Methods for Expression of Recombinant Proteins Using a *Pichia pastoris* Cell-Free System

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Cell-free protein synthesis is a powerful tool for engineering biology and has been utilized in many diverse applications, from biosensing and protein prototyping to biomanufacturing and the design of metabolic pathways. By exploiting host cellular machinery decoupled from cellular growth, proteins can be produced in vitro both on demand and rapidly. Eukaryotic cell-free platforms are often neglected due to perceived complexity and low yields relative to their prokaryotic counterparts, despite providing a number of advantageous properties. The yeast *Pichia pastoris* (also known as *Komagataella phaffii*) is a particularly attractive eukaryotic host from which to generate cell-free extracts, due to its ability to grow to high cell densities with high volumetric productivity, genetic tractability for strain engineering, and ability to perform post-translational modifications. Here, we describe methods for conducting cell-free protein synthesis using *P. pastoris* as the host, from preparing the cell lysates to protocols for both coupled and linked transcription-translation reactions. By providing these methodologies, we hope to encourage the adoption of the platform by new and experienced users alike. © 2020 The Authors.

Basic Protocol 1: Preparation of *Pichia pastoris* cell lysate
Basic Protocol 2: Coupled in vitro transcription and translation
Basic Protocol 3: Determining luciferase production from cell-free protein synthesis reactions
Alternate Protocol 1: Linked in vitro transcription and translation
Alternate Protocol 2: Quantifying HSA protein concentration
Support Protocol 1: Preparation of mRNA by in vitro transcription for linked transcription and translation

Keywords: cell-free protein synthesis • in vitro transcription-translation • *Pichia pastoris* • *Komagataella phaffii* • rapid prototyping • synthetic biology • eukaryotic cell-free expression system

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INTRODUCTION

Cell-free protein synthesis (CFPS) has experienced a revival in the past 20 years thanks to the explosion in the field of synthetic biology (Carlson, Gan, Hodgman, & Jewett, 2012). Since the first demonstration of CFPS in the early 1960s (Nirenberg & Matthaei, 1961), vast strides have been made in the development of cell-free systems and their potential applications (Silverman, Karim, & Jewett, 2020). CFPS offers a number of distinct advantages over recombinant protein production in vivo. The ability to rapidly produce protein on demand allows users to avoid lengthy cell handling procedures associated with in vivo expression protocols, such as cloning and transformation. Additionally, due to the truly open nature of the reaction environment, reactions can be controlled, modulated, and monitored with ease in real time (Swartz, 2012). As a result, cell-free systems are well suited to high-throughput screening and rapid prototyping approaches for characterization and optimization purposes.

Because of these advantageous properties, cell-free systems are increasingly being employed for a diverse and developing range of applications. Continued efforts have led to greatly improved yields and significant work on scale-up processes. It is now possible to use CFPS for the manufacturing of products, particularly those that are difficult to make in vivo, including toxic products (Katzen, Chang, & Kudlicki, 2005), and for decentralized manufacture of personalized medicines (Ogonah, Polizzi, & Bracewell, 2017). Additionally, cell-free systems have been used in the de novo design of metabolic pathways (Hodgman and Jewett, 2013), in biosensing (Pardee et al., 2016), and in education (Huang et al., 2018; Stark et al., 2018).

An advantage of the resurgence of CFPS is the ability to produce extracts from an ever-expanding variety of organisms, as theoretically any host can be used as the source for extract generation. Typically, the most commonly used cell-free systems are from *Escherichia coli* (Chen & Zubay, 1983), Chinese hamster ovary (CHO) cells (Brodel, Sonnabend, & Kubick, 2014), wheat-germ extract (WGE; Anderson, Straus, & Dudock, 1983; Madin, Sawasaki, Ogasawara, & Endo, 2000), rabbit reticulocyte lysate (RRL; Jackson & Hunt, 1983), and insect (Sf9) cells (Stech et al., 2014). However, the variety and number of developed CFPS extracts is continually being expanded and now includes HEK293 human embryonic kidney cells (Bradrick, Nagyal, & Novatt, 2013), *Saccharomyces cerevisiae* (Gan & Jewett, 2014), *BY-2* tobacco cells (Buntru, Vogel, Spiegel, & Schillberg, 2014), *Streptomyces* species (Moore, Lai, Needham, Polizzi, & Freemont, 2017; Li, Wang, Kwon, & Jewett, 2017), *Bacillus megaterium* (Moore et al., 2018), and *Vibrio natriegens* (Failmezger, Scholz, Blombach, & Siemann-Herzberg, 2018; Wiegand, Lee, Ostrov, & Church, 2019). This method describes the established cell-free system derived from *Pichia pastoris* (syn. *Komagataella phaffii*; Aw & Polizzi, 2019).

Pichia pastoris has been reported to be the second most used expression system after *E. coli* (Bill, 2014). Its popularity as a recombinant expression platform is due to its ability to grow to high cell densities, which means that high volumetric productivity is achieved (Ahmad, Hirz, Pichler, & Schwab, 2014). As a Crabtree-negative yeast, *P. pastoris* does not release any toxic products during growth and therefore concentrations of up to 135 g/liter wet cell weight have been reported in bioreactors (Cregg, 2007). It is this ability to reach such high cell densities that make it an attractive host for cell lysate production. There are specific advantages of using a eukaryotic system over the *E. coli* commercial kits available, including the ability to perform post-translational modifications, such as disulfide bond formation. The system outlined below is capable of producing 116.2 μg/ml of human serum albumin (HSA; Spice, Aw, Bracewell, & Polizzi, 2020a), a complex biopharmaceutical containing numerous disulfide bonds. These yields exceed the reported GFP production in HeLa (Mikami, Masutani, Sonenberg, Yokoyama, & Imataka, 2006) and RRL systems (Kobs, 2008), and luciferase in CHO Brodel et al.,

2014), insect (Ezure et al., 2006) and *S. cerevisiae* platforms (Gan & Jewett, 2014). Additionally, we have demonstrated the production of Hepatitis B core antigen virus-like particles (VLPs), highlighting the versatility of the *P. pastoris* cell-free platform for complex protein production (Spice, Aw, Bracewell, & Polizzi, 2020b).

This methods paper will cover the preparation of cell lysate through high-pressure homogenization, the production of recombinant protein either by coupled transcription and translation or by linked transcription and translation where the mRNA is produced in vitro before the reaction mix, and finally methods to determine the productivity of the CFPS reaction for two model proteins.

