find Pierce660 to be simple and reproducible, with a wide dynamic range (50-2000 ug/mL), and robust to buffer composition, including detergents and reducing agents. Prepare a standard curve within the assay's working range (125 ug / mL to 2000 ug / mL). Remember to dilute the BSA stock in the same buffer used for your sample. The standards can be stored at -20C for future assays.

At your bench

Item	Quantity	Temperature	Location	Notes
Strip tubes	1		Bench	
Pierce660 Reagent	1	RT	Shared bench	
Protein Storage Buffer	1 mL	4C	Shared 4C	
BSA standard	1x ampule	RT	Shared bench	
Clear 96-well optical plate	1		Shared bench	

Protocol

- [ ] **Make BSA standard curve**

☐ Prepare a stand curve in a strip tube from BSA stock solution according to the table below:

Concentration	Volume of BSA Stock (2 ug/uL)	Volume of Buffer
2 ug/uL	200 uL	0 uL
1.5 ug/uL	150 uL	50 uL
1 ug/uL	100 uL	100 uL
0.75 ug/uL	75 uL	125 uL
0.50 ug/uL	50 uL	150 uL
0.25 ug/uL	25 uL	175 uL
0.125 ug/uL	12.5 uL	187.5 uL
0 ug/uL	0	200 uL

☐ **Prepare a twofold (2x) dilution series of your samples in the same buffer**

☐ Array 20 uL of Protein Storage Buffer (30% glycerol) in all 8 tubes of a PCR strip tube

☐ Add 20 uL of your sample to the first tube and mix by pipetting.

☐ Transfer 20 uL of your diluted sample from Tube 1 to Tube 2 and mix by pipetting. Repeat through Tube 8.

▼ **Notes: only a few dillutions will be within the linear range**

Eight twofold dilutions ($2^8 \sim 100\text{x}$) fit on a single column of a 96-well plate for each sample and tend to give us at least 3 dilutions in the linear range of the assay.

☐ **Pierce 660 Assay**

☐ Mix Pierce660 Reagent well by inverting the bottle before use.

☐ Array 150 uL of Pierce660 Reagent on a clear 96-well optical plate.

☐ Add 10 uL of each sample (BSA standard series and sample dilution series) column-wise (e.g., BSA standard in Column 12, Sample 1 dilution series in column 1, ...) to the optical plate.

▼ **Notes: adjust your sample volume as needed**

You can adjust your sample volume as needed, as long as you adjust the volume of Pierce660 to keep the ratio of Pierce660 reagent to sample at 15:1. We have shown that 75 uL of Pierce660 reagent and 5 uL of sample works well.

- [ ] Cover your plate with aluminum foil and mix on a plate shaker at medium speed for 1 minute.
- [ ] Incubate your plate at 25C / 5 min. Samples should turn from brown to green.
- [ ] Using a plate reader, measure the absorbance of the samples at 660 nm.

- [ ] **Analyze results**
  - [ ] Subtract the absorbance of blank samples (i.e., BSA standard = 0 ug / uL) from all other samples (”background subtracted absorbance”).
  - [ ] Plot the standard curve by plotting the background subtracted absorbance vs. concentration for each BSA standard. Fit a line to your standard curve.
  - [ ] For each sample dilution series, choose a well with a background subtracted absorbance in the linear range of the standard curve.
  - [ ] Using the linear fit of your standard curve, calculate the concentration of the sample.

Notes

Use this page to record any important details and reflections

Measure - Optimize Mg++ Concentration

Overview

The goal of this experiment is to measure how sensitive *your* protein mix is to magnesium concentration and identify an optimal value for expression. These experiments will use b.next ribosomes and tRNA because they are prepared using Nucleus protocols that are designed to interoperate with your protein mix. We will include controls for these components.

