Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62625R2
Full Title:	OnePot PURE cell-free system
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Bioengineering
Please indicate whether this article will be Standard Access or Open Access.	Open Access ($3900)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lausanne, Vaud, Switzerland
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TITLE:

OnePot PURE Cell-Free System

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KEYWORDS:

cell-free transcription translation, PURE, OnePot pure, synthetic biology

SUMMARY:

We present a fast and cost-effective method to produce the recombinant PURE cell-free TX-TL system using standard laboratory equipment.

ABSTRACT:

The defined PURE (protein synthesis using recombinant elements) transcription-translation system provides an appealing chassis for cell-free synthetic biology. Unfortunately, commercially available systems are costly, and their tunability is limited. In comparison, a home-made approach can be customized based on user needs. However, the preparation of home-made systems is time-consuming and arduous due to the need for ribosomes as well as 36 medium scale protein purifications. Streamlining protein purification by coculturing and co-purification allows for minimizing time and labor requirements. Here, we present an easy, adjustable, time- and cost-effective method to produce all PURE system components within 1 week, using standard laboratory equipment. Moreover, the performance of the OnePot PURE is comparable to commercially available systems. The OnePot PURE preparation method expands the accessibility of the PURE system to more laboratories due to its simplicity and cost-effectiveness.

INTRODUCTION:

Cell-free transcription-translation (TX-TL) systems constitute a promising platform for investigating and engineering biological systems. They provide simplified and tunable reaction conditions, as they no longer rely on life-sustaining processes, including growth, homeostasis, or regulatory mechanisms[1]. Thus, it is anticipated that cell-free systems will contribute to the investigation of biomolecular systems, offer a framework to test rational biodesign strategies[2], and provide a chassis for a future synthetic cell[3,4]. The fully recombinant PURE system offers an especially appealing chassis due to its defined and minimal composition, as well as its adjustability and tuneability[5].

Since the first functional, fully recombinant PURE system was established in 2001[5], efforts have been made to expand the system limits and optimize the system's composition to improve the system yields[6–8], allow for transcriptional regulation[9], membrane[10,11] and secretory protein synthesis[12], and to facilitate protein folding[13,14]. Nowadays, there are three commercially available systems: PUREfrex (GeneFrontier), PURExpress (NEB), and Magic PURE (Creative Biolabs). However, those systems are costly, their exact composition is proprietary and thus unknown, and adaptability is limited.

PURE systems prepared in-house proved to be the most cost-effective and tunable option[15,16]. However, the required 37 purification steps for protein and ribosome fractions are time-consuming and tedious. Several attempts have been made to improve the efficiency of the PURE system preparation[17–19]. We recently demonstrated that it is possible to coculture and co-purify all required non-ribosomal proteins present in the PURE system. This OnePot method has proved to be cost-effective and time-efficient, cutting down preparation time from several weeks to 3 working days. The approach generates a PURE system with a protein production capacity comparable to the commercially available PURExpress system[20]. Contrary to the previous approaches to simplify the PURE preparation[17–19], in the OnePot approach all proteins are still expressed in separate strains. This enables the user to tune the composition of the OnePot PURE system by merely omitting or adding specific strains or adjusting the inoculation volumes, thus generating dropout PURE systems or altering the final protein ratios, respectively.

The protocol presented here provides a detailed method for creating the OnePot PURE system as described previously[20], although β-mercaptoethanol was replaced with tris(2-carboxyethyl)phosphine (TCEP). Moreover, two methods for ribosome purification are described: traditional tag-free ribosome purification using hydrophobic interaction and sucrose cushion, adapted from Shimizu et al.[15], and Ni-NTA ribosome purification based on Wang et al.[18] and Ederth et al.[21] but significantly modified. The latter method further facilitates the preparation of the PURE system and makes it accessible to more laboratories, as only standard laboratory equipment is required.

The experimental protocol summarizes the preparation of a versatile PURE cell-free TX-TL system to provide a simple, tunable, cost-effective cell-free platform, which can be prepared using standard laboratory equipment within a week. Besides introducing the standard PURE composition, we indicate how and where it can be adjusted, with a primary focus on critical steps in the protocol to ensure the system's functionality.

PROTOCOL:

NOTE: This protocol describes the preparation of cell-free TX-TL system from recombinant components. For convenience, the work is separated into five parts. The first part describes preparation steps, which should be done before starting the protocol. The second part describes the preparation of the OnePot protein solution. The third part describes ribosome purifications, the fourth part details the preparation of the energy solution, and the last part provides a manual for setting up a PURE reaction. For convenience, the protocols are divided into days and summarized in daily schedules in **Table 1**. Following the schedule, the whole system can be prepared in 1 week by one person.

1. Preliminary work

1.1. Prepare the bacterial culture media and media supplements as described in **Supplementary Table 1**. Prepare and sterilize the materials required, including pipette tips, 96 deep-well plates.

1.2. Strain preparation

1.2.1. Transform the expression strains indicated in **Table 2** with the corresponding expression vectors using the heat shock method.

1.2.1.1. Add purified plasmid to the chemically competent bacteria and incubate on ice for 20–30 min.

1.2.1.2. Place the mixture at 42 °C for 30 s (heat shock) and then place it back on ice for 2 min.

1.2.1.3. Pipette 20 µL of the bacteria directly onto agar plates containing ampicillin (AMP) and incubate at 37 °C overnight. Store the plates at 4 °C for up to 1 week.

1.2.1.4. Inoculate 3 mL of LB media containing AMP with a single colony of bacteria from the agar plates. Incubate at 37 °C while shaking at 260 rpm overnight.

1.2.1.5. Mix 250 µL of the culture with 250 µL of 50% (v/v) glycerol and store at -80 °C.

NOTE: For faster preparation in the future, store the strains in a 96-well plate as glycerol stocks.

1.2.2. Confirm all vector transformations by colony PCR and sequencing. Sequence the gene, promoter region, and ribosome binding site.

1.3. Expression test

1.3.1. Inoculate 300 µL of LB media containing AMP with around 1 µL of the prepared glycerol stocks in a 1.3 mL deep-well plate. Seal the plate with a breathable membrane and then incubate at 37 °C while shaking at 260 rpm overnight.

NOTE: All expressions are done separately at this point.

1.3.2. Inoculate 300 µL of fresh LB media containing AMP with 1 µL of the overnight cultures. Incubate at 37 °C while shaking at 260 rpm overnight. After 2 h, induce the cells with 100 µM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) and grow for an additional 3 h.

1.3.3. Mix 10 µL of the culture with 10 µL of 2x Laemmli buffer and heat to 95 °C for 10 min. Spin the samples for 1 min using a table centrifuge and load 10 µL of the supernatant on a PAGE gel. Run the gel in Tris/Glycine/SDS buffer at 200 V for 30 min. Rinse it well with deionized water. Cover the gel with a Coomassie protein stain and incubate for 1 h. Destain the gel in water if necessary (representative results for the expression test in **Figure 1**).

NOTE: Use gradient (4%–15% or 4%–20%) PAGE gels to achieve a good separation.

1.4. IMAC Sepharose resin restoration and cleaning

1.4.1. Column preparation.

1.4.1.1. Mix the Sepharose resin well by vortexing.

1.4.1.2. Pipette the required amount of resin into an empty gravity flow column.

NOTE: The amount of resin required varies between His-ribosome purification and protein purification and is specified in the respective sections.

1.4.1.3. Wash the resin with 30 mL of deionized water.

1.4.1.4. Proceed with column re-charge as specified in section 1.4.4.

NOTE: Always let all the liquid pass through the column before continuing with the next step. However, make sure that the column never runs dry. Whenever running any liquid through the column, ensure to stop the flow or continue to the next step as soon as the liquid reaches the resin.

1.4.2. Restoration.

1.4.2.1. Wash the column with 30 mL of deionized water.

1.4.2.2. Apply 10 mL of a 0.2 M EDTA and 0.5 M NaCl solution.

1.4.2.3. Add 30 mL of a 0.5 M NaCl solution.

1.4.2.4. Wash the column with 50 mL of deionized water.

1.4.2.5. Store in 20% (v/v) ethanol at 4 °C or continue with the next step.

1.4.3. Cleaning.

CAUTION: Wear protective equipment.

1.4.3.1. Wash the column with 30 mL of 0.5 M NaOH.

1.4.3.2. Wash the column with 30 mL of deionized water.

1.4.3.3. Wash the column with 30 mL of 0.1 M acetic acid.

1.4.3.4. Wash the column with 30 mL of deionized water.

1.4.3.5. Wash the column with 30 mL of 70% (v/v) ethanol.

1.4.3.6. Wash the column with 50 mL of deionized water.

1.4.3.7. Store in 20% (v/v) ethanol at 4 °C or continue with the next step.

1.4.4. Re-charging.

1.4.4.1. Add 10 mL of 0.1 M nickel sulfate solution to the column.

CAUTION: Nickel sulfate is toxic. Nickel sulfate waste needs to be discarded with the precautions indicated by the supplier.

1.4.4.2. Wash the column with 50 mL of deionized water.

1.4.4.3. Store in 20% (v/v) ethanol at 4 °C or continue with the column equilibration.

NOTE: If the column is stored in ethanol between steps, make sure to remove all traces of ethanol by washing the column with water.

2. OnePot protein solution expression and purification

NOTE: The protocol consists of three parts divided into days (**Figure 2**). An ideal preparation procedure produces 1.5 mL of 13.5 mg/mL OnePot protein solution, which corresponds to more than one thousand 10 µL PURE reactions. However, the amount and the ideal concentration of the solution will vary from batch to batch. Experienced users can perform multiple OnePot PURE preparations at a time.

Day 1:

2.1. Prepare bacterial culture media and media supplements as described in **Supplementary Table 1**.

2.2. Prepare and sterilize the required materials, including pipette tips, two 96 deep-well plates, and one 1 L baffled Erlenmeyer flask.

2.3. Prepare buffers and supplements as described in **Supplementary Table 2**. Filter sterilize all buffers using bottle top filters (0.45 µm) and store them at 4 °C. Supplement all the buffers with 1 mM TCEP right before use, unless indicated otherwise.

2.4. Use 2 mL of sepharose resin for the OnePot protein purification. Prepare the column as described in section 1.4.

2.5. To prepare the starter cultures, combine 20 mL of LB media with 20 µL of AMP. In a sterile 96, 1.3 mL deep-well plate, add 300 µL of the media into 35 wells. Inoculate each of them with its respective strain, except elongation factor thermo unstable (EF-Tu), and seal the plate with a breathable membrane.

NOTE: Inoculate the plate using a 96-well replicator (see **Table of Materials**). The well volume of the deep-well plate and the volume of the starter culture are essential. Larger media volumes or smaller well volumes will lead to a different bacterial density due to aeration inconsistencies.

2.6. For the EF-Tu culture, inoculate 3 mL of LB media in a 14 mL culture tube with a snap cap. A single 3 mL of culture for EF-Tu is sufficient for one OnePot expression culture.

2.7. Incubate at 37 °C while shaking at 260 rpm overnight.

Day 2:

NOTE: Perform all the steps at room temperature unless indicated otherwise.

2.8. Transfer 500 mL of LB media and 500 µL of AMP into the sterile baffled flask.

2.9. Inoculate the OnePot PURE culture with 1675 µL of the EF-Tu culture and 55 µL of each of the cultures from the deep-well plate (**Table 2**).

NOTE: During this step, the overall protein composition can be adjusted by tuning the inoculation ratios. Make sure that the overall inoculation volume remains constant at 3.6 mL.

OPTIONAL: To confirm that all strains have grown overnight, measure the optical density of the overnight cultures at 600 nm (OD_{600}) in a 96-well plate using a plate-reader. Use a dilution of 10x for the optical density measurement.

2.10. Incubate the culture for 2 h at 37 °C with a shaking of 260 rpm, or until the OD_{600} of the culture reaches 0.2–0.3.

