A Simplified and Robust Protocol for Immunoglobulin Expression in *Escherichia coli* Cell-Free Protein Synthesis Systems

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Cell-free protein synthesis (CFPS) systems allow for robust protein expression with easy manipulation of conditions to improve protein yield and folding. Recent technological developments have significantly increased the productivity and reduced the operating costs of CFPS systems, such that they can compete with conventional in vivo protein production platforms, while also offering new routes for the discovery and production of biotherapeutics. As cell-free systems have evolved, productivity increases have commonly been obtained by addition of components to previously designed reaction mixtures without careful re-examination of the essentiality of reagents from previous generations. Here we present a systematic sensitivity analysis of the components in a conventional Escherichia coli CFPS reaction mixture to evaluate their optimal concentrations for production of the immunoglobulin G trastuzumab. We identify eight changes to the system, which result in optimal expression of trastuzumab. We find that doubling the potassium glutamate concentration, while entirely eliminating pyruvate, coenzyme A, NAD, total tRNA, folinic acid, putrescine and ammonium glutamate, results in a highly productive cell-free system with a 95% reduction in reagent costs (excluding cell-extract, plasmid, and T7 RNA polymerase made in-house). A larger panel of other proteins was also tested and all show equivalent or improved yields with our simplified system. Furthermore, we demonstrate that all of the reagents for CFPS can be combined in a single freeze-thaw stable master mix to improve reliability and ease of use. These improvements are important for the application of the CFPS system in fields such as protein engineering, high-throughput screening, and biotherapeutics. © 2015 The Authors Biotechnology Progress published by Wiley Periodicals, Inc. on behalf of American Institute of Chemical Engineers Biotechnol. Prog., 31:823–831, 2015
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Introduction

Cell-free protein synthesis (CFPS) systems provide a robust platform for protein production and offer unique advantages over traditional cell-based protein expression.[1–3] Elimination of the cell wall enables direct control of important experimental conditions such as pH, redox potential and ionic strength, which can influence protein folding and stability.[4,5] This enables rapid production of proteins from plasmid DNA or PCR products without the need to manipulate cells. Cell-free platforms also readily facilitate supplementation of the protein translation reaction with additional components, such as isotope-labeled and non-natural amino acids, which can serve as site-specific probes,[6–9] as well as chaperones, which can enhance folding and solubility of expressed proteins.[5,10]

CFPS systems have previously been limited to specialized research roles due to their high cost and relatively low productivity. However, improvements to the cell-free platforms during the past two decades have greatly expanded the applications of the technology. These important contributions include improvements to the strain[11,12] and growth and lysis protocols[13–17] to give highly productive extracts. Furthermore, changes in ATP regeneration systems also account for greatly improved productivity in CFPS reactions. While the first cell-free systems used expensive high-energy phosphate containing molecules, such as acetyl-phosphate or phosphoenolpyruvate to directly generate ATP,[18] subsequent generations have harnessed the reactions of central metabolism, including glycolysis and oxidative phosphorylation. These improvements allowed inexpensive secondary energy sources such as pyruvate, glucose and glutamate to be utilized.[19,20] Finally, the ability to produce correctly folded and disulfide-bonded proteins in a prokaryotic cell-free system[5,10] has been critical for the high yield production of diverse proteins in CFPS reactions. These improvements have expanded the use of the CFPS platform into the fields of protein engineering, high-throughput screening,[21] synthetic biology,[22–24] and

biotherapeutics.[2] Indeed, we have recently demonstrated the potential for cell-free production at industrial scales by expression of a correctly folded human cytokine at 0.6 g/L using a 100 L CFPS bioreactor.[25]

Of the many exciting applications of cell-free technologies, one of the most promising is the rapid screening and production of therapeutic antibodies. Monoclonal antibodies are an important class of biotherapeutics for the treatment of cancer, autoimmune, and inflammatory disorders due to their high specificity, low immunogenicity, and long serum half-life.[26] The production of antibodies can be challenging since they require correct folding and disulfide bond assembly of a 150 kDa heterotetramer. The most common platform for therapeutic antibody production is mammalian cell culture, which is both time and resource intensive. In contrast, CFPS offers many unique advantages for the rapid discovery and production of these important therapeutic molecules. Correctly folded and disulfide bonded antibodies can be expressed in high yield in *Escherichia coli* based CFPS systems.[5,10] Ribosome display in conjunction with CFPS enables the rapid screening of Fab antibody fragment from PCR libraries with reduced concerns for solubility and folding *in vivo*.[27] CFPS systems also offer easy access to site-specific antibody drug conjugates using non-natural amino acids as chemical handles.[28]

Increases to the productivity, reproducibility, ease of use, and per liter cost of CFPS reactions will be integral to future applications of the technology. Previous generations of the CFPS platform have enhanced productivity by supplementing the reaction with additional reagents; however, as the system has evolved these components may no longer be required for efficient protein synthesis. Here, we perform a sensitivity analysis to individually evaluate components of an *E. coli* CFPS system using the immunoglobulin G (IgG) protein trastuzumab as a model. Trastuzumab IgG is a humanized antibody approved for adjuvant therapy of Her2-positive breast cancer and has recently been expressed in our cell-free system at yields of 1 g/L.[10] In the present work, we develop a simplified CFPS reaction mixture, which doubles glutamate concentration while entirely removing seven commonly used reagents from the system, resulting in a 95% decrease in reagent costs (excluding cell-extract, plasmid, and T7 RNA polymerase made in-house) without a loss in productivity. A larger panel of proteins was tested, including IgGs as well as Fab, scFv antibody fragments, and other nonantibody proteins, and all constructs showed equivalent or increased yields with the simplified protocol. In addition, we demonstrate that all of the reagents for CFPS can be combined in a single freeze thaw stable master mix solution, which greatly simplifies routine lab work and screening. This reoptimized CFPS protocol allows for simplified experimental applications and dramatically reduced costs compared with previous systems.