PURE reactions overview

Master mix

Sample	Exp (8x) (uL per reaction)
Workshop SMS	3
b.next tRNA (40 ug/uL)	1
Workshop Protein Mix (___ ug/uL)	1.2
b.next Ribosomes (10 uM)	1.8
Mg Supplement	1
RNse Inhibitor	0.5
DNApT7-plamGFP plasmid (120 ng/uL)	0.5
Ultrapure water	1
Total	10

Controls

NOTE: we are running controls in duplicate (2x). The build volumes below will give 25 uL PURE assemblies, which can be split into two wells.

Component	b.next tRNA Control (uL)	b.next Ribosome Control (uL)	NEB +DNA (uL)	NEB -DNA (uL)
NEB Sol A -tRNA -AA	5			
NEB AA	2.5			
b.next tRNA (40 ug/uL)	2.5			
NEB Sol B -Ribos		3		
b.next Ribosomes (10 uM)		4.5		
NEB Sol A		10	10	10
NEB Sol B	7.5		7.5	7.5
RNase Inhibitor	1.25	1.25	1.25	1.25
DNApT7-plamGFP plasmid (120 ng/uL)	1.25	1.25	1.25	
Ultrapure water	5	5	5	6.25
Total	25	25	25	25

Protocol

PURE reaction set up

- [ ] Gather reagents from the table.
- [ ] Thaw reagents on ice.
- [ ] Make Magnesium supplement from Magnesium Acetate (1M) stock solution.
  - [ ] Working on ice, prepare the following wells in a PCR strip tube:

Tube (#)	[Mg++] additional (mM)	Vol Mg(OAc)2 (uL)	Vol Ultrapure water (uL)
1	0	0	200

Tube (#)	[Mg++] additional (mM)	Vol Mg(OAc)2 (uL)	Vol Ultrapure water (uL)
2	0.5	1	199
3	1	2	198
4	1.5	3	197
5	2	4	196
6	2.5	5	195
7	3	6	194
8	3.5	7	193

☐ Prepare Magnesium Sweep Master Mix (”MM”): working on ice, add each component listed following table at the specified volume in a single PCR tube.

Component	Exp Master Mix (uL)
Workshop SM Mix	27
b.next tRNA	9
Workshop Protein Mix	10.8
b.next Ribosomes (10 mM)	16.2
Mg Supplement	**omit**
RNAse Inhibitor	4.5
DNA	4.5
Ultrapure water	9
Total	**81**

☐ Vortex SM Mix aggressively (≥ 1 min, 10s on/10s on ice). Spin down, pipette mix, and add to the master mix.

☐ Briefly vortex and spin down tRNA and Protein Mix. Add each to the master mix as specified above.

☐ Briefly spin down Ribosomes, *gently* pipette mix, and add to the master mix. **Do not vortex.**

☐ Add RNase Inhibitor to the master mix.

- [ ] Vortex and spin `pT7-plamGFP` plasmid (120 ng / uL), pipette mix, and add to the master mix.
- [ ] Add Ultrapure water to the reaction tubes.

- [ ] **Assemble final reactions for the Magnesium Sweep**
  - [ ] Aliquot 9 uL of master mix into each tube of a PCR strip tube. These will be your reaction tubes.
  - [ ] Add 1 uL of each Magnesium Supplement the corresponding reaction tube.

- [ ] Using a multichannel pipette, mix your samples by pipetting and array onto a column of a black 384-well optical plate. Note which column has your reactions.
- [ ] Measure.
- [ ] Return reagents to their appropriate storage locations.
  - [ ] Add a black dot to the lid of each of PURE Solution A and B. The number of dots indicates freeze–thaw cycles.

Notes

Use this page to record any important details and reflections

DevNote - Analyze and Draft

Overview

This protocol will introduce you to Nucleus Hub which provides easy access to the b.next Cell Development Kit (CDK). These tools will allow you to use a standardized software workflow to analyze experimental data. The data can be quickly incorporated into a DevNote, enabling you to structure research artifacts associated with an experiment and share them with the community.