2.11. Induce the culture with 500 µL of 0.1 mM IPTG and grow for an additional 3 h.

2.12. Harvest the cells by centrifugation at 4 °C and 3220 x *g* for 10 min and store the cell pellet at -80 °C until further use.

NOTE: To optimize the timing, prepare the energy solution described in section 4 during the incubation times on day 2 (**Table 1**).

Day 3:

2.13. Measure the amounts of buffers needed for the purification described in the steps below and add TCEP to all of them as indicated in **Supplementary Table 2**. Store the remaining buffers without TCEP at 4 °C for future purifications.

2.14. Equilibrate the charged column (section 2.4) with 30 mL of buffer A. After 25 mL of buffer A has passed through, close the column from the bottom. In parallel, continue with steps 2.15–2.17.

2.15. Thaw the cells and use a serological pipette to resuspend the cell pellet in 7.5 mL of buffer A.

2.16. Lyse the cells using a 130-watt probe sonicator (see **Table of Materials**, probe tip diameter: 6 mm) with the following parameters: 4 x 20 s pulse on, 20 s pulse off, 70% amplitude. If sonication is successful, the solution will turn darker (**Figure 2**).

NOTE: Make sure to keep the cells on ice during sonication. 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.

2.17. Remove the cell debris by centrifugation at 21130 x *g* for 20 min at 4 °C immediately after sonication. Keep the lysate on ice.

2.18. Add the supernatant to the equilibrated column. Close the column from the top and make sure there is no leakage. Incubate the column for 3 h at 4 °C under rotation using a tube rotator.

2.19. Elute unbound components from the column and wash with 25 mL of buffer A.

2.20. Wash the column with 25 mL of 25 mM imidazole buffer (23.95 mL of buffer A and 1.25 mL of buffer B).

2.21. Elute the proteins with 5 mL of 450 mM imidazole buffer (0.5 mL of buffer A and 4.5 mL of buffer B). Keep the eluted proteins on ice at all times.

2.22. Dilute the eluate with 25 mL of HT buffer, keep the mixture on ice. Add 15 mL to a 15 mL centrifugal filter and concentrate to a volume of 1.5 mL. Add the remaining 15 mL to the filter with the concentrated solution and concentrate to 1.5 mL once more.

2.23. Add 10 mL of HT buffer to the concentrated sample and concentrate to 1 mL. Add an equal amount of stock buffer B and store at -80 °C until further use.

NOTE: One round of exchange/concentration takes about 60 min spinning at 3220 x *g* at 4 °C.

2.24. During the buffer exchange, restore the column as specified in section 1.4.

Day 4:

2.25. Measure the protein concentration using the Bradford assay as described by the supplier. Concentrate the sample with a 0.5 mL of 3 kDa cutoff centrifugal filter to 20 mg/mL.

NOTE: Dilute the protein solution 25-fold or 50-fold before the concentration measurements to avoid oversaturating the Bradford assay.

2.26. To establish the ideal protein concentration, perform an expression test at this stage (section 5.2) with different concentrations of the protein solution. To perform the titration, keep the total volume of the solution constant and pipette the OnePot protein solution, including stock buffer B, at five different ratios (**Supplementary Table 7**).

2.27. Verify the OnePot PURE protein composition using SDS-PAGE (**Figure 3A**). Dilute 2.5 µL of the sample with 7.5 µL of water, mix with 10 µL of 2x Laemmli buffer and then load 5 µL and 2.5 µL of the samples to the gel. Run the SDS-PAGE as specified in section 1.3.3.

2.28. Aliquot the protein solution into 50 µL aliquots after verifying the expression and adjusting the concentration. Store the OnePot PURE protein solution at -80 °C until further use.

NOTE: If a protein component is suspected not to be present, or is present in a lower-than-expected concentration in the OnePot PURE, perform the following steps.

2.29. Check whether the overnight culture of the respective strain has grown at a comparable rate to the other cultures by performing optical density measurements (OD_{600}) of all cultures.

2.30. Perform an additional expression test of the specific strain to verify the expression of the suspect protein.

3. Ribosome solution

NOTE: Two different ribosome purification strategies are introduced, one for hexahistidine-tagged and one for non-tagged ribosomes. The major advantage of the purification method using His-purification on a standard affinity Ni-NTA gravity flow column is that the purification is easy, fast, and does not require additional laboratory equipment, such as a FPLC system and an ultracentrifuge. However, the protein production capacity in OnePot PURE reactions is around one-third compared to tag-free ribosomes. Therefore, choose the method for ribosome production based on whether a high yield is important for the given application.

3.1. His-tagged ribosome purification

NOTE: This protocol utilizes the *E. coli* RB1 strain, a gift from Professor Wang (Columbia University, USA)[18]. This strain has a genomic insertion of a hexa-histidine tag on the C terminus of 50S ribosomal protein (L7/L12), allowing for purification using a Ni-NTA gravity-flow column. The usual yield is around 0.5 mL of 3.45 µM ribosomes, which is sufficient for more than five hundred 10 µL PURE reactions.

Day 1:

3.1.1. Prepare bacterial culture media and media supplements as described in **Supplementary Table 1**.

3.1.2. Prepare and sterilize the required materials, including pipette tips, one 5 L Erlenmeyer flask, and one 100 mL Erlenmeyer flask.

3.1.3. Prepare buffers and supplements as described in **Supplementary Table 2**. Filter sterilize all the buffers using bottle top filters (0.45 µm) and store them at 4 °C.

Day 2:

3.1.4. Pipette 5 mL of resin to a column and prepare the column as specified in section 1.4.

NOTE: Due to the higher volume of the resin, the restoration and purification take significantly longer. Use a different column for ribosome purification to avoid cross-contamination and thoroughly clean it before the purification.

3.1.5. Prepare an overnight culture of *E. coli* RB1 strain by inoculating 35 mL of LB media in a 100 mL Erlenmeyer flask. Incubate at 37 °C while shaking at 260 rpm.

Day 3:

NOTE: Perform all the steps at room temperature unless indicated otherwise.

3.1.6. Add 2 L of LB media into a 5 L sterile flask, inoculate with 12 mL of the overnight culture, and then incubate for 3–4 h at 37 °C while shaking at 260 rpm.

NOTE: Alternatively, perform bacterial culturing in 4 x 500 mL of cultures in 1 L baffled flasks.

3.1.7. Pellet the cells by centrifugation for 10 min at 3220 x *g* and 4 °C. Store at -80 °C until further use.

Day 4:

3.1.8. Equilibrate the column prepared in step 3.1.4. with 30 mL of lysis buffer.

3.1.9. Resuspend the pellet in 20 mL of lysis buffer using a serological pipette.

3.1.10. Lyse the cells with a 130-watt probe sonicator (see **Table of Materials**, probe tip diameter: 6 mm) on ice with the following parameters: 11 x 20 s pulse on; 20 s pulse off, 70% amplitude (see step 2.16 for procedure details).

3.1.11. Immediately after sonication, remove the cell debris by centrifugation for 20 min at 21130 x *g* at 4 °C. Keep the lysate on ice.

3.1.12. Load the supernatant to the columns and let it pass through.

3.1.13. Wash the column with the following mixtures of lysis and elution buffers.

3.1.13.1. Wash 0: use 30 mL of lysis buffer.

3.1.13.2. Wash 1: use 30 mL of 5 mM imidazole (29 mL of lysis buffer, 1 mL of elution buffer).

3.1.13.3. Wash 2: use 60 mL of 25 mM imidazole (50 mL of lysis buffer, 10 mL of elution buffer).

3.1.13.4. Wash 3: use 30 mL of 40 mM imidazole (22 mL of lysis buffer, 8 mL of elution buffer).

3.1.13.5. Wash 4: use 30 mL of 60 mM imidazole (18 mL of lysis buffer, 12 mL of elution buffer).

3.1.14. Elute the ribosomes with 7.5 mL of the elution buffer. Keep the eluted proteins on ice at all times.

3.1.15. Add 22 µL of pure β-mercaptoethanol to 45 mL of ribosome buffer.

CAUTION: β-mercaptoethanol is toxic. Take safety precautions and work in a fume hood.

3.1.16. Add the eluate to a 15 mL centrifugal filter and concentrate to 1 mL.

3.1.17. Add 15 mL of ribosome buffer to the concentrated sample and concentrate again to 1 mL.

3.1.18. Repeat the previous step twice.

3.1.19. Store at -80 °C until further use.

NOTE: One round of exchange/concentration takes about 60 min of centrifugation at 3220 x *g* at 4 °C.

3.1.20. During the buffer exchange, restore the column as specified in section 1.4.

Day 5:

3.1.21. Determine the ribosome concentration by measuring the absorbance at 260 nm of a sample diluted 1:100 in ribosome buffer. An absorbance value of 10 of the diluted solution corresponds to 23 µM of undiluted solution as previously described[16].

3.1.22. Implement a final stock concentration of 3.45 µM. To adjust the concentration, dilute the ribosomes with ribosome buffer or concentrate them further by centrifugation at 14000 x *g* in a 3 kDa 0.5 mL centrifugal filter at 4 °C.

NOTE: To achieve optimal system expression, perform a ribosome concentration titration (section 5.2, **Supplementary Table 7**).

3.1.23. Verify the ribosome composition using SDS-PAGE (**Figure 3A**) as specified in section 1.3.3. Dilute 2.5 µL of the sample with 7.5 µL of water, mix with 10 µL of 2x Laemmli buffer, and then load 5 µL and 2.5 µL of the samples onto the gel.

3.2. Tag-free ribosome purification

NOTE: Tag-free ribosome purification is performed using a FPLC system (**Table of Materials**) and is based on hydrophobic interaction chromatography using 2 x 5 mL Butyl columns (**Table of Materials**). Although ribosomes may be purified from any strain, using the *E. coli A19* (*E. coli* Genetic Resources at Yale CGSC) strain is advantageous due to its RNase I deletion[22]. Perform the purification at 4 °C in either a cold room or a cooling cabinet. The usual yield is around 0.5 mL of 10 µM ribosomes, which corresponds to more than five hundred 10 µL PURE reactions.

Day 1:

3.2.1. Prepare bacterial culture media and media supplements as described in **Supplementary Table 1**.

3.2.2. Prepare and sterilize the required materials, including pipette tips, 5 L Erlenmeyer flask, and 100 mL Erlenmeyer flask.

3.2.3. Prepare buffers and supplements as described in **Supplementary Table 2**. Filter sterilize all the buffers using bottle top filters (0.45 µm) and store them at 4 °C.

Day 2:

3.2.4. To prepare an overnight culture of the *E. coli* A19 strain, inoculate 35 mL of LB media in a 100 mL Erlenmeyer flask. Incubate at 37 °C while shaking at 260 rpm.

Day 3:

3.2.5. Transfer 2 L of LB media into the 5 L sterile baffled flask, inoculate with 30 mL of the overnight culture, and then incubate for 3–4 h at 37 °C while shaking at 200 rpm.

3.2.6. Pellet the cells by centrifugation at 4000 x *g* for 15 min at 4 °C. Resuspend the pellet in 25 mL of suspension buffer and store at -80 °C until further use.

Day 4:

3.2.7. Perform steps 3.2.8–3.2.12 in parallel with steps 3.2.13–3.2.19.

3.2.8. Thaw and lyse the cells using a 130-watt probe sonicator (see **Table of Materials** and probe tip diameter: 6 mm) on ice with the following parameters: 12 x 20 s pulse on; 20 s pulse off, 70% amplitude (see step 2.16 procedure details).

3.2.9. Immediately remove the cell debris by centrifugation at 20000 x *g* for 20 min at 4 °C.

3.2.10. Aspirate the supernatant and measure the volume. Add an equal volume of suspension buffer (high salt) to adjust the final concentration of ammonium sulfate to 1.5 M and mix well.

3.2.11. Remove the precipitate by centrifugation at 20000 x *g* for 20 min at 4 °C.

3.2.12. Filter the supernatant using a 0.45 µm polyethersulfone membrane syringe filter before FPLC purification and collect the filtrate in a 100 mL glass bottle. Keep the supernatant at 4 °C at all times.

