FINAL SCRIPT: APPROVED FOR FILMING

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Title: Refinement of OnePot PURE and Crude Ribosome Production for Reproducible Cell-Free Protein Synthesis

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

3. **Filming location:** Will the filming need to take place in multiple locations? **YES**

   Roger Land Building and Swann Building on Kings Buildings Campus (around 300m apart)

Current Protocol Length
Number of Steps: 18
Number of Shots: 35

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Nadanai Laohakunakorn:** We refined the OnePot PURE system and crude ribosome preparation to improve reproducibility and accessibility, enabling more labs to build and troubleshoot robust cell-free systems for synthetic biology research.

1.1.1. **INTERVIEW:** Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 1*

What are the current experimental challenges?

1.2. **Sahan Liyanagedera:** Experimental challenges include batch-to-batch variability, expression vector instability, and slow-growing strains, all of which hinder reproducibility and consistent performance in preparing in-house PURE systems.

1.2.1. **INTERVIEW:** Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What significant findings have you established in your field?

1.3. **Christoph Wagner:** We established that careful control of strain growth, inoculation, and glycerol concentration significantly improves reproducibility and yield in OnePot PURE and ribosome preparations across users and biological replicates.

1.3.1. **INTERVIEW:** Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 4*

What research gap are you addressing with your protocol?

1.4. **Sahan Liyanagedera:** We address the lack of a reproducible, accessible protocol for preparing PURE and ribosomes in-house, helping labs overcome technical barriers to adopt cell-free systems for synthetic biology research.

1.4.1. **INTERVIEW:** Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What advantage does your protocol offer compared to other techniques?

1.5. **Christoph Wagner:** Our protocol improves reproducibility and flexibility by standardising growth conditions and glycerol use, and streamlines ribosome preparation using only ultracentrifugation—avoiding more complex or costly purification methods.

1.5.1. **INTERVIEW:** Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Sahan Liyanagedera, Post Doctoral Researcher, University of Edinburgh**: (authors will present their testimonial statements live)

1.6.1. **INTERVIEW:** Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Sahan Liyanagedera, Post Doctoral Researcher, University of Edinburgh**: (authors will present their testimonial statements live)

1.7.1. **INTERVIEW:** Named talent says the statement above in an interview-style shot, looking slightly off-camera. NOTE: Not filmed

Protocol

2. Crude Ribosome Preparation

Demonstrator: Sahan Liyanagedera

2.1. To begin, thaw the frozen BL21 *(B-L-Twenty-One)* cell pellets on ice **[1]** and add 50 milliliters of Ribosome Buffer A into a clean conical tube **[2]**.

2.1.1. WIDE: Talent placing frozen cell pellet tubes on ice for thawing.

2.1.2. Talent pouring Ribosome Buffer A into a labeled, sterile 50-milliliter conical tube.

2.2. Using a pipette, take 100 microliters of Ribosome Buffer A from the conical tube and add it to a No-Weigh DTT *(D-T-T)* vial **[1]**. Aspirate gently until the DTT pellet is completely dissolved to create a 0.5 molar DTT stock solution **[2]**.

2.2.1. Talent pipetting Ribosome Buffer A and dispensing it into the No-Weigh DTT vial. NOTE: 2.2.1, 2.2.2 and 2.3.1 were filmed in one shot

2.2.2. Talent gently aspirating the solution until the DTT is fully dissolved.

2.3. Then, pipette 100 microliters of the 0.5 molar DTT stock solution back into the 50-milliliter conical tube **[1-TXT]** and place the tube on ice **[2]**.

2.3.1. Talent adding 100 microliters of DTT stock into the conical tube**. TXT: Final Concentration: 1 mM DTT in 50 mL Ribosome Buffer A**

2.3.2. Talent placing the conical tube back on ice.

2.4. Using a vortex mixer, resuspend each thawed cell pellet in Ribosome Buffer A with DTT at a ratio of 2 milliliters per 1 gram of cell pellet **[1]**. Combine the resuspended samples into a conical tube and check that the total slurry volume is 10 milliliters **[2]**.

2.4.1. Talent using vortex to thoroughly mix buffer into each thawed pellet.

2.4.2. Talent pouring all resuspended mixtures into a single conical tube, checking volume, and topping up if required.

2.5. Now, divide the 10-milliliter slurry equally into two separate 5-milliliter samples in labeled conical tubes **[1]**.

2.5.1. Talent measuring and transferring 5 milliliters of slurry into each of two clean

conical tubes.