Protocol

Intro to Collaboration Hub

- [ ] **Getting started**
  - [ ] Navigate to workshop.nucleus.engineering.
  - [ ] Create an account (requires admin approval).
- [ ] **Setting up your DevNote**
  - [ ] In the File Directory (left panel), navigate to the `/devnotes/` directory and make a copy of the `/template/` directory and rename it.
  - [ ] Inspect the output of the directory. In the Launcher Window (Main Panel), click Curvenote Preview and then select your renamed template file.
  - [ ] Change the title and author of your DevNote by opening the file `curvenote.yml` from the File Directory and modify the corresponding fields:

```
# Ensure your title is the same as in your `main.md`
  title: An Unexpected Enzyme in PURE
  subtitle: The enzyme MTHFS is needed for 36 pot PURE
  description:
```

```
# Add any supporting files to the toc, but ensure that your main manuscript
# is first is the list. The title field is optional.
# Authors should have affiliations, emails and ORCIDs if available
  authors:
    - name: Sam Cell
      email: samcell@bnext.bio
      orcid: 0000-0001-1234-1234
      affiliations:
        - b.next
```

▼ **Note: a complete Curvenote writing guide is available online**

Curvenote writing guide: https://curvenote.com/docs/write

☐ Verify that the changes have been made by clicking Curvenote Preview.

☐ Edit your DevNote by making changes to `main.md`. Refer to https://mystmd.org/guide for a comprehensive guide on MyST MD syntax.

☐ **Analyze platereader data**

☐ Navigate to the `/shared/` directory and find the data corresponding to your experiment.

☐ Copy your experimental directory into `devnotes/my-devnote/experiments/`.

☐ In your current directory, open a Launcher Window by creating a new tab ("+" button) from the main panel. Click the Template icon, select `/analysis/platereader.ipynb`, and click GO.

☐ Link this notebook to your DevNote by including a reference to it in your `curvenote.yml` file as follows:

```
# Add any supporting files to the toc, but ensure that your main manuscript
# is first is the list. The title field is optional.
  toc:
    - file: main.md
    - file: ./experiments/experiment-01/my-platereader.ipynb
      title: Analysis Notebook
```

- [ ] In the newly created Jupyter Notebook, adjust the paths to your platemap and data file respectively.
- [ ] Run the notebook cells.
- [ ] The figure output of a cell can be referenced in two ways. We recommend using both.
  - [ ] Option 1: save the output of a cell to the `/analysis/` directory by adding the following code to the end of the cell:

    ```
    plt.savefig(./analysis/my-fig.png)
    ```

  - [ ] Option 2: label the cell by adding the following syntax to the top of the cell:

    ```
    #| label: fig:name-of-figure
    ```

    ▼ **Note: complete documentation of MyST Markdown is available online**

    MyST specification: https://mystmd.org/spec
- [ ] The table output of a cell can be referenced as follows:
  - [ ] Label the cell by adding the following syntax to the top of the cell:

    ```
    #| label: tbl:name-of-table
    ```

- [ ] **Analyze Pierce660 data**
  - [ ] Open the Launch Window and click the Template icon again. Select `/analysis/pierce660.ipynb`.
  - [ ] Adjust the paths to your platemap and data file respectively.
  - [ ] Run the notebook cells.
  - [ ] If desired, save the outputs as before.
- [ ] **Reference figures in `main.md`**

- [ ] A figure can be generated in two ways depending on how you want to reference it:
    - [ ] Option 1: reference the saved file:

```
:::{figure} ./experiments/experiment-01/my-fig.png
:name: name-of-fig
:align: center
:width: 50%

My caption
:::
```

    - [ ] Option 2: reference the output of a labelled jupyter cell:

```
:::{figure} #fig:name-of-figure
:kind: table
:name: name-of-fig
:width: 50%

My caption
:::
```

    - [ ] Multiple figures can be handled elegently by using tabs:

```
:::::{tab-set}

::::{tab-item} Tab Title 1
:sync: tab1
:::{figure} #fig:name-of-figure-1
:name: name-of-fig-1
:align: center
:width: 50%

My caption 1
:::
```

````
::::

::::{tab-item} Tab Title 2
:sync: tab2
:::{figure} ./experiments/experiment-01/my-fig-2.png
:name: name-of-fig-2
:align: center
:width: 50%

My caption 2
:::
::::

:::::
````

- [ ] A table output can be included from a labelled jupyter cell as follows:

````
:::{figure} #tbl:name-of-table
:kind: table
:name: name-of-table

My caption
:::
````

- [ ] Figures and tables can be referenced in documents using the following syntax:

````
This point is illustrated in {ref}`name-of-fig`.
````

- [ ] Verify that the changes have been made by clicking Curvenote Preview.