Materials and Methods

Cell-free protein synthesis reaction

All reagents were purchased from Sigma-Aldrich unless otherwise indicated. Bacterial cell-free S30 extract with 2× DsbC was prepared as described previously.[25] Briefly, plasmids carrying two tandem copies of *DsbC* were transformed into *E. coli* strain SBJY001, which was then grown to log phase in a fermenter and harvested for the production of cell extracts. The doubling time is ~1.3 h on average, and cells

were harvested at an OD$_{595}$ of ~45. Cells were lysed in a homogenizer (Avestin). A run-off reaction was performed by pre-incubating the lysate for about 1 to 2 h at 30 °C. The extract was then clarified by centrifugation, flash frozen in liquid nitrogen, and stored at −80 °C before use. All experiments in this study were performed from the same extract preparation. His$_6$-tagged T7 RNA polymerase was expressed in *E. coli* and purified by Ni-IMAC chromatography.[25] All expressed proteins were cloned into the pYD317 plasmid under a T7 promoter using the NdeI and SalI restriction sites as described previously.[29]

The conventional cell-free reaction mixture has been reported previously[10,20,30] and contains: 30% S30 *E. coli* extract, 10 μg/mL plasmid DNA, 33 mM sodium pyruvate, 130 mM potassium glutamate, 10 mM ammonium glutamate, 8 mM magnesium glutamate, 4 mM sodium oxalate, 1 mM putrescine, 1.5 mM spermidine, 15 mM potassium phosphate, 220 nM T7 RNA polymerase, 170 μg/mL total *E. coli* tRNA (Roche Diagnostics, 10109550001), 34 μg/mL folinic acid, 270 μM coenzyme A, 330 μM NAD, 2 mM GSSG, 1.2 mM AMP, 0.86 mM each of GMP, UMP and CMP and 2 mM 19 amino acids with 1 mM tyrosine. Thawed extract was treated for 30 minutes with 50 μM iodoacetamide to stabilize the redox environment for disulfide bond formation. Master mixes containing the cell-free reagents were created using stock solutions (see Supporting Information for details) to facilitate the titration of the individual reaction components in the present work. However, with the exception of tyrosine that has low solubility at neutral pH, all master mix components can be combined in a single solution directly from powder.

Cell-free reactions were performed in 100 μL volumes and run at 30 °C for 14 h with 600 rpm shaking in V-bottom polypropylene 96-well microtiter plates (VWR International) using a temperature controlled plate mixer (Thermomixer R, Eppendorf). Plates were sealed with breathable sealing film (BF-400, Axygen Scientific) to ensure adequate aeration. Evaporation was controlled by filling all unused wells with water as well as placing a second water filled plate in the Thermomixer chamber. The reaction was cooled to 4 °C after 14 h and processed for analysis within 3 h. Optimization was performed by varying the concentration of each reagent between zero and two to threefold higher than the conventional cell-free protocol. During each titration, the concentrations of all other reagents in the reaction were fixed according to the standard concentration.

In order to eliminate the heavy to light chain plasmid ratio as a variable affecting IgG yields in the CFPS sensitivity analysis titration experiments,[31] 1 mg/mL of purified trastuzumab light chain (LC) protein was added to cell-free reactions containing only the heavy chain (HC) DNA to yield full-length IgG or Fab fragment assembly. Trastuzumab light chain (LC) protein was pre-expressed under standard cell-free expression conditions, purified via KappaSelect affinity capture chromatography (GE Healthcare) and dialyzed into 10 mM Tris-acetate (pH 8.2), 60 mM potassium acetate and stored at 4 °C before use. When scaling up trastuzumab in a stirred tank reactor, coexpression of HC and LC was achieved by simultaneously adding two plasmids at a ratio of 3:1 HC to LC at a total DNA concentration of 10 μg/mL.

Determination of protein yield

To quantify protein yield, the reaction mixture was supplemented with a small amount of ^{14}C labeled leucine (3 μL

per 100 μL reaction, PerkinElmer: NEC279E001MC, 0.1 mCi/mL). A 4 μL aliquot of each reaction was spotted on a Filtermat A (PerkinElmer: 1450-421) before and after centrifugation at 6,100g for 15 min and dried on a hot plate at 100 °C for 10 min. The Filtermat with supernatant samples was washed three times for 15 min with 5% trichloroacetic acid on ice to remove unincorporated ^{14}C leucine, rinsed with absolute ethanol and dried on the hot plate. The Filtermat with noncentrifuged samples was not washed. All Filtermats were coated with MeltiLex melt-in scintillate (PerkinElmer: 1450-441) and counted in a Wallac MicrobetaTrilux liquid scintillation and luminescence counter (model 1450). Soluble protein yield was calculated according to:

$$P_{\text{sol}} = [\text{Leu}]_{\text{total}} \times \frac{\text{MW}}{(\# \text{ Leu})} \times \frac{C_{\text{sol}}}{C_{\text{full}}}$$