3.2.13. Set up the FPLC system for hydrophobic-interaction chromatography purification using a double Butyl column (2 x 5 mL) as follows. For this setup, one column volume (CV) refers to a volume of 10 mL.

3.2.14. Three inlets will be needed: two as buffer lines and one as the sample line. Due to the default settings of the purifier, it is convenient to choose lines A1 and B1 for buffer C and buffer D, respectively, and line A2 as the sample line. Apply a default flow rate of 4 mL/min, except for pump washes (10 mL/min) or unless indicated otherwise.

NOTE: As TCEP is a costly reagent, add the corresponding amount to buffers C and D only after the equilibration step.

3.2.15. Perform a system pump wash in 20% (v/v) ethanol to clean the system and remove potential contamination from previous purifications. Manually set a flow rate of 0.2 mL/min and mount the column. Stop the flow.

3.2.16. Execute a system pump wash with water. Wash the column with 3 CV of water.

3.2.17. Equilibration: place inlets A1 and A2 in buffer C and inlet B1 in buffer D without TCEP. Execute a pump wash and equilibrate the column with 4 CV of buffer C.

3.2.18. Add TCEP to buffers C and D.

3.2.19. Prepare 15 mL tubes or clear round fraction collector tubes to the fraction collector to collect 4–5 mL elution fractions.

3.2.20. Loading: Place the inlet A2 into the bottle with the filtered sample. Load approximately 90% of the sample volume onto the column. Dilute the sample with 20 mL of TCEP-containing buffer C, and load 10 mL of the sample onto the column. Repeat the dilution step at least twice and load as much sample onto the column as possible. It is critical to ensure that no air is sucked into the machine.

3.2.21. Washing step 1: wash with 3 CV of buffer C to remove the unbound components.

3.2.22. Washing step 2: wash with 5 CV of 80% buffer C and 20% buffer D.

3.2.23. Elution: elute the product by applying 50% of buffer C and 50% of buffer D, with a total elution volume of 5 CV. Collect this fraction in the collector tubes.

3.2.24. Washing step 3: Elute all strongly interacting contaminants using 100% buffer D with a total volume of 5 CV.

3.2.25. Analyze the absorption spectrum of the sample fraction at 260 or 280 nm (**Figure 4**). The first peak shows the non-absorbed proteins eluted during loading and the first washing step; the second peak shows contaminants that have been eluted during the second washing step. The third peak monitors the final product, and the last peak shows the strongly interacting contaminants. Pool all sample fractions corresponding to the third peak for further processing. Keep the eluted proteins on ice at all times.

3.2.26. Gently overlay the recovered fraction onto 15 mL of the cushion buffer in four polycarbonate ultracentrifugation tubes. Add a maximum of 15 mL of the sample to 15 mL of the cushion buffer. Make sure to balance the weight of the tube well. Pellet the ribosomes by ultracentrifugation at 100000 x *g* at 4 °C for 16 h.

NOTE: Ensure that no cracks are present in the ultracentrifugation tubes.

3.2.27. Clean and reset the column as follows. A flow rate of 5 mL/min works well. Place all the inlets into the water and execute a pump wash. Wash the column with 2 CV of water.

3.2.27.1. Place the inlet into a 0.5 M NaOH solution, perform a pump wash, and subsequently wash the column with 3 CV of NaOH.

3.2.27.2. Place the inlet into water, perform a pump wash, and then wash the column in 2 CV of water.

3.2.27.3. Place the inlet to a 0.1 M acetic acid solution, perform a pump wash, and subsequently wash the column with 3 CV of acetic acid solution.

3.2.27.4. Pump wash and wash the column with 2 CV of water.

3.2.27.5. Place all inlets into 20% (v/v) ethanol, execute a pump wash step, and store the column in 20% (v/v) ethanol by washing it with 3 CV of a 20% (v/v) ethanol solution.

NOTE: Ensure that the system never runs dry or sucks in air. Never apply buffer directly to ethanol, or ethanol to buffer. Always add a water washing step in between, as otherwise there is a risk of precipitates clogging the column. Make sure to add enough sample collection tubes.

Day 5:

3.2.28. Discard the supernatant and carefully, without disturbing the translucent pellet, wash each pellet with 0.5 mL of ice-cold ribosome buffer. Repeat this step twice.

3.2.29. Resuspend each of the clear pellets in 100 µL of ribosome buffer on ice using a magnetic stir bar (3 mm diameter, 10 mm length) on a magnetic stirrer using the lowest possible speed. Collect the resuspended ribosomes and wash the tubes with an additional 50 µL of ribosome buffer.

NOTE: The translucent pellet is difficult to see. Therefore, carefully wash the pellet from the sides of the tube.

3.2.30. Determine the ribosome concentration by measuring the absorbance at 260 nm of the sample diluted at a ratio of 1:100 in ribosome buffer. An absorbance of 10 of the diluted solution corresponds to 23 µM of undiluted solution as previously described[16].

3.2.31. Implement a final stock concentration of 10 µM. To adjust the concentration, dilute the ribosomes with ribosome buffer or concentrate them further by centrifugation at 14000 x *g* in a 3 kDa centrifugal filter at 4 °C.

NOTE: To achieve optimal system expression, perform ribosome titration (section 5.2, **Supplementary Table 7**).

3.2.32. Verify the ribosome composition with SDS-PAGE (**Figure 3A**) as specified in section 1.3.3. Dilute 2.5 µL of the sample with 7.5 µL of water, mix with 10 µL of 2x Laemmli buffer, and then load 5 µL and 2.5 µL of the samples to the gel.

4. Energy solution

NOTE: The composition for the 2.5x energy solution introduced here is an example of a solution that worked well for a standard TX-TL reaction. To optimize the timing, prepare the energy solution during day 2. The preparation of the amino acid solution is explained in detail, followed by the final preparation procedure.

4.1. Amino acid solution

NOTE: Prepare the amino acid solution in bulk. Preparing the amount of amino acid stock solutions required for a final volume of at least 2000 µL will reduce the weighing error for the otherwise very small amounts. The overall concentration of the amino acid solution is limited by the solubility of the amino acids and the respective stock solution concentrations. For the standard PURE system, prepare a solution with a final concentration of 3.25 mM. Use the amino acid solution calculation table (**Supplementary Table 3**) as a template. Use cysteine in the salt form to ensure sufficient solubility. Avoid using KOH-based amino acid preparation methods. It is possible to directly weigh the exact amounts of amino acids into the final amino acid solution without preparing stock solution for all the amino acids. However, this is more challenging and less precise.

4.1.1. Prepare stock solutions for each amino acid as described in **Supplementary Table 3**, except for Tyrosine.

NOTE: Due to the different solubilities of the amino acids in water, the respective suggested concentrations of the stock solution differ.

4.1.2. Minimal mass [mg] provides the approximate minimum mass required to obtain a sufficient amount of stock solution for the target overall volume, as a reference.

NOTE: The minimal mass is calculated with a surplus of 10%.

4.1.3. For an easier preparation of the solutions, do not weigh the exact amount of amino acid, but instead, for the mass at hand, adjust the amount of water to achieve the desired concentration. Calculate the amount of deionized water (Water to add [µL]) needed, based on the actual mass filled in (light yellow cells) and the desired concentration using the spreadsheet in **Supplementary Table 3**.

4.1.4. Solubilize the amino acid stock solutions by vortexing until all precipitate has dissolved. The individual amino acid stock solutions can be stored at -20 °C for several weeks.

NOTE: Some amino acids are difficult to dissolve in water; the process may take some time.

4.1.5. Weigh the exact amount of tyrosine required to obtain a final concentration of 3.25 mM directly into the tube for the amino acid solution.

NOTE: Tyrosine is very difficult to dissolve in water. Add it directly instead of preparing a stock solution.

4.1.6. Add the corresponding amounts of amino acid stock solutions and water as indicated in the Final volume to add [µL] column (light blue cells) and vortex the solution well. Store the completed amino acid solution at -80 °C until further use.

4.2. Preparation of the energy solution

NOTE: In total, the 2.5x energy solution contains 0.75 mM of each amino acid, 29.5 mM of magnesium acetate, 250 mM of potassium glutamate, 5 mM of ATP and GTP each, 2.5 mM of CTP, UTP, and TCEP, respectively, 8.75 mg/mL of tRNA from *E. coli* MRE 600, 50 mM of creatine phosphate, 0.05 mM of folinic acid, 5 mM of spermidine, and 125 mM of HEPES. First-time users prepare the energy solution in small batches of 200 µL. Store the individual solutions prepared according to **Supplementary Table 4** at -20 °C or -80 °C for later use.

4.2.1. Thaw all aqueous solutions mentioned in the **Supplementary Table 5** on ice.

4.2.2. Meanwhile, prepare the stock solutions for the remaining components listed in **Supplementary Table 4**. Keep all the solutions on ice after preparation.

NOTE: Add 500 µL of RNase and DNase-free water directly to the vial to dissolve the lyophilized tRNAs. Mix well by gentle vortexing; limit pipetting to avoid introducing RNases.

4.2.3. Add the calculated volumes (**Supplementary Table 5**) of stock solutions and water and mix well using a vortex. Keep the solution on ice at all times.

4.2.4. Measure the pH of the solution by pipetting 1 µL onto a pH strip, to ensure that the pH of the solution is neutral.

4.2.5. Aliquot the energy solution at 50–100 µL per tube on ice and store at -80 °C until further use. While aliquoting, vortex the main stock frequently to prevent the components from precipitating.

NOTE: Optionally, conduct an activity assay of the newly made energy solution against commercial energy solutions, e.g., Solution A in PURExpress. If a significantly lower performance of the system with the energy solution is observed, optimizing the ion concentrations, especially magnesium ions, by titration (5–20 mM) may be advantageous.

5. OnePot PURE reaction

5.1. DNA template

NOTE: Proteins encoded downstream of the T7 promoter can be expressed in PURE from either linear or circular DNA. By generating a linear DNA template using extension PCR, tedious cloning steps can be omitted. The linear templates for this study were generated by PCR as described below, using a high-fidelity DNA polymerase (**Table of Materials**). Primer sequences, melting temperatures, and the thermocycler settings used in this study are specified in **Supplementary Table 6**. The preparation of the DNA template is not included in the daily schedule.

5.1.1. Set up a PCR reaction as recommended by the polymerase supplier.

NOTE: Optimized parameters for a high-fidelity DNA polymerase (**Table of Materials**) are given in **Supplementary Table 6**.

5.1.2. Amplify the target gene (e.g., eGFP) as a linear template from a plasmid or genome using gene-specific primers (500 nM) (for the parameters, see **Supplementary Table 6**).

5.1.3. The amplification generates short extensions to provide annealing sequences for the following extension PCR steps.

5.1.4. Check the amplicon on an agarose gel for correct size and purity.

5.1.5. Use the amplified DNA as a template for the subsequent extension steps. Set up a reaction of at least 50 µL.

5.1.6. Run 10 PCR amplification cycles with the extension primers (2.5 nM). After completing the amplification cycles, immediately add the final primers (500 nM) to the same reaction and run 30 cycles to amplify the extended PCR product. Find the melting temperatures and primer sequences in **Supplementary Table 6**.

5.1.7. Purify the DNA fragments using a DNA purification kit and elute the DNA in nuclease-free water instead of EDTA containing elution buffer.

5.1.8. Check the linear template on an agarose gel for correct size and purity.

5.1.9. Measure the DNA concentration in ng/µL using an UV-Vis spectrophotometer.

5.2. Setting up the PURE reaction

NOTE: The final reaction composition is 1x energy solution, tag-free ribosomes or His-tag ribosomes, OnePot PURE proteins, and DNA template. The reaction volume ratio comprises 40% energy solution, 30% protein and ribosome solution, and 30% DNA and water. Typical reaction volumes vary between 5 µL and 25 µL. Quantify the expression of a fluorescent protein continuously on a plate-reader. Use a Green Lys *in vitro* Translation Labeling System, which incorporates fluorescently labeled Lysine residue into newly synthesized proteins, to verify the expression of non-fluorescent proteins on a SDS-PAGE gel. An example reaction template is given in **Supplementary Table 7** to help establish a PURE cell-free expression reaction. Cells in yellow indicate user-input values, and cells in orange indicate additional reagents to be optionally added to the reaction. Keep the volume ratios of the components precise to ensure the correct ion balance. For instance, to achieve a higher protein concentration, increase the OnePot protein solution concentration; however, do not increase the volume of protein solution added to the reaction.