- [ ] **Submit Draft**

  - [ ] From the JupyterHub Launcher, click DevNote Draft Submit and follow the dialogue to view a preview of your DevNote from the publication server.

Notes

Use this page to record any important details and reflections

Ribosomes - Purify and Ultracentrifuge

Overview

Ribosomes are large complexes of RNA and proteins (MW ~2.7 MDa) that are the site of protein synthesis. Ribosomes coordinate the decoding of mRNA transcripts by tRNA and catalyze the formation of each peptide bond in new proteins, making them a key component of any protein synthesis system. Ribosomes can be purified from *E. coli* biomass by a variety of methods (e.g., His-tagged ribosomes can be purified by Ni-His chromatography, as in ✪ Purify Proteins by Ni2+ Gravity Column), but we recommend a two step process: (1) initial, tag-free purification by hydrophobic interaction chromatography (HIC) and (2) size-selective precipitation by ultracentrifugation.

This protocol will show you how to perform sucrose cushion ultracentrifugation of FPLC-purified *E. coli* A19 (HIC). Note: this protocol is heavily abridged. A complete version is available at https://nucleus.bnext.bio/pure-protocols/make-ribosomes.

‼️All reagents and materials must be prepared RNase-free. Use RNaseZap or 10% bleach to decontaminate plastic and glassware and rinse with nuclease-free water. We find ultrapure water (18.2 MOhm) is often sufficient for RNase-free work.

At your bench

Item	Quantity	Temperature	Location	Notes
Ribosome eluent	35 mL		Bench	Prepared beforehand from FPLC

Item	Quantity	Temperature	Location	Notes
Ribosome Cushion Buffer	35 mL	RT	Bench	Prepared beforehand
70 mL polycarbonate ultracentrifuge tube	1		Bench	

Protocol

- [ ] **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 (Δm ≤ 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.

Notes

Use this page to record any important details and reflections

b.next

Day 4

1. Ribosomes - Resuspend and Concentrate
2. tRNA - Precipitations and Dialysis

Ribosomes - Resuspend and Concentrate

Overview

Ribosomes are large complexes of RNA and proteins (MW ~2.7 MDa) that are the site of protein synthesis. Ribosomes coordinate the decoding of mRNA transcripts by tRNA and catalyze the formation of each peptide bond in new proteins, making them a key component of any protein synthesis system. Ribosomes can be purified from *E. coli* biomass by a variety of methods (e.g., His-tagged ribosomes can be purified by Ni-His chromatography, as in ✪ Purify Proteins by Ni2+ Gravity Column), but we recommend a two step process: (1) initial, tag-free purification by hydrophobic interaction chromatography (HIC) and (2) size-selective precipitation by ultracentrifugation.

This protocol will show you how to resuspend your ribosome pellet and concentrate your sample for use in PURE reactions.

‼️All reagents and materials must be prepared RNase-free. Use RNaseZap or 10% bleach to decontaminate plastic and glassware and rinse with nuclease-free water. We find ultrapure water (18.2 MOhm) is often sufficient for RNase-free work.