PREPARATION OF *PICHIA PASTORIS* CELL LYSATES

BASIC PROTOCOL 1

Although theoretically any organism can be used for cell-free protein synthesis, the preparation of the lysate needs to be optimized for the organism being used. There are many considerations to ensure that lysis is effective and that the cellular machinery is kept as active as possible, most commonly by ensuring that the lysate is kept cold throughout the entire protocol. This basic protocol involves growing cells to an optimum growth phase before they are harvested and washed. The cells are then lysed using a high-pressure cell disrupter before being dialyzed and flash frozen (Fig. 1). High yields of cellular protein from lysates are necessary for the production of proteins in vitro, and therefore it is important that the protein concentration of the lysate is checked

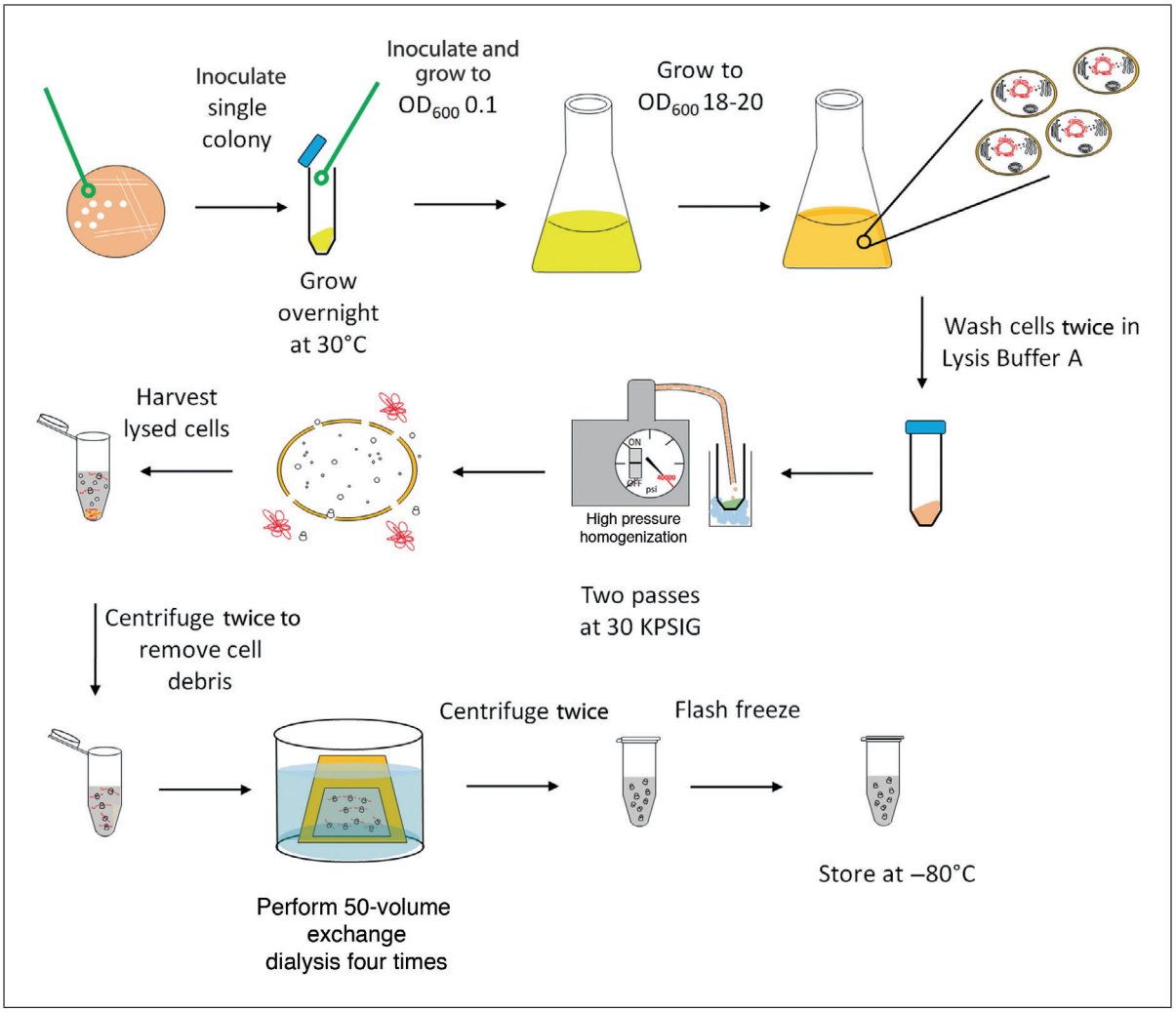


Figure 1 Overview of cell lysate preparation (Basic Protocol 1).

at the end (Fujiwara & Doi, 2016). Successfullysis will result in 15-25 mg/ml of protein (Aw & Polizzi, 2019). Although the strain we use is a modified ribosome-overexpressing strain, FHL1 (Aw & Polizzi, 2019), we have also successfully used wild-type X33 for CFPS. Theoretically any *P. pastoris* strain should be compatible with our protocols, but it is important to note that when using a strain with different growth characteristics, this may require further optimization of harvest time.

Materials

Fresh single colonies from a YPD agar plate at 30°C
YPD liquid medium (see recipe)
YPD agar (see recipe)
Buffer A (see recipe), ice cold
Dry ice (optional)
Methanol (optional)
Lysis buffer A (see recipe), ice cold
Bradford assay kit (or other commercial protein quantification kit)

1-liter baffled glass flask
Orbital shaking incubator, 30°C
UV/visible spectrophotometer
1.5-ml cuvettes
50-ml centrifuge tubes
High-speed refrigerated centrifuge
High-pressure cell disruptor (e.g., CF1 model, Constant Systems Ltd., Daventry, England), precooled in a 4°C refrigerator if possible
10-ml Stripette disposable pipets
Syringe with 18-G needle
3.5 K MWCO Slide-A-Lyzer™ G2 dialysis cassette (ThermoFisher Scientific)
1.5-ml microcentrifuge tubes

1. Select a single colony from a fresh YPD agar plate (grown at 30°C, static) and culture in 5 ml YPD medium in a 50-ml centrifuge tube overnight at 30°C, shaking at 250 rpm. Antibiotic may be included in both the plate and the liquid culture if selecting for a specific strain.

   It is recommended that a fresh YPD agar plate be made for each lysate preparation; however, a plate may be reused for ∼2 weeks if it has been stored at 4°C after the initial overnight growth.

2. Dilute the overnight culture into 200 ml YPD medium in a 1-liter baffled glass flask to OD_{600} 0.1.

   Typically, this culture is set up at ∼6 p.m., to ensure cultures grow adequately so that the lysis steps can be begun at ∼8 or 9 a.m. the next day.

   If desired, the culture volume can be scaled up to exceed 200 ml, but subsequent steps would need to be scaled as well. We recommend dividing larger cultures into 200-ml batches that are processed simultaneously but separately. The remainder of the protocol details the handling of a single 200-ml batch.

3. Regularly check the OD_{600} of the culture, and harvest when cells have reached OD_{600} of 18.0-20.0.

4. Weigh a single blank 50-ml centrifuge tube and record the weight of the tube. Store this tube and three additional 50-ml centrifuge tubes on ice until ready.

5. Once the cells reach the correct OD_{600}, pour the cultures into four 50-ml centrifuge tubes, and keep cells and reagents on ice as much as possible.

6. Centrifuge 10 min at 3000 × *g*, 4°C.
7. Pour off the supernatant and resuspend in 50 ml ice-cold buffer A.

   At this step, the cultures should be condensed into the centrifuge tube that has been weighed out.