2.6. Secure the conical tubes in a stand and lyse the cells using a cooled sonication probe in an ice water bath **[1]**. Set the sonicator to 30 percent amplitude with a cycle of 20 seconds on and 20 seconds off, for a total of 10 cycles **[2]**.

2.6.1. Talent placing tubes into a secured stand submerged in an ice bath and positioning the sonication probe. 2.6.1 + 2.6.2 were filmed in one shot

2.6.2. Talent operating the sonicator with the programmed settings for 10 cycles.

2.7. After sonication, top up each lysed sample to 25 milliliters using Ribosome Buffer A with DTT **[1]** and briefly vortex to mix **[2]**.

2.7.1. Talent pipetting additional Ribosome Buffer A with DTT into each sonicated sample.

2.7.2. Talent vortexing the tubes to mix the contents.

2.8. Transfer the 25-milliliter lysed samples into two pre-chilled S30 *(S-Thirty)* centrifugation tubes **[1]**. Place the tubes in a centrifuge and spin at 30,000 *g* for 1 hour at 4 degrees Celsius **[2]**.

2.8.1. Talent pouring each lysed sample into a cooled S30 centrifuge tube.

2.8.2. Talent loading tubes into the centrifuge and setting parameters to 30,000 g for 1 hour at 4 degrees Celsius.

2.9. Then, carefully recover up to 75 percent of the pellet-free supernatant from each S30 tube to prevent membrane contamination **[1]**. Transfer the recovered supernatant into two clean, precooled S30 tubes **[2]**.

2.9.1. Talent pipetting only the upper 75 percent of the supernatant from each centrifuged S30 tube. NOTE: 2.9.1 + 2.9.2 were filmed in one shot. Ignore take 2 of 2.9.1

2.9.2. Talent dispensing the recovered supernatant into new precooled S30 tubes.

2.10. After centrifuging the tubes again for 30 minutes, recover up to 75 percent of the pellet-free supernatant and transfer it into two clean 50-milliliter conical tubes **[1]**. Add Ribosome Buffer A with DTT to each tube until the volume reaches 25 milliliters **[2]**.

2.10.1. Talent pipetting supernatant from the S30 tubes and transferring it into clean 50-milliliter conical tubes.

2.10.2. Talent topping up each tube with Ribosome Buffer A plus DTT to reach the 25-milliliter mark.

2.11. Transfer the 25-milliliter samples into two clean, prechilled S100 *(S-One-Hundred)* ultracentrifuge tubes **[1]**. Load the tubes into the ultracentrifuge and spin at 100,000 *g* for 4 hours at 4 degrees Celsius **[2]**.

2.11.1. Talent pouring sample into S100 tubes on ice.

2.11.2. Talent placing S100 tubes in ultracentrifuge, setting to 100,000 g for 4 hours at 4 degrees Celsius.

2.12. Afterward, discard the supernatant **[1]**. Use a pipette to remove any residual buffer while working quickly to prevent drying of the pellet **[2]**.

2.12.1. Talent pouring off the supernatant from each S100 tube into a waste container.

2.12.2. Talent pipetting away the last traces of buffer from around the pellet.

2.13. Then, add 400 microliters of ice-cold Ribosome Buffer C with DTT into each S100 tube containing the ribosomal pellet **[1]**. Incubate the tubes on ice overnight in a cold room to allow the ribosome pellet to soak in the buffer **[2]**.

2.13.1. Talent pipetting cold Ribosome Buffer C plus DTT directly onto the ribosomal pellet in each tube.

2.13.2. Talent placing the tubes into a rack in the cold room.

2.14. The following day, use a 1-milliliter pipette and gentle swirling to fully resuspend the clear halo ribosome pellet in buffer **[1]**. Make sure only the clear halo is dissolved and avoid disturbing any yellow insoluble pellet **[2]**.

2.14.1. Talent using a 1-milliliter pipette to gently aspirate and swirl the solution around the ribosomal pellet.

2.14.2. Close-up of the tube showing only the clear halo being resuspended, leaving any yellow pellet untouched. NOTE: 2.14.2 take 2 is file 2Y8A7736.mp4

2.15. Then, transfer the resuspended ribosome solution into two sterile 2-milliliter microcentrifuge tubes **[1]**. Centrifuge the tubes at 20,000 *g* for 10 minutes at 4 degrees Celsius to remove insoluble material **[2]**. Carefully transfer the pellet-free supernatant into a clean microcentrifuge tube and keep it on ice **[3]**.