At your bench

Item	Quantity	Temperature	Location	Notes
Ribosome Buffer	1 mL	4C	Shared 4C	
TCEP-HCl (0.5M, pH 7.0)	Reagent bottle	RT	Shared bench	
3 kDa MWCO, 0.5 mL centrifugal	1		Bench	

Item	Quantity	Temperature	Location	Notes
filters				
100kDA MWCO, 4 mL centrifugal filters	1		Bench	
Magnetic stir bar (3 × 10 mm)	1		Bench	
Quartz cuvette	1		Shared bench	

Protocol

- [ ] **Ultracentrifugation (continued)**
  - [ ] Finish Ribosome Buffer by adding 2 uL of TCEP (0.5 M). Vortex to mix.
  - [ ] Discard the supernatant. Carefully, wash each pellet with 0.5 mL cold ribosome buffer. Repeat this step twice.

    ▼ **Notes - Don’t disturb the ribosome pellet during washing**

    The ribosome pellet is fairly compact and stable, but some ribosomes can get resuspended during washing and be lost in the process. Be careful!

    First, find the pellet. Next, wash the pellet by gently pipetting Ribosome Buffer down the sides of the tube, allowing the buffer to run over the pellet. Tilt the ultracentrifuge bottle gently so that the buffer falls away from the pellet, aspirate the buffer, add fresh buffer as before, and repeat.
  - [ ] Resuspend the clear pellets in 100 uL 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 uL of Ribosome Buffer to resuspend any remaining ribosomes.
- [ ] **Quality Control**

- [ ] Determine the ribosome concentration by measuring the absorbance at 260 nm at a 100x dilution in Ribosome Buffer. 10 absorbance units from a 100x dilution corresponds to 23 uM of undiluted solution. Record your concentration.

- [ ] Dilute to final stock of 10 uM. 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.

- [ ] **Storage**

- [ ] Aliquot your ribosomes in 0.5 mL protein low bind microfuge tubes in 20 uL aliquots to reduce freeze / thaw cycles and store at -80C.

Notes

Use this page to record any important details and reflections

tRNA - Precipitations and Dialysis

Overview

Transfer RNAs (tRNAs) are small RNA molecules (76 - 90) nt that carry amino acids to ribosomes during protein synthesis. They are essential for translation. In this protocol, we will purify by precipitation and perform dialysis to prepare tRNAs for use in PURE reactions.

▼ ⚠️ Hazardous Materials: Acid Phenol

- Corrosive, toxic, rapidly absorbed through skin, & respiratory irritant
- Use in fume hood, wear two layers of gloves, & PPE (labcoat, goggles as necessary)

At your bench

Item	Quantity	Temperature	Location	Notes
A19 biomass	1 g	-80C	Bench	
tRNA extraction buffer	35 mL		Bench	
Acid phenol (pH ≤ 5.0)	Reagent bottle	4C	Fume hood	Long term storage at 4C
NaCl (5M)	2 mL	RT	Bench	
NaCl (1M)	20 mL	4C	Shared 4C	
Isopropanol	30 mL	RT	Bench	
Ethanol (70%)	35 mL	-20C	Shared -20C	
Ethanol (100%)	35 mL	-20C	Shared -20C	

Item	Quantity	Temperature	Location	Notes
NaOAc (300 mM, pH 5.0)	8 mL	RT	Bench	
Ultrapure water	50 mL	RT	Bench	
TCEP-HCl (500 mM, pH 7)	Reagent bottle	RT	Shared bench	
3.5 kDa MWCO, 3 mL dialysis cassette	1		Bench	
3.5 kDa MWCO 0.5 mL dialysis cassette	1		Bench	
10 mL glass serological pipette	1		Bench	
Parafilm	4		Bench	
Ultrapure water	1 L		Shared Bench	For dialysis
4 L bucket	1		Shared bench	

Protocol

- [ ] **First Nucleic Acid Extraction**
  - [ ] Set centrifuge to 4C and set shaking incubator to 37C.
  - [ ] Resuspend 2 g of biomass into 18 mL of Extraction Buffer in a 50 mL centrifuge tube by vortexing.
  - [ ] Add 18 mL of Acid Phenol (pH 4.5) using a glass serological pipette in a fume hood.
  - [ ] Cap the 50 mL centrifuge tube and seal with parafilm to prevent spillage.
  - [ ] Incubate at 37C / 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 / 4C / 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.