5.2.1. Fill in the concentration [ng/µL] and length [base pairs] of the DNA in the corresponding yellow cells in the spreadsheet. Use 2–10 nM of DNA for the reaction.

5.2.2. Fill in the desired total reaction volume in µL.

5.2.3. Remove the required reagents from the freezer and thaw them on ice.

NOTE: Refreezing of the components is possible without a decrease in functionality. However, minimize the number of freeze-thaw cycles and the time samples are stored on ice as much as possible.

5.2.4. Pipette the calculated amounts of water, DNA, and energy solution to one side of the PCR tube or one corner of a well on the 384-well plate. Add the required amount of any additional reagent on the same side. Minimize the number of samples per experiment to avoid sample evaporation and experimental start time bias.

NOTE: It is crucial to keep the energy component physically separated from the protein components to avoid premature consumption of the energy sources and lower yields.

5.2.5. Pipette the calculated amounts of protein and ribosome solution to the other side of a PCR tube or the opposite corner of the 384-well plate.

NOTE: Use master mixes whenever possible to reduce the impact of pipetting errors. After initial testing, the ribosome and protein solutions can be mixed and stored as one solution.

5.2.6. Spin for a short time (30 s) to merge the reaction components. To prevent evaporation during plate-reader experiments, add 35 µL of liquid wax and seal the plate with a transparent sealant (see **Table of Materials**).

5.2.7. Incubate for a minimum of 3 h at 37 °C.

5.2.8. For readout on a plate-reader, measure the fluorescence intensity at the required wavelength every 2 min (representative results are shown in **Figure 3B**).

5.2.9. Perform the following steps for Green Lys labeled samples.

5.2.10. After the cell-free expression, incubate the sample with 0.16 µg/µL of RNase A for 30 min at 37 °C to remove the fluorescent background of the Green Lys labeling kit.

NOTE: Use RNase A, as other types of RNases do not remove the background sufficiently well.

5.2.11. Visualize the protein expression by running SDS-PAGE as specified in section 1.3.3. Wash the unstained gel gently in deionized water, and image it on a fluorescent imager using an excitation wavelength of 488 nm.

5.2.12. Subsequently, stain the gel using conventional Coomassie staining methods. For the suitable parameters see section 1.3.3.

NOTE: Perform a titration of the protein solution with the recommended ribosome concentration and, if required, titrate ribosomes with the optimal OnePot protein concentration afterward. Use the commercial PURExpress ΔRibosome kit as a positive control. Solution A, Factor Mix, and the ribosome solution correspond to the prepared energy, the OnePot protein solution, and the purified ribosomes, respectively.

REPRESENTATIVE RESULTS:

The above protocol is designed to facilitate establishing the PURE cell-free TX-TL system in any laboratory. The protocol includes a detailed description of the preparation of the three distinct parts of the PURE system: the OnePot protein, ribosome, and energy solution. A detailed daily schedule, which optimizes the workflow, is shown in **Table 1**. The workflow is optimized for the purification of His-tagged ribosomes, and time frames may differ slightly if tag-free ribosome purification is performed. One preparation provides a sufficient amount of PURE for a minimum of five hundred 10 µL reactions. Moreover, the prepared solutions are stable for more than a year at -80 °C and can withstand multiple freeze-thaw cycles.

Adequate overexpression levels for all strains are crucial for the functionality of the final protein solution. **Figure 1** shows successful overexpression in all 36 individual strains used subsequently for the OnePot protein preparation. Variation in the over-expressed proteins' band intensities occurred most probably due to a bias in loading volumes onto the SDS-PAGE gel. The expected protein sizes are summarized in **Table 2**. GlyRS and PheRS consist of two subunits of various molecular weights; the remaining 34 proteins consist of a single subunit. Key to this protocol's simplicity and time-effectiveness is the coculturing and co-purification step (**Figure 2**). The OnePot protein solution was prepared by increasing the ratio of EF-Tu strain with respect to all the other expression strains. The overall composition of the final proteins was analyzed by SDS-PAGE (**Figure 3A**). From the gels (lanes 2, 3), it is noticeable that EF-Tu (43.3 kDa) is present in a higher concentration compared to the other proteins, as expected. While the gel provides a good first indication of protein expression ratios, it is difficult to determine whether and at which level each individual protein was expressed. Therefore, it is highly recommended to confirm the overexpression in each strain before coculturing, as shown above.

The *E. coli* ribosome is a complex molecular machine composed of over 50 individual protein subunits[23]. A representative absorption spectrum at 260 nm for tag-free ribosome purification is shown in **Figure 4**; the third peak is characteristic of successful ribosome elution. For both ribosome purification methods, the expected running pattern on the SDS-PAGE gel (**Figure 3A**)[18] was observed. We did observe contaminations for both purifications, albeit in small quantities (<10%). Notably, different contaminants were present in the tag-free (lanes 5, 6) and His-tagged (lanes 11, 12) ribosomes due to the variation in the method. For user reference, the SDS-PAGE gels for the combined systems are also included (lanes 8, 9, and 14, 15).

Lastly, the performance of the prepared systems (**Figure 3**) using the different ribosome variants are compared. The time courses of *in vitro* eGFP expression show that both PURE systems are functional and produce fluorescent eGFP. However, the OnePot protein solution combined with the His-tagged ribosomes, using the ribosome concentration optimized by titration, yielded only one-third of the expression level of the non-tagged ribosome version (**Figure 3B**). Similar results were observed when three proteins of different sizes were expressed and labeled using the Green Lys tRNA *in vitro* labeling system (**Figure 3C**). As seen on the fluorescent gel, full-length products were successfully expressed in both systems; however, only around half of the expression level was achieved with the His-tag ribosome system. In addition to the fluorescence labeling, the expected bands for all three proteins are distinguishable on a Coomassie-stained gel (**Figure 3D**). The results show that the introduced expression system, which can be prepared within a week in a laboratory with standard equipment, can be used for the *in vitro* expression of proteins encoded downstream of the T7 promoter from linear templates.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative results for the overexpression test for all expression strains of the PURE system. PURE protein numbers and sizes are summarized in **Table 2**. Protein numbers 21, 24, and 27 are marked with a star for better visualization.

Figure 2: OnePot protein purification. The schematic depiction and corresponding photographs of all steps involved in the production of the OnePot protein solution.

Figure 3: Performance of the prepared systems using the different ribosome variants. (**A**) Coomassie blue stained SDS-PAGE gels of the OnePot protein solution (lanes 2, 3), tag-free ribosomes without protein solution (lanes 5, 6) and with protein solution (lanes 8, 9), His-tagged ribosomes without protein solution (lanes 11, 12) and with protein solution (lanes 14, 15). Two different concentrations were loaded per sample. (**B**) Comparison of eGFP expression of His-tagged ribosomes and tag-free ribosomes. The fluorescence intensity of *in*

vitro eGFP expression is monitored over time for a PURE reaction using tag-free ribosomes (1.8 µM, blue) and His-tagged ribosomes (0.62 µM, red). The concentrations of the linear template and the OnePot protein solution were 4 nM and 2 mg/mL, respectively. Panels (**C**) and (**D**) show the SDS-PAGE gel of proteins synthesized in OnePot with tag-free (1.8 µM, blue, lanes 3, 4, 5) and His-tag ribosomes (0.62 µM, red, lanes 6, 7, 8) labeled with a GreenLys *in vitro* labeling kit (**C**) and stained with Coomassie blue (**D**), respectively. The black arrows indicate the expected bands of synthesized proteins: eGFP (26.9 kDa), ArgRS (64.7 kDa), T7 RNAP (98.9 kDa). The linear template and OnePot protein solution concentrations were 4 nM and 1.6 mg/mL, respectively.

Figure 4: Absorbance spectra at 260 nm. Representative results of absorbance spectra at 260 nm during hydrophobic interaction purification of tag-free ribosomes.

Table 1: A daily time-optimized schedule for the preparation of all the OnePot PURE solutions.

Table 2: PURE protein list

Supplementary Table 1: Reagents. The table lists concentrations, volumes, and other specific details of the reagents and components used during this study.

Supplementary Table 2: Buffers. The spreadsheet lists the exact buffer compositions for protein, tag-free ribosome, and His-tag ribosome purifications, as well as the concentrations of the stock solutions used for their preparation. In addition, it calculates the required amounts of components based on the buffer volume.

Supplementary Table 3: Amino acid calculations. The spreadsheet lists the amino acids and their recommended stock solution concentrations required for the energy solution. It calculates the amount of water to be added to each amino acid based on the actual weighed mass, and also calculates the volume of the amino acid solution to be added to the final amino acids' mixture.

Supplementary Table 4: Stock solutions for the energy solution. The table lists the concentrations and volumes of stock solutions needed for the energy solution and indicates further details, including storage conditions.

Supplementary Table 5: Energy solution. The table lists the energy solution components and their recommended concentrations. In addition, it calculates their required volumes to be added to the final solution based on their stock solution concentrations and the volume of the energy solution.

Supplementary Table 6: Primers. The table lists sequences and concentrations of the primers used for the extension PCR and indicates melting temperatures and thermocycler steps optimized for a high-fidelity DNA polymerase.

Supplementary Table 7: PURE reaction. The spreadsheet shows an example setup of a PURE reaction. It lists the used concentrations and volumes of the components for a PURE reaction using tag-free ribosomes or His-tag ribosomes. Moreover, it calculates the volume ratios for protein and ribosome titrations.

DISCUSSION:

The protocol presented here describes a simple, time- and cost-effective method to prepare a versatile PURE expression system[20] based on the standard composition[15]. By utilizing the protocol together with the supplied daily schedules (**Table 1**), all components can be prepared in 1 week and yield amounts sufficient for up to five hundred 10 µL PURE reactions. Since the proteins used in this protocol are overexpressed from high copy plasmids and have low toxicity to *E. coli*, good expression levels are observed for all the required proteins (**Figure 1**). This allows for the easy adjustment of strains, and therefore also protein composition in cocultures, simply by modifying the ratios of the inoculation strains[20]. Besides the ribosomal proteins, the concentration of EF-Tu showed to be of fundamental importance for expression yields[6]. In contrast, changes in the concentration of the other protein components had a relatively low impact on the robustness of the PURE system[7,24]. Therefore, by adjusting the inoculation ratio of EF-Tu with regard to all the other components, a comparable composition to the standard PURE composition can be achieved, and a PURE system with a similar yield[20] can be attained. In preparing the protein solution, it is crucial to ensure that all strains grow well and overexpress the encoded protein after induction (**Figure 1**).

Ribosome function is key for the overall performance of the PURE system[24]. In this protocol, two different methods for preparing the ribosome solution are demonstrated, i.e., tag-free and His-tagged ribosome purification. The tag-free ribosome purification is based on hydrophobic interaction chromatography followed by centrifugation with a sucrose cushion, which requires access to a FPLC purification system and an ultracentrifuge[15]. In contrast, the method utilizing His-tagged ribosomes[18] and gravity flow affinity chromatography purification does not require specialized equipment and can be performed in most laboratories. The latter method, therefore, brings advantages such as simplicity and accessibility. However, we observed a significantly lower synthesis yield when using the His-tagged ribosomes in the OnePot PURE compared to the tag-free variant (**Figure 3**). Based on the type of application, this lower yield may be acceptable.