2.15.1. Talent pipetting ribosome solution into labeled 2-milliliter tubes.

2.15.2. Talent loading tubes into centrifuge and setting it to 20,000 g for 10 minutes at

4 degrees Celsius.

2.15.3. Talent transferring clear supernatant into a fresh microcentrifuge tube and placing it on ice.

3. Setting up CFPS Experiments

3.1. Thaw the necessary aliquots of PURE system proteins, ribosomes, energy solution, and matrix solution on ice **[1]**.

3.1.1. Talent placing frozen aliquots of PURE components into an ice bucket for thawing. NOTE: 3.1.1. + 3.2.1. were filmed in one shot

3.2. Assemble a master mix for triplicate reactions by adding energy solution, PURE proteins, ribosomes, matrix solution, and nuclease-free water in the specified order **[1-TXT]**. Set a pipette to 5.1 microliters and aspirate the master mix 5 to 6 times to ensure the solution is well mixed and homogeneous **[2]**.

3.2.1. Talent pipetting each reagent sequentially into a PCR tube while checking off a labeled tube rack or protocol list. **TXT: Total volume: 17 µL**

3.2.2. Talent mixing the contents of the PCR tube by gently aspirating and dispensing 5.1 microliters several times.

3.3. Using the pipette set to 5.1 microliters, dispense the master mix into three separate wells of a 384-well optical plate while avoiding bubbles **[1]**. Seal the 384-well plate using an adhesive plate seal **[2]**.

3.3.1. Talent pipetting 5.1 microliters of master mix into the first, third, and fifth wells of a 384-well plate. **TXT: Leave a well gap between replicates to reduce cross-talk**

3.3.2. Talent applying and smoothing down an adhesive seal over the entire surface of the well plate. **TXT: Proceed to incubation or plate reading as per the protocol**

Results

4. Results

4.1. All four PURE reaction batches prepared by User 1 showed consistent GFP fluorescence kinetics, peaking around 2 hours **[1],** and were comparable to the GFP signal from the independently prepared batch by User 2.1 **[2],** while no fluorescence was observed in the negative control lacking DNA **[3].**

4.1.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the four overlapping red and orange curves labeled User 1.1 to User 1.4 that rise steeply and level off around the 2-hour mark.*

4.1.2. LAB MEDIA: Figure 4A. *Video editor: Highlight the blue curve labeled User 2.1, which follows a similar trajectory to the User 1 curves.*

4.1.3. LAB MEDIA: Figure 4A. *Video editor: Highlight the flat grey curve labeled "-DNA" at the bottom of the graph.*

4.2. At the 2-hour timepoint, the GFP fluorescence for all user-prepared PURE batches was similarly high, while the minus DNA control remained near baseline **[1].**

4.2.1. LAB MEDIA: Figure 4B. Video editor*: Highlight the group of tall bars for Users 1.1 through 2.1, which are all similarly high*

4.3. In ribosome preparations, GFP production over time varied slightly more across users, with User 1.2 showing the highest activity **[1],** followed by Users 2.1 and 3.1 **[2]**, while User 1.1 had the lowest among active batches **[3].**

4.3.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the red line for User 1.2 that peaks highest among all user curves.*

4.3.2. LAB MEDIA: Figure 5A. *Video editor: Highlight the blue and green curves for Users 2.1 and 3.1*

4.3.3. LAB MEDIA: Figure 5A. *Video editor: Highlight the orange curve for User 1.1*

4.4. At 1.5 hours, User 1.2's ribosome preparation yielded the highest GFP fluorescence, while Users 2.1 and 3.1 had similar but slightly lower values **[1].** User 1.1 had the lowest among active samples **[2],** and the minus DNA control remained near zero **[3].** The overall variation between Users 1.1 and 1.2 is around 30%. **[4]**

4.4.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the tallest red bar labeled User 1.2, followed by slightly shorter blue and green bars labeled Users 2.1 and 3.1*.

