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A High-Yield Streptomyces Transcription–Translation Toolkit for Synthetic Biology and Natural Product Applications

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TITLE:

A High-Yield *Streptomyces* Transcription–Translation Toolkit for Synthetic Biology and Natural Product Applications

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SUMMARY:

This protocol details an enhanced method for synthesizing high yields of recombinant proteins from a *Streptomyces venezuelae* cell-free transcription-translation (TX-TL) system.

ABSTRACT:

Streptomyces spp. are a major source of clinical antibiotics and industrial chemicals. *Streptomyces venezuelae* ATCC 10712 is a fast-growing strain and a natural producer of chloramphenicol, jadomycin, and pikromycin, which makes it an attractive candidate as a next-generation synthetic biology chassis. Therefore, genetic tools that accelerate the development of *S. venezuelae* ATCC 10712, as well as other *Streptomyces* spp. models, are highly desirable for natural product engineering and discovery. To this end, a dedicated *S. venezuelae* ATCC 10712

cell-free system is provided in this protocol to enable high-yield heterologous expression of high G+C (%) genes. This protocol is suitable for small-scale (10–100 mL) batch reactions in either 96-well or 384-well plate format, while reactions are potentially scalable. The cell-free system is robust and can achieve high yields (~5–10 mM) for a range of recombinant proteins in a minimal setup. This work also incorporates a broad plasmid toolset for real-time measurement of mRNA and protein synthesis, as well as in-gel fluorescence staining of tagged proteins. This protocol can also be integrated with high-throughput synthetic biology workflows or bespoke studies on biosynthetic pathways or single enzymes derived from high G+C (%) genes present in Actinomycetes genomes.

INTRODUCTION:

Cell-free transcription-translation (TX-TL) systems provide an ideal prototyping platform for synthetic biology to implement rapid design-build-test-learn cycles, the conceptual engineering framework for synthetic biology[1]. In addition, there is growing interest in TX-TL systems for high-value recombinant protein production in an open-reaction environment[2], for example, to incorporate non-standard amino acids in antibody-drug conjugates[3]. Specifically, TX-TL requires a cell extract, plasmid or linear DNA, and an energy solution to catalyze protein synthesis in batch or semicontinuous reactions. While *Escherichia coli* TX-TL is the dominant cell-free system, a number of emerging non-model TX-TL systems have attracted attention for different applications[4–8]. Key advantages of TX-TL include flexible scalability (nanoliter to liter scale)[9,10], strong reproducibility, and automated workflows[8,11,12]. In particular, automation of TX-TL permits the accelerated characterization of genetic parts and regulatory elements[8,12,13].

In terms of reaction setup, TX-TL requires primary and secondary energy sources, as well as amino acids, cofactors, additives, and a template DNA sequence. Nucleotide triphosphates (NTPs) provide the primary energy source to drive initial mRNA (ATP, GTP, CTP, and UTP) and protein synthesis (only ATP and GTP). To increase TX-TL yields, NTPs are regenerated through the catabolism of a secondary energy source, such as maltose[14], maltodextrin[15], glucose[14], 3-phosphoglycerate (3-PGA)[16], phosphoenolpyruvate[17], and L-glutamate[18]. This inherent metabolic activity is surprisingly versatile, yet poorly studied, especially in emerging TX-TL systems. Each energy source has distinct properties and advantages in terms of ATP yield, chemical stability, and cost, which is an important consideration for scaled-up TX-TL reactions. So far, current protocols for *E. coli* TX-TL have reached up to 4.0 mg/mL (~157 mM) for the model green fluorescent protein (GFP) using a blend of 3-PGA (30 mM), maltodextrin (60 mM), and D-ribose (30 mM) as the secondary energy source[19].

Recently, there has been a rising interest in studying secondary metabolite biosynthetic pathways in TX-TL systems[20–22]. Specifically, Actinobacteria are a major source of secondary metabolites, including antibiotics and agricultural chemicals[23,24]. Their genomes are enriched with so-called biosynthetic gene clusters (BGCs), which encode enzymatic pathways for secondary metabolite biosynthesis. For the study of Actinobacteria genetic parts and biosynthetic pathways, a range of *Streptomyces*-based TX-TL systems have recently been developed[5,6,25,26]. These specialized *Streptomyces* TX-TL systems are potentially beneficial for the following reasons: [1] provision of a native protein folding environment for enzymes from *Streptomyces* spp.[26]; [2] access to a high

G+C (%) tailored tRNA pool for optimal high G+C (%) gene expression; [3] active primary metabolism, which potentially can be hijacked for the supply of biosynthetic precursors; and [4] tailoring of enzymes, precursors, or cofactors from secondary metabolism present in the native cell extract. Hence, a high-yield *S. venezuelae* TX-TL toolkit has recently been established to harness these unique capabilities[5].

Streptomyces venezuelae is an emerging host for synthetic biology with a rich history in industrial biotechnology[5,27–29] and as a model system for studying cell division and genetic regulation in Actinobacteria[30–32]. The main type strain, *S. venezuelae* ATCC 10712, has a relatively large genome of 8.22 Mb with 72.5% G+C content (%) (Accession number: CP029197), which encodes 7377 coding sequences, 21 rRNAs, 67 tRNAs, and 30 biosynthetic gene clusters[27]. In synthetic biology, *S. venezuelae* ATCC 10712 is an attractive chassis for the heterologous expression of biosynthetic pathways. Unlike most other *Streptomyces* stains, it provides several key advantages, including a rapid doubling time (~40 min), an extensive range of genetic and experimental tools[5,28], lack of mycelial clumping, and sporulation in liquid media[28,33]. Several studies have also demonstrated the use of *S. venezuelae* for heterologous production of a diverse array of secondary metabolites, including polyketides, ribosomal and nonribosomal peptides[34–38]. These combined features make this strain an attractive microbial host for synthetic biology and metabolic engineering applications. While *S. venezuelae* is not the dominant *Streptomyces* model for heterologous gene expression, with further developments, it is primed for broader use within natural product discovery.

This manuscript presents a detailed protocol (**Figure 1**) for a high-yield *S. venezuelae* TX-TL system, which has been updated from the original previously-published protocol[26]. In this work, the energy solution and reaction conditions have been optimized to increase protein yield up to 260 mg/mL for the mScarlet-I reporter protein in a 4 h, 10 mL batch reaction, using a standard plasmid, pTU1-A-SP44-*mScarlet-I.* This plasmid has been specifically designed to enable various methods of detecting protein expression. The protocol is also streamlined, while the energy system has been optimized to reduce the complexity and cost of setting up cell-free reactions without compromising the yield. Along with the optimized TX-TL system, a library of genetic parts has been developed for fine-tuning gene expression and as fluorescent tools for monitoring TX-TL in real time, thereby creating a versatile platform for prototyping gene expression and natural product biosynthetic pathways from *Streptomyces* spp. and related Actinobacteria.

In this work, the recommended standard plasmid (pTU1-A-SP44-*mScarlet-I*) can be used to establish the *S. venezuelae* TX-TL workflow in a new laboratory and is available on AddGene (see **Supplemental Table S1**). pTU1-A-SP44-*mScarlet-I* provides the user with the flexibility to study other open-reading frames (ORFs). The mScarlet-I ORF is codon-optimized for *S. venezuelae* gene expression. The SP44 promoter is a strong constitutive promoter that is highly active in both *E. coli* and *Streptomyces* spp.[39]. The plasmid has two unique restriction enzyme sites (NdeI, BamHI) to allow the sub-cloning of new ORFs in-frame with a joint C-terminal FLAG-tag and fluorescein arsenical hairpin (FlAsH) binder tag system. Alternatively, both tags can be removed with the inclusion of a stop codon after sub-cloning a new gene. With this base vector, the high-yield expression of a range of proteins has been demonstrated, namely proteins from the

oxytetracycline biosynthesis pathway and an uncharacterized nonribosomal peptide synthetase (NRPS) from *Streptomyces rimosus* (**Figure 2**). In terms of mRNA detection, the pTU1-A-SP44-*mScarlet-I* standard plasmid contains a dBroccoli aptamer (in the 3'-untranslated region) for detection with the 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) probe. For increased flexibility, a toolset of EcoFlex[40]-compatible MoClo parts has also been made available on AddGene, including an EcoFlex-compatible *Streptomyces* shuttle vector (pSF1C-A-RFP/pSF2C-A-RFP) and a range of pTU1-A-SP44 variant plasmids expressing superfolder green fluorescence protein (sfGFP), mScarlet-I, mVenus-I, and β-glucuronidase (GUS). In particular, the pSF1C-A plasmid is derived from pAV-*gapdh*[28] and is cured of BsaI/BsmBI sites for MoClo assembly. pSF1C-A-RFP/pSF2C-A-RFP is equivalent to pTU1-A-RFP/pTU2-A-RFP from EcoFlex[40] but contains additional functionality for conjugation and chromosomal integration in *Streptomyces spp*. using the *phiC31* integrase system[28].

The first stage of the protocol involves the growth of the *S. venezuelae* ATCC 10712 or a closely related strain, cell harvest at mid-exponential phase, cell wash steps, and equilibration in S30A and S30B buffers. This stage requires three days, and the time for cell growth can be used to prepare the remaining components as described below. The harvested cells are then lysed by sonication, clarified, and undergo a run-off reaction. At this final stage of preparation, the cell extracts can be prepared for long-term storage at -80 °C to minimize loss of activity. For the assembly of TX-TL reactions using this protocol, a *Streptomyces* Master Mix (SMM) is presented, with the option of a Minimal Energy Solution format (MES) that gives comparable yields. Further, it is recommended to streak a fresh culture of *S. venezuelae* ATCC 10712 from a -80 °C glycerol stock onto a GYM agar plate and incubate at 28 °C for at least 48–72 h until single colonies are visible. Only fresh cultures should be used for the following steps.

PROTOCOL:

NOTE: See **Table 1** and **Table 2** for recipes for GYM medium and agar plate and S30A and S30B wash buffers.

1. Preparation of solutions and general guidance

1.1. Keep all solutions, cells (post-growth), and cell extracts on ice after preparation, unless an exception is stated.

1.2. Store stocks for 1 M Mg-glutamate, 4 M K-glutamate, 40% (w/v) PEG 6000, 1.11 g/mL polyvinylsulfonic acid at room temperature, and all other stocks at -80 °C. Minimize the number of freeze-thaw cycles to avoid chemical degradation.

1.3. For the preparation of energy solution stocks (see **Table 3**) such as 3-PGA (requires pH adjustment), follow the guidance provided in the *E. coli* TX-TL protocol[41].

NOTE: All components are fully soluble in ddH_2O and stored as aliquots in the -80 °C freezer.

1.4. Defrost individual stocks or energy solutions (described later) on ice. Heat the amino acids stock at 42 °C with vortexing for ~15–30 min to solubilize all amino acids.

1.5. As some amino acids (L-Cys, L-Tyr, L-Leu) precipitate on ice, while minimizing rest time, leave this solution at room temperature and use a vortex to dissolve.

1.6. Add the calculated volumes (**Table 3**) of stock solutions and water and mix well using a vortex.

1.7. Aliquot the energy solution as 20–100 µL aliquots per tube, or as desired, on ice and store at -80 °C until further use.

2. Preparation of *S. venezuelae* ATCC 10712 cells

2.1. Day 1—Media/buffer preparation and overnight pre-culture

2.1.1. Prepare 1 L of sterile GYM liquid medium in a 2 L baffled flask, as described in **Table 1**. See the **Table of Materials** for equipment/chemical/reagent sources.

2.1.2. Prepare 1 x 50 mL of sterile GYM liquid medium in a 250 mL Erlenmeyer flask, as described in **Table 1**.

2.1.3. Prepare 100 mL of 1 M HEPES, 100 mL of 1 M $MgCl_2$, and 500 mL of 4 M NH_4Cl solutions to make 1 L of S30A and 1 L of S30B wash buffers. See **Table 2** for the recipes.