The energy solution provides the low molecular weight components and tRNAs required to fuel *in vitro* TX-TL reactions. This protocol provides a recipe for a typical energy solution, which can be easily adjusted based on user needs. Together with tRNA, NTP, and creatine phosphate, the abundance and concentration of Mg^{2+} ions have been crucial for the overall performance of the PURE system[8], as they are critical cofactors for transcription and translation. In some cases, the titration of ions can, therefore, greatly enhance the overall PURE performance. DNA integrity is crucial for PURE performance. Thus, sequence verifying the promoter region, ribosome binding site, and target gene and ensuring that an adequate DNA concentration (<2 nM) will help troubleshoot issues that may arise while setting up a PURE reaction.

The PURE system is a minimal TX-TL system, and specific applications may thus require additional adjustments[25]. These may include incorporating different RNA polymerases[9,26], chaperones[13], and protein factors such as EF-P or ArfA[8]. Although the expression strains for these proteins can be included in the cocultures, adding them separately to the prepared system may provide better control of the required protein levels. Furthermore, the inclusion of vesicles is essential to the production of membrane proteins[10,11]. Oxidizing rather than reducing environments and a disulfide bond isomerase facilitate proper disulfide bond formation, which are, for example, required for secretory proteins[12].

It is essential to ensure that any additional components do not interfere with the reaction. The most important factors to pay attention to when setting up a reaction or adding other components are listed below. Ensure that neither incompatible buffers are used nor the ion concentrations are disturbed. Avoid solutions containing glycerol, high concentrations of potassium, magnesium, calcium ions, osmolytes, pyrophosphate, antibiotics, or EDTA, as much as possible. For example, replacing an elution buffer with water during DNA purification can be beneficial as EDTA is a common additive in this buffer. Supplying the solutions with additional negatively charged molecules such as NTP or dNTP requires adjusting the magnesium concentration[8], as the negatively charged molecules behave as chelating agents and bind positively charged molecules. A neutral pH is ideal for the reaction. Accordingly, all components should be buffered to the corresponding pH; this is especially important for highly acidic or basic molecules such as NTPs. Lastly, temperature and volume are key parameters for the reaction. To achieve a good yield, one should implement a temperature around 37 °C, as temperatures below 34 °C will significantly reduce the yield[27].

It is relevant to note that before preparing the OnePot PURE, one should consider the target application and the associated requirements, such as volume, purity, ease of modification, and inclusion or omission of components. For many applications, the system will be an excellent choice, but others may require yields, adjustability, and other factors, which the OnePot system cannot provide. Irrespectively, the introduced protocol will be beneficial for the preparation of any home-made system, as all critical steps for such preparation are summarized here.

One of the main advantages of the OnePot system is its compatibility with the commercially available PURExpress system, which provides the possibility of testing the functionality and integrity of all components separately by sequentially replacing each PURExpress component with its OnePot equivalent. The advantages of the OnePot PURE system, such as tunability and easy, fast, and cost-effective preparation, will make cell-free TX-TL accessible to more laboratories worldwide and contribute to expanding the implementation of this powerful platform in cell-free synthetic biology.

ACKNOWLEDGMENTS:

This work was supported by the European Research Council under the European Union’s Horizon 2020 Research and Innovation Program Grant 723106, a Swiss National Science Foundation Grant (182019), and EPFL.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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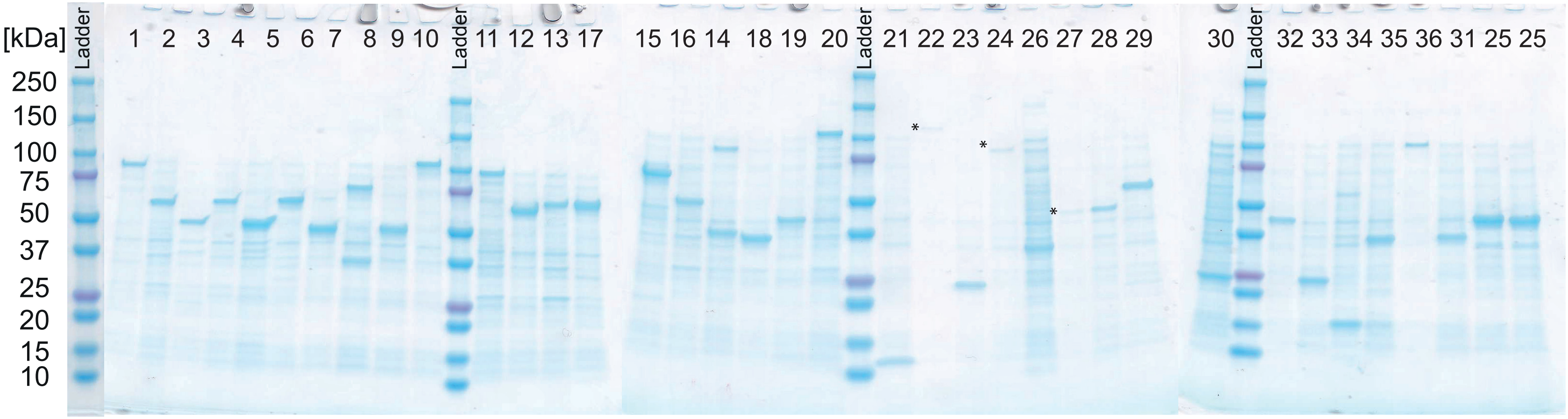
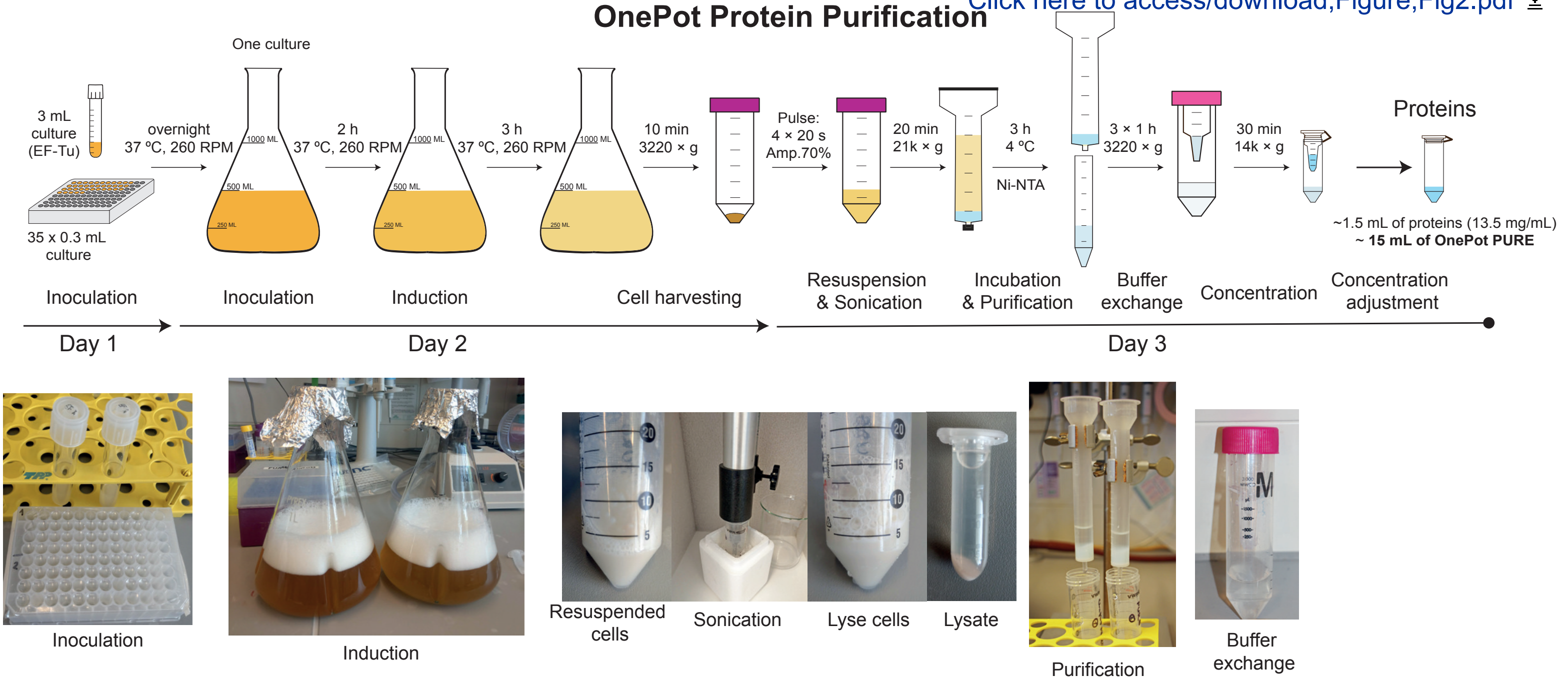
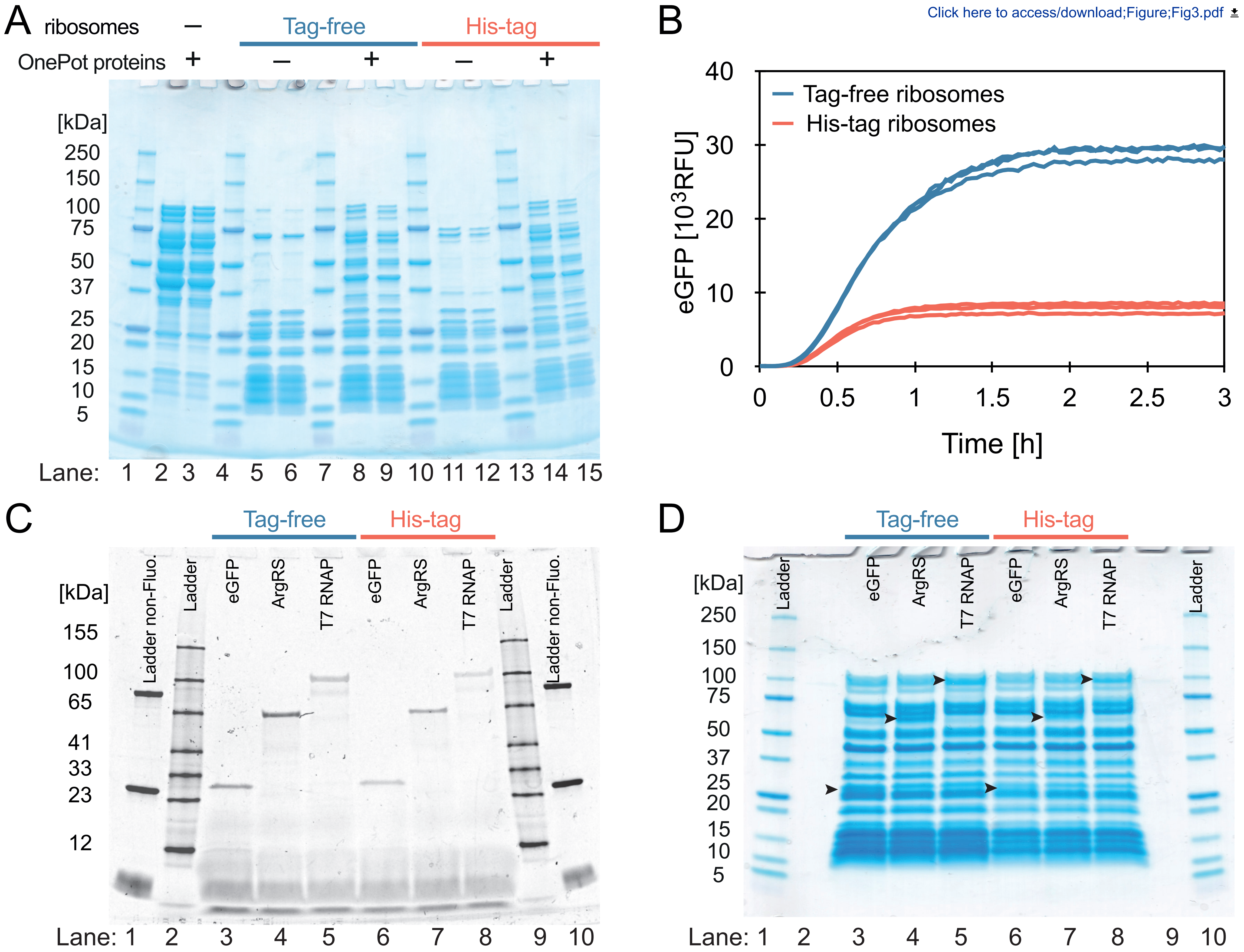
Expression Test