where P_{sol} is the soluble protein yield in mg/mL, $[\text{Leu}]_{\text{total}}$ is the total leucine concentration in the reaction (commonly 2 mM), $\frac{\text{MW}}{(\# \text{ Leu})}$ is the ratio of molecular weight to number of leucine residues in the proteins and $\frac{C_{\text{sol}}}{C_{\text{full}}}$ is the ratio of counts measured by the scintillation counter in the soluble fraction and full reaction mixture. For products with quaternary structure (Fab, scFvFc, IgG), autoradiograms were run using 4 to 12% Bis-Tris SDS-PAGE gels (Invitrogen) to determine assembly of the proper complex. Both reducing and nonreducing gels were run following the manufacturer's instructions. Exposed phosphor screens were scanned by Typhoon FLA 7000 (GE Healthcare life sciences) and the intensity of the bands was quantified by ImageQuant software (GE). The final yield of assembled complex was calculated according to the equation:

$$P_{\text{complex}} = P_{\text{sol}} \times I_{nr}^{\text{compex}} \Big/ \sum I_r$$

where P_{complex} is the yield of the correctly assembled complex in mg/mL, P_{sol} is the soluble protein yield, I_{nr}^{compex} is the intensity of the correctly assembled complex band on the non-reducing gel and $\sum I_r$ is the sum of intensities of all bands on the reducing gel. A sample gel used for protein yield calculation is displayed in the Supporting information Figure S1.

Results and Discussion

Extensive re-examination of the conventional CFPS components

The components of the CFPS can be categorized into seven groups: machinery, building blocks, transcription, translation, energy, polyamines and folding (Table 1). Though many of these components—S30 extract, DNA template and the building blocks for instance—are no doubt essential for the CFPS reaction, some of the others are included because they have enhanced protein synthesis in previous generations of the system. As the cell-free system has evolved, many of the components have been retained due to their historical performance increases and may no longer be required in the reaction. Indeed, CFPS systems have been reported in which some of the reaction components enumerated in Table 1 have been eliminated,[25] confirming that they are not all essential for high yield protein synthesis. However, a systematic investigation simultaneously addressing all components of cell-free reactions has never been reported in the literature and elimination of nonessential components from CFPS reactions has often

Table 1. Summary of the CFPS Components Grouped by Function

Function	Components
Machinery	S30 extract
Building blocks	AMP, GMP, CMP, UMP
	20 amino acids
Transcription	DNA template
	T7 RNA polymerase
Translation	*E. coli* total tRNA
	Folinic acid
Energy related	Ammonium glutamate
	Magnesium glutamate
	Potassium glutamate
	Potassium oxalate
	Sodium pyruvate
	NAD
	Coenzyme A
	Potassium phosphate buffer
Polyamines	Putrescine
	Spermidine
Folding	Glutathiones
	Chaperones

been associated with reduced yields.[20,30] As CFPS systems become more widely adopted it is important to re-evaluate the components of the reaction to increase ease of use and reproducibility, while maintaining productivity and reducing complexity and operating cost. To this end, we have subjected the individual CFPS reactions components to a sensitivity analysis, from a zero to twofold increase in concentration, while monitoring the production of trastuzumab IgG. In cases where the highest tested concentration of a reagent showed improvements in protein expression the titration range was further extended to include higher concentrations.

Several of the components in the standard CFPS reaction (Table 1) such as magnesium and phosphate are known to be essential or have already been extensively optimized and thus were not included in the present study. Cell extract, DNA template and the building blocks are all essential components of the reaction mixture and were not re-optimized. T7 RNA Polymerase can be produced in large amounts with relatively low cost, either as a purified protein[25] or in crude extract,[30] and does not require reoptimization, especially when it is overexpressed in cell extract. Oxalate was not re-evaluated as it has been shown to significantly increase CFPS yields since it inhibits phosphoenoylpyruvate synthetase and eliminates an ATP consuming futile cycle in the cell-free reaction.[32–34] Extensive optimization has recently been performed to determine the optimal glutathione and chaperone concentrations of CFPS reactions for the production of antibodies and antibody fragments in this system [10,25,29] and has not been repeated here.

Optimization of translation related components

Exogenous total *E. coli* tRNA and folinic acid are components of the CFPS reaction and have long been included in cell-free reactions and comprise ~30% of the reagent cost (Supporting Information Table S1). Total tRNA is added to supplement the endogenous *E. coli* tRNA in the cell-free system while folinic acid is included as a precursor for formylmethione (fMet) synthesis, which is required for prokaryotic translation initiation. The concentration of each of these components was titrated from zero to twofold of the standard concentration (Figure 1). For both reagents the highest yields were observed when they were eliminated from the CFPS reaction entirely, while the standard concentrations (denoted with an asterisk) gave a 10 to 20% reduction in yield. This
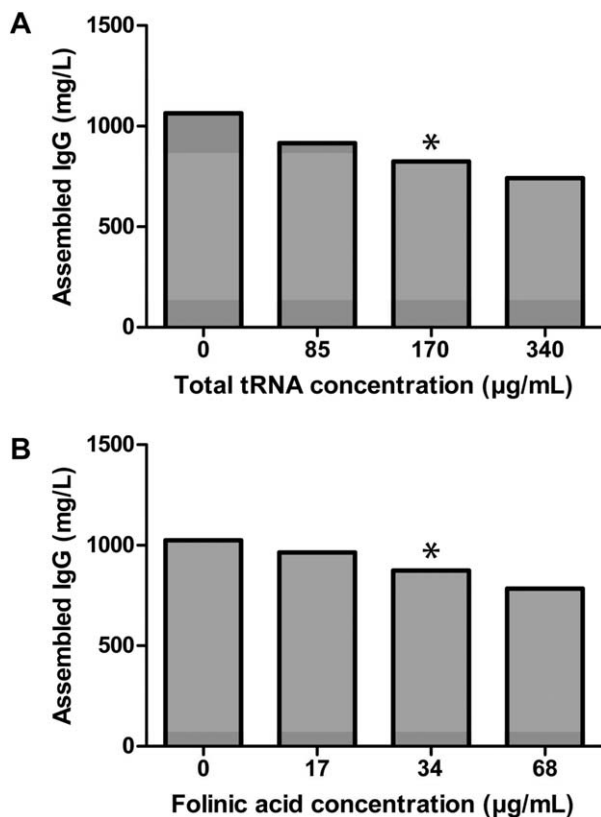
Figure 1. **Effects of *E. coli* total tRNA and folinic acid on cell-free production of trastuzumab IgG.**

Reagent concentrations found in the conventional cell-free protocol are indicated by asterisks above the bars. (A) Elimination of exogenous *E. coli* total tRNA results in a 20% increase in trastuzumab IgG yield. (B) Elimination of folinic acid results in a 20% increase in trastuzumab IgG yield. A representative experiment is shown. Experiment has been reproduced with similar results.