8. Centrifuge 10 min at 3000 × *g*, 4°C.
9. Wash the cells in 20 ml ice-cold buffer A.
10. Centrifuge 10 min at 3000 × *g*, 4°C.
11. Wash the cells a second time in 20 ml ice-cold buffer A.
12. Centrifuge 10 min at 3000 × *g*, 4°C.
13. Blot on a towel to remove extra buffer.
14. Weigh the 50-ml centrifuge tube containing the cell pellet, and calculate wet cell weight by subtracting the original weight of the centrifuge tube (from step 4).

    At this stage it is possible to either flash freeze the pellet or continue with lysis.

15. To flash freeze, use a dry ice and methanol bath. Hold the tube in the dry ice and methanol bath until the color has changed throughout the whole pellet. The pellet should be stored at −80°C.
16. To continue with lysis, resuspend the pellet in 1 ml ice-cold lysis buffer A per 1 g wet cell weight

    If continuing from this point using a flash-frozen pellet, make sure the pellet has fully defrosted before continuing with lysis.

17. Perform two passes using a high-pressure homogenizer at 30,000 psi. The homogenizer should be precooled in advance by refrigeration, if possible. All samples should be kept cold as much as possible and collected on ice.

    If possible, a one-shot adapter should be used to process small volumes (≤10 ml). If this is not available, it would be possible to scale up the volumes (using multiple flasks) to ensure adequate lysis.

    We use 10-ml Stripettes to transfer the lysate to the high-pressure homogenizer and remove it, before re-adding it for the second pass. This will depend on the machine available and the setup.

    Efficiency of lysis is usually determined by the protein concentration at the end of the lysis procedure. When optimizing the efficiency of homogenizer, it is possible to perform serial dilutions (to 10^5) onto YPD plates (containing no antibiotic) to determine the optimum conditions for the specific machine being utilized. Plates should be left to grow for 3-5 days at 30°C and then a colony count performed.

18. Collect lysed sample into a 50-ml centrifuge tube and centrifuge 30 min at 18,000 × *g*, 4°C.
19. Transfer the supernatant to a fresh 50-ml centrifuge tube and centrifuge again 30 min at 18,000 × *g*, 4°C.

Dialysis

20. Load the supernatant from the lysis into a 3.5 K MWCO Slide-A-Lyzer™ G2 dialysis cassette using a syringe.

    An 18-G needle is required for the Slide-A-Lyzer G2 cassettes. Follow the manufacturer's instructions on rehydrating the cassette before use.

Table 1 Example Protein Yields of Lysate After Extract Preparation

Lysate	Protein content (mg/ml)
Lysate 1	22.72 ± 1.91
Lysate 2	17.91 ± 0.106

21. Buffer exchange the sample four times for 30 min with at least 50 vol lysis buffer A at 4°C.

    Buffer exchanging in larger volumes to ensure that the cassette is fully submerged is possible; this will depend on the equipment that is available.

    The PMSF present in the buffer inhibits a large number of serine proteases, but this could be substituted for other protease inhibitors if desired.

22. After four buffer exchanges, remove the sample from the dialysis cassette using a syringe and an 18-gauge needle, and transfer to a 50-ml centrifuge tube.

23. Centrifuge 1 hr at 18,000 × *g*, 4°C.

24. Remove the supernatant and immediately divide into aliquots in 1.5-ml microcentrifuge tubes. These frozen aliquots are single use, and the volume should be determined accordingly.

    The 1.5-ml microcentrifuge tubes should be precooled for at least 30 min in a −80°C freezer. Based on six standard runs, aliquots are often made in 175 µl volume.

25. Immediately flash freeze the samples in a dry ice and methanol bath (as described previously) and transfer to a −80°C freezer.

26. Keep one aliquot to test for lysate protein concentration using a Bradford assay kit (or other protein determination assay of choice) according to the manufacturer's instructions.

    High yields of protein lysate concentration are essential for successful in vitro transcription/ translation. We would expect yields of 15-25 mg/ml (Table 1).

BASIC PROTOCOL 2

COUPLED IN VITRO TRANSCRIPTION AND TRANSLATION

The second critical aspect of cell-free protein synthesis is the reaction mix. This is where the components required for protein synthesis are added to the cell lysate. The reaction mix outlined below has been improved using Design of Experiments (DOE) to result in increased protein synthesis (Spice et al., 2020a). Alternatively, a standard reaction mix more closely aligned to that used for CFPS with *S. cerevisiae* and *P. pastoris* in other published papers (Aw & Polizzi, 2019; Hodgman & Jewett, 2013; Zhang, Liu, & Li, 2020) can be used. This protocol requires precise pipetting, and the order in which components are added is essential for the function of the CFPS reaction (Fig. 2). When establishing a CFPS system, it is recommended that the luciferase protein be used as a reporter assay.

Materials

40 nM DNA plasmid prepared by extraction with Qiagen Plasmid Maxi Kit, resuspended in TE buffer (see recipe)
1 M HEPES•KOH, pH 7.4
2 M potassium glutamate (if following alternate reaction mix recipe in Table 2)
1 M magnesium glutamate
0.1 M dithiothreitol (DTT)
RTS Amino Acid Mix (biotechrabbit GmbH, Hennisdorf, Germany)