OnePot Protein Purification
3 mL
culture
(EF-Tu)
35 x 0.3 mL
culture
One culture
overnight
37 ºC, 260 RPM
1000 ML
500 ML
250 ML
2 h
37 ºC, 260 RPM
3 h
37 ºC, 260 RPM
10 min
3220 × g
Pulse:
4 × 20 s
Amp.70%
20 min
21k × g
3 h
4 ºC
Ni-NTA
3 × 1 h
3220 × g
30 min
14k × g
Proteins
~1.5 mL of proteins (13.5 mg/mL)
~ 15 mL of OnePot PURE
Inoculation
Inoculation
Induction
Cell harvesting
Resuspension
& Sonication
Incubation
& Purification
Buffer
exchange
Concentration
Concentration
adjustment
Day 1
Day 2
Day 3
Inoculation
Induction
Resuspended
cells
Sonication
Lyse cells
Lysate
Purification
Buffer
exchange


A
ribosomes
–
Tag-free
His-tag
OnePot proteins
+
–
+
–
+
[kDa]
250
150
100
75
50
37
25
20
15
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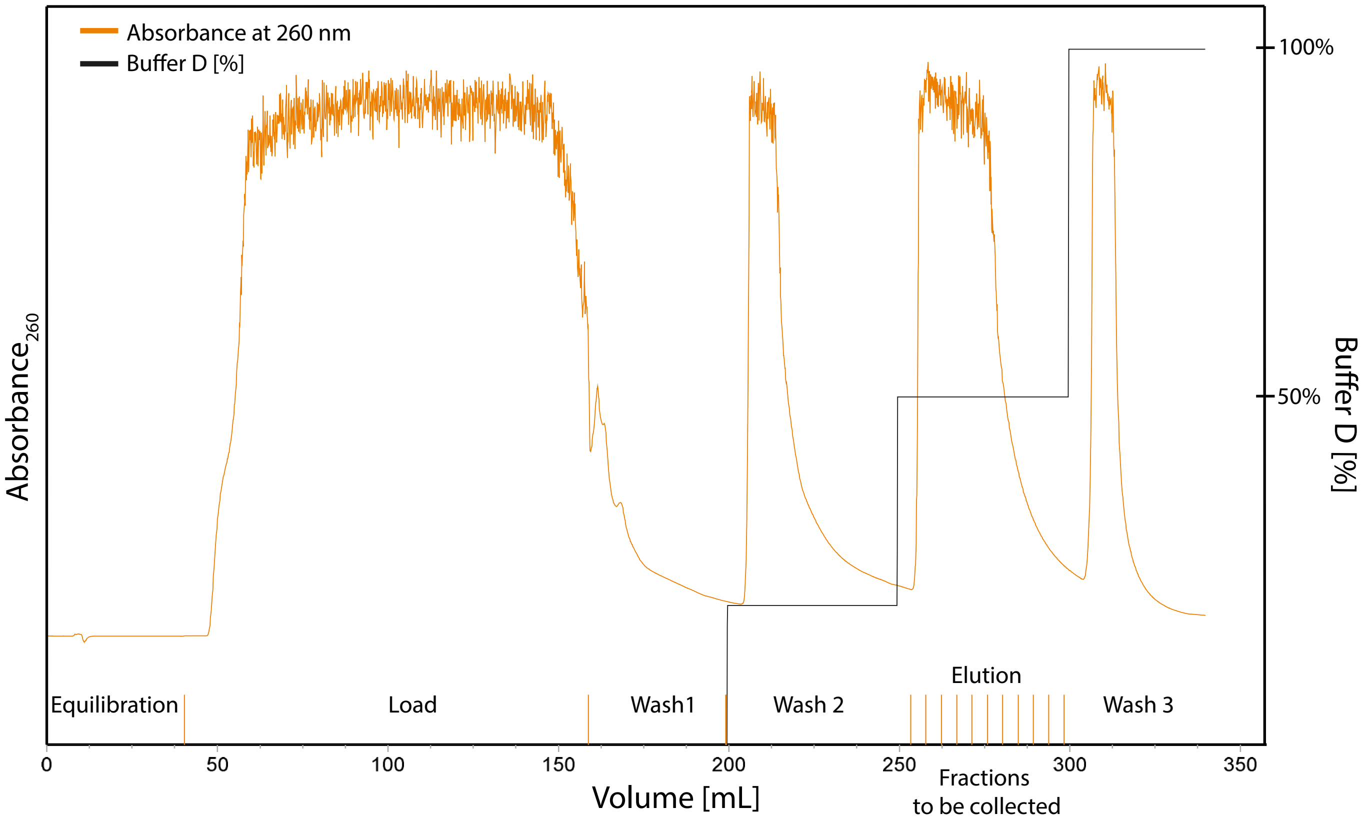
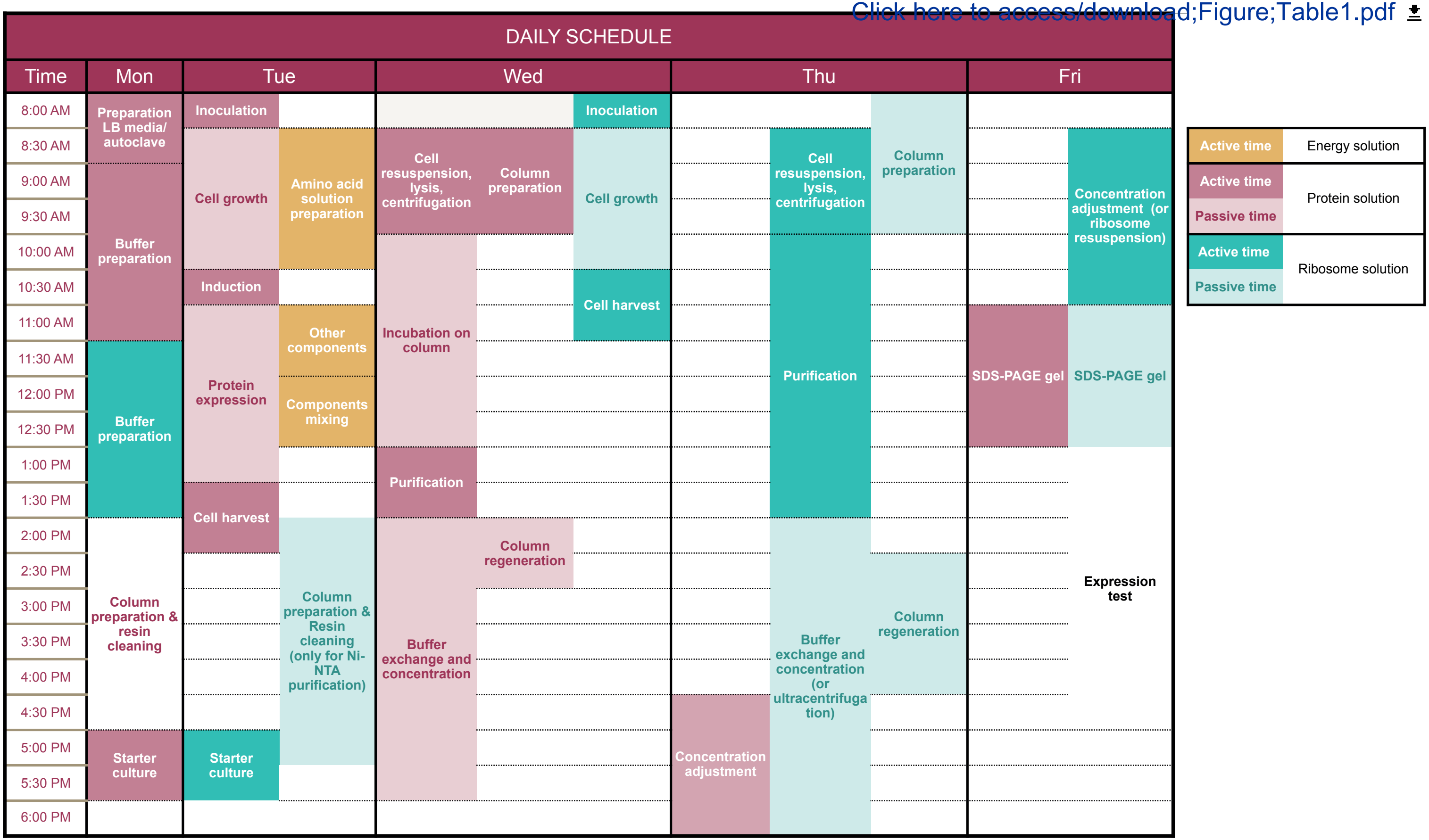
5
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
40
30
20
10
0
eGFP [10^3RFU]
Tag-free ribosomes
His-tag ribosomes
0
0.5
1
1.5
2
2.5
3
Time [h]
C
Tag-free
His-tag
[kDa]
155
100
65
41
33
23
12
Ladder non-Fluo.
Ladder
eGFP
ArgRS
T7 RNAP
eGFP
ArgRS
T7 RNAP
Ladder
Ladder non-Fluo.
Lane: 1 2 3 4 5 6 7 8 9 10
D
Tag-free
His-tag
[kDa]
250
150
100
75
50
37
25
20
15
10
5
Ladder
eGFP
ArgRS
T7 RNAP
eGFP
ArgRS
T7 RNAP
Ladder
Lane: 1 2 3 4 5 6 7 8 9 10


Absorbance at 260 nm
Buffer D [%]
Absorbance$_{260}$
Buffer D [%]
100%
50%
Equilibration
Load
Wash1
Wash 2
Elution
Wash 3
0
50
100
150
200
250
300
350
Volume [mL]
Fractions
to be collected



DAILY SCHEDULE

Time	Mon	Tue		Wed			Thu			Fri	
8:00 AM	Preparation LB media/ autoclave	Inoculation				Inoculation			Column preparation		
8:30 AM		Cell growth	Amino acid solution preparation	Cell resuspension, lysis, centrifugation	Column preparation	Cell growth		Cell resuspension, lysis, centrifugation			Concentration adjustment (or ribosome resuspension)
9:00 AM	Buffer preparation										
9:30 AM											
10:00 AM				Incubation on column				Purification			
10:30 AM		Induction				Cell harvest					
11:00 AM		Protein expression	Other components							SDS-PAGE gel	SDS-PAGE gel
11:30 AM	Buffer preparation										
12:00 PM			Components mixing								
12:30 PM											
1:00 PM				Purification							Expression test
1:30 PM		Cell harvest									
2:00 PM	Column preparation & resin cleaning		Column preparation & Resin cleaning (only for Ni-NTA purification)	Buffer exchange and concentration	Column regeneration			Buffer exchange and concentration (or ultracentrifugation)			
2:30 PM									Column regeneration		
3:00 PM											
3:30 PM											
4:00 PM											
4:30 PM							Concentration adjustment				
5:00 PM	Starter culture	Starter culture									
5:30 PM											
6:00 PM											

Active time	Energy solution
Active time Passive time	Protein solution
Active time Passive time	Ribosome solution