2.1.4. Prepare the overnight pre-culture. Pre-warm the sterile 50 mL of GYM liquid medium in a 250 mL Erlenmeyer flask to 28 °C for 30 min.

2.1.5. Inoculate a single colony of *S. venezuelae* ATCC 10712 (or related strain) from a GYM agar plate into prewarmed 50 mL of GYM liquid medium and incubate at 28 °C, 200 rpm for 16 h (pre-culture 1).

2.2. Day 2—Prepare daytime pre-culture and main growth culture.

2.2.1. Pre-warm 50 mL of sterile GYM liquid medium in a 250 mL Erlenmeyer flask at 28 °C for 30 min.

2.2.2. Transfer 1 mL of overnight pre-culture into pre-warmed 50 mL of GYM liquid medium and incubate at 28 °C, 200 rpm for 8 h (pre-culture 2).

2.2.3. After this growth period, check the OD_{600} in a spectrophotometer using a 1:10 dilution with sterile GYM medium in a 1 mL (1 cm path length) plastic cuvette.

NOTE: The OD_{600} should have reached at least 3–4. If there is poor growth, it is advisable to repeat steps 2.1–2.2.2.

2.2.4. Sub-culture 0.25 mL of pre-culture 2 into 1 L of liquid GYM medium in 2 L baffled flasks.

2.2.5. Shake overnight at 28 °C, 200 rpm for 14 h.

2.3. Day 3—Harvest cells

2.3.1. After the previous incubation period (14 h), record the OD_{600} of the main culture. Dilute the overnight culture 1:10 with fresh GYM medium for OD_{600} measurement.

NOTE: The OD_{600} should have reached 3.0–4.0 at this stage.

2.3.2. If OD_{600}<3.0, increase the shaking speed to 250–300 rpm and grow until an OD_{600} of 3.0 is reached. Grow for no longer than an additional 2 h (16 h in total).

2.3.3. If OD_{600}>3.0, transfer the cultures to centrifugation containers and rapidly cool on wet ice for 30 min.

2.3.4. While waiting for the cell culture to cool on ice, prepare 4 mL of fresh 1 M dithiothreitol (DTT), S30A and S30B buffers, as described in **Table 1**, and keep them on ice. See the **Table of Materials** for chemical/reagent source.

2.3.5. Pre-weigh an empty 50 mL centrifuge tube and pre-chill at -20 °C.

2.3.6. Add 2 mL of 1 M DTT to 1 L of S30A buffer on ice and mix well.

NOTE: Add DTT to the S30A and S30B wash buffers only before using them.

2.3.7. Centrifuge cells at 6,000 × g, 4 °C, 10 min, and carefully discard the supernatant in a quick and single motion.

NOTE: If the pellet is disturbed, maximize cell retention with residual GYM medium and continue the protocol.

2.3.8. Add 500 mL of S30A buffer and resuspend the cells by shaking the centrifugation bottles vigorously until the cell clumps are homogeneously dispersed.

2.3.9. Centrifuge the cells at 6,000 × g, 4 °C, 6 min, and carefully discard the supernatant.

NOTE: Although the cell pellet will be firmer at this point, some cells will remain in suspension (see **Figure 1**). Treat as described in 1.3.7 and retain as many cells as possible.

2.3.10. Repeat steps 2.3.8–2.3.9.

2.3.11. Add 2 mL of 1 M DTT to 1 L of S30B buffer on ice and mix well. Add 500 mL of S30B buffer to the cells. Repeat step 2.3.9.

2.3.12. Resuspend the cell pellet in 10 mL of S30B buffer and transfer to the pre-weighed, pre-chilled 50 mL centrifuge tube. If required, transfer the residual cells with an additional 5–10 mL of S30B buffer. Fill to 50 mL with S30B.

2.3.13. Centrifuge cells at 6,000 × *g*, 4 °C, 10 min, and carefully discard the supernatant.

2.3.14. Repeat step 2.3.13.

2.3.15. Carefully aspirate the remaining S30B supernatant with a 100–200 µL pipette.

2.3.16. Weigh the wet cell pellet.

NOTE: Typical wet cell pellet weight for 1 L of overnight GYM culture (OD_{600} = 3.0) is ~4.5 g.

2.3.17. For every 1 g of wet cells, add 0.9 mL of S30B buffer. Resuspend the cells using either a Pasteur pipette or vortex.

2.3.18. Centrifuge briefly (~10 s) up to 500 × *g* to sediment the cells.

NOTE: The protocol can be paused at this point, and cells can be frozen on either liquid nitrogen or dry ice and stored at -80°C. **For safety**, wear appropriate personal protective equipment (PPE) when handling liquid nitrogen, including face shields and gloves.

3. Cell lysis by sonication to obtain the crude cell extract

NOTE: At this stage, the user can choose to disrupt the cells by sonication either in 1 mL fractions (option 2.1) or as a larger cell suspension (5 mL) in a 50 mL tube (option 2.2). Both options have been detailed below to ensure reproducibility, as the final volume of the cell suspension can change due to the loss of cells between steps 2.3.1–2.3.18. A new user should attempt option 2.1 first to establish the protocol.

3.1. Cell Lysis by sonicating in 1 mL fractions

3.1.1. Using a 1 mL pipette tip (cut off the end of the tip to increase the bore size), transfer 1 mL of the cell suspension into 2 mL microcentrifuge tubes.

NOTE: If the cells are frozen, rapidly thaw the 50 mL tube containing the pellet in lukewarm water prior to cell lysis. Transfer the tube to wet ice as soon as the pellet has begun to defrost, and chill for 10 min.

3.1.2. Place each microcentrifuge tube in a beaker of ice water, using a plastic tube rack to hold the tube for sonication.

NOTE: Due to the sensitivity of the cell extract to overheating, it is critical to ensure that the tubes do not warm up to prevent protein precipitation and reduced enzymatic activity.

3.1.3. Use a sonicator probe with a 3 mm diameter tip and clean it with 70% (v/v) ethanol and double-distilled water (ddH_2O). Lower the sonicator tip into the cell suspension until it is ~1 cm below the liquid surface.

3.1.4. Input the following settings into the sonicator: 20 kHz frequency, 65% amplitude, 10 s pulse ON time, 10 s pulses OFF time, 1 min total sonication time.

3.1.5. Run the sonication protocol. Move the tube up/down and sideways during the first two resting cycles to ensure the cells are evenly sonicated. Record the energy input.

NOTE: **For safety**, wear appropriate hearing protection during sonication. The viscosity will decrease as cells are disrupted, and the pale cream wet cell pellet should turn into a homogenous brown fluid. The recommended energy input is 240 J per mL of wet cells. If the cells are only partially lysed, the suspension will still appear cream-colored with viscous clumps of cells, particularly on the sides of the tube.

3.1.6. Invert the tube 2–3 times and repeat the sonication for an additional one or two 10 s cycles, mixing frequently until the cells are fully disrupted.

3.2. Cell Lysis by sonicating a 5 mL cell suspension

3.2.1. If the cells are frozen, rapidly thaw the 50 mL tube containing the pellet in lukewarm water with shaking before cell lysis. Transfer the tube to wet ice as soon as the pellet has begun to defrost, and chill for 10 min.

3.2.2. Briefly spin the tube at 500 x *g* to sediment the cells.

3.2.3. Place the 50 mL tube in a beaker of ice water for sonication.

NOTE: Due to the sensitivity of the cell extract to overheating, it is critical to ensure that the tubes do not warm up to prevent protein precipitation and reduced enzymatic activity.

3.2.4. Use a sonicator probe with a 6 mm diameter tip and clean it with 70% (v/v) ethanol and ddH_2O (see the visual schematic of 6 mm probe in **Figure 1**). Lower the sonicator tip into the cell suspension (~5 mL) until it is ~1 cm below the liquid surface.

3.2.5. Input the following settings into the sonicator: 20 kHz frequency, 65% amplitude, 10 s pulse ON time, 10 s pulses OFF time, 1 min total sonication time per mL of wet cells (5 min in total).

3.2.6. Run the sonication protocol. Move the tube up/down and sideways during the first two resting cycles to ensure the cells are evenly sonicated.

NOTE: **For safety**, wear appropriate hearing protection during sonication. The viscosity will decrease as the cells are disrupted, and the pale cream wet cell pellet should turn into a homogenous brown fluid. Record the energy input. An optimal energy input of 240 J per mL of wet cells (~1200 J in total from 5 min sonication) is recommended.

3.2.7. If some cells remain intact, follow the guidance from step 3.1.5.

3.2.8. Transfer the cell extracts into 2 mL microcentrifuge tubes.

4. Cell extract clarification and run-off reaction

4.1. Centrifuge the lysed cells at 16,000 × *g* for 10 min at 4 °C to remove the cell debris. Transfer the supernatant into 1.5 mL microcentrifuge tubes as 1 mL aliquots.

4.2. Perform the run-off reaction for the cell extracts. Incubate the 1.5 mL tubes containing the cell extracts at 30 °C for 60 min on a heat block or incubator without shaking.

4.3. Centrifuge the cell extracts at 16,000 × *g* for 10 min at 4 °C. Pool the supernatants into a 15 mL centrifuge tube. Mix the supernatant by inverting the tube five times until homogenous, then keep it on ice. Invert gently to avoid the formation of air bubbles.

4.4. Dilute 10 µL of the cell extract 100-fold with S30B buffer and measure the total protein concentration using a Bradford assay with three technical repeats (see **Supplemental Material S2** for Bradford assay guidance).

4.5. If the protein concentration is 20–25 mg/mL, transfer the cell extracts as 100 µL aliquots into new 1.5 mL tubes, flash-freeze in liquid nitrogen, and store at -80 °C.

NOTE: **For safety**, wear appropriate PPE when handling liquid nitrogen, including face shields and gloves.

4.6. If the protein concentration is <20 mg/mL, repeat the crude extract preparation steps to ensure high-quality cell extract and TX-TL yields comparable to the previously published work[5].

5. Preparation of plasmid DNA template

5.1. Purify the pTU1-A-SP44-*mScarlet-I* plasmid (pUC19 origin) from a freshly transformed *E. coli* plasmid strain (DH10b, JM109) grown in 50 mL of LB culture (with 100 mg/mL carbenicillin) using an appropriate plasmid DNA purification kit as per manufacturer's instructions.

5.2. Elute the plasmid in 2 x 300 µL of nuclease-free water and combine the fractions.

5.3. Add 0.1 volumes (66 mL) of 3 M sodium acetate (pH 5.2).

5.4. Add 0.7 volumes (462 mL) of isopropanol.

5.5. Incubate the DNA at -20 °C for 30 min.

5.6. Centrifuge at 16,000 × *g* for 30 min at 4 °C and discard the supernatant.

5.7. Add 2 mL of 70% (v/v) ethanol to the DNA pellet.

5.8. Invert the tube 3–4 times to resuspend the plasmid DNA pellet.

5.9. Centrifuge at 16,000 × *g* for 5 min at 4 °C and discard the supernatant.

5.10. Repeat steps 5.7–5.9 and remove all visible liquid.

5.11. Air-dry the DNA pellet for 10–30 min or dry for 5 min with a vacuum centrifuge.

5.12. Resuspend the dried pellet with 600 µL of nuclease-free ddH_2O.

5.13. Measure the DNA concentration and purity using a spectrophotometer.

5.14. Prepare 50–100 µL aliquots and store at -20 °C.

NOTE: A high DNA concentration in the range of 500–1000 ng/µL is recommended due to the tight volume constraints of cell-free reactions. Dilute the plasmid DNA stock to 80 nM; 168 ng/µL pTU1-A-SP44-*mScarlet-I* plasmid is equivalent to 80 nM.

6. Preparation of the *Streptomyces* Master Mix (SMM) solution

6.1. Amino acid solution

6.1.1. Use the amino acid sampler kit to avoid manual errors and reduce preparation time, following the manufacturer's instructions provided online.