Figure 2. **Polyamine effects on the cell-free production of trastuzumab IgG.**

Reagent concentrations found in the conventional cell-free protocol are indicated by asterisks above the bars. (A) Cell-free production of trastuzumab IgG with varying amounts of spermidine with and without 1 mM putrescine. Optimal IgG yields are obtained at 1.5 mM spermidine. (B) Further titration of putrescine in the presence of 1.5 mM spermidine confirms that the highest IgG yields are achieved without putrescine. A representative experiment is shown. Experiment has been reproduced with similar results.

finding indicates that neither exogenous tRNA nor folinic acid is required for efficient translation in CFPS reactions. Since both of these molecules are required for translation in the system, we conclude that the cell extract produced with the current protocols must contain sufficient tRNA and fMet or its precursors to support translation and translation initiation over the course of a 10+ h CFPS reaction. It is also possible that the glycine cleavage system, which is capable of producing the fMet precursor N^5,N^{10}-methylenetetrahydrofolate from glycine and tetrahydrofolate,[35] is active in the cell extract.

Polyamine requirements for cell-free IgG production

E. coli cells have two types of polyamines, spermidine and putrescine, that participate in many cellular processes including transcriptional regulation, mRNA stabilization, and ribosome assembly.[36] Accordingly, both spermidine and putrescine have been included in the cell-free system in order to mimic the cytoplasmic environment of *E. coli*.[37] We titrated these two polyamines and found that putrescine was not required while 1.5 mM spermidine gave the highest trastuzumab yield (Figure 2). The majority of polyamines exist as polyamine-nucleic acids complexes, which can affect ribosomal structure and translational elongation rates.[36] Putrescine is believed to be a global regulator of gene expression[36] which may rationalize our finding that it is not
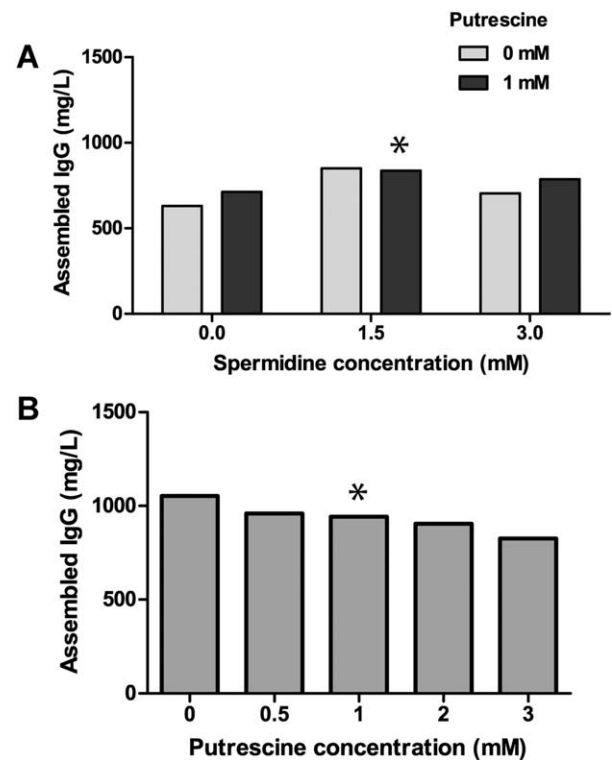
essential since transcriptional regulation and cell viability are irrelevant in CFPS. Spermidine is primarily associated with mRNA stabilization and probably helps to perform this function in our cell-free system.[36]