#Addgene	Number	Protein	Protein name	Size [kDa]	Vector	Strain	Induction volume [µL]	Note
#124103	**1**	**AlaRS**	Alanyl-tRNA synthetase	96.0	pQE30	M15	**55**	
#124104	**2**	**ArgRS**	Arginyl-tRNA synthetase	64.7	pET16b	BL21(DE3)	**55**	
#124105	**3**	**AsnRS**	Asparaginyl-tRNA synthetase	52.6	pQE30	M15	**55**	
#124106	**4**	**AspRS**	Aspartate-tRNA synthetase	65.9	pET21a	BL21(DE3)	**55**	
#124107	**5**	**CysRS**	Cysteinyl-tRNA synthetase	52.2	pET21a	BL21(DE3)	**55**	
#124108	**6**	**GlnRS**	Glutaminyl-tRNA synthetase	63.5	pET21a	BL21(DE3)	**55**	
#124109	**7**	**GluRS**	Glutamyl-tRNA synthetase	53.8	pET21a	BL21(DE3)	**55**	
#124110	**8**	**GlyRS**	Glycyl-tRNA synthetase	34.8 & 76.8	pET21a	BL21(DE3)	**55**	2 subunits
#124111	**9**	**HisRS**	Histidyl-tRNA synthetase	47.0	pET21a	BL21(DE3)	**55**	
#124112	**10**	**IleRS**	Isoleucyl-tRNA synthetase	104.3	pET21a	BL21(DE3)	**55**	
#124113	**11**	**LeuRS**	Leucyl-tRNA synthetase	97.2	pET21a	BL21(DE3)	**55**	
#124114	**12**	**LysRS**	Lysyl-tRNA synthetase	57.8	pET21a	BL21(DE3)	**55**	
#124115	**13**	**MetRS**	Methionine--tRNA ligase	76.3	pET21a	BL21(DE3)	**55**	
#124116	**14**	**PheRS**	Phenylalanyl-tRNA synthetase	36.8 & 87.4	pQE30	M15	**55**	2 subunits
#124117	**15**	**ProRS**	Prolyl-tRNA synthetase	63.7	pET21a	BL21(DE3)	**55**	
#124118	**16**	**SerRS**	Seryl-tRNA synthetase	48.4	pET21a	BL21(DE3)	**55**	
#124119	**17**	**ThrRS**	Threonyl-tRNA synthetase	74.0	pQE30	M15	**55**	
#124120	**18**	**TrpRS**	Tryptophanyl-tRNA synthetase	37.4	pET21a	BL21(DE3)	**55**	
#124121	**19**	**TyrRS**	Tyrosyl-tRNA synthetase	47.5	pET21a	BL21(DE3)	**55**	
#124122	**20**	**ValRS**	Valyl-tRNA synthetase	108.2	pET21a	BL21(DE3)	**55**	
#124123	**21**	**IF1**	Initiation factor 1	8.3	pQE30	M15	**55**	
#124124	**22**	**IF2**	Initiation factor 2	97.4	pQE30	M15	**55**	
#124125	**23**	**IF3**	Initiation factor 3	20.6	pQE30	M15	**55**	
#124126	**24**	**EF-G**	Elongation factor G	77.6	pQE60	M15	**55**	
#124127	**25**	**EF-Tu**	Elongation factor Tu	43.3	pQE60	M15	**1675**	
#124128	**26**	**EF-Ts**	Elongation factor Ts	30.4	pQE60	M15	**55**	
#124129	**27**	**RF1**	Release factor 1	40.5	pQE30	M15	**55**	
#124130	**28**	**RF2**	Release factor 2	41.2	pET15b	BL21(DE3)	**55**	
#124131	**29**	**RF3**	Release factor 3	59.6	pQE30	M15	**55**	
#124132	**30**	**RRF**	Ribosome recycling factor	20.6	pQE60	M15	**55**	
#124133	**31**	**MTF**	Methionyl-tRNA formyltransferase	34.2	pET21a	BL21(DE3)	**55**	
#124134	**32**	**CK**	Creatine kinase	37.1	pQE30	M15	**55**	
#118977	**33**	**MK**	Adenylate kinase (Myokinase)	21.7	pET21a	BL21(DE3)	**55**	
#124136	**34**	**NDK**	Nucleotide diphosphate kinase	15.5	pQE30	M15	**55**	
#118978	**35**	**PPiase**	Inorganic pyrophosphatase	32.3	pET21a	BL21(DE3)	**55**	
#124138	**36**	**T7 RNAP**	T7 RNA polymerase	98.9	pQE30	M15	**55**	

Name of Material/ Equipment	Company	Catalog Number
10x Tris/Glycine/SDS buffer	Bio-Rad Laboratories	1610732
15 mL centrifuge tubes	VWR International	525-0309
384-well Black Assay Plates	Corning	3544
4-20% Mini-PROTEANRTM TGXTM Precast Protein Gels	Bio-Rad Laboratories	4561096
50 mL centrifuge tubes	VWR International	525-0304
96-Well Polypropylene DeepWell plate	Nunc	260252
Acetic acid, 99.8 %	Acros	222140010
Äkta purifier	GE Healthcare	
AMICON ULTRA 0.5 mL - 3 KDa	Merck Millipore	UFC500324
AMICON ULTRA 15 mL - 3 KDa	Merck Millipore	UFC900324
Amino acids	Sigma-Aldrich	LAA21-1KT
Ammonium chloride	Sigma-Aldrich	09718-250G
Ammonium sulfate	Sigma-Aldrich	A4418
Ampicillin	Condalab	6801
BenchMark Fluorescent Protein Standard	ThermoFisher	LC5928
Breathe-Easy sealing membrane	Diversified Biotech	Z380059-1PAK
Centrifuge tubes polycarbonate	Beckman	355631
Chill-out Liquid Wax	Bio-Rad Laboratories	CHO1411
Creatine phosphate	Sigma-Aldrich	27920
DNA Clean & Concentrator-25 (Capped)	Zymo	ZYM-D4034-200TS
DTT	SantaCruz Biotech	sc-29089B
Econo-Pac Chromatography Columns	Bio-Rad Laboratories	7321010
EDTA (Ethylenediaminetetraacetic acid)	Sigma-Aldrich	03609-250G
Eppendorf Protein LoBind microcentrifuge tubes	VWR International / Eppendorf	525-0133
Falcon 14 mL Round Bottom Polystyrene Test Tube, with Snap Cap	Falcon	352051
Flasks, baffled 1000 mL 4 baffles, borosilicate glass	Scilabware	9141173
FluoroTect Green Lys in vitro Translation Labeling System	Promega	L5001
Folinic acid	Sigma-Aldrich	PHR1541
Glycerol	Sigma-Aldrich	G7757-1L
HEPES	Gibco	15630-056

HiTrap Butyl HP Column	GE Healthcare	28411005
IMAC Sepharose 6 Fast Flow	GE Healthcare	17-0921-07
Imidazole	Sigma-Aldrich	I2399
InstantBlue	Expedeon	ISB1L-1L
IPTG (Isopropyl-beta-D-thiogalactoside)	Alfa Aesar	B21149.03
Laemmli buffer (2x), sample buffer	Sigma-Aldrich	S3401-1VL
Lysogeny broth (LB) media	AppliChem	A0954
Magnesium acetate	Sigma-Aldrich	M0631
Magnesium chloride	Honeywell Fluka	63020-1L
Nickel Sulfate	Alfa Aesar	15414469
NTP	ThermoFisher	R0481
Phusion High-Fidelity DNA Polymerase (2 U/µL)	ThermoFisher	F530S
Potassium chloride	Sigma-Aldrich	P5405-1KG
Potassium glutamate	Sigma-Aldrich	49601
PURExpress In Vitro Protein Synthesis Kit	NEB	E6800S
PURExpress Δ Ribosome Kit	NEB	E3313S
Quick Start Bradford 1x Dye Reagent	Bio-Rad Laboratories	5000205
Rapid-Flow Sterile Single Use Vacuum Filter Units	ThermoFisher	564-0020
RNaseA solution	Promega	A7973
SealPlate film	Excel Scientific	Z369659-100EA
Sodium hydroxide	Sigma-Aldrich	6203
Spermidine	Sigma-Aldrich	S2626
Sucrose	Sigma-Aldrich	84097
TCEP (Tris(2-carboxyethyl)phosphin -hydrochlorid)	Sigma-Aldrich	646547-10X1mL
Thickwall Polycarbonate Tube	Beckman	355631
Trichloroacetic acid	Sigma-Aldrich	T0699
Tris base	ThermoFisher	BP152-500
tRNA	Roche	10109541001
Ultracentrifuge Optima L-80	Beckman	
Whatman GD/X syringe filters	GE Whatman	WHA68722504
β-mercaptoethanol	Sigma-Aldrich	M6250-100mL

Comments/Description
purification of tag free ribosomes
purification of tag free ribosomes
optional

purification of tag free ribosomes

purification of tag free ribosomes

1. The manuscript file has been formatted to fit the journal standard. Please take this opportunity to thoroughly proofread the manuscript to ensure no spelling or grammar issues.

2. The highlighting is above the 3- page limit. Please consider removing the highlights from some "Notes" to fit the highlighting to the 3-page limit.

We revised the manuscript accordingly.

3. Please discuss Figure 4 in the representative results section.

We revised the manuscript accordingly.

4. Please include a title and description for each supplementary table and list it under the Figure and Table Legends.
For example:
Supplementary Table 1: List of Reagents. The table lists the concentration, volume, and other specific details of the reagents/compounds used in the study.

We revised the manuscript accordingly.

5. Please ensure that the references are correctly cited. Reference numbers 18 and 25 are the same (marked in red). Please replace one of the references or adjust the reference numbers in the text accordingly.

We revised the manuscript accordingly.

6. Figure 1: Please maintain a single space between the numeral and (abbreviated) unit (e.g., 2 h, 5 mL, 70%, 37 °C, 3220 x g, 13.5 mg/mL, etc.).

We revised the manuscript accordingly.

7. Figure 3: Please label the line/lane numbers in the figure to make the figure more informative (Figure 3A).

We revised the manuscript accordingly.

Compound	Concentration	Volume
Lysogeny broth (LB) media	25 g/L	3 L
Ampicilin (AMP)	100 mg/mL in 50% (v/v) ethanol	2 mL
50 % Glycerol	50% (v/v)	100 mL
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	100 mM (23.8 mg/mL)	2 mL
Nickel sulfate	0.1 M	0.5 L
EDTA buffer	0.2 M EDTA, 0.5 M NaCl	1 L
NaCl	0.5 M	1 L
Acetic acid	0.1 M	1 L
NaOH	0.5 M	1 L
20% Ethanol	20% (v/v)	1 L
70% Ethanol	70% (v/v)	1 L

Sterilization
Autoclave
Filter sterilize (0.22 µM)
Autoclave
Filter sterilize (0.22 µM)

Note
2 L for ribosome solution preparation, 0.5 L for OnePot PURE, 0.5 L surplus
Can be made in larger volumes and stored at -20 °C for later use.
Can be made in larger volumes and stored at -20 °C for later use.
Dilute 100 mL of 0.5 M nickel sulfate in 400 mL water .
Weigh 29.22 g of NaCl, 58.4 g of EDTA and add 700 mL water. EDTA will not dissolve completely at this pH. Adjust to pH =7 with NaOH to dissolve EDTA, then add water to 1 L.
Prepare 29.22 g of NaCl and add water to 1 L.
Add 5.72 mL of glacial acetic acid to 994.3 mL of water. Caution: Add acid to water, not in the opposite o
Dissolve 20 g of NaOH in 800 mL water on ice and add water to 1 L

order.