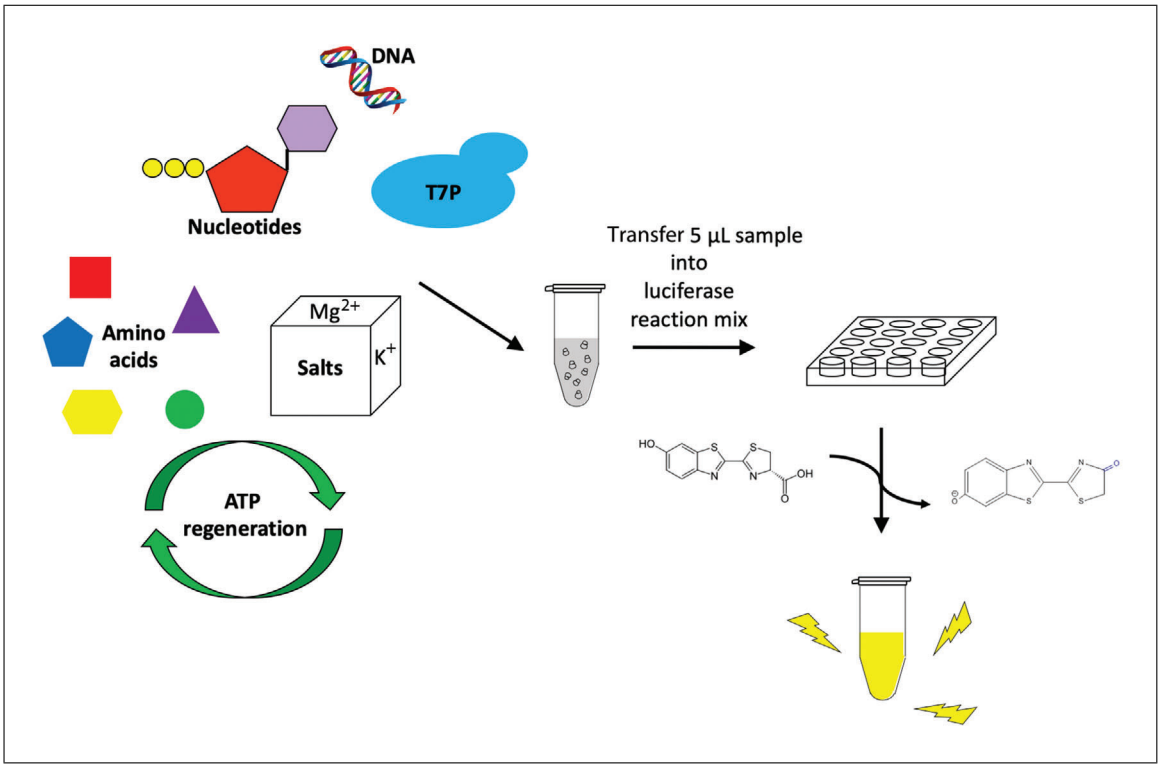


Figure 2 Overview of coupled in vitro transcription and translation (Basic Protocol 2, left) which can be monitored via luciferase production (Basic Protocol 3, right).

NTP solution set, 100 mM each (ThermoFisher Scientific)
500 mM creatine phosphate
4 mg/ml creatine phosphokinase (Sigma-Aldrich) in 25 mM glycylglycine (pH 7.5)
40 U/µl murine RNase inhibitor (New England Biolabs)
T7 polymerase, HC (200 U/µl, ThermoFisher Scientific)
Nuclease-free H_2O (Life Technologies)
Cell lysate (see Basic Protocol 1)

1.5-ml microcentrifuge tubes
Microcentrifuge
Water bath or heat block, 60°C
Water bath or incubator, 21°C

1. Extract DNA plasmid from Qiagen Plasmid Maxi Kit (Fig. 3).

   Ensure the plasmid is resuspended in TE buffer at a concentration of 40 nM. We have optimized our system for this concentration of DNA; therefore, if using a different concentration of DNA, changes to the reaction mix may be required. Furthermore, using a Maxiprep Kit results in higher CFPS yields than using a miniprep kit and is preferred. The plasmid is used without linearization.

2. Prepare the reaction components (see step 4) and store as indicated.

   The individual components can be made up in advance and stored under the following conditions: HEPES•KOH, magnesium glutamate, and potassium glutamate at 4°C; lysate and RTS amino acid mix at −80°C; all other components at −20°C

   The HEPES•KOH, magnesium glutamate, potassium glutamate, DTT, creatine phosphate, and creatine phosphokinase should be filter sterilized before use.

3. Defrost the components, including the lysate, and incubate on ice.

   Ensure the lysate has fully defrosted. Leave to defrost for at least 15 min.

Table 2 Comparative Protocol for Improved or Standard Reaction Mix

Component	Improved reaction mix (µl)	Standard reaction mix (µl)
1 M HEPES•KOH, pH 7.4	3	1.25
2 M potassium glutamate	0	2
1 M magnesium glutamate	0.3	1
100 mM DTT	1	1
RTS amino acid mix	7.5	5
100 mM ATP	0.5	0.75
100 mM GTP	0.5	1
100 mM UTP	0.5	1
100 mM CTP	0.5	1
500 mM creatine phosphate	4.5	2.5
4 mg/ml creatine phosphokinase	3.38	6.75
40 U/µl RNase inhibitor	0.25	0.25
200 U/µl T7 polymerase	0.5	0.5
DNA (40 nM)	*X* (depends on purification yield)	*X* (depends on purification yield)

The RTS amino acid solution must be fully dissolved before use. Heat it for 5 min at 60°C and allow it to cool to room temperature again before use (it must not go on ice or it will precipitate).

4. Mix the components on ice in a 1.5-ml centrifuge tube in the following order, to a total volume of 25 µl:

   3 µl 1 M HEPES•KOH, pH 7.4
   0.3 µl 1 M magnesium glutamate
   1 µl 100 mM DTT
   7.5 µl RTS amino acid mix (0.9 mM each of 19 amino acids, 0.75 mM leucine)
   0.5 µl 100 mM ATP
   0.5 µl 100 mM GTP
   0.5 µl 100 mM UTP
   0.5 µl 100 mM CTP
   4.5 µl 500 mM creatine phosphate
   3.38 µl 4 mg/ml creatine phosphokinase
   0.25 µl 40 U/µl RNase inhibitor
   0.5 µl 200 U/µl T7 polymerase
   Plasmid DNA to 40 nM.

   A negative control reaction should be run simultaneously for each condition. This consists of the full reaction mix described above but with the DNA replaced by nuclease-free water.

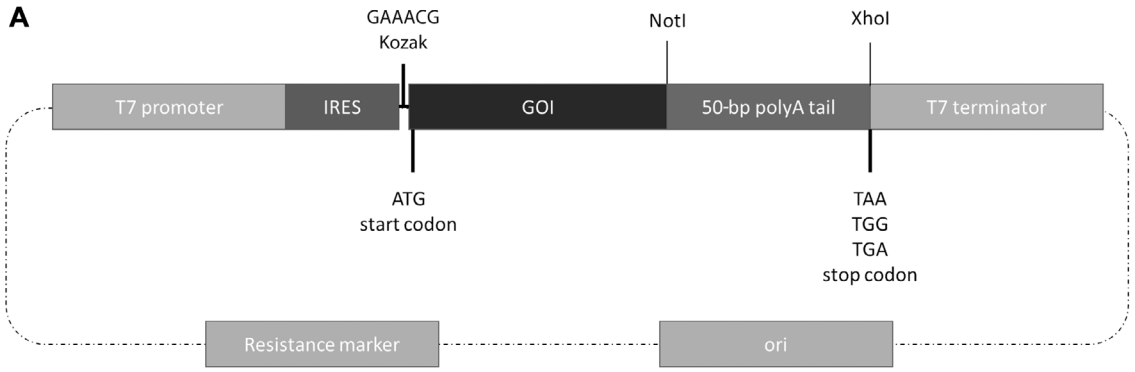
5. Flick the tube to mix and centrifuge briefly; vortexing is not recommended.