Secondary energy sources for cell-free IgG production

Glutamate was originally added to the cell-free reaction mixture as a counter ion to the physiologically relevant cations ammonium (NH₄Glu), magnesium (MgGlu) and potassium (KGlu).[32] However, more recent iterations of the CFPS system are able to use glutamate as a secondary energy source for ATP regeneration by oxidative phosphorylation.[20] The original choice for the concentration of the three glutamate salts in the cell-free reaction mixture was guided by the intracellular concentrations of the desired cations in the *E. coli* cytoplasm. While magnesium is clearly required for nucleic acid stabilization,[38] it is unclear whether ammonium ions are essential for the CFPS reaction or whether the overall glutamate concentration has been fully optimized given its new role as a secondary energy source. As the concentration of KGlu (130 mM) is much higher than that of NH₄Glu (10 mM), we optimized the KGlu concentration, while testing whether NH₄Glu is essential for efficient CFPS production of trastuzumab IgG. Figure 3A shows a titration of five different KGlu concentrations between 130 mM and 330 mM while NH₄Glu was varied between 0 and 20 mM.
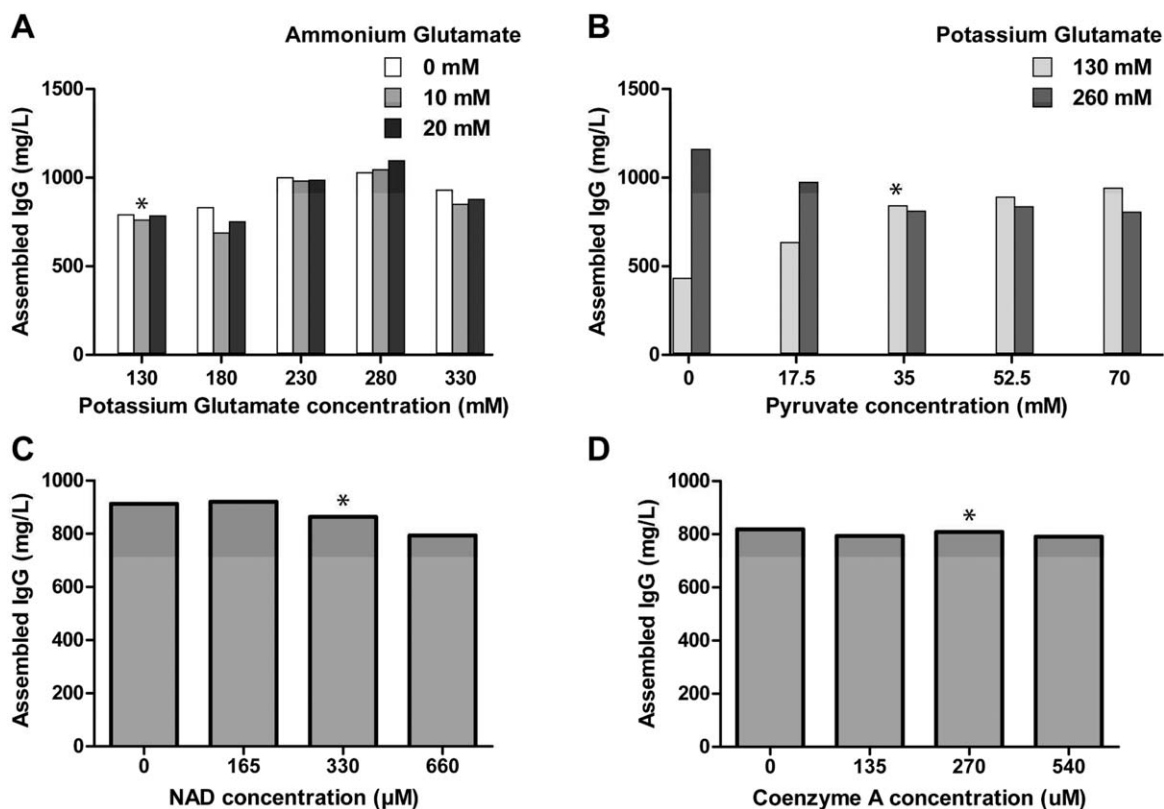
Figure 3. **The effect of energy-related reagents on the yield of trastuzumab IgG.**

Reagent concentrations found in the conventional cell-free protocol are indicated by asterisks above the bars. (A) Combined sensitivity analysis of ammonium glutamate and potassium glutamate. Elimination of ammonium glutamate shows no significant yield differences at any potassium gluta-mate concentrations. A 25% increase in trastuzumab IgG yields is observed when the potassium glutamate concentrations are increased from 130 mM to 230 to 280 mM. (B) Titration of pyruvate at two potassium glutamate concentrations. A decrease in IgG yields is observed when pyru-vate is eliminated at low glutamate concentrations. Elimination of pyruvate results in higher IgG yields at higher glutamate concentrations. (C) The yield of IgG is insensitive to NAD concentrations between 0 to 660 µM. (D) The yield of IgG is insensitive to Coenzyme A concentrations between 0 and 540 µM. A representative experiment is shown. Experiment has been reproduced with similar results.

For all the tested KGlu concentrations, the addition of NH$_4$Glu did not improve yields by >10% leading us to con-clude that it could be eliminated from the CFPS reaction mixture. In contrast, we observed a 25% increase in trastuzu-mab IgG yields when the concentration of glutamate was increased from 130 to 230–280 mM.

Since increased glutamate concentrations supported a higher IgG yield in the CFPS reaction, we next focused on pyruvate, which is also included in the conventional reaction mixture as a secondary energy source for ATP regeneration. The pyruvate titration was performed at both low (130 mM) and high (260 mM) KGlu concentrations (Figure 3B). While higher pyruvate concentrations were found to increase IgG yields at the low glutamate condition, additional pyruvate was observed to decrease IgG yields at higher glutamate concentrations. Reduced CFPS yields have previously been reported when pyruvate was eliminated from the reaction mixture at 130 mM KGlu,[20] however, the combined effect of re-optimizing the glutamate as secondary energy source and eliminating pyruvate from the reaction results in a 40% increase in trastuzumab IgG yield. Furthermore, the removal of pyruvate from the system may obviate the need for oxa-late though this was not tested in the present work.

Cofactor requirements for cell-free IgG production

The cofactors NAD and coenzyme A (CoA) together rep-resent 60% of the reagent cost in the CFPS reaction (Sup-

porting Information Table S1). They were initially found to increase protein synthesis yields when pyruvate was used as a secondary energy source since they are cosubstrates for the oxidative decarboxylation of pyruvate to acetyl-CoA cata-lyzed by the pyruvate dehydrogenase complex. However, since these experiments, the cell-free system has switched from substrate level phosphorylation to oxidative phospho-rylation as the primary source for ATP regeneration.[20] Fur-thermore, a dialysis step, which may deplete small molecule cofactors, has also been eliminated from the current cell extract preparation protocol.[25] Given these changes and the high cost of the cofactors, we retested the essentiality of these reagents to the cell-free reaction. Both NAD and CoA were tested in concentrations ranging from zero to twice the conventional reaction concentration (Figures 3C,D). Within the tested range, we detected no significant difference in the final yield of the assembled IgG. The lack of improvement in IgG yields with added NAD and CoA suggests that the current lysate preparations already contain sufficient amounts of these molecules, since both cofactors are required for the oxidative phosphorylation of ATP using glutamate.