4.4.2. LAB MEDIA: Figure 5B. *Video editor: Highlight the orange bar labeled User 1.1, which is visibly shorter than the others.*

4.4.3. LAB MEDIA: Figure 5B. *Video editor: Highlight the nearly flat grey bar labeled "-DNA".*

4.4.4. LAB MEDIA: Figure 5B. *Video editor: Highlight difference between the red bar labelled User 1.2 and the orange bar labelled User 1.1.*

4.5. GFP production in CFPS *(C-F-P-S)* reactions decreased progressively with increasing polyethylene glycol concentrations above 0.0%, with 6.0% showing the lowest expression **[1].**

4.5.1. LAB MEDIA: Figure 6. *Video editor: Highlight the gradual decline from the top curve labeled "0.0%" down to the lowest red curve labeled "6.0%".*

1. **BL21**
   Pronunciation link: https://www.howtopronounce.com/bl21
   IPA: /ˌbiːˌɛlˈtwɛnti wʌn/
   Phonetic Spelling: bee-ell-twen-tee-one
2. **Ribosome**
   Pronunciation link: https://www.merriam-webster.com/dictionary/ribosome
   IPA: /ˈraɪbəˌsoʊm/ or /ˈraɪbəˌzoʊm/
   Phonetic Spelling: rye-boh-sohm or rye-boh-zohm
3. **Conical**
   Pronunciation link: https://www.merriam-webster.com/dictionary/conical
   IPA: /ˈkɑːnɪkəl/
   Phonetic Spelling: kah-nih-kul
4. **Pipette**
   Pronunciation link: https://www.merriam-webster.com/dictionary/pipette
   IPA: /paɪˈpɛt/
   Phonetic Spelling: pie-pet
5. **Microliter**
   Pronunciation link: https://www.merriam-webster.com/dictionary/microliter
   IPA: /ˈmaɪkroʊˌliːtər/
   Phonetic Spelling: my-kroh-lee-ter
6. **Molar** (chemistry concentration unit)
   Pronunciation link: https://www.merriam-webster.com/dictionary/molar

IPA: /ˈmoʊlər/
Phonetic Spelling: moh-ler

7. **Vortex**
   Pronunciation link: https://www.merriam-webster.com/dictionary/vortex
   IPA: /ˈvɔːrˌtɛks/
   Phonetic Spelling: vor-teks
8. **Sonication**
   Pronunciation link: https://www.merriam-webster.com/dictionary/sonication
   IPA: /ˌsɑːnɪˈkeɪʃən/
   Phonetic Spelling: sah-nih-kay-shun
9. **Centrifuge**
   Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
   IPA: /ˈsɛntrəˌfjuːdʒ/
   Phonetic Spelling: sen-truh-fyooj
10. **Supernatant**
    Pronunciation link: https://www.merriam-webster.com/dictionary/supernatant
    IPA: /ˌsuːpərˈneɪtənt/
    Phonetic Spelling: soo-per-nay-tuhnt
11. **Ultracentrifuge**
    Pronunciation link: https://www.merriam-webster.com/dictionary/ultracentrifuge
    IPA: /ˌʌltrəˈsɛntrəˌfjuːdʒ/
    Phonetic Spelling: uhl-truh-sen-truh-fyooj
12. **Aliquot**
    Pronunciation link: https://www.merriam-webster.com/dictionary/aliquot
    IPA: /ˈælɪˌkwɑːt/
    Phonetic Spelling: al-ih-kwot
13. **Homogeneous**
    Pronunciation link: https://www.merriam-webster.com/dictionary/homogeneous
    IPA: /ˌhoʊməˈdʒiːniəs/ or /ˌhɑːməˈdʒiːniəs/
    Phonetic Spelling: hoh-muh-jee-nee-us or hah-muh-jee-nee-us
14. **Nuclease**
    Pronunciation link: https://www.merriam-webster.com/dictionary/nuclease
    IPA: /ˈnjuːkliˌeɪs/ or /ˈnuːkliˌeɪs/
    Phonetic Spelling: nyoo-klee-ace or noo-klee-ace
15. **Optical**
    Pronunciation link: https://www.merriam-webster.com/dictionary/optical
    IPA: /ˈɑːptɪkəl/
    Phonetic Spelling: op-ti-kul
16. **Fluorescence**
    Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence
    IPA: /flʊˈrɛsəns/ or /flɔːˈrɛsəns/
    Phonetic Spelling: floo-res-ens or flaw-res-ens

17. **Kinetics**
    Pronunciation link: https://www.merriam-webster.com/dictionary/kinetics
    IPA: /kɪˈnɛtɪks/ or /kaɪˈnɛtɪks/
    Phonetic Spelling: kih-ne-tiks or kye-ne-tiks
18. **Polyethylene glycol**
    Pronunciation link: https://www.merriam-webster.com/dictionary/polyethylene%20glycol
    IPA: /ˌpɑːliˈɛθɪˌliːn ˈglaɪˌkɔːl/
    Phonetic Spelling: pah-lee-eh-thuh-leen gly-kawl