6. Add 25 µl cell lysate to the mix.

7. Flick the tube and centrifuge briefly to ensure all the components are well mixed.

8. Place the tubes in either a water bath or a static incubator at 21°C.

   Depending on the protein of interest, the reaction will take between 2 and 8 hr. For more complex proteins, such as those with post-translational modifications including disulfide



B **>Firefly Luciferase**

MEDAKNIKKGPAPFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAHIEVNITYA
EYFEMSVRLAEAMKRYGLNTNHRIVVCSENSLQFFMPVLGALFIGVAVAPANDI
YNERELLNSMNISQPTVVFVSKKGLQKILNVQKKLPIIQKIIIMDSKTDYQGFQ
SMYTFVTSHLPPGFNEYDFVPESFDRDKTIALIMNSSGSTGSPKGVALPHRTAC
VRFSHARDPIFGNQIIPDTAILSVVPFHHGFGMFTTLGYLICGFRVVLMYRFEE
ELFLRSLQDYKIQSALLVPTLFSFFAKSTLIDKYDLSNLHEIASGGAPLSKEVG
EAVAKRFHLPGIRQGYGLTETTSAILITPEGDDKPGAVGKVVPFFEAKVVDLDT
GKTLGVNQRGELCVRGPMIMSGYVNDPEATNALIDKDGWLHSGDIAYWDEDEHF
FIVDRLKSLIKYKGCQVAPAELESILLQHPNIFDAGVAGLPGDDAGELPAAVVV
LEHGKTMTEKEIVDYVASQVTTAKKLRGGVVFVDEVPKGLTGKLDARKIREILI
KAKKGGKSKL

C **>Human Serum Albumin**

MRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVT
EFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNEC
FLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELL
FFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGER
AFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKY
ICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCK
NYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYA
KVFDEFKPLVEEPQNLIKQNCELFKQLGEYKFQNALLVRYTKKVPQVSTPTLVE
VSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTE
SLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELV
KHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL

Figure 3 Circular (plasmid) DNA template design and DNA sequences. (**A**) The plasmid uses a T7 promoter and terminator. There is an internal ribosome entry site (IRES) upstream of the gene of interest (GOI) to allow cap-independent translation, although a Kozak sequence is also included. A poly(A) tail ensures the stability of the mRNA. (**B**) DNA sequence of firefly luciferase. (**C**) DNA sequence of HSA.

bonds or particularly large proteins, it is possible to leave the reaction running overnight. Initial optimization may be required to determine the length of reaction time. Excessive incubation of reactions may lead to a decrease in yield of active recombinant protein.

9. The completed reaction can be analyzed directly using the chosen methodology (see Basic Protocol 3 or Alternate Protocol 2). Alternatively, it may be possible to

freeze the reaction for further analysis, depending on the stability of the protein produced.

The protocol above uses an improved reaction mix that was developed using a Design of Experiments approach (Spice et al., 2020a). Alternatively, it is possible to use the alternative recipe, which was used for the initial development of the system. The reaction should be set up as described above, using the concentrations given in Table 2.

BASIC PROTOCOL 3

DETERMINING LUCIFERASE PRODUCTION FROM CELL-FREE PROTEIN SYNTHESIS REACTIONS

Depending on the protein of interest, yields of protein can be observed via fluorescence, luminescence, or a protein-specific assay. Here we describe the use of luminescence assays to determine luciferase production as a convenient model protein (Fig. 3B) for troubleshooting the CFPS platform. To allow the reaction to be observed over a time course, the luciferase assay is set up in 96-well, half-well white microtiter plates to allow small sample sizes.

Materials

CFPS reaction from Basic Protocol 2 or Alternate Protocol 1
200 mM tricine, pH 7.6
25 mM ATP
50 mM $MgSO_4$
10 mM $MgCO_3$
10 mM EDTA, pH 7.6
500 mM DTT
25 mM D-luciferin or luciferin salt

1.5-ml microcentrifuge tubes
96-well, half-area, flat-bottom white microtiter plates (Grenier Bio cat no. 675075)
Plate reader for luminescence

1. Assemble the luciferase assay buffer on ice as follows for a 600-µl reaction volume:

   50 µl 200 mM tricine
   5 µl 25 mM ATP
   26.7 µl 50 mM $MgSO_4$
   53.4 µl 10 mM $MgCO_3$
   5 µl 10 mM EDTA
   17 µl 500 mM DTT
   5 µl 25 mM D-luciferin
   337.9 µl water.

   Due to the small volumes, batches can be made in advance and stored on ice but should be protected from light sources using foil. DTT and ATP solutions should be freshly made before use and kept on ice. All other solutions can be prepared beforehand and stored at room temperature, aside from the D-luciferin, which must be stored at −20°C.

2. Pipet 30 µl luciferase assay buffer into a clean 96-well half-area flat-bottom white microtiter plate and add 5 µl of CFPS reaction.

3. Read immediately using a plate reader able to detect luminescence. Readings should be taken over a 20-min period, reading 10 times. See example data for this assay in Figures 4 and 5.

   The average luminescence values are collected once the signal has plateaued.

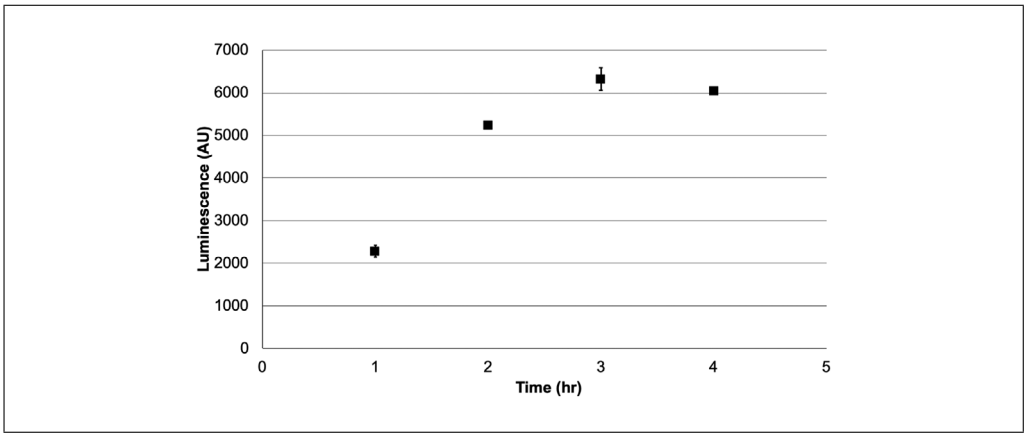


Figure 4 Example time course of luciferase production in a coupled CFPS reaction (Basic Protocol 3). Luciferase production was monitored over 5 hr, using FHL1 to produce the cell extract with a vector containing the cricket paralysis virus (CrPV) IRES. Error bars represent the standard deviation of the mean of three biological repeats (corrected for the negative control reactions), calculated using error propagation.