6.1.2. Dilute the 20x amino acid stock solution using ddH_2O to a final concentration of 6 mM (5 mM L-Leu).

6.1.3. Further dilute to 2.4 mM (2 mM L-Leu) within the **2.4x SMM** solution (see **Table 3**).

NOTE: The final concentration in the TX-TL reaction is 1 mM 19x amino acids and 0.83 mM L-Leu.

6.2. Energy solution and additives

6.2.1. Prepare the other components in the **2.4x SMM** solution by following the recipe described in **Table 3**.

6.2.2. Alternatively, prepare a **2.4x MES**, following the recipe described in **Table 3**.

7. Setting up a standard *S. venezuelae* TX-TL reaction

7.1. Thaw the cell extract, **SMM** (or **MES**) solution, and plasmid DNA on ice. Pre-chill a 384-well plate at -20 °C.

7.2. Set up TX-TL reactions where 25% of the volume is plasmid DNA, 33.33% is cell extract, and 41.67% is **SMM** solution; keep them on ice to avoid start time bias.

NOTE: A standard TX-TL template has been provided (**Table 4**) to calculate the volume of reagents needed based on the number of reactions. The standard volume for a 33 µL reaction is as follows: 11 µL of cell extract, 13.75 µL of **SMM**, and 8.25 µL of plasmid DNA.

7.3. Gently vortex the mixture for ~5 s at a low-speed setting to ensure the solution is homogenous. Avoid foaming/bubble formation.

7.4. Transfer 10 µL aliquots into three wells of a 384-well plate as a technical triplicate without introducing air bubbles. Seal the plate with a transparent cover and spin at 400 × *g* for 5 s.

7.5. Incubate the reaction at 28 °C either in an incubator (for end-point readings) or a plate-reader without shaking.

NOTE: Reactions typically require 3–4 h to reach completion. See **Supplemental Material S2** for guidance on a plate reader and mScarlet-I standard measurements.

REPRESENTATIVE RESULTS:

This detailed protocol is provided as an example to help the user establish a *Streptomyces* TX-TL system based on the *S. venezuelae* ATCC 10712 model strain (**Figure 1**). The user may seek to study other *Streptomyces* strains; however, the growth/harvesting stages of other strains with longer doubling times or growth preferences will need to be custom-optimized to achieve peak results. For the representative result, the mScarlet-I fluorescent protein from the pTU1-A-SP44-*mScarlet-I* standard plasmid (**Figure 2** and **Figure 3**) was optimized to provide high-yield expression in *S. venezuelae* TX-TL with a range of detection methods (SDS-PAGE, fluorescence). In addition, this standard plasmid was modified to demonstrate the synthesis of a range of

secondary metabolite enzymes from *S. rimosus* (**Figure 2**)[5]. Finally, a potential workflow for scaled-up natural product biosynthesis is shown as a schematic workflow using a model pathway for the early-stages of heme biosynthesis. The workflow is potentially adaptable to other secondary metabolite biosynthetic pathways. As a guideline, this protocol should provide a minimum yield of 2.8 μM for sfGFP and 3.5 μM for mScarlet-I/mVenus from the expression plasmids provided on AddGene. These figures allow for typical batch variation (up to 28%) observed in previous data[5], although yields greater than 10 μM mScarlet-I have been achieved with optimal batches (unpublished data).

Measuring *S. venezuelae* TX-TL of the mScarlet-I gene using five distinct methods

The expression of the pTU1-A-SP44-*mScarlet-I* standard plasmid is shown, with the measurement of mScarlet-I expression using five different methods: 1. real-time fluorescence measurement of mRNA using the dBroccoli aptamer, 2. real-time fluorescence measurement of immature mScarlet-I protein using the FlAsH tag system, 3. real-time fluorescence measurement of mature mScarlet-I protein, 4. in-gel fluorescence staining of mScarlet-I using FlAsH tag, and 5. Coomassie blue staining of total cell-free proteins. For this data, the reactions were set up in 2 mL microcentrifuge tubes as 33 μL reactions (for end-point samples) or as a 10 mL technical triplicate in 384-well plates in a plate reader. A triple-tagged (N-terminal His_6, C-terminal Flag and C-terminal FlAsH) mScarlet-I protein was separately purified to create a calibration standard for measurements, using the pET15b-*mScarlet-I* plasmid, which is described further in **Supplemental Material S2**. The data for these experiments are shown in **Figure 3**. Further details of the in-gel fluorescence staining method are available in **Supplemental Material S3**.

***S. venezuelae* TX-TL of early-stage heme biosynthesis**

To serve as a model natural product biosynthetic pathway, the 'one-pot' biosynthesis of uroporphyrinogen III (uro'gen III) was performed using the pTU1-A-SP44-*hemC-hemD*/*cysG*^{A}-*hemB* expression plasmid[5]. This model biosynthetic pathway was chosen sas uro'gen III is highly oxygen-sensitive and rapidly oxidizes (loss of 6 electrons) to uroporphyrin III, which displays strong red fluorescence. This enables easy detection of the reaction in real time using fluorescence measurements and/or HPLC-MS (**Figure 4**), as previously described[5]. In addition, these reactions were studied using either a batch or semicontinuous method. A semicontinuous reaction is a strategy, which uses a micro-dialysis device[42,43] that provides additional energy (NTPs, secondary energy source) and amino acids to prolong the reaction time and increase protein synthesis yields. Here, the semicontinuous method is used to scale up the heme model reaction and separate the TX-TL proteins from the reaction product to facilitate the purification and analysis by HPLC-MS. Further details of methods are available in **Supplemental Material S4** or for data, see previous work[5]. Semicontinuous cell-free reactions are also described in earlier work[42,43]. The example schematic workflow demonstrated here (**Figure 4**) is potentially adaptable to other natural product biosynthetic pathways.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the *Streptomyces venezuelae* TX-TL protocol. A protocol summary is illustrated, including a recommended time frame of three days. The protocol is broken down into

distinct stages of cell growth, cell harvest, cell wash, cell lysis by sonication, clarification, run-off reaction, energy solution (SMM) preparation, plasmid DNA preparation, and the TX-TL reaction assembly. The full protocol is described in detail within the text, along with helpful guidance and practical tips. Abbreviations: SMM = *Streptomyces* Master Mix; TX-TL = transcription-translation.

Figure 2: High-yield protein synthesis from high G+C (%) genes. (**A**) Synthesis of sfGFP, mVenus-I, and mScarlet-I fluorescent proteins. (**B**) Synthesis of biosynthetic enzymes from *Streptomyces rimosus.* The figure is modified from [5]. Please see the protocol and supplemental files for reaction setup and methodology.

Figure 3: Measurement of TX-TL five-ways with the pTU1-A-SP44-*mScarlet-I* plasmid. (**A**) Plasmid design including the following features: SP44 is a strong constitutive promoter active in *Streptomyces* spp. and *E. coli*; pET-RBS is derived from the pET expression plasmids and is highly active in both *Streptomyces* spp. and *E. coli*[5,40]; *Streptomyces* codon-optimized *mScarlet-I* gene, which encodes a rapid folding red-fluorescent protein derivative[44]; C-terminal FLAG-tag for affinity chromatography purification or western blotting detection; C-terminal FlAsH tag for fluorescent labeling for in-gel staining or real-time measurement of nascent protein synthesis; dBroccoli aptamer for real-time mRNA measurement using the DFHBI probe; Bba_B0015 transcription terminator, which are highly efficient in *S. venezuelae* ATCC 10712[5]; ampicillin resistance marker; and pUC19 origin of replication. (**B**) Real-time mRNA expression, detected with the dBroccoli aptamer and the DFHBI probe (excitation 483-14 nm, emission 530-30 nm). (**C**) Real-time nascent protein synthesis detection with FlAsH-EDT_2 fluorescent probe (excitation 500-10 nm, emission 535-10 nm). (**D**) Real-time fluorescence measurement of mScarlet-I synthesis (excitation 565-10 nm, emission 600-10 nm). (**E**) In-gel staining with the FlAsH-EDT_2 fluorescent probe. (**F**) Coomassie blue staining of total TX-TL proteins with purified His_6-mScarlet-I standard for comparison. Reactions were run under the conditions described in the protocol with 40 nM of plasmid DNA template. All fluorescence data are represented as RFU, and error bars (standard deviation of three technical repeats) are represented within a grey shaded area. Abbreviations: TX-TL = transcription-translation; FlAsH = fluorescein arsenical hairpin; RFU = relative fluorescence units.

Figure 4: Schematic workflow for the *S. venezuelae* TX-TL semicontinuous reaction. An example workflow for natural product TX-TL, using the early-stage heme biosynthetic operon and downstream analysis by HPLC-MS. Reactions and analysis are detailed in the supplemental material. The figure is modified from [5]. Abbreviations: SMM = *Streptomyces* Master Mix; TX-TL = transcription-translation; ALA = 5-aminolevulinic acid; SPE = solid phase extraction; ESI-MS = electron spray ionization-mass spectrometry; HPLC-MS = high-performance liquid chromatography-mass spectrometry.

Table 1: Recipe for GYM bacterial growth medium and GYM agar plate.

Table 2: Reagents for preparing S30A and S30B wash buffers. This information was adapted from Kieser et al.[45] Abbreviation: DTT = dithiothreitol.

Table 3: Recipe for making the *S. venezuelae* MES and SMM solutions. Abbreviations: MES = Minimal Energy Solution; SMM = *Streptomyces* Master Mix; NTP = nucleoside triphosphate; PEG 6000 = polyethylene glycol 6000; 3-PGA = 3-phosphoglycerate; G6P = glucose-6-phosphate; PVSA = polyvinylsulfonic acid.

Table 4: Recipe for *S. venezuelae* TX-TL reaction. Abbreviations: MES = Minimal Energy Solution; SMM = *Streptomyces* Master Mix; TX-TL = transcription-translation.

Supplemental Table S1: Plasmids for *S. venezuelae* TX-TL workflow. Abbreviation: TX-TL = transcription-translation.

Supplemental Material S2: mScarlet-I calibration standard preparation and plate reader measurements.

Supplemental Material S3: FlAsH-tag methods. Abbreviation: FlAsH = fluorescein arsenical hairpin.

Supplemental Material S4: Semicontinuous reaction, purification, and HPLC-MS.

DISCUSSION:

In this manuscript, a high-yield *S. venezuelae* TX-TL protocol has been described with detailed steps that are straightforward to conduct for both experienced and new users of TX-TL systems. Several features from existing *Streptomyces*[45] and *E. coli* TX-TL[41] protocols have been removed to establish a minimal, yet high-yield protocol for *S. venezuelae* TX-TL[5,26]. The workflow recommended here is to ensure that *S. venezuelae* is growing rapidly in the chosen rich medium, to be able to inoculate the final culture in the evening. This allows cell harvest at peak growth the following morning and allows the user to harvest and prepare the active cell extract on the same day. By following this streamlined protocol, it is expected that a single researcher can complete the protocol conveniently in a three-day framework. A complementary plasmid toolkit has also been provided for the *S. venezuelae* TX-TL system, including a strong expression plasmid system (pTU1-A-SP44-*mScarlet-I*), which provides broad functionality for mRNA/protein analysis. This standard plasmid is powered by the constitutive SP44 promoter that is highly active in a range of *Streptomyces spp*. and in *E. coli*[39]. To demonstrate the initial potential of the *S. venezuelae* TX-TL toolkit, the representative results show the high-yield synthesis of a range of fluorescent proteins, secondary metabolite enzymes, and the biosynthesis of a model natural product pathway (from heme biosynthesis).

