

Research Article

A combined cell-free transcription-translation system from *Saccharomyces cerevisiae* for rapid and robust protein synthesis

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Cell-free protein synthesis (CFPS) provides a valuable platform for understanding, using, and expanding the capabilities of the translation apparatus. For example, high-throughput CFPS is helping to address the increasing discrepancy between genome sequence data and their translation products. Here, we report the development of a combined cell-free transcription-translation (Tx/TI) system from *Saccharomyces cerevisiae* that is suitable for such efforts. First, we show the ability to enable translation initiation in a cap-independent manner. The performance of various genetic elements was assessed, including 5'-UTR, 3'-UTR, and length of poly(A) tail. A specific vector harboring the 5'-UTR fragment of the Ω sequence from the tobacco mosaic virus and a poly(A) tail of 50 nucleotides led to optimal performance. Second, we developed a simple, two-step polymerase chain reaction (PCR) method for high-throughput production of linear templates for yeast CFPS. This procedure allows all functional elements needed for Tx/TI to be added to an open-reading frame directly by overlap extension PCR. Our two-step PCR method was successfully applied to three reporter proteins: luciferase, green fluorescence protein, and chloramphenicol acetyl transferase, yielding 7 to 12.5 $\mu\text{g mL}^{-1}$ active protein after 1.5-h batch reactions. Surprisingly, the linear templates outperformed plasmid DNA by up to 60%. Hence, the presented CFPS method has the potential to rapidly prepare tens to thousands of DNA templates without time-consuming cloning work. Further, it holds promise for fast and convenient optimization of expression constructs, study of internal ribosome entry site, and production of protein libraries for genome-scale studies.

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Abbreviations: CAT, chloramphenicol acetyltransferase; CFPS, cell-free protein synthesis; CrPV, cricket paralysis virus; DTT, dithiothreitol; Tx/TI, transcription-translation; IRES, internal ribosome entry site; PMSF, phenylmethanesulfonyl fluoride; TEV, tobacco etch virus; TMV, tobacco mosaic virus; UTR, untranslated region; WGE, wheat germ extract

1 Introduction

Affordable, simple, and efficient protein production technologies are of growing fundamental importance for science and society. This is especially true given the ever-increasing demand for therapeutic proteins, enzyme variants, and functional genomics analysis. Cell-free protein synthesis (CFPS) is an emerging technology that is helping to address this demand [1]. While prokaryotic systems are the most productive [2, 3], eukaryotic systems have shown advantages for producing complex proteins with multiple subdomains and activating co-translational fold-

ing not found in bacteria [4]. Post-translational modifications have also been shown (i.e. isoprenylation, acetylation, *N*-myristoylation, ubiquitin-conjugation [5], and core glycosylation [6]). Wheat germ extract (WGE), rabbit reticulocyte lysate (RRL), and insect cell extract (ICE) systems are the most widely used eukaryotic CFPS systems with yields ranging from tens to hundreds of micrograms protein per milliliter in batch and semi-continuous reactions [1]. Unfortunately, several features limit the most widely used eukaryotic CFPS systems: (i) extract preparation is costly and difficult to scale; (ii) complex pre-treatments are needed before making extract from highly developed tissues or cells; and (iii) genetic tools for host strain modification are not as developed as in classical model organisms, like yeast, limiting comprehensive strain improvement programs. For these reasons, there has been resurgence in the development of new eukaryotic CFPS platforms. For example, Mureev et al. [7] reported the development of a eukaryotic platform from *Leishmania tarentolae*. Further, Kubick and colleagues [8] recently reported the development of a high yielding (~50 µg mL⁻¹) batch CFPS derived from Chinese hamster ovary (CHO) cells that promises to speed development pipelines for the pharmaceutical industry.

Our laboratory is developing a CFPS system derived from yeast. Compared to other eukaryotic cells mentioned above, yeast is a model organism with facile genetic tools that has played a prominent role in elucidating fundamental biochemical knowledge. In addition, yeast can be grown quickly and inexpensively under precise conditions, is generally regarded as safe (GRAS), and is an important biomanufacturing production organism. Approximately 18.5% of all FDA and EMA licensed recombinant protein biopharmaceuticals, as of January 2009, were produced in yeast [9]. While there has been some development in the realm of yeast CFPS [10–13], a robust, high yielding combined transcription-translation (Tx/Tl) system has not been established. Further, efforts to streamline extract preparation methods, optimize the reaction conditions, and characterize reaction substrates quantitatively and systemically have not been performed. Here we describe efforts to active combined Tx/Tl, with the latter points being addressed in recently reported parallel work [14, 15].