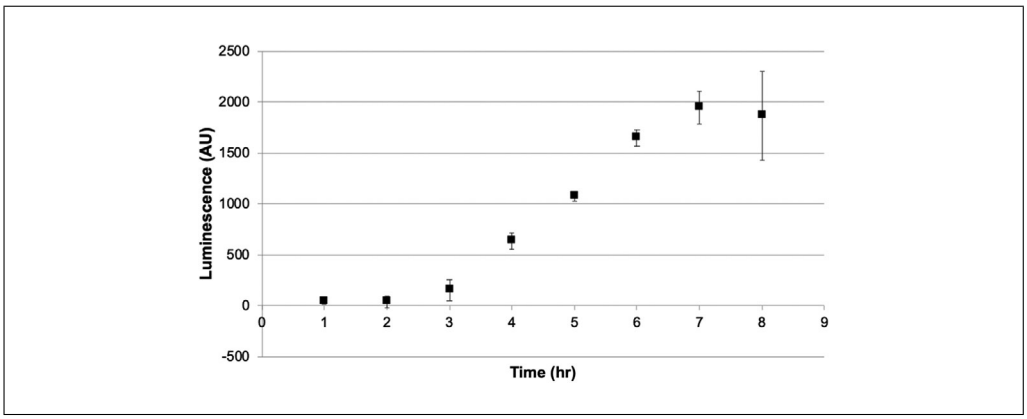


Figure 5 Example time course of luciferase production using a standard CFPS reaction mix. Luciferase production was monitored over 8 hr, using FHL1 to produce the cell extract with a vector containing the CrPV IRES. Error bars represent the standard deviation of the mean of three biological repeats (corrected for the negative control reactions), calculated using error propagation.

LINKED IN VITRO TRANSCRIPTION TRANSLATION

ALTERNATE PROTOCOL 1

It is possible to perform cell-free protein synthesis as separate reactions—first performing the in vitro transcription using a commercial kit and then adding RNA directly to the in vitro translation reaction. This method can be used for troubleshooting if the coupled reaction is not functioning well and will allow the user to determine whether the issue lies with the plasmid or gene design (transcription) or is a problem with the protein production itself. The in vitro translation reaction is similar to that of the coupled reaction, except that no T7 polymerase is used in the reaction mix. It has been reported that a modified reaction mix can be used in which nonessential components are removed or the concentrations are modified; however, we have found that our standard reaction mix protocol works efficiently for production.

Materials

1 M HEPES•KOH, pH 7.4
2 M potassium glutamate
1 M magnesium glutamate
0.1 M DTT
RTS Amino Acid Mix (biotechrabbit GmbH, Hennisdorf, Germany)
NTP set (100 mM solutions, ThermoFisher Scientific)

500 mM creatine phosphate
Creatine phosphokinase (4 mg/ml, Sigma-Aldrich) dissolved in glycylglycine (pH 7.5)
RNase inhibitor, murine (40 U/µl, New England Biolabs)
T7 polymerase, HC (200 U/µl, ThermoFisher Scientific)
Nuclease-free H_2O (Life Technologies)
600 ng in vitro–transcribed RNA (see Support Protocol 1)
Cell lysate (see Basic Protocol 1)

1.5-ml microcentrifuge tubes
Microcentrifuge
Water bath or heat block, 60°C
Water bath or incubator, 21°C

1. Prepare all the reaction components and store as indicated.

   The individual components can be made up in advance and stored under the following conditions: HEPES•KOH, magnesium potassium, and potassium glutamate at 4°C; lysate, RNA, and RTS amino acid mix at −80°C; all other components at −20°C.

   The HEPES•KOH, magnesium glutamate, potassium glutamate, DTT, creatine phosphate, and creatine phosphokinase should be filter sterilized before use.

2. Defrost the components, including the lysate, and incubate on ice.

   Ensure the lysate has fully defrosted. Leave to defrost for at least 15 min.

   The RTS amino acid solution must be fully dissolved before use. Heat it for 5 min at 60°C and allow it to cool to room temperature again before use (it must not go on ice or it will precipitate).

3. Mix the components on ice in a 1.5-ml centrifuge tube in the following order to a total volume of 25 µl:

   1.25 µl 1 M HEPES•KOH, pH 7.4
   3 µl 2 M potassium glutamate
   0.3 µl 1 M magnesium glutamate
   1 µl 100 mM DTT
   5 µl RTS amino acid mix (0.6 mM each of 19 amino acids, 0.5 mM leucine)
   0.75 µl 100 mM ATP
   1 µl 100 mM GTP
   1 µl 100 mM UTP
   1 µl 100 mM CTP
   2.5 µl 500 mM creatine phosphate
   6.75 µl creatine phosphokinase (4 mg/ml)
   0.25 µl RNase inhibitor (40 U/µl)
   600 ng in vitro–transcribed RNA.

   A negative control reaction should be run simultaneously for each condition. This consists of the full reaction mix described above but with the RNA replaced by nuclease-free water.

4. Flick the tube and centrifuge briefly.

5. Add 25 µl cell lysate to the mix.

6. Flick the tube and centrifuge briefly to ensure all the components are well mixed.

7. Place the tubes in either a water bath or a static incubator at 21°C. Example data obtained using the standard mix is shown in Figure 6.

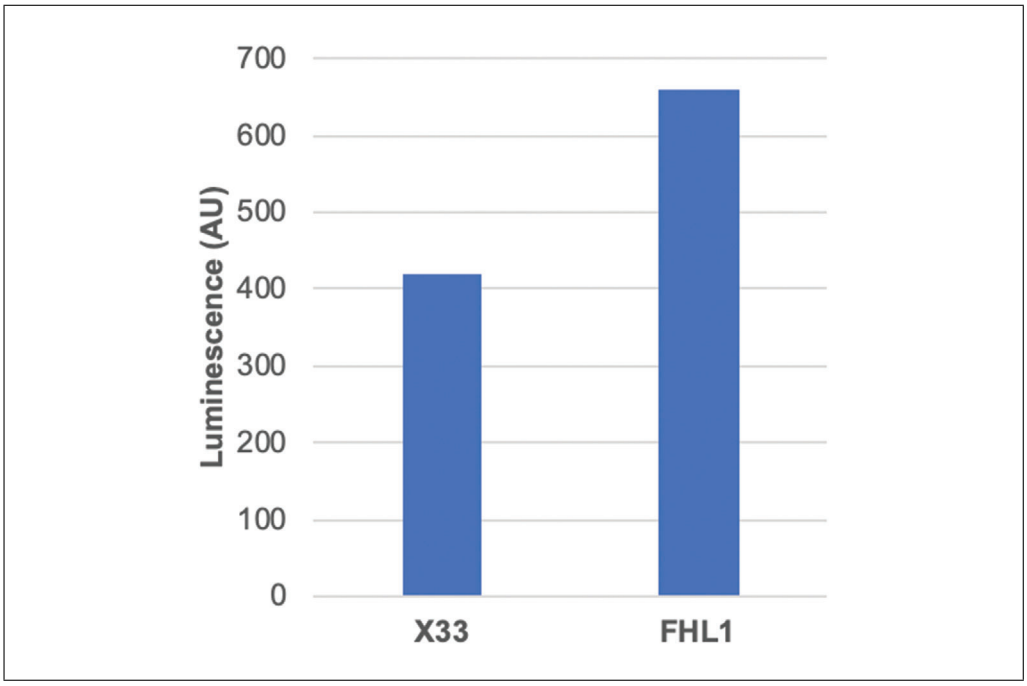


Figure 6 Example yields from a linked CFPS reaction using luciferase as the reporter protein. The reaction was run for 5 hr using two different strains, X33 and FHL1. Samples were corrected for the negative control.