Overall, the protocol contains a detailed description of the *S. venezuelae* TX-TL system, as well as practical tips for preparing the three essential components of the TX-TL reaction: (1) cell extract, (2) *Streptomyces* Master Mix (SMM) solution, and (3) plasmid DNA. This protocol does not require specialized equipment and only requires routine microbiology and biochemistry skills; hence, it is accessible to most laboratories. The protocol is suited for small-scale (10–100 μL) and larger-scale reactions (~2.5 mL), although some optimization of reaction size/aeration may influence protein yield. The recommended reaction volume is 33 μL in a 2 mL tube or 10 μL in a

384-well plate. The crude extract takes five days to prepare by a single person starting from a glycerol stock. Each liter (L) of culture yields at least 5 mL of cell extract (equivalent to ~1500 x 10 µL TX-TL reactions)—this is a conservative estimate and accounts for sample loss during wash steps and cell extract clarification. Each stage of the protocol is independent and can be optimized by the user to meet their needs. A major limitation for all cell-free systems is batch variation[46,47]. Generic factors include pipetting error, user experience, media batch variation, and equipment differences. We specifically introduce a master mix to minimize pipetting error and provide detailed instructions that cover media and equipment use. To date, the protocol is reproducible by a range of users in at least five UK research groups. However, it is unknown what role biological variation contributes to cell-free batch variability. Alongside global gene expression regulation differences, genome plasticity in *Streptomyces* spp. is widely reported and is a potential contributor[48]. To investigate batch variation, it is recommended to grow up to four separate 1 L cultures derived from four single colonies grown overnight. Previously, up to 28% variation (in terms of standard deviation) was observed between four biological batches (4 L per batch provided ~20 mL of cell extract)[5]. Based on these data, a reasonable minimal target for a new user is 2.8 mM for sfGFP and 3.5 mM mScarlet-I/mVenus-I using the plasmids that are available on AddGene—these targets are 30% lower than the average observed in previous data. If downstream HPLC-MS analysis is desired, the PEG 6000 can be removed from the master mixes, although a decrease in the overall TX-TL yield can be expected by up to 50%.

In terms of the potential of specialized *Streptomyces* cell-free systems[5,6], there is a growing desire to develop new wet-laboratory tools for bioprospecting applications such as natural products. The *Streptomyces* genus is steeped in the history of natural product discovery, including antibiotics, herbicides, and pharmaceutical drugs[49]. The increasing knowledge gained from whole-genome sequencing projects and the latest bioinformatic tools[50–52] has revealed an unprecedented level of natural products encoded by BGCs within microbial genomes[53]. Unlocking this genetic information—which is anticipated to hold new drugs/chemicals and enzymes useful to biotechnology—will require the development of new synthetic biology strategies, including novel expression systems and a range of metabolic engineering tools[54]. Specialized *Streptomyces*-based TX-TL systems are advantageous to study genes and regulatory elements from Actinobacteria and related genomes for the following reasons: [1] availability of a native protein folding environment[26], [2] access to an optimal tRNA pool for high G+C (%) gene expression, and [3] an active primary metabolism for the potential supply of biosynthetic precursors. In addition, a key advantage of cell-free systems is the high-throughput characterization of genetic parts and gene expression, using next-generation sequencing[13] and acoustic liquid handling robotics[8,11,12]. In summary, the *S. venezuelae* TX-TL toolkit[5] provides a complementary tool in the field of synthetic biology for natural products. The *S. venezuelae* TX-TL toolkit will support the further development of *S. venezuelae* as a model system and provide a method to engineer novel synthetic biology parts/tools and explore secondary metabolite biosynthetic pathways and enzymes.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

REFERENCES:

1. Carbonell, P. et al. An automated Design-Build-Test-Learn pipeline for enhanced microbial production of fine chemicals. *Communications Biology.* **1**, 66 (2018).
2. Gregorio, N. E., Levine, M. Z., Oza, J. P. A user's guide to cell-free protein synthesis. *Methods Protocols.* **2** (1), 24 (2019).
3. Zimmerman, E. S. et al. Production of site-specific antibody-drug conjugates using optimized non-natural amino acids in a cell-free expression system. *Bioconjugate Chemistry.* **25** (2), 351–361 (2014).
4. Wiegand, D. J., Lee, H. H., Ostrov, N., Church, G. M. Cell-free protein expression using the rapidly growing bacterium *Vibrio natriegens*. *Journal of Visualized Experiments: JoVE.* (145) doi:10.3791/59495 (2019).
5. Moore, S. J. et al. A *Streptomyces venezuelae* cell-free toolkit for synthetic biology. *ACS Synthetic Biology.* **10** (2), 402–411 (2021).
6. Xu, H., Liu, W.-Q., Li, J. Translation related factors improve the productivity of a *Streptomyces*-based cell-free protein synthesis system. *ACS Synthetic Biology.* **9** (5), 1221–1224 (2020).
7. Yim, S. S. et al. Multiplex transcriptional characterizations across diverse bacterial species using cell-free systems. *Molecular Systems Biology.* **15** (8), e8875 (2019).
8. Moore, S. J. et al. Rapid acquisition and model-based analysis of cell-free transcription-translation reactions from nonmodel bacteria. *Proceedings of the National Academy of Sciences of the United States of America.* **115** (19), E4340–E4349 (2018).
9. Zawada, J. F. et al. Microscale to manufacturing scale-up of cell-free cytokine production--a new approach for shortening protein production development timelines. *Biotechnology and Bioengineering.* **108** (7), 1570–1578 (2011).
10. Geertz, M., Shore, D., Maerkl, S. J. Massively parallel measurements of molecular interaction kinetics on a microfluidic platform. *Proceedings of the National Academy of Sciences of the United States of America.* **109** (41), 16540–16545 (2012).
11. McManus, J. B., Emanuel, P. A., Murray, R. M., Lux, M. W. A method for cost-effective and rapid characterization of engineered T7-based transcription factors by cell-free protein synthesis reveals insights into the regulation of T7 RNA polymerase-driven expression. *Archives of Biochemistry and Biophysics.* **674**, 108045 (2019).
12. McManus, J. B. et al. A method for cost-effective and rapid characterization of genetic parts. *bioRxiv*. doi:10.1101/2021.04.30.440836 (2021).
13. Park, J., Yim, S. S., Wang, H. H. High-throughput transcriptional characterization of regulatory sequences from bacterial Biosynthetic Gene Clusters. *ACS Synthetic Biology.* doi:10.1021/acssynbio.0c00639 (2021).

14. Caschera, F., Noireaux, V. Synthesis of 2.3 mg/ml of protein with an all *Escherichia coli* cell-free transcription-translation system. *Biochimie.* **99**, 162–168 (2014).
15. Caschera, F., Noireaux, V. A cost-effective polyphosphate-based metabolism fuels an all *E. coli* cell-free expression system. *Metabolic Engineering.* **27**, 29–37 (2015).
16. Shin, J., Noireaux, V. Efficient cell-free expression with the endogenous *E. coli* RNA polymerase and sigma factor 70. *Journal of Biological Engineering.* **4**, 8 (2010).
17. Karim, A. S., Heggestad, J. T., Crowe, S. A., Jewett, M. C. Controlling cell-free metabolism through physiochemical perturbations. *Metabolic Engineering.* **45**, 86–94 (2018).
18. Cai, Q. et al. A simplified and robust protocol for immunoglobulin expression in *Escherichia coli* cell-free protein synthesis systems. *Biotechnology Progress.* **31** (3), 823–831 (2015).
19. Garenne, D., Thompson, S., Brisson, A., Khakimzhan, A., Noireaux, V. The all-*E. coli* TXTL toolbox 3.0: New capabilities of a cell-free synthetic biology platform. *Synthetic Biology.* doi:10.1093/synbio/ysab017 (2021).
20. Goering, A. W. et al*. In vitro* reconstruction of nonribosomal peptide biosynthesis directly from DNA using cell-free protein synthesis. *ACS Synthetic Biology.* **6** (1), 39–44 (2017).
21. Khatri, Y. et al. Multicomponent microscale biosynthesis of unnatural cyanobacterial indole alkaloids. *ACS Synthetic Biology.* **9** (6), 1349–1360 (2020).
22. Zhuang, L. et al. Total in vitro biosynthesis of the nonribosomal macrolactone peptide valinomycin. *Metabolic Engineering.* **60**, 37–44 (2020).
23. Hoskisson, P. A., Seipke, R. F. Cryptic or silent? The known unknowns, unknown knowns, and unknown unknowns of secondary metabolism. *mBio.* **11** (5), e02642-20 (2020).
24. Bentley, S. D. et al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature.* **417** (6885), 141–147 (2002).
25. Li, J., Wang, H., Kwon, Y.-C., Jewett, M. C. Establishing a high yielding *Streptomyces*-based cell-free protein synthesis system. *Biotechnology and Bioengineering.* **114** (6), 1343–1353 (2017).
26. Moore, S. J., Lai, H.-E., Needham, H., Polizzi, K. M., Freemont, P. S. *Streptomyces venezuelae* TX-TL - a next generation cell-free synthetic biology tool. *Biotechnology Journal.* **12** (4), doi: 10.1002/biot.201600678 (2017).
27. Kim, W. et al. Comparative genomics determines strain-dependent secondary metabolite production in *Streptomyces venezuelae* strains. *Biomolecules.* **10** (6), 864 (2020).
28. Phelan, R. M. et al*.* Development of next generation synthetic biology tools for use in *Streptomyces venezuelae*. *ACS Synthetic Biology.* **6** (1), 159–166 (2017).
29. Song, J. Y. et al. Complete genome sequence of *Streptomyces venezuelae* ATCC 15439, a promising cell factory for production of secondary metabolites. *Journal of Biotechnology.* **219**, 57–58 (2016).
30. Bush, M. J., Bibb, M. J., Chandra, G., Findlay, K. C., Buttner, M. J. Genes required for aerial growth, cell division, and chromosome segregation are targets of WhiA before sporulation in *Streptomyces venezuelae*. *mBio* **4** (5), e00684-13 (2013).
31. Schumacher, M. A. et al. The crystal structure of the RsbN-σBldN complex from *Streptomyces venezuelae* defines a new structural class of anti-σ factor. *Nucleic Acids Research.* **46** (14), 7405–7417 (2018).
32. Ramos-León, F. et al. A conserved cell division protein directly regulates FtsZ dynamics in filamentous and unicellular actinobacteria. *Elife.* **10**, e63387 (2021).