- [ ] **Second Nucleic Acid Extraction**
  - [ ] Add 14 mL of Extraction Buffer to Acid Phenol, parafilm the 50 mL centrifuge tube, and incubate at 37C / 225 rpm / 15 min.
  - [ ] Centrifuge at 4000 rcf / 4C / 15 min.
  - [ ] Collect the aqueous fraction and combine with the first nucleic acid extraction (total volume between 30 mL and 32 mL).

- [ ] **Precipitate Nucleic Acids (RNA & DNA)**
  - [ ] Set centrifuge to 25C.
  - [ ] 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 2× 50 mL centrifuge tubes.
  - [ ] Precipitate nucleic acids by adding one volume of isopropanol (~17 mL) to each tube and incubate at RT for 10 min. The mixture should turn cloudy.
  - [ ] Pellet nucleic acid precipitate via centrifugation at 14 500 rcf / 25C / 15 min.
  - [ ] Wash the pellet with EtOH (70%):
    - [ ] Decant supernatant and wash nucleic acid pellet with 10 mL cold (-20C) EtOH (70%). Break apart pellet by pipetting or vortexting to ensure salt is removed.
    - [ ] Re-pellet nucleic acid pellet by centrifugation at 14 500 rcf / 25C / 5 min.
    - [ ] Decant the supernatant and allow the pellet to air dry for 10 minutes.

- [ ] **Remove rRNA by precipitation**

- [ ] Resuspend each pellet into 15 mL of cold (4C) 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 RT to help the pellet dissolve.
- [ ] Precipitate rRNA by centrifugation at 9500 / 4C / 20 min.
- [ ] Decant the supernatant to a new 50 mL centrifuge tube.

- [ ] **Precipitate remaining DNA and tRNA nucleic acids**
  - [ ] Add 2 volumes (~30 mL) of cold (-20C) EtOH (100%) to the supernatant and incubate at -20C / >30 min to precipitate remaining nucleic acids. You can perform this step overnight.
  - [ ] Centrifuge at 14 500 rcf / 4C / 5 min.
  - [ ] Wash the pellet with EtOH (70%) as above.

- [ ] **Remove DNA by precipitation**
  - [ ] Set centrifuge to 25C.
  - [ ] 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 60C, 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 together, totaling 6 mL.
  - [ ] Add 0.56 volumes of isopropanol (~3.4 mL) to each nucleic acid solution and incubate at RT / 10 min.
  - [ ] Centrifuge at 14 500 rcf / RT / 5 min. Decant the supernatant to a 15 mL centrifuge tube.

- [ ] **Precipitate tRNAs**
  - [ ] Set the centrifuge to 4C.
  - [ ] Add 2.3 mL of isopropanol to the supernatant (supernatant:isopropanol is 100:95) and incubate at -20C / >30 min. This step can be performed overnight.
  - [ ] Centrifuge the suspension at 14 500 rcf / 4C / 15 min.
  - [ ] Wash the pellet with EtOH (70%) as above.
  - [ ] Resuspend tRNA pellet in 1.5 mL of nuclease-free water and keep on ice.

- [ ] **Dialyze tRNAs to remove salts**
    - [ ] Hydrate membrane: Remove the cassette from its protective pouch and hydrate the membrane by immersing the cassette in nuclease-free water for 2 minutes.
    - [ ] Add Sample:
        - [ ] Open the cassette by twisting the cap counter-clockwise (~45 degrees) and pull out the cap.
        - [ ] Carefully pipette 1.5 mL of resuspended tRNAs into the cassette without 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 4C / 45 min. We do this by putting our beaker in a bucket of ice on a stir plate.
        - [ ] Change the dialysis buffer and dialyze for another 45 min.

        ▼ **Notes - you can dialyze overnight.**

        Dialysis can be done overnight after changing the dialysis buffer at least once. We have found three dialysis steps of 45 minutes each is sufficient.