Simplified protocol for CFPS reactions

In the previous titration experiments, components were tested individually to identify their essentiality and optimal concentrations. This led us to design a simplified cell-free protocol that doubled the concentration of KGlu, while
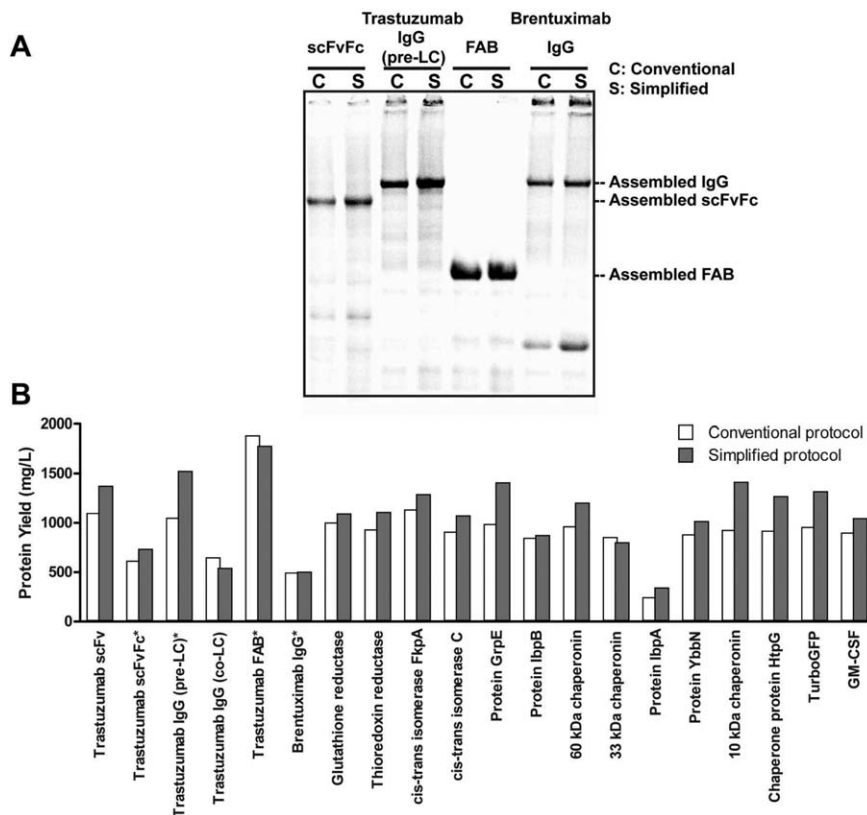
Figure 4. **Comparison of the protein yields using the conventional and simplified CFPS reaction protocols.**

(A) Autoradiogram analysis of antibodies in various scaffolds. Both the conventional and the simplified protocols result in similar product profiles. (B) A panel of 20 proteins used to characterize the performance of the simplified protocol. Proteins include 6 antibodies in different scaffolds, 12 *E. coli* chaperones, TurboGFP and human GM-CSF. Proteins from the autoradiogram in part A are denoted with an asterisk. The simplified protocol shows equivalent or improved yields for all proteins tested compared with the conventional protocol. A representative experiment is shown. Experiment has been reproduced with similar results.

entirely eliminating exogenous addition of NAD, CoA, total tRNA, folinic acid, putrescine, NH$_4$Glu, and pyruvate. We next proceeded to evaluate the performance of the simplified protocol by simultaneously making all eight changes in a single reaction. Different scaffolds of trastuzumab antibodies, such as scFv, scFvFc, Fab and IgG, and brentuximab IgG were tested using the conventional and simplified protocols. To demonstrate that the simplified protocol is not limited to immunoglobulins, fourteen other proteins were tested in parallel including 12 *E. coli* chaperones, TurboGFP and human granulocyte macrophage-colony-stimulating factor (GM-CSF). Figure 4 summarizes the results of the comparison between the conventional and simplified protocols. Out of the twenty candidates tested in the simplified protocol, all proteins had yields at least comparable with the conventional cell-free reaction mixture, while fourteen (74%) showed higher yields (Figure 4B). Trastuzumab IgG expression was tested in the presence of prefabricated LC (pre-LC) and by coexpressing both chains (co-LC). The pre-LC experiments have a higher yield, since only the HC is being expressed. While the pre-LC trastuzumab shows an improved yield with the simplified protocol the co-LC expression showed an equivalent yield for both the conventional and simplified protocols.

To test the scalability of the simplified protocol, trastuzumab coexpression experiments were conducted in DASGIP DASbox stirred tank reactors, at 100 mL scale as previously described.[25] Time point samples were collected. The IgG product was captured by Protein A Phytips using a Biomek

robotic liquid handling system and then quantified by A$_{280}$ and SDS-PAGE.[10] Data suggested that both the conventional and simplified protocols had equivalent trastuzumab IgG synthesis kinetics and final yields (Supporting Information Figure S2).

All seven components we identified as nonessential have previously been eliminated from CFPS reactions although a reduction in productivity was also observed.[20,30] This implies that the increased glutamate concentration must be the critical element, which is responsible for maintaining CFPS productivity in our simplified system. While the changes proposed are likely specific to our *E. coli* cell-free platform, the method for systematic sensitivity analysis of all reaction components will likely be useful for optimization of other cell-free systems including those utilizing different energy generation sources or alternative organisms.