33. Bush, M. J., Tschowri, N., Schlimpert, S., Flärdh, K., Buttner, M. J. c-di-GMP signalling and the regulation of developmental transitions in *Streptomycetes*. *Nature Reviews. Microbiology.* **13** (12), 749–760 (2015).
34. Ehrlich, J., Gottlieb, D., Burkholder, P. R., Anderson, L. E., Pridham, T. G. *Streptomyces venezuelae*, n. sp., the source of chloromycetin. *Journal of Bacteriology.* **56** (4), 467–477 (1948).
35. Inahashi, Y. et al. Watasemycin biosynthesis in *Streptomyces venezuelae*: thiazoline C-methylation by a type B radical-SAM methylase homologue. *Chemical Science.* **8** (4), 2823–2831 (2017).
36. Jakeman, D. L. et al. Antimicrobial activities of jadomycin B and structurally related analogues. *Antimicrobial Agents and Chemotherapy.* **53** (3), 1245–1247 (2009).
37. Kodani, S., Sato, K., Hemmi, H., Ohnish-Kameyama, M. Isolation and structural determination of a new hydrophobic peptide venepeptide from *Streptomyces venezuelae*. *Journal of Antibiotics.* **67** (12), 839–842 (2014).
38. Akey, D. L. et al. Structural basis for macrolactonization by the pikromycin thioesterase. *Nature Chemical Biology.* **2** (10), 537–542 (2006).
39. Bai, C. et al. Exploiting a precise design of universal synthetic modular regulatory elements to unlock the microbial natural products in *Streptomyces. Proceedings of the National Academy of Sciences of the United States of America.* **112** (39), 12181–12186 (2015).
40. Moore, S. J. et al. EcoFlex: A multifunctional MoClo kit for *E. coli* synthetic biology. *ACS Synthetic Biology.* **5** (10), 1059–1069 (2016).
41. Sun, Z. Z. et al. Protocols for implementing an *Escherichia coli* based TX-TL cell-free expression system for synthetic biology. *Journal of Visualized Experiments: JoVE.* (79) e50762 (2013).
42. Kim, D. M., Choi, C. Y. A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane. *Biotechnology Progress.* **12** (5), 645–649 (1996).
43. Liu, Y., Fritz, B. R., Anderson, M. J., Schoborg, J. A., Jewett, M. C. Characterizing and alleviating substrate limitations for improved *in vitro* ribosome construction. *ACS Synthetic Biology.* **4** (4), 454–462 (2015).
44. Bindels, D. S. et al. mScarlet: a bright monomeric red fluorescent protein for cellular imaging. *Nature Methods.* **14**, 53–56 (2017).
45. Hopword, D. A., Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. Practical Streptomyces genetics. John Innes Foundation (2000).
46. Hunter, D. J. B., Bhumkar, A., Giles, N., Sierecki, E., Gambin, Y. Unexpected instabilities explain batch-to-batch variability in cell-free protein expression systems. *Biotechnology and Bioengineering.* **115** (8), 1904–1914 (2018).
47. Dopp, J. L., Jo, Y. R., Reuel, N. F. Methods to reduce variability in *E. coli*-based cell-free protein expression experiments. *Synthetic and Systems Biotechnology.* **4** (4), 204–211 (2019).
48. Hoff, G., Bertrand, C., Piotrowski, E., Thibessard, A., Leblond, P. Genome plasticity is governed by double strand break DNA repair in *Streptomyces*. *Scientific Reports.* **8**, 5272 (2018).
49. Bibb, M. J. Regulation of secondary metabolism in streptomycetes. *Current Opinion in Microbiology.* **8** (2), 208–215 (2005).
50. Weber, T. et al. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Research.* **43** (W1), W237–W243 (2015).

51. Navarro-Muñoz, J. C. et al. A computational framework to explore large-scale biosynthetic diversity. *Nature Chemical Biology.* **16**, 60–68 (2020).
52. Alanjary, M. et al. The Antibiotic Resistant Target Seeker (ARTS), an exploration engine for antibiotic cluster prioritization and novel drug target discovery. *Nucleic Acids Research.* **45** (W1), W42–W48 (2017).
53. Medema, M. H., Fischbach, M. A. Computational approaches to natural product discovery. *Nature Chemical Biology.* **11** (9), 639–648 (2015).
54. Whitford, C. M., Cruz-Morales, P., Keasling, J. D., Weber, T. The Design-Build-Test-Learn cycle for metabolic engineering of *Streptomycetes*. *Essays in Biochemistry.* EBC20200132, doi:10.1042/EBC20200132 (2021).

Figure 1

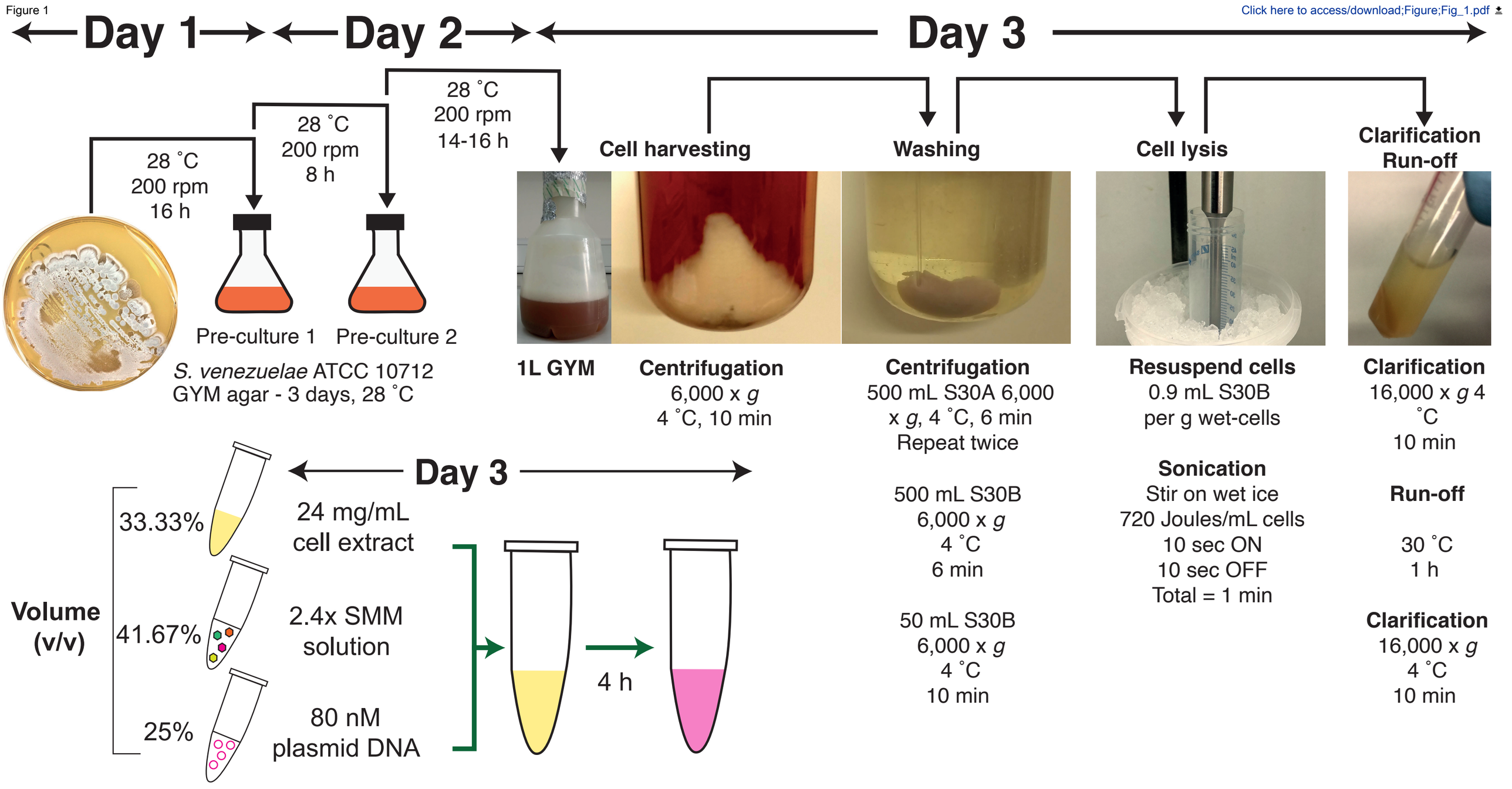


Figure 2

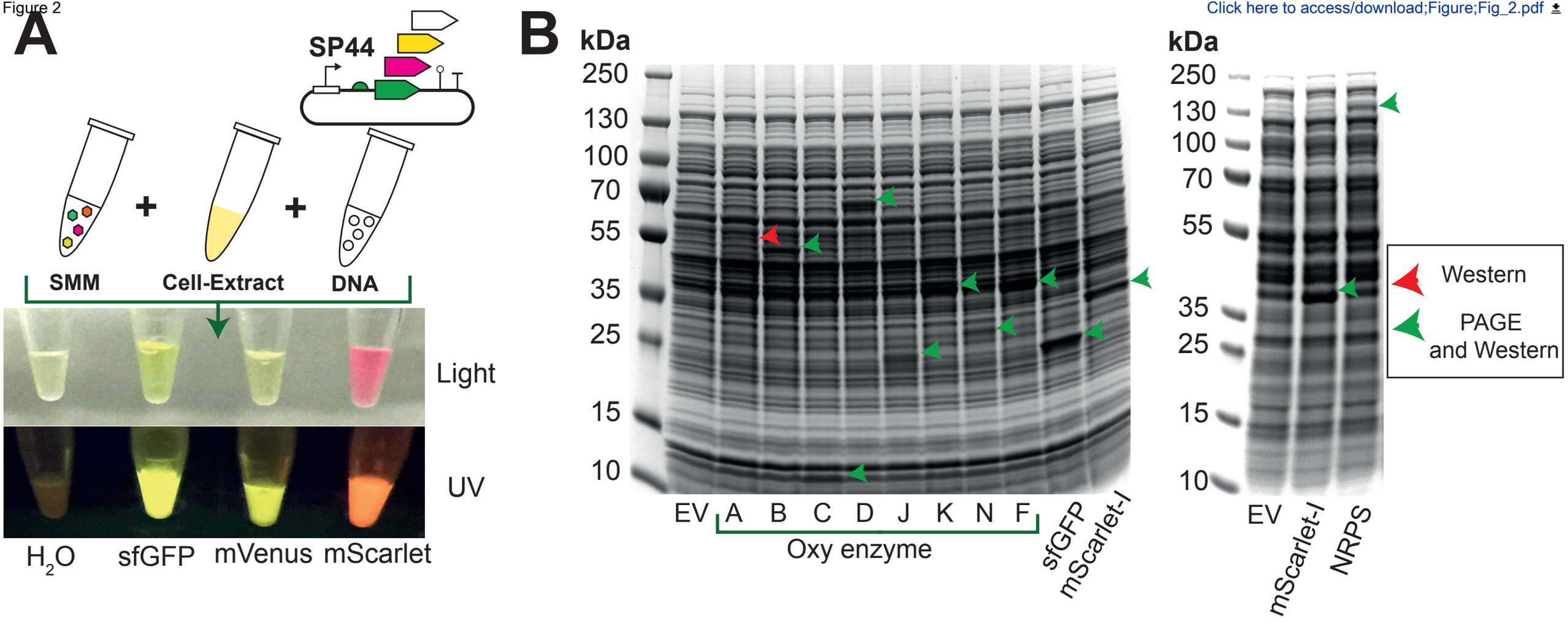
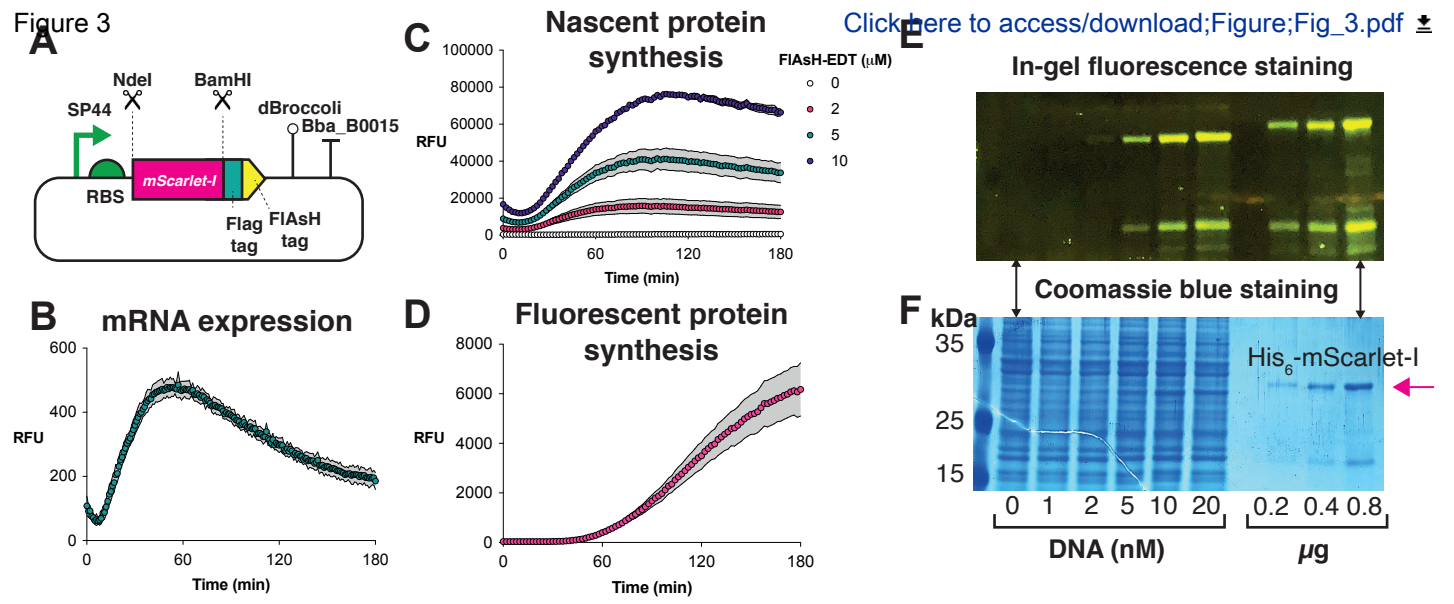