As described in Basic Protocol 2, the length of the reaction will depend on the protein produced. For smaller, less complex proteins (such as those with no disulfide bonds), 2-8 hr is sufficient for production. It is possible to run the reactions longer (up to overnight), but for some proteins, this may lead to a decrease in yield.

QUANTIFYING HSA PROTEIN CONCENTRATION

ALTERNATE PROTOCOL 2

The advantage of a *P. pastoris* CFPS platform is that it is capable of performing post-translational modifications, such as disulfide bond formation. Human serum albumin (HSA) is a relevant biotherapeutic product that contains 17 disulfide bonds and can be used as a convenient model protein for troubleshooting the CFPS platform for proteins that require post-translational modification (Fig. 3C). HSA can be produced using either the coupled (Basic Protocol 2) or linked (Alternate Protocol 1) in vitro transcription translation method. Because of the complexity of this product, the CFPS reactions are run overnight to allow the disulfide bonds to form, before being quantified using an Albumin Blue Fluorescence Kit.

Materials

Albumin Blue Fluorescence Assay Kit (Active Motif, Belgium)
CFPS reaction product

96-well, flat-bottom black microtiter plates
Plate reader for fluorescence

1. Remove all contents of the kit and allow to equilibrate to room temperature.
2. Set up a standard curve as described in the manufacturer's instructions
3. Pipet 25 µl CFPS reaction product to each sample well.
4. Add 150 µl Dye Reagent working solution and mix by pipetting.
5. Incubate 5 min with gentle shaking at room temperature.

Table 3 Example Protein Yields of HSA Using the Albumin Blue Fluorescence Kit

Strain/plasmid/mix	Yield (μg/ml)
FHL1/CrPV HSA/improved	96.8 ± 10.4
FHL1/CrPV HSA/standard	48.1 ± 7.89
X33/CrPV HSA/standard	20.1 ± 11

6. Measure the fluorescence (excitation 560 nm, emission 620 nm). Example yields are shown in Table 3.

   Samples must be read within 30 min, but are most accurate when read within the first 5 min.

SUPPORT PROTOCOL 1

PREPARATION OF MESSENGER RNA BY IN VITRO TRANSCRIPTION FOR LINKED TRANSCRIPTION AND TRANSLATION

This protocol describes the preparation of mRNA by in vitro transcription. For eukaryotic CFPS, it is essential that the mRNA is both capped and tailed, unless an internal ribosome entry site (IRES) is used instead to allow cap-independent translation. Therefore, in this procedure the T7 ARCA mRNA kit (with tailing) from NEB is used in order to ensure that the mRNA is stable and cap-dependent translation can occur without the need for an additional IRES. The kit incorporates Anti-Reverse Cap Analog (ARCA) using a T7 polymerase.

Materials

DNA template containing the protein sequence
Appropriate restriction enzyme to linearize the gene of interest
Gel DNA Recovery Kit (Zymo Research) or other commercial gel extraction kit
HiScribe™ T7 ARCA mRNA Kit (with tailing; NEB) containing:
  2× ARCA/NTP Mix
  T7 RNA Polymerase Mix
  DNase I
  10× Poly(A) Polymerase Reaction Buffer
  Poly(A) Polymerase
RNA Clean & Concentrator-5 (Zymo Research) or other commercial mRNA cleanup kit
Nuclease-free H_2O (Fisher Scientific)
Bleach
Agarose
10× Orange-G Buffer (40% sucrose, 0.2% Orange G)

PCR machine
0.2-ml PCR tubes
Microcentrifuge
RNase-free 1.5-ml microcentrifuge tubes
Nanodrop spectrophotometer
Agarose gel electrophoresis apparatus

1. Linearize the DNA containing the gene of interest and the T7 promoter upstream of the T7 promoter using an appropriate restriction enzyme.
2. Gel extract the linearized DNA and quantify using a Nanodrop instrument or equivalent

3. Assemble the in vitro transcription reaction in PCR tubes on ice in 20-µl reactions as described in the HiScribe T7 ARCA mRNA Kit manufacturer's protocols as follows:

   10 µl 2× ARCA/NTP mix (supplied with the kit)
   1 µg linearized DNA
   2 µl T7 RNA polymerase mix (supplied with the kit)
   Nuclease-free water to 20 µl.

4. Mix thoroughly and briefly centrifuge. Incubate at 37°C for 30 min.

   The reaction time will be dependent on the amount of template and the quality and length of the RNA transcript. The reaction is stable up to 16 hr overnight. For transcripts >500 bp, incubation for 30 min results in the optimum yield; however, for transcripts <500 bp the reaction should be left for 1 hr.

   A PCR machine, water bath, or heat block can be used. If reactions are left for >1 hr, a PCR machine should always be used to ensure that the reaction is not affected by evaporation.

5. Remove the DNA by add 2 µl DNase I from the kit. Mix the reaction, centrifuge briefly, and incubate 15 min at 37°C.

6. Poly(A) tail the reaction in a fresh PCR tube by setting up a 50-µl reaction on ice:

   20 µl reaction from step 5
   5 µl 10× Poly(A) Polymerase reaction buffer (supplied with the kit)
   5 µl 10× Poly(A) Polymerase (supplied with the kit)
   20 µl with nuclease-free water.

7. Mix thoroughly and briefly centrifuge. Incubate the reaction at 37°C for 30 min.

8. Purify the RNA using the RNA Clean & Concentrator-5 kit according to manufacturer's instructions.

9. Quantify the RNA using a Nanodrop spectrophotometer.

10. Analyze the quality of the RNA by gel electrophoresis using either a 1% bleach gel (Aranda, LaJoie, & Jorcyk, 2012) or TBE/urea gel.

11. Store the RNA at −80°C. An example of luciferase data from a linked CFPS assay is shown in Figure 6.

REAGENTS AND SOLUTIONS

Buffer A

20 mM HEPES•KOH, pH 7.4
100 mM potassium acetate
2 mM magnesium acetate

Prepare fresh.

Lysis buffer A

20 mM HEPES•KOH, pH 7.4
100 mM potassium acetate
2 mM magnesium acetate
2 mM DTT
0.5 mM PMSF

Prepare fresh.

TE buffer

100 mM Tris•Cl, pH 8.0
10 mM EDTA, pH 8.0
Suitable for storage at room temperature.

YPD liquid medium

1% yeast extract
2% peptone
2% dextrose
Suitable for storage at room temperature.

YPD agar

1% yeast extract
2% peptone
2% dextrose
2% agar
Add antibiotic as appropriate if needed for selection of a specific strain.