Ease of use and reproducibility for simplified CFPS reactions

The conventional cell-free protocol required individual pipetting of over 10 reagents for each reaction. Factors such as the order of reagent addition, pipetting error, and duration of reaction setup time could thus affect the ultimate performance of the system. This complicated setup procedure reduces ease of use and makes batch-to-batch consistency difficult to maintain. It has been shown that many of the cell-free reagents can be combined into a single master mix to address ease of use and reproducibility.[30]
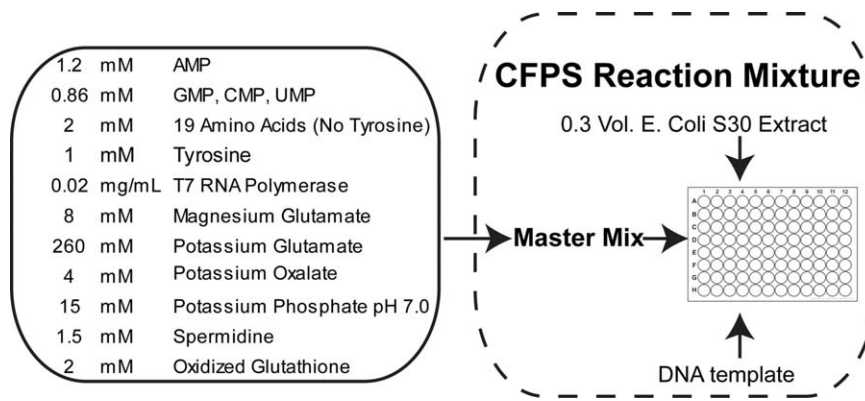
1.2	mM	AMP
0.86	mM	GMP, CMP, UMP
2	mM	19 Amino Acids (No Tyrosine)
1	mM	Tyrosine
0.02	mg/mL	T7 RNA Polymerase
8	mM	Magnesium Glutamate
260	mM	Potassium Glutamate
4	mM	Potassium Oxalate
15	mM	Potassium Phosphate pH 7.0
1.5	mM	Spermidine
2	mM	Oxidized Glutathione

Figure 5.   **Summary of the simplified CFPS reaction protocol.**

All components except cell extract and DNA template are included in a single freeze-thaw stable master mix. Compared with the conventional protocol, 260 mM KGlu serves as the sole energy source and seven components have been eliminated.
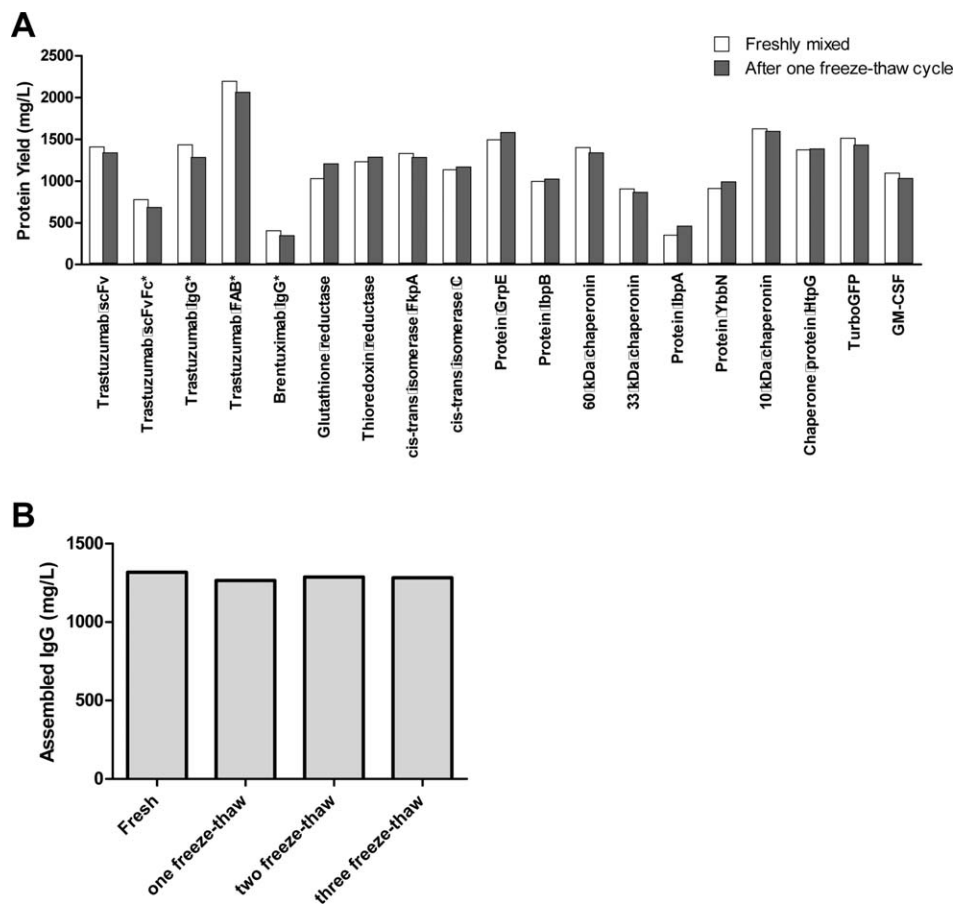

Figure 6.   **Performance of the new protocol does not decrease or diminish after a freeze-thaw cycle.**

(A) Nineteen protein candidates, including 5 antibodies in various scaffolds, 12 *E. coli* chaperones, TurboGFP and human GM-CSF, were used to characterize the productivity of previously frozen master mix and fresh mix from individual components. None of the proteins tested showed significant yield decrease after one freeze-thaw cycle. (B) To characterize the effect of multiple freeze-thaw cycles, trastuzumab IgG was produced by identical aliquots of master mix undergoing 0 (fresh), 1, 2, and 3 freeze-thaw cycles. The fully assembled IgG yield does not decrease even after 3 freeze-thaw cycles. A representative experiment is shown. Experiment has been reproduced with similar results.