Figure 3



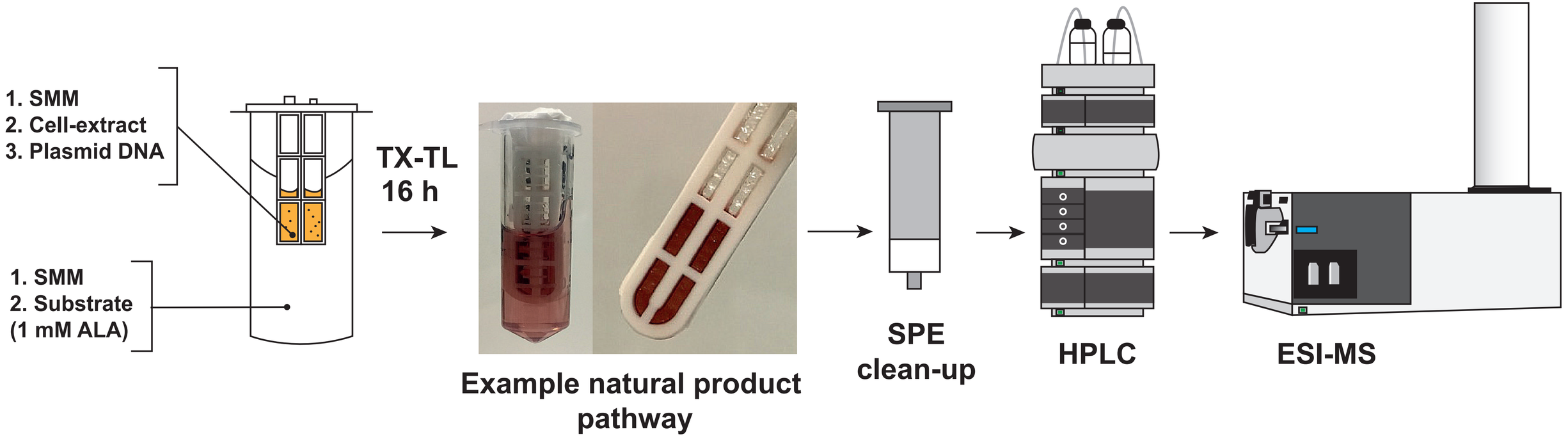


Table 1

Media	Concentration (per L)	Volume
GYM agar plate	0.8 g D-glucose	200 mL
	0.8 g yeast extract	
	2 g malt extract	
	0.4 g $CaCO_3$	
	2.4 g technical agar	
GYM medium	4 g D-glucose	1 L
	4 g yeast extract	
	10 g malt extract	

These components are required for routine *S. venezuelae* growth. For prepa
recommend using standard media/protocols from Sa

Notes
Dissolve with ddH_2O. Adjust pH to 7.2 with 5 M NaOH before adding agar.
Dissolve with distilled water and adjust pH to 7.2 with 5 M NaOH.
aration of plasmid DNA from *E. coli* , we ımbrook *et al.* [46].

Table 2 Click here to access/download;Table;Table 2.xlsx

Reagent	Concentration	Amount
HEPES	1 M	0.1 L
$MgCl_2$	1 M	0.1 L
NH_4Cl	4 M	0.5 L
ddH_2O for S30A	55.5 M	730 mL
ddH_2O for S30B	55.5 M	927.5 mL
DTT	1 M	5 mL
The above reagents are required to prepare 1 L of the S30A and S30B buffer We recommend using a pen to mark the side of container (Duran flask) be sterile ddH_2O if rec		
S30A buffer		
On the day of use, add the followin		

Component	Stock concentration	Volume
Deionized water	55.5 M	730 mL
HEPES	1 M	10 mL
$MgCl_2$	1 M	10 mL
NH_4Cl	4 M	250 mL
DTT	1 M	2 mL
S30B buffer		

On the day of use, add the followin

Component	Stock concentration	Volume
ddH_2O for S30B	55.5 M	927.5 mL
HEPES	1 M	50 mL
$MgCl_2$	1 M	10 mL
NH_4Cl	4 M	12.5 mL
DTT	1 M	2 mL

Additional Notes
Adjust pH to 7.5 with 5 M KOH
Takes time to dissolve (endothermic). Use a stir bar and let the solution equilibrate to room temperature.
For S30A
For S30B
Make fresh on the day of use and keep on ice.
rs. Dissolve all components fully in ddH_2O. Autoclave separately. efore autoclaving to check for evaporation. Adjust volume with quired.
g in order. Keep on ice.

Final concentration
10 mM
10 mM
1 M
2 mM

g in order. Keep on ice.

Final concentration
50 mM
10 mM
50 mM
2 mM

Table 3

MES	Stock (mM)	2.4x solution
HEPES pH 8	2000	60
NTP	25	7.2
Amino acids	6	2.4
Mg-glutamate	1000	9.6
K-glutamate	4000	360
PEG 6000	40	2.4
3PGA	1400	72
ddH_2O		
Total volume (µL)		

Note: Amino acids, K-glutamate, and PEG 6000 can be omitted bu

SMM	Stock (mM)	2.4x solution
HEPES pH 8	2000	60
NTP	25	2.4
Amino acids	6	2.4
Mg-glutamate	1000	9.6
K-glutamate	4000	360
PEG 6000	40	2.4
3-PGA	1400	72
G6P	1000	12
PVSA (mg/mL)	1000	12
ddH_2O		

Total volume (µL)
Aliquot 50 µL aliquots and store at -80 °C
Troubleshooting:
We have provided instructions for making 1 mL of MES/SMM, which minim freeze-thaw cycles of stocks.
100 µL (1/10 volume) MES/SMM aliquots can be prepared to initially valida
If activity is low, optimization of the Mg-glutamate and K-glutamate levels extract batches. This is a guideline that existing TX-TL/CFPS protocols recon

Volume to pipette (µL)
30
288
400
9.6
90
60
51.4
71
1000

ıt expect reduced activity.

Volume to pipette (µL)
30
96
400
9.6
90
60
51.4
12
12
239

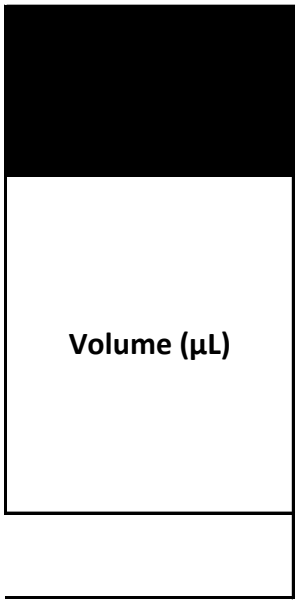
1000
ɔ
ıizes pipetting error and reduces
ıte the protocol.
may be required between cell ınmend performing.

Table 4

Component	Volume (fraction)		
		1	3
Extract (24 mg/mL)	1/3	3.67	11
DNA (80 nm)	1/4	2.75	8.25
MES/SMM (2.4x)	5/12	4.58	13.75
Total volume (µL)		11	33

Note: 10% extra (dead volume) is added to allow for pipetting error

Reactions - Equivalent to one 10 µL reaction			
5	**10**	**50**	**100**
18.3	36.67	183.33	366.67
13.75	27.5	137.5	275
22.96	45.83	229.17	458.33
55	111	555	1110


Volume (µL)

We would like to thank the reviewers and editor for their shared comments that improve the overall quality of the protocol. All comments are in blue. We would like to add an additional middle author (Miss Tanith Hanson – Kent) for assisting with extra experiments and supplementary protocols. Please note the line numbers in the track change file are offset by the track changes. We would also like to update the introduction within the manuscript based on a recent publication that advances *E. coli* cell-free yields.

"So far, current protocols for *E. coli* TX-TL have reached up to 4.0 mg/mL (~157 μM) for the model green fluorescent protein (GFP) using a blend of 3-PGA (30 mM), maltodextrin (60 mM) and d-ribose (30 mM) as the secondary energy source [1]."

Reviewer #1:

Summary

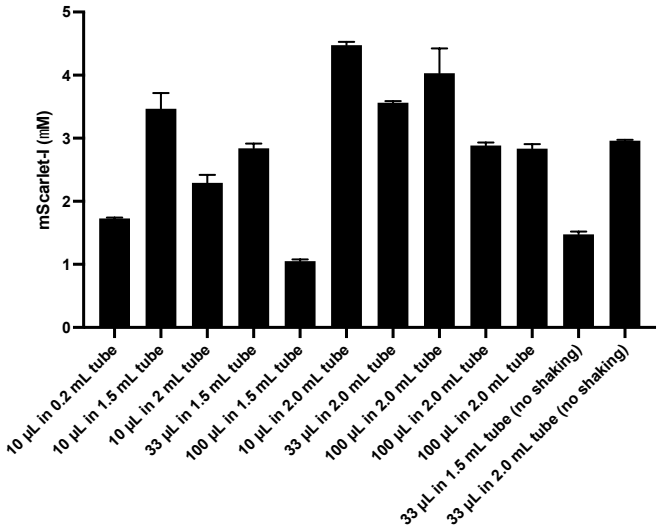
Toh et al. detail a protocol for producing and measuring high-yields of recombinant protein from Streptomyces venezuelae based crude extracts. In general, the steps listed in the procedure are clear and easy to follow with side notes for complicated steps for users with minimal CFPS extract preparation experience. In addition to the 3-day extract preparation instructions, the authors provide a set of plasmids that allow the user to quantify and visualize their TX-TL reactions. Using this toolkit, they then demonstrate various uses of their crude extract system, including producing proteins with high G+C(%) and one-pot synthesis of uroporphyrinogen III. In addition to the detailed protocol, specific advantages to using cell-free TX-TL systems as well as S. venezuelae for natural product-based discovery are noted. I would recommend this manuscript for publication in JoVE if the authors address the questions and comments listed below.

We would like to thank for the reviewer for their time and comments.

Major Questions/Comments

1. Line 40: The authors state that reactions are "…also scalable since reactions do not require aeration for activity." Other CFPS based methods have noted that increasing reaction size in the same reaction vessel may decrease protein yields due to surface to volume effects. Have the authors demonstrated that the reaction vessel used for their CFPS reactions does not impact protein yields? If altering the reaction vessel does improve yields, the authors should consider adding a section describing the importance of optimizing reaction vessel for obtaining highest protein yields.

The reviewer makes an excellent point. Previously we have run our reactions in 384-well plates (10 μL) or 2 mL tubes (33 μL) and had not extensively optimised this variable. To address this point, we have performed some preliminary experiments, since we lacked this data. See below.



Interestingly, there is some variability as the reviewer suggests, however, all reactions in a 2 mL tube provide the highest yields. This variable requires further investigation and may not be due to aeration alone – we have unpublished data that suggests the TCA cycle is not fully functional. We have modified the manuscript in line with the reviewers recommendation.

Discussion
"The protocol is suited for both small-scale (10-100 mL) and larger-scale reactions (~2.5 mL), although some optimisation of reaction size/aeration may influence protein yield. The recommended reaction volume is 33 µL in a 2 mL tube, or 10 µL in a 384-well plate"

2. Figure 4 includes a workflow with semi-continuous reactions. While the use of semi-continuous reactions is mentioned a couple of times, the authors should consider including a brief description of semi-continuous reactions as well as the potential benefits to volumetric protein yield.