Notes

Use this page to record any important details and reflections

b.next

Day 5

1. tRNA - Concentrate
2. Measure - Assemble PURE Reactions
3. Measure - tRNA Gel
4. DevNote - Publish

tRNA - Concentrate

Overview

Transfer RNAs (tRNAs) are small RNA molecules (76 - 90) nt that carry amino acids to ribosomes during protein synthesis. By now, you have purified tRNAs and dialyzed them. In this protocol, we will prepare working stocks of your tRNAs (40 ug / uL) that can be directly added to PURE reactions by concentrating them and measuring their concentration by A260.

At your bench

Item	Quantity	Temperature	Location	Notes
3 kDa MWCO, 4 mL centrifugal filter	1		Bench	
DNA low bind tubes	4		Bench	
Quartz cuvette	1		Shared bench	

Protocol

- [ ] **Estimate the total tRNA in your sample by A260**
  - [ ] Prepare a 1:100 dilution of your tRNAs in water.
  - [ ] Estimate your concentration by A260 by microvolume photospectroscopy. The concentration of your undiluted sample is approximately 4 mg / mL per 1 unit of A260 absorbance of your 1:100 dilution.

- [ ] Calculate the total mass of tRNA in your sample. Aiming to concentrate your sample to 40 mg / mL, calculate your target volume.

- [ ] **Concentrate tRNAs by centrifugal filtration**
  - [ ] Pipette dialyzed tRNAs to the upper chamber of an 3 kDa MWCO, 4 mL centrifugal filter.
  - [ ] Centrifuge at 14 000 rcf / 4C / 10 min
  - [ ] Check the remaining volume in the upper chamber. Repeat until you hit your target volume.

- [ ] **Quality Control**
  - [ ] Prepare a 1:1000 dilution of your tRNAs in water.
  - [ ] Measure absorbance at 260 nm, 280 nm, and 230 nm using a quartz cuvette.
  - [ ] Estimate your concentration (approximately 40 mg / mL per 1 unit of A260 absorbance of your 1:1000 dilution). Record your concentration.

- [ ] **Storage**
  - [ ] Aliquot your tRNAs into DNA low bind tubes. Ensure each aliquot has ≥ 20 uL of tRNA.
  - [ ] Store your tRNAs at -80C.

Notes

Use this page to record any important details and reflections

Measure - Assemble PURE Reactions

Overview

The goal of this experiment is to quickly check if your PURE components work together. This PURE reaction will include a Magnesium Supplement with a concentration identified during your previous Magnesium Sweep. Based on previous unit testing, we will supplement non-functional components with validated references.

PURE reactions overview

Component	OnePot (uL)	NEB +DNA (uL)	NEB -DNA (uL)
Workshop SM Mix	7.5		
Mg Supplement (___ mM)	2.5		
Workshop tRNA (___ ug/uL)	2.5		
Workshop Protein Mix (___ ug/uL)	3		
Workshop Ribosome (___ mM)	4.5		
NEB Sol A		10	10
NEB Sol B		7.5	7.5
RNase Inhibitor	1.25	1.25	1.25
pT7-plamGFP plasmid (120 ng/uL)	1.25	1.25	
Ultrapure water	2.5	5	6.25
Total	**25**	**25**	**25**

Protocol

PURE reaction set up

- [ ] Make Magnesium Supplement.
    - [ ] Look at your Magnesium Sweep data. Identify the Magnesium Supplement concentration with which your Protein Mix had the highest activity. Note this concentration.
    - [ ] Make 200 uL of Magnesium Supplement at your previously identified optimal concentration.
        - [ ] Add X uL Mg(OAc)2 (1 mM) to (200 - X) uL ultrapure water.
        - [ ] Mix by pipette or vortexing.
- [ ] **Assemble PURE reactions**
    - [ ] Gather reagents from the table.
    - [ ] Thaw reagents on ice.
    - [ ] Prepare a PCR tube on ice to assemble reactions into.
    - [ ] For each reaction, assemble by adding the volume of reagents from the table in the order listed:
        - [ ] Vortex SM Mix aggressively (≥ 1 min, 10s on/10s on ice). Spin down, pipette mix, and add to your reaction tube.
        - [ ] Add Magnesium Supplement to the reaction tube as specified above
        - [ ] Briefly vortex and spin down Protein Mix. Add to the reaction tube.
        - [ ] Briefly spin down Ribosomes , *gently* pipette mix, and add to the reaction tube. **Do not vortex.**
        - [ ] Add RNase Inhibitor to the reaction tube.
        - [ ] Vortex and spin `pT7-plamGFP` plasmid (120 ng / uL), pipette mix, and add to the reaction tube.
        - [ ] Add Ultrapure water to the reaction tube.