COMMENTARY

Background Information

The methodology for generating the *P. pastoris* cell-free system described here was initially developed with a biosensor-assisted approach to engineer and improve the productivity of the strain from which the extract is derived (Aw & Polizzi, 2019). The reaction mix composition was initially based on the protocol for the earlier reported *S. cerevisiae* cell-free system (Gan & Jewett, 2014). Later, the *P. pastoris* CFPS system was used for the production of virus-like particles (VLPs), specifically those derived from the model hepatitis B core antigen VLP (Spice et al., 2020b). Most recently, the productivity of the system was increased using a minimized Design of Experiments (DOE) approach to improve the composition of the reaction mix (Spice et al., 2020a). The main effects influencing the productivity of the system were elucidated, and protein synthesis of firefly luciferase and the biopharmaceutical human serum albumin (HSA) was increased by 4.8-fold and 3.5-fold, respectively, using the improved reaction mix (Spice et al., 2020a). Although the composition of the reaction mix was modified in our most recent publication, the methodology for extract preparation and reaction setup was consistent throughout. It is worth noting that a *P. pastoris* cell-free system was very recently developed by another group using the protease-deficient strain SMD1163. Although the extract preparation methodologies and reaction mix composition they describe have similarities to the protocol described here, the yields reported for superfolder green fluorescent protein (sfGFP) in their system are considerably lower, potentially due to their use of the more readily accessible sonication method for lysis (Zhang et al., 2020).

Critical Parameters

During the extract preparation process, experimentalists must aim to keep the temperature of the extract as low and as consistent throughout the extract preparation process as reasonably possible, by ensuring that tubes are kept on ice throughout the entire process. Furthermore, the recording of the wet cell weight (WCW) is critical for correctly resuspending the harvested cells in the optimum volume of lysis buffer to ensure the correct total protein concentration of the extract after lysis. Before conducting the lysis step, it is highly advisable to precool the sample cooling jacket of the cell disruptor to prevent a significant increase in the temperature of the sample, which may negatively affect the viability of the extract. After preparation and processing of the lysate, Eppendorf tubes are used to store the extract in small aliquots to minimize freeze-thaw cycles. We have observed stability of the extract for up to 1 year at −80°C. Although this step is time consuming, it is far more economical and avoids multiple freeze-thaw cycles. The storage volume of these aliquots is a matter of personal preference; in our case 175 µl was generally used to support two sets of three replicates per aliquot of lysate. A more concentrated lysate will result in higher CFPS yields. The protein concentration of the lysate can be increased by increasing the lysis efficiency; however, care must be taken not to denature essential cellular proteins during lysis.

For example, an increased number of passes using the homogenizer could cause damage to the cellular machinery, and in that case would not be beneficial, despite potentially providing a higher protein concentration. Additionally, the ratio of lysis buffer to pellet has been optimized to account for the viscosity of the lysate. We would expect a minimum of 15 mg/ml of protein concentration using the methodology described, which is sufficient for CFPS.

Reaction setup

Concentrated stock mixes of each reaction component must be prepared initially that can then be combined to form the master mix. A large proportion of the reaction mix components are water soluble; however, those that are not (such as the amino acid mixture) require additional attention during their preparation and it is necessary to ensure their complete thawing and resuspension before use. A key point that we have observed, and has been noted previously in other systems, is that reaction mix preparation is the greatest source of variability observed when conducting CFPS (Cole et al., 2019; Dopp, Jo, & Reuel, 2019). As such, particular care must be taken when formulating the reaction mix. When setting up cell-free reactions, the reaction mix components and lysate must be fully thawed on ice before use, and they should always be stored at −80°C after use if repeat usage of components is desired. We have found that the order of addition of reaction mix components described in the reaction mix protocol must be followed exactly as described, as incorrect combination can result in failed or impaired protein synthesis. Our method for preparing the reaction mix involves creating a master mix that is used to seed multiple reactions. For the master mix, all the components are combined into a single mixture tube, which is mixed by flicking the tube multiple times (it is important to not vortex or mix by pipetting), and then individual replicates from this mixture are aliquoted into separate tubes. This methodology helps mitigate the potential error introduced by pipetting small volumes, as each replicate originates from the same mixture. The method does unfortunately require slightly more reagent than desired for any given number of samples or replicates. For example, in order to prepare four replicates, we would create a master mix containing the necessary volume for five replicates. Finally, as is true in all cell-free systems, the quality of the DNA template, its overall design, and the quantity added to the reaction will vastly impact the yield of protein synthesis. Although we have not evaluated the benefit of codon optimization with this system, the reporter proteins used here, luciferase and HSA, are not codon optimized. If translation blockages are observed when troubleshooting (using the linked CFPS reaction), it may be worth investigating codon optimization. However, codon optimizing genes for use in *P. pastoris* in vivo does not guarantee an increase in yield, and the same may be observed for CFPS. The additional components required (promoter, IRES, poly(A) tail, transcription terminator; Fig. 3) are standard components and should not require alteration.

Troubleshooting

See Table 4 for potential issues and suggestions for troubleshooting these protocols.

Understanding Results

The average yield will vary depending on the complexity of the protein produced and the method, including the reaction mix chosen. In general, it will not be possible to visualize the proteins on an SDS-PAGE gel because of the high concentration of host proteins present in the cell lysate. Therefore, an alternative visualization method needs to be established. For complex proteins such as HSA, which contains multiple disulfide bonds protein yields above 100 μg/ml have been recorded using an optimized strain and reaction mix. For assessing the *P. pastoris* CFPS system, a convenient model protein such as luciferase is used. It is not possible to use a GFP reporter, unless it is allowed to fold correctly into a refolding buffer before reading for at least 2 hr (Zhang et al., 2020). As this is more time consuming than a luciferase assay in our hands, luciferase remains our preferred reporter.

Time Considerations

All of the buffers and reaction mix components can be made in advance and frozen. The RTS amino acid mix should be dissolved as described by the manufacturer. This should be done slowly in order to ensure each amino acid is fully dissolved. Once combined, the RTS mix should be stored in the −80°C freezer. All other components can be stored at −20°C, or at 4°C in the case of the buffers.

Plasmid DNA or mRNA can be prepared in advance. RNA should always be stored at −80°C and freeze-thaw cycles should be avoided.

For the generation of active cell lysate, the cultures are grown overnight and the cell lysate preparation takes a full working day. There is a natural break point after washing the

Table 4 Potential Problems and Suggestions for Troubleshooting

Issue	Suggestion
Low protein yield from extract	Insufficient or incomplete lysis. The conditions of lysis may need to be optimized based on the method the user chooses. Additional passes or higher pressures can be used as long as the sample remains cold.
Inconsistent yields across replicates	The reaction mix may not be sufficiently mixed. Ensure that master mixes are made to avoid pipetting small volumes.
Reactions no longer producing proteins	The reagents, in particular creatine phosphate and creatine phosphokinase, do deteriorate over time even when stored at −20°C. Fresh reagents should be made if a noticeable drop in yield is observed.

cells, so this procedure can be split into 2 days, if necessary.

The CFPS reaction length will vary depending on the protein produced. Five-hour reactions are often performed for reporter proteins, but more complex proteins may require overnight incubation to fold correctly.

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Author Contributions

Rochelle Aw: Conceptualization; data curation; formal analysis; investigation; methodology; supervision; writing-original draft; writing-review & editing. **Alex J. Spice:** Conceptualization; data curation; formal analysis; investigation; methodology; writing-original draft; writing-review & editing. **Karen M. Polizzi:** Conceptualization; funding acquisition; supervision; writing-review & editing.

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