See below.

3. Lines 414- 417: In the representative results section, the authors note that they show the expression of the m-Scarlet-I fluorescent protein from the pTU1-A-SP44-mScarlet-I plasmid as well as the expression of a natural product pathway from the early-stages of haem biosynthesis. However, in figure 2B, the enzymes expressed are instead from the oxytetracycline BGC. Furthermore, while the authors do include Figure 4 as a workflow for the haem BGC, they do not provide any expression results or data. The authors should consider amending lines 414-417 to more clearly layout which enzyme expression results are demonstrated in the figures.

Thank you for highlighting this point. Since the protocol is focused on detailing the methodology for cell-free batch reactions, we did not provide extensive details on the semi-continuous methodology. We believe comment 2 and 3 can be addressed as follows:

"To serve as a model natural product biosynthetic pathway, the 'one-pot' biosynthesis of uroporphyrinogen III (uro'gen III) was performed using the pTU1-A-SP44-*hemC-hemD*/*cysG*^{A}-*hemB* expression plasmid [2]. This model biosynthetic pathway was chosen since uro'gen III is highly oxygen sensitive and rapidly oxidizes (loss of 6-electrons) to uroporphyrin III, which displays strong red fluorescence. This enables the reaction to be easily detected in real-time using fluorescence measurements, as well as analysis by HPLC-MS (**Figure 4**), as we have previously described [2]. In addition, these reactions were studied using either a batch or semi-continuous method. A semi-continuous reaction is a strategy that uses a micro-dialysis device

[3,4] that provides additional energy (NTPs, secondary energy source) and amino acids in order to prolong the reaction time period and increase protein synthesis yields. Here, we used the semi-continuous method to scale-up the haem model reaction and separate the TX-TL proteins from the reaction product to facilitate purification and analysis by HPLC-MS. Full details of this specific methodology is available in our previous work [2] or by others [3,4]. We propose our example schematic workflow (**Figure 4**) is adaptable to other natural product biosynthetic pathways."

4. The authors should consider more strongly contrasting the use of cell-free TX-TL with in vivo protein production, particularly with regards to the open reaction environment and ease of expressing and assaying enzymes/genetic parts in parallel. Authors may also consider mentioning the use of high-throughput equipment that are amenable to cell-free systems, such as automated liquid handlers.

These are excellent points. We have added some remarks/references in the introduction and discussion to highlight these key advantages of cell-free.

Introduction

"…In addition, there is growing interest in TX-TL systems for high-value recombinant protein production in an open-reaction environment[2], for example, to incorporate non-standard amino acids in antibody-drug conjugates[3]."

"…strong reproducibility and automated workflows[5–7]. Automation of TX-TL permits the accelerated characterisation of genetic parts and regulatory elements[5,7,8]."

Discussion
"…In addition, the high-throughput characterisation of genetic parts and gene expression can be assisted using next-generation sequencing [8] and acoustic liquid handling robotics [5,7]."

Specifically, we have also added a pre-print for a protocol that describes the use of liquid handling robotics assisted cell-free reactions from the Murray and Lux groups:

A Method for Cost-Effective and Rapid Characterization of Genetic Parts
https://www.biorxiv.org/content/10.1101/2021.04.30.440836v1.full

Minor Questions/Comments

- The abstract submitted to JoVE and the abstract presented in the manuscript differ with regards to the units provided for the volume of small scale reactions and the expected yields for proteins. Line 39 in manuscript indicates a range of 10 -100 uL for small scale reactions while the abstract submitted to JoVE indicates 10-100 mL. Line 41 in the manuscript estimates protein yields of ~5-10 uM while the abstract submitted to JoVE indicates ~5-10 mM.

This is part of the online submission and conversion, which does recognise special characters (Greek mu into plain text geometry "\mu"). Since there is no opportunity in the online submission, we can only clarify this to the reviewer.

- Lines 58-62: Sentence starting with "To increase TX-TL yields…" should remove the words "can increase TX-TL yields" at end of sentence due to repetition.

Thank you for highlighting – deleted.

- Line 159: Step 1.1 should not be included in the list of requirements for Day 1 as the

prepared plate should be produced at least 48-72 hours prior to day 1 as indicated in the preparation notes.

Thank you for highlighting – deleted.

- Lines 185-187: For steps 1.13 and 1.14, there are no instructions for instances where the main culture OD600 is between 2.0-3.0. Should step 1.13 read "If OD600<3.0…"?

Thank you for highlighting this typo – we have modified as stated.

- Lines 250-252: Sonication instructions do not indicate that the cell suspension should be placed in an ice bath when being sonicated (Figure 1 image shows cell suspension placed on ice). Authors should consider emphasizing that cell suspension/lysed cells should be placed on ice to prevent enzyme degradation due to overheating.
- Lines 267-270: Same comment as above for lines 250-252 regarding specifying ice bath for cell suspension/lysed cells during sonication.

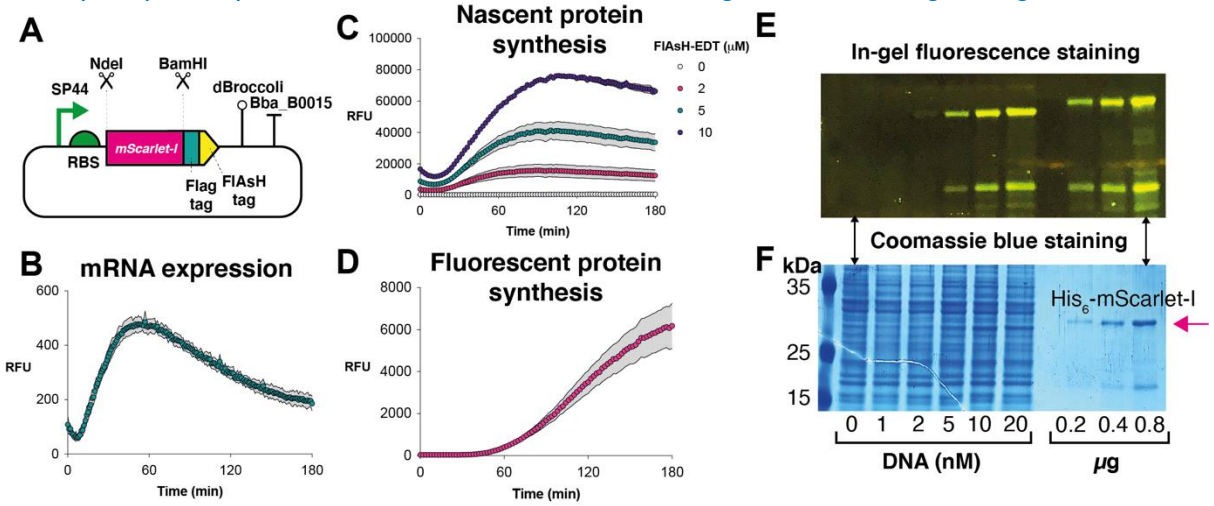
Thank you for highlighting – we have added comments and clarify why this is important.

"Place each microcentrifuge tube in a beaker of ice water, using a plastic tube rack to hold the tube for sonication. NOTE: Due to sensitivity of cell extract to over-heating, it is critical to ensure that the tubes do not warm up to prevent protein precipitation and reduced enzymatic activity."

- The link for Figure 3 directs the reviewer to the excel file for Table 1. For panel C, the authors should consider changing the colors of the three different FlAsH-EDT2 conditions to larger differences in shading or different colors.

The Fig 3 link the reviewer refers to is created within the JoVE submission process, and beyond our control.

We agree the colour format for panel C is unsuitable – modified version shown below. We have also increased the size of panel B-D and altered the y-axis label to relative fluorescence units (RFU) and specified the excitation/emission settings used in the figure legend.



- Table 3: Authors should consider adding (mM) into the heading of 2.4X solution in Table 3 for additional clarity.

Thank you for highlighting this point - we have made this modification.

Reviewer #2:
Manuscript Summary:
The manuscript describes methods for implementing a S. venezuelae based TX-TL system. The intoduction provides useful context, rationale, and utility of this host in the rapidly expanding cell-free field. The manuscript is well written, easy to follow, and provides many useful suggestions for new users to implement the methods. The manuscript also describes multiple methods to evaluate the success of the TX-TL reactions, which can also be helpful for new users. This manuscript is going add a lot of value to the growing community of cell-free researchers.

We would like to thank for the reviewer for their time and comments.

Reviewer #3:
Manuscript Summary:
This manuscript described a very detailed protocol for the preparation of Streptomyces CFPS system, which can be adapted by new users in other labs. The optimized system was able to synthesize proteins with relatively high yields, showing potential for the expression of high G+C (%) genes involved in natural product biosynthesis. This manuscript makes a valued contribution to the scientific community. This reviewer recommends publication after clarifying a minor point:

We would like to thank for the reviewer for their time and comments.

In sections 2A and 2B: When sonicating in 1 mL fractions, the input energy was 240 J per mL of wet cells (lines 258-259). When the entire cell suspension (~6-8 mL) was sonicated together, the energy was used the same as in 1 mL suspension: 240 J per mL of wet cells (lines 276-277). Does this mean the total energy input is about 1440-1920 J? If yes, please consider adding the total energy input. Also in line 280: 720 J per mL is not the total energy input.

An excellent point. The volume can be variable depending on yield. To keep our protocol precise, we have modified our recommendation to a maximum of 5 mL of cell-suspension and included total energy input to 1200 J over 5 min of sonication.

Reviewer #4:
Manuscript Summary:

The manuscript presented by Toh et al provides a clear protocol of all the steps required for the preparation TX-TL system from Streptomyces spp. Although the manuscript shows the preparation for S. venezuelae ATCC 10712, the authors provide details on how to utilize the protocol for other Streptomyces spp. This protocol will be beneficial to the growing cell-free community. Our comments mainly focus on the representative result presented in Fig.3. However, we believe that the protocol and representative results would benefit from additional details and tips for troubleshooting. We leave it to the authors if they want to add more details only to the representative results, which is essential to understanding the presented results, or also to the protocol part which we highly encourage the authors to do.

We would like to thank for the reviewer for their time and comments.

We agree with the reviewer for highlighting these specific points on the representative results that use additional protocols. Based on related JoVE cell-free protocols (https://www.jove.com/t/50762/protocols-for-implementing-an-escherichia-coli-based-tx-tl-

cell-free), we have included some separate protocols as supplementary files and provided tips for troubleshooting.

Supplementary Material S3. mScarlet-I calibration standard preparation and platereader measurements
Supplementary Material S4. FlAsH-tag methods
Supplementary Material S5. Semi-continuous reaction, purification, and HPLC-MS

Main comments:

Line 143: What batch variation do you expect, how do you verify it, and what batch variation is considered to be tolerable? We propose to use "investigate" rather than "verify" in this context

Based on the editors comments, this section has been moved to the discussion. Batch variation is an critical consideration in cell-free that we have previously discussed and provided batch variation data in Figure S2 of our recent publication[2]. With four tested batches, the average yield of protein (40 nM sfGFP plasmid) was 4.28 μM sfGFP with a standard deviation of 1.20 μM (28%). Based on this data, we suggest setting a minimum threshold for a new user with 30% less yield – this corresponds to 2.8 μM sfGFP and 3.5 μM mScarlet-I/mVenus-I. Increasing yields and improving batch variability is a key consideration for our future work. We have introduced some recommended targets into the manuscript text, along with some extended discussion on batch variation.

REPRESENTATIVE RESULTS
"As a guideline, this protocol should provide a minimum yield of 2.8 μM for sfGFP and 3.5 μM for mScarlet-I/mVenus from the expression plasmids provided on AddGene. These figures allow for typical batch variation (up to 28%) observed in previous data[3], although yields greater than 10 μM mScarlet-I have been achieved with optimal batches (unpublished data)."