Thus, we have created an analogous master mix using our simplified cell-free protocol that contains all reagents except the S30 cell extract and DNA template (Figure 5). Although individual stock solutions have been made for each of the reagents to facilitate sensitivity analysis, all of the components, other than tyrosine, which has low solubility at neutral pH, can be added directly to a single solution to facilitate ease of preparation. The extract may also be included in the master mix, however, we find it more convenient to store the two separately in order to reduce the number of freeze thaw cycles to which the extract is subjected. Moreover, this allows the optional treatment with iodoacetamide in order to stabilize the cell-free redox environment.
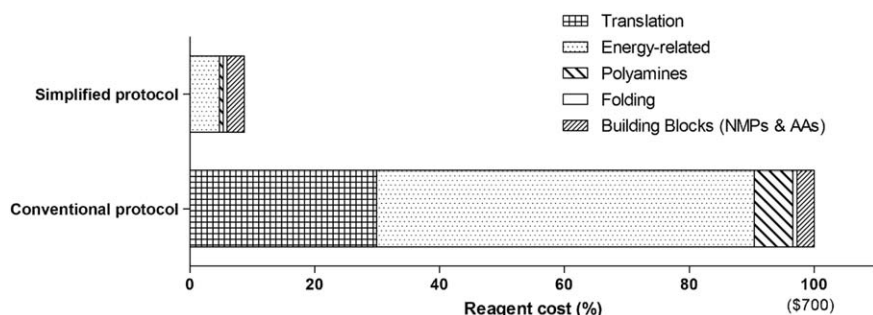
Figure 7. The costs of cell-free reagents plotted according to their category in Table 1.

Cell-free S30 extract, T7 RNA polymerase and DNA template can be readily produced in-house and have been excluded from the cost analysis.

In contrast to previous work,[30] our master mix contains purified recombinant T7 RNA polymerase. Therefore it is important to demonstrate the freeze-thaw stability of the solution. Figure 6A compares the performance of freshly made solution before and after a single freeze-thaw cycle. Out of a total of 19 proteins tested, we observed no significant reduction in yield after a single freeze-thaw cycle of the master mix solution. We subsequently tested the yield of trastuzumab IgG after three freeze-thaw cycles and found no significant reduction in yield using the master mix (Figure 6B). Multiple batches of experiments have confirmed that the master mix solution is stable for more than 6 months when stored at -80 °C (data not shown). This demonstrates that most of the components of our simplified cell-free system can be formulated into a freeze-thaw stable master mix, which streamlines the setup procedure and improves reproducibility for CFPS reactions.

Dramatically reduced cost for simplified CFPS reactions

By re-examining the essentiality of the reagents in the conventional cell-free reaction mixture, we have determined that seven components could be eliminated entirely. The list of nonessential components includes many of the most expensive reagents on a per liter of reaction basis including tRNA, NAD, and CoA. Elimination of these seven compounds reduces the reagent cost for CFPS reactions by 95%, a $620 per liter reduction (Figure 7 and Supporting Information Table S1). This calculation does not take into account the costs of the S30 extract, T7 RNA polymerase or DNA template, which can be produced in-house with a relatively low cost over a wide range of scales.[25,30]

Though the simplified CFPS system we developed is adequate for lab-scale applications, it may still be desirable to perform a protein dependent optimization of the remaining essential components for production of protein therapeutics. The NMP and amino acid building blocks now represent $\sim$50% of the reagent cost in the simplified protocol (Supporting Information Table S1) and may be supplied in excess in the current system. Optimal amino acid concentrations in the cell-free reaction may be a function of the amino acid content of the target protein. In addition, the cell extract may contain enzymes sufficient for the production of some of the amino acids from glutamate rendering them nonessential to the CFPS reaction. Previous work has shown that aspartate, lysine and tyrosine are produced during the CFPS reaction[20] suggesting that some amino acids might also be eliminated altogether. Aspartate is readily produced from the tricarboxylic acid cycle intermediates by aspartate ammonia lyase (aspA)[39] and aspartate aminotransferase

(aspC).[40] The biosynthetic pathway for lysine synthesis from aspartate and pyruvate requires the activity of 11 enzymes as well as ATP, NADPH, succinyl-CoA, and glutamate, all of which are present in the cell-free reaction. However, the route for tyrosine production in the cell-free system presents the most interesting case. Since *E. coli* lacks phenylalanine hydroxylase activity, the two remaining possibilities are production of tyrosine through a route including gluconeogenesis, the pentose phosphate pathway and the aromatic amino acid synthesis pathway, which would involve more than 30 enzyme activities and many cofactors or the presence of an unknown pathway in the *E. coli* extract for directly producing aromatic amino acids from glutamate or one of its metabolites. The optimization of aspartate, lysine, tyrosine, and other amino acids is planned for future work. We believe that the optimized minimum concentration of the amino acids concentration will be a function of the product sequence.

Conclusions

In summary, we developed an improved and simplified protocol for CFPS IgG production by doubling the glutamate concentration and eliminating seven components from the conventional reaction mixture. This simplified protocol greatly reduces the reagent cost for CFPS reactions by eliminating several of the most expensive components and improves ease of use and reproducibility for cell-free reactions since the reagents can be stored as a single freeze-thaw stable master mix.

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