- [ ] Close lids on the PCR tube and briefly spin down to eliminate bubbles.
- [ ] Hold assembled reactions on ice until ready for measurement.
- [ ] Add your experimental sample onto 384 well plate and take note of your plate map.
- [ ] Measure.

- [ ] **Return reagents to their appropriate storage locations.**
    - [ ] Add a black dot to the lid of each of PURE Solution A and B. The number of dots indicates freeze–thaw cycles.

Notes

Use this page to record any important details and reflections

Measure - tRNA Gel

Overview

The goal of this experiment is to determine the purity of your tRNA preps. Gel electrophoresis is a standard molecular biology assay that uses an electric field to push your sample through a gel, separating the components of that sample by migration rate. For samples that are composed of one type of molecule (e.g., tRNA preps), migration rate is determined predominantly by the molecular weight of the sample, allowing you to check your sample for contaminants that are above or below your desired weights. Pure samples should give distinct, bright bands. Here, we will use a gel to determine if your tRNA prep have contaminating gDNA or other RNAs (high weight bands) as well as if your prep has any degradation (low weight bands, or smeary bands at your target weight).

Item	Quantity	Temperature	Location	Notes
TBE-Urea 10% gel	1	4C	Shared 4C	
TBE Running Buffer	1L	RT	Shared Bench	
2x TBE-Urea sample buffer	1x bottle	4C	Shared 4C	
Low range ssRNA ladder	1x tube	-20C	Shared -20C	
Sybr Green	1x tube	-20C	Shared -20C	

Protocol

- [ ] **Visualize purity by TBE-Urea 10% Gel:**

- [ ] (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 or pipette. You should be able to see urea displaced from the well by change in refractive index.
- [ ] Prepare tRNA samples:
    - [ ] Dilute tRNAs to 40 ng / uL tRNA (~1000x) in nuclease-free water.
    - [ ] Prepare 20 ng / uL sample by adding 10 uL of 40 ng / uL tRNA to 10 uL 2x TBE-Urea sample buffer.
    - [ ] Prepare an ssRNA ladder. We use the NEB low range ssRNA (2 uL ladder + 2 uL 2x sample buffer).
    - [ ] Incubate sample and and ladder at 65C / 3 min → 4C / hold using a thermal cycler.
- [ ] Load 200 ng of tRNA (10 uL at 20 ng / uL) onto the TBE-Urea gel and run at 125V / 2.5 hr.
- [ ] Meanwhile, prepare SYBR-Green stain (4 uL 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.

▼ **Notes - tRNA degradation → smear on gel**

Your tRNA sample may degrade over time, either due to RNase contamination or due to self cleaving. A clear sign of tRNA degradation are poorly defined, smeared bands. A clean tRNA prep should have distinct bands.

Notes

Use this page to record any important details and reflections

DevNotes - Publish

Overview

You have now made your own OnePot PURE and characterized all of its components. Use Nucleus Hub to analyze the remaining data and record any experimental details that are important to understand your observations. This protocol show you how to submit a draft of your work.

Protocol

- [ ] **Analyze and write up data**
  - [ ] Use the CDK tools to analyze and generate figures from newly generated data.
  - [ ] Record thoughts and obersations in `main.md`.
- [ ] **Submit Draft!**
  - [ ] From the JupyterHub Launcher, click DevNote Draft Submit and follow the dialogue to view a preview of your DevNote from the publication server.

Notes

Use this page to record any important details and reflections