DISCUSSION
"A major limitation for all cell-free systems is batch variation[44,45]. Generic factors include pipetting error, user experience, media batch variation and equipment differences. We specifically introduce a master mix to minimise pipetting error and provide detailed instructions that cover media and equipment use. To date, we report that the protocol is reproducible by a range of users in at least five UK research groups. However, it is unknown what role biological variation contributes to cell-free batch variability. Alongside global gene expression regulation differences, genome plasticity in *Streptomyces* spp. is widely reported and a potential contributor[46]. To safeguard against batch variability, key fundamental targets have been set in this protocol for new users to aim for. This includes reaching a recommended optical density (OD_{600} = 3.0) for cell-harvest within 16 hours of growth and achieving a minimum protein yield (within 30% of previous batch variation data) for the standard proteins. This is equivalent to 2.8 μM for sfGFP and 3.5 μM for mScarlet-I/mVenus."

Lines 389-390: Aerobic and anaerobic conditions are very different; do you experience any difference in protein expression or functionality of the expressed proteins? Do you have recommended applications for either aerobic or anaerobic conditions? Furthermore: What kind of mineral oil do you use? To our knowledge some of them may still be gas permeable.

We used mineral oil (Sigma, UK) to limit oxygen diffusion in an attempt to prevent oxidation of the colourless uroporphyinogen III into the coloured and fluorescent porphyin oxidation product[2]. Mineral oil is used[12] to quickly replicate anaerobic conditions for microbial cell cultures. However, without degassing or using an artificial reductant, we can't guarantee that some oxygen is present – especially if it is not consumed during the reaction. We believe there

are some significant differences (vesicles, ETC) between Gram-positive and Gram-negative TX-TL systems, which require further investigation. Therefore, the TX-TL reactions do not seem to require oxygen at the current level of protein production. We have unpublished data that shows succinate accumulates in the reactions, which suggests the ETC/oxidative phosphorylation may not be functional. Since it is not a major point within the protocol, we shall change all instances of “anaerobic” to “microaerobic”.

Paragraph 419 - Figure 3: The results presented in this paragraph and Figure 3 are highly valuable and show that the produced lysate is effective. However, the exact details of the experiments are missing (DNA concentration, additional components necessary, explanation of the presented results). Please add these details to the manuscript. The different methods presented in this part will be very valuable to any new user. Therefore, as stated above, we would suggest to add how to set up the different methods of detection to the protocol.

We agree with the reviewer and have addressed the comment above.

Minor comments:

Line 49: Specify the TX-TL the first time used in text

Corrected

Line 138, 141, 229: You specify 2 numbers for the volume of lysate that should be produce. The minimal volume of 5 mL does not seem to be matching the expected pellet size.

We agree – we have corrected both numbers to 5 mL as a conservative estimate, to account for loss of material over the processing steps.

Lines 101 / 109: in line 101 you are writing about a "standard" plasmid, in line 109 about an "optimized" plasmid, at the same time you are writing about the same plasmid, as far as we understand it. Please specify.

Thank you highlighting – it is accurate to use “standard” plasmid and have corrected.

Line 156: How long can the plate be stored? Or do you recommend to make a new plate before each preparation?

We have not studied this variable in extract preparation, although *S. venezuelae* will survive on a GYM plate for months. Therefore, for consistency it is best use a fresh plate. In line with editor comments, we have provided some commentary on this point prior to the numbered protocol steps.

“…Also It is also recommended to streak a fresh culture of *S. venezuelae* ATCC 10712 from a -80°C glycerol stock onto a GYM agar plate and incubate at 28°C for at least 48-72 h until single colonies are clearly visible. Only fresh cultures should be used for the following steps.”

Line 245: Although the two versions for lysis are beneficial to the protocol, the reasoning seems to be flawed as the sonication is based on the volume of resuspended cells and therefore any losses during the previous steps are not relevant to the sonications.

Since it takes significant time and preparation to make the extracts, we have only optimised individual 1 mL sonication reactions. This provides a standard workflow, which we recommend a first time to try first. The volumes of the wet cells will differ. We agree with the reviewer and suggest it is more reasonable to modify this section to a set volume of 5 mL in a 50 mL falcon.

"**Cell lysis by sonication to obtain the crude cell extract**
The user can choose to disrupt the cells by sonication either in 1 mL fractions (**option 2.1**) or as a larger cell-suspension (5 mL) in a 50 mL tube (**option 2.2**). Both options have been detailed below to ensure reproducibility, since the final volume of the cell-suspension can change due to loss of cells between steps 1.17-1.32. We recommend a first-time user attempts option 2.1 to first establish the protocol."

Line 260: How do fully disrupted cells compare to not fully disrupted cells? Do you have any tips to help new users.

Thank for you highlighting this. We have modified the protocol steps to help new users.

2.1.5. Run the sonication protocol. During the first two resting cycles, move the tube up/down and sideways, to ensure the cells are evenly sonicated. Record the energy input. Safety: Wear appropriate hearing protection during sonication. NOTE: The viscosity will decrease as cells are disrupted, and the pale cream wet cell pellet should turn into a homogenous brown fluid. The recommended energy input is 240 J per mL of well cells.
2.1.6. If the cells are only partially lysed the suspension will still appear cream-coloured with viscous clumps of cells. Repeat the sonication for an additional one or two 10-sec cyles, mixing frequently, until cells are fully disrupted.

Line 419-431: The same style is used for numbering and citations.

Thank you for highlighting this, we have changed the numbering format: 1.

Line 399: typo: as follows

Corrected

Line 495: Do you have any experience if one can also express from linear templates?

Yes – very poor (~95% less active). Although we have not tried protective recombinase proteins such as GamS. Data is available in the supplementary of our original work. Since it is not a significant finding, we prefer not to highlight this in the discussion.

Editorial comments:
Editorial Changes
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

To the best of our ability

2. Please provide an institutional email address for each author.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Corrected

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Ultra Yield, Thomson, Falcon, Qiagen, etc.

Corrected

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Corrected

6. Line 134-146: Please move the discussion of the protocol to the Discussion section.

Corrected

7. Lines149-152/241-245/284-288: Please ensure that the Protocol section consists of numbered steps. We cannot have non-numbered paragraphs/steps/headings/subheadings.

Corrected – for the cell lysis stage, it is important to clarify the user has two options. We have added an extra note before introducing the numbered steps.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Corrected

9. Line 155-156: Add the sentence as a “NOTE”

Corrected

10. Line 176-177: Please specify how the absorbance is measured. Is a spectrophotometer used? How much sample (volume) is used to check the OD?

Corrected

11. Line 233-237/255-263/273-281: In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

We have moved some of notes into the discussion section.

12. 338-364/384-393: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Corrected

13. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Corrected

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have included additional paragraphs that covers these points.

16. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Corrected

17. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

We have used the an equivalent reference style from PaperPile reference manager that adopts this format.

18. Figure 1: Please maintain a single space between the numeral and the unit (e.g., revise “4°C” to “4 °C”).

Corrected

19. Figure 4: Revise “16 hr” to “16 h”

Corrected

20. Table 3: Replace “uL” with “µL” amd “mg/ml” with “mg/mL”.

Corrected

21. Table 4: Replace “uL” with “µL”.

Corrected

22. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the Table in alphabetical order.α

Corrected

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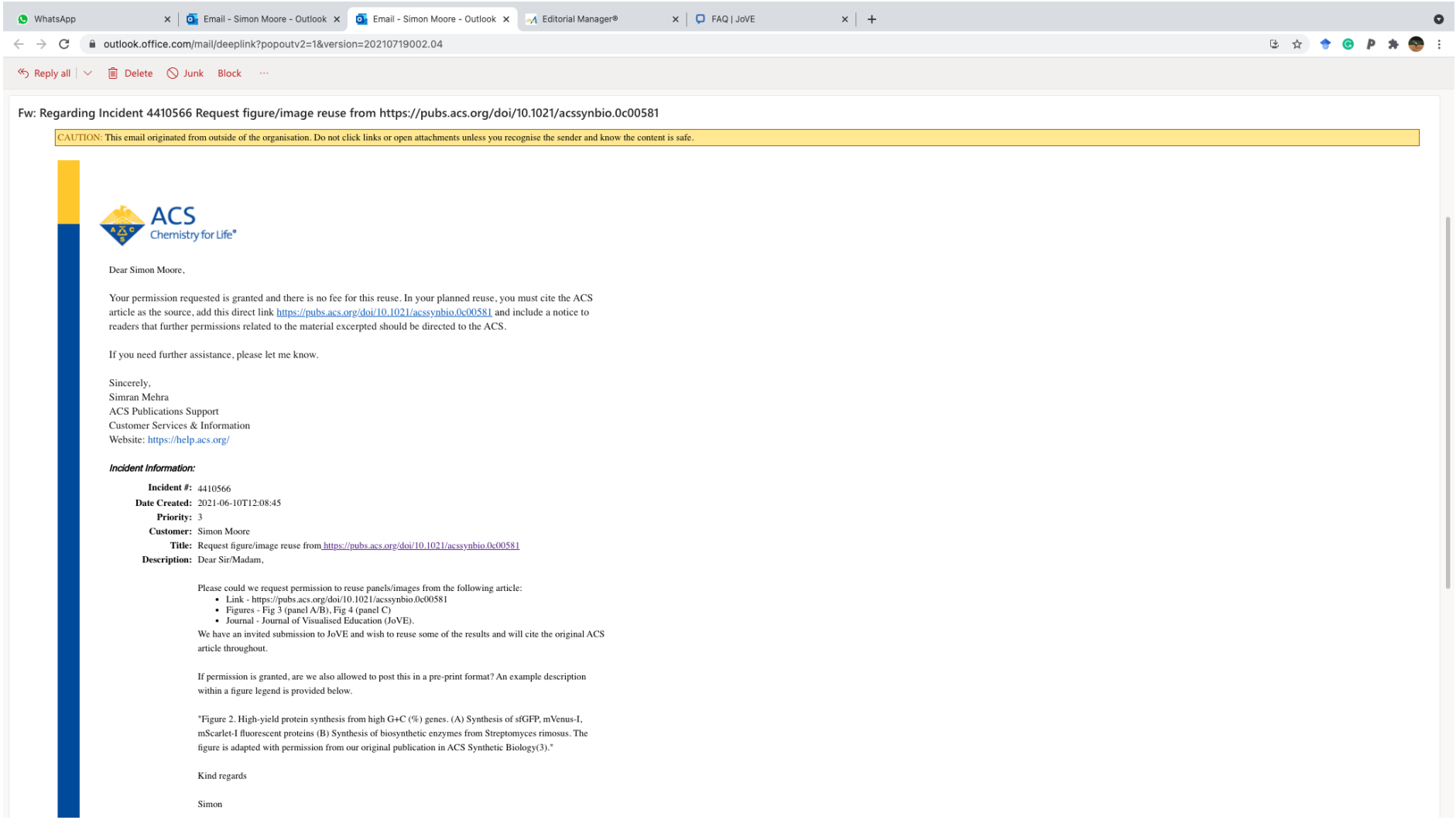

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- Journal - Journal of Visualised Education (JoVE).

We have an invited submission to JoVE and wish to reuse some of the results and will cite the original ACS article throughout.

If permission is granted, are we also allowed to post this in a pre-print format? An example description within a figure legend is provided below.

"Figure 2. High-yield protein synthesis from high G+C (%) genes. (A) Synthesis of sfGFP, mVenus-I, mScarlet-I fluorescent proteins (B) Synthesis of biosynthetic enzymes from Streptomyces rimosus. The figure is adapted with permission from our original publication in ACS Synthetic Biology(3)."

Kind regards

Simon

Supplemental Table S1

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Supplemental Coding Files

Table S1.xlsx

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Supplemental Coding Files

SupMat S2.docx