Supplemental File 2

Stock solutions	Mw [g/mol]	Concentration [M]	Volume [L]
Magnesium acetate	214.45	1	0.25
Potassium chloride	74.55	2	1
Ammonium chloride	53.49	1	1
Imidazole (pH=7)	68.08	1	1
TrisHCl (pH=7.6) (TrisBase, HCl)	121.136	1	0.5

Protein solution purification components	Stock solution		
	MW [g/mol]	Concentration [M]	concen
HEPES**		1	**50**
Ammonium chloride*	53.49	1	**1000**
Magnesium chloride		1	**10**
Potassium chloride	74.55	2	
Imidazole (pH=7)	68.08	1	
Glycerol			
TCEP**		0.5	**1**
total buffer volume			

Ribosome purification components	Stock solution		
	MW [g/mol]	Concentration [M]	concen
HEPES**		1	**10**
Magnesium acetate	214.45	1	**10**
Potassium chloride	74.55	2	**50**
Ammonium chloride	53.49	1	
Ammonium sulfate*	132.14		
Sucrose*	342.30		
TCEP**		0.5	**1**
total buffer volume			

His-ribosome purification components	Stock solution		
	MW [g/mol]	Concentration [M]	concen
HEPES**		1	
TrisHCl (pH=7.6)		1	**20**

Ammonium chloride	53.49	1	**30**
Magnesium acetate	214.45	1	
Magnesium chloride		1	**10**
Potassium chloride	74.55	2	**150**
Imidazole (pH=7)	68.08	1	
Glycerol			
β-mercaptoethanol**		14.3	
total buffer volume			

* weighed directly to the buffer without preparing a prior stock solution

**add just before use

Stock solution

Mass [g]	Notes
53.6	Add the required mass, fill with water to the required volume. Filter
149.1	Add the required mass, fill with water to the required
53.5	Add the required mass, fill with water to the required
68.1	Add the required mass, fill with water to 80%, adjust the pH to 7 with HCl, and fil
60.6	Add the required mass of TrisBase, fill with water to 80%, adjust the pH to 7.6 with volume.

Buffer A			Buffer B				Buffe	
tration	volume		concentration		volume		concentration	
mM	25	mL	**50**	**mM**	25	mL	**50**	**mM**
mM	26.7	g						
mM	5	mL	**10**	**mM**	5	mL	**10**	**mM**
			100	**mM**	25	mL	**100**	**mM**
			500	**mM**	250	mL		
mM	1	mL	**1**	**mM**	1	mL	**1**	**mM**
	500	mL			500	mL		

Suspension buffer			Suspension buffer (high salt)				Buffe	
tration	volume		concentration		volume		concentration	
mM	2.5	mL	**10**	**mM**	1	mL	**20**	**mM**
mM	2.5	mL	**10**	**mM**	1	mL	**10**	**mM**
mM	1.25	mL	**50**	**mM**	2.5	mL		
			3000	**mM**	39.6	g	**1500**	**mM**
mM	0.50	mL	**1**	**mM**	0.20	mL	**1**	**mM**
	250	mL			100	mL		

Lysis buffer			Elution buffer				Ribosom	
tration	volume		concentration		volume		concentration	
							20	**mM**
mM	20	mL	**20**	**mM**	20	mL		

mM	30	mL	**30**	**mM**	30	mL		
							6	**mM**
mM	10	mL	**10**	**mM**	10	mL		
mM	75		**150**	**mM**	75	mL	**30**	**mM**
			150	**mM**	150	mL		
							7	**mM**
	1000	mL			1000	mL		

sterile and store at 4ºC
l volume.
l volume.
l with water to the required volume.
HCl, and fill with water to the required

r HT		**Stock buffer A**				**Stock buffer B**			
volume		concentration		volume		concentration		volume	
10	mL	**50**	**mM**	5	mL	**50**	**mM**	5	mL
2	mL	**10**	**mM**	1	mL	**10**	**mM**	1	mL
10	mL	**100**	**mM**	5	mL	**100**	**mM**	5	mL
		30%	**(v/v)**	30	mL	**60%**	**(v/v)**	60	mL
0.4	mL	**1**	**mM**	0.20	mL	**1**	**mM**	0.20	mL
200	mL			100	mL			100	mL

er C		**Buffer D**				**Cushion buffer**			
volume		concentration		volume		concentration		volume	
10	mL	**10**	**mM**	3	mL	**20**	**mM**	5	mL
5	mL	**10**	**mM**	3	mL	**10**	**mM**	2.5	mL
		50	**mM**	7.5	mL			0	mL
						30	**mM**	7.5	
99.1	g								
						30%	**(w/v)**	75	g
1.00	mL	**1**	**mM**	0.60	mL	**1**	**mM**	0.50	mL
500	mL			300	mL			250	mL

e buffer	
volume	
5	mL

1.5	mL
3.75	mL
0.12	mL
250	mL

Ribosome buffer			
concentration		volume	
20	**mM**	5	mL
6	**mM**	1.5	mL
30	**mM**	3.75	mL
1	**mM**	0.50	mL
		250	mL

Amino acid	Density [g/mL]	MW [g/mol]	Stock concentration [mM]
Ala	1.42	89.1	**168**
Arg	1.42	210.66	**500**
Asn	1.54	132.1	**100**
Asp	1.7	133.1	**12.5**
Cys	1.54	157.62	**200**
Glu	1.46	147.1	**25**
Gln	1.47	146.2	**100**
Gly	1.61	75.1	**500**
His	1.49	209.63	**500**
Ile	1.23	131.2	**100**
Leu	1.17	131.2	**150**
Lys	1.1	182.65	**500**
Met	1.34	149.2	**250**
Phe	1.34	165.2	**100**
Pro	1.38	115.1	**200**
Ser	1.6	105.1	**500**
Thr	1.3	119.1	**100**
Trp	1.4	204.2	**12.5**
Tyr	1.46	181.2	**3.25**
Val	1.32	117.1	**200**
H2O			

Amino acid solution	**final concentration**
total	**3.25**

Minimal required mass [mg]	Actual weighed mass [mg]	Water to add [µL]	Final volume to add [µL]
0.7		0	**38.7**
1.6		0	**13.0**
1		0	**65.0**
1		0	**520.0**
1.2		0	**32.5**
1.1		0	**260.0**
1.1		0	**65.0**
0.6		0	**13.0**
1.5		0	**13.0**
1		0	**65.0**
1		0	**43.3**
1.4		0	**13.0**
1.1		0	**26.0**
1.2		0	**65.0**
0.9		0	**32.5**
0.8		0	**13.0**
0.9		0	**65.0**
1.5		0	**520.0**
	1.18		
0.9		0	**32.5**
			104.5

			final volume **2000**

Supplemental File 4

Compound				
	Mw [mol/g]	Concentration [M]	Volume [mL]	Mass [g]
Magnesium acetate	214.45	2	50	21.4
Potassium glutamate	203.23	2	50	20.3
Creatine phosphate	327.14	1	1	0.327
Folinic acid	511.50	0.034	1	0.017
Spermidine	145.25	1	0.5	0.073
tRNA			0.5	0.050

Stock solution
Note
Filter sterilize, Store at -20 °C
Filter sterilize, Store at -20 °C
Store at -20 °C
Store at -20 °C
Warm to 34 °C, pipette 79 µL of spermidine and fill to 500 µL with water. Store at -20 °C
Add water directly to the vial and vortex gently. Store at -20 °C

Component	Stock concentration [mM]	Concentration of components in reaction [mM]	Concentration in Energy solution [mM]
HEPES	1000	50	**125**
Potassium glutamate	2000	100	**250**
Magnesium acetate	2000	11.8	**29.5**
ATP*	100	2	**5**
GTP*	100	2	**5**
CTP	100	1	**2.5**
UTP	100	1	**2.5**
tRNA [mg/mL]	200	3.5	**8.75**
Creatine phosphate	1000	20	**50**
TCEP	500	1	**2.5**
Folinic acid	34	0.02	**0.05**
Spermidine	500	2	**5**
Amino Acid solution	3.25	0.3	**0.75**
Water			

	Final concentration [fold]
Energy solution total	**2.5**

*For energy solutions volumes above 5 mL order double the amount of these components

Final volume to add [µL]
625.0
625.0
73.8
250.0
250.0
125.0
125.0
218.8
250.0
25.0
7.4
50.0
1153.8
1221.3

Final volume
5000

Primer name
5'final
3'final
5'extension
3'extension
5' Gene specific
3' Gene specific

* determine by user

Primer sequence
5' GATCTTAAGGCTAGAGTAC 3'
5' CAAAAAACCCCTCAAGAC 3'
5’ GATCTTAAGGCTAGAGTAC**TAATACGA CTCACTATAGG**GAGACCACAACGGTTT *CCCTCTAG*
5’ **CAAAAAACCCCTCAAGACCCGTTTA GAGGCCCCAAGGGGTTATGCTA**GTTTTTTTTTTTTTTT
5’ *CCTCTAGAAATAATTTTGTTTAAC* TT**AAGAAG**GAGGAAAAAAAA NNNNNNNNNNNNNN
5’ *GTAGCAGCCTGAGTCG* **TTA** TTANNNNNNNNNNNNNNNNNNNN 3'

Primer working concentration	Melting temperature
500 nM	47 °C
500 nM	47 °C
2.5 nM	58 °C
2.5 nM	58 °C
500 nM	Tm*
500 nM	Tm*

Notes	
	Cylcle number Temperature, Time
T7 promoter **T7 terminator**	Cylcle number Temperature, Time
RBS, Modify based on 5'-terminus of the target gene Modify based on 3'-terminus of the target gene	Cylcle number Temperature, Time

Thermocycler setting

Step 1	**Step 2**		
1x	30x		
98 °C, 180 s	98 °C, 20 s	47 °C, 30 s	72 °C, 30 s per 1 kb
1x	10x		
98 °C, 30 s	98 °C, 8 s	58 °C, 20 s	72 °C, 30 s per 1 kb
1x	30x		
98 °C, 180 s	98 °C, 20 s	Tm °C, 30 s	72 °C, 30 s per 1 kb

Step 3
1x 72 °C, 420 s
1x 72 °C, 420 s
1x 72 °C, 420 s

PURE reaction setup for Tag-free ribosomes

Component	Input concentration	Unit	Final concentration
Energy solution 2.5x	2.50	×	1
Protein solution**		mg/mL	
Ribosome solution ***	10	µM	1.8
DNA 1	100	ng/µL	4
DNA 2	100	ng/µL	0
tRNA Lys*	25	×	
Additional components			
Water			

Total volume [µL]
10

Calculation for DNA concentration	DNA length [bp]	Unit	Avg. MW of bp
eGFP	990	bp	650
		bp	650

PURE reaction setup for His-Taged ribosomes

Component	Input concentration	Unit	Final concentration
Energy solution 2.5x	2.50	×	1
Protein solution**		mg/mL	
Ribosome solution (His-tag)***	3.45	µM	0.6
DNA 1	100	ng/µL	4
DNA 2	100	ng/µL	
tRNA Lys*	25	×	
Additional components			
water			

Total volume [µL]
10

Calculation for DNA concentration	DNA length	Unit	Avg. MW of bp
eGFP	990	bp	650
		bp	650

Unit	Volume for one reaction [µL]	
×	4.00	
mg/mL	1.20	** determine the optimal protein concentration by titration
µM	1.80	*** ribosome concentration can be optimised by titration
nM	0.26	
nM	0.00	
×	0.00	* use at final concentration 1x
	2.74	

Unit
g/mol
g/mol

Unit	Volume for one reaction [µL]	
×	4.00	
mg/mL	1.20	** determine the optimal protein concentration by titration
µM	1.80	*** ribosome concentration can be optimised by titration
nM	0.26	
nM	0.00	
×	0.00	* use at final concentration 1x
	2.74	

Unit
g/mol
g/mol

Protein solution titration **

Input proteins concetration (determined by Bradfpord assay):	20	mg/mL
Final protein concentration [mg/mL]	**2.4**	**2.1**
Protein solution [µL]	1.20	1.05
Stock buffer B [µL]	0.00	0.15

Total concetration of protein solution and stock buffer B is kept constant

Optional: Ribosome solution titration (Tag-free)***

Input ribosome concetration:	15	µM
Final ribosome concentration [mg/mL]	**2.7**	**2.25**
Ribosome solution [µL]	1.80	1.50
Ribosom buffer [µL]	0.00	0.30

Optional: Ribosome solution titration (His-tag)***

Input ribosome concetration:	10	µM
Final ribosome concentration [mg/mL]	**1.8**	**1.4**
Ribosome solution [µL]	1.80	1.40
Ribosom buffer [µL]	0.00	0.40

1.8	1.5	1.2
0.90	0.75	0.60
0.30	0.45	0.60

1.8	1.35	0.9
1.20	0.90	0.60
0.60	0.90	1.20

1	0.6	0.3
1.00	0.60	0.30
0.80	1.20	1.50