Typically, for CFPS to be active in the previously developed yeast systems, capped mRNA (with m⁷GpppG or its analog) must be used to initiate protein synthesis [10, 13]. The capping reaction is a time-consuming and expensive method with unpredictable results. Therefore, most developed eukaryotic CFPS systems seek to utilize cap-independent methods to initiate translation [16]. Most known cap-independent sequences are located in the 5'-end untranslated region (5'-UTR) of genes, but a few are also found in the 3'-UTR and intergenic regions [17]. For example, internal ribosome entry site (IRES) sequences can recruit eukaryotic ribosomes to initiate translation in

a cap-independent method. IRES sequences are able to recruit eukaryotic ribosomes by short sequence patterns, complex secondary structures, or aided by trans-acting factors [18, 19]. Other cap-independent methods, such as the Ω gene from tobacco mosaic virus (TMV) are able to recruit initiation factors such as eIF4F to initiate translation [20]. In WGE, for example, the 5'-UTR fragment of Ω gene from TMV is able to efficiently initiate translation [21]. ICE systems use the 5'-UTR fragment of polyhedrin gene from *Malacosoma neustria nucleopolyhedrovirus* (MnNPV) [22]. RRL systems use a 5'-UTR fragment from encephalomyocarditis virus [23, 24]. Recently, Mureev et al. engineered artificial sequences termed species-independent translational sequences (SITS) with AT-rich sequences at the 5' end of mRNA. This IRES sequence, termed species-independent translational sequences (SITS), showed dramatically enhanced efficiency of cap-independent translation in almost all known eukaryotic CFPS systems [7].

Here, we investigated the ability of different 5'-UTRs to enable combined Tx/Tl in yeast. Specifically, we evaluated the impact of different expression constructs and further report a rapid and robust CFPS platform from linear DNA molecules, i.e. PCR products. By use of overlap extension PCR, we show that Tx/Tl elements (i.e. T7 promoter, cap-independent translation sequence, and poly(A) tail) can be rapidly added to the coding region of gene. By doing so, the templates for a number of proteins can be prepared in parallel within a short time using a cloning-free method. In addition to their rapid construction, PCR templates yielded up to 60% more protein per DNA molecule when compared to plasmid vectors in the yeast CFPS system. This work opens the way to using yeast CFPS for high-throughput production of protein libraries.

2 Materials and methods

2.1 Strains and reagents

Yeast strain S288c was used. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise noted. DNA polymerase, T4 polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). T7 polymerase was prepared in lab (following the protocol developed by Swartz et al. [25]). Plasmids were extracted using Omega Kits (Omega Bio-Tek, Norcross, GA). All DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa).

2.2 Preparation of yeast S60 extract

Yeast colonies were cultivated in rich media (2% peptone, 1% yeast extract, 2% glucose), shaking at 250 rpm at 30°C.

The seeding culture was used to inoculate 1 L of fresh rich media with 1:1000 in 2.5 L Tunair (Sigma–Aldrich), shaking at 250 rpm at 30°C. Cells were harvested at mid-logarithmic phase (OD₆₀₀ 10–12) by centrifugation at 3000 *g* for 10 min. Cell pellets were resuspended and washed three times in Buffer A (20 mM HEPES–KOH pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate). The wet pellet was weighed and suspended by vortex in lysis buffer (20 mM HEPES–KOH pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonylfluoride (PMSF)) with 1 mL buffer per gram of wet cell weight. Cells were lysed using an Avestin EmulsiFlex-C5 High Pressure Homogenizer (Avestin, Ottawa, ON, Canada) one time under 30 000 psig. The lysate was centrifuged at 4°C and 30 000 *g* for 30 min, the supernatant was removed, placed in a clean spherical bottom high-speed centrifuge bottle and clarified again. Supernatant was desalted using dialysis tubing (Spectra/Por 3 MWCO 3500, Spectrum Labs, Rancho Dominguez, CA) against four exchanges of 50-volumes of lysis buffer (20 mM HEPES–KOH pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.5 mM PMSF) for 30 min each at 4°C. After dialysis, extract was centrifuged at 60 000 *g* for 20 min at 4°C. Final extract was distributed

into 100 µL aliquots in 1.5-mL Eppendorf tubes, frozen in liquid nitrogen and stored at –80°C. The protein concentration was determined using Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA).

2.3 Cloning and plasmid construction

The structures of all expression templates described below are listed in Fig. 1. All primers for template construction are shown in Supporting information, Table S1. The luciferase-coding region was amplified from pK7LUC plasmid [26] using primers pET23LucA-f/pET23LucA-r, and inserted into pET23c plasmid with *NdeI* and *XhoI* sites to construct plasmid pET23LucA. Poly(A) tails with 25 and 50 nt were introduced into pET23LucA plasmid to replace 90 nt poly(A) using the primer pairs: PolyA-f/PolyA25-r and PolyA-f/PolyA50-r. We were unable to produce a plasmid with a correctly identified 170 nt poly(A) due to the poor efficiency of PCR and sequencing for a 170 nt poly(A) region. However, we introduced a 170 nt poly(A) into linear DNA template by PCR using the primer PolyA170-r, purchased from IDT with PAGE purification. Three yeast native IRES sequences, the 5′-UTR of TFIID, HAP270, and YAP1 genes [27], were amplified from yeast genomic DNA using TF5UTR-f/TF5UTR-r, HAP270-f/

Construct	← 5′ UTR →	← ORF →	← 3′ UTR →	← PolyA →
CappA90	Cap analog	luciferase	None	90
HAPpA90	HAP270	luciferase	None	90
TFIIDpA90	TFIID	luciferase	None	90
p150pA90	p150	luciferase	None	90
YAP1pA90	YAP1	luciferase	None	90
IGRpA90	IGR	luciferase	None	90
A64pA90	PolyA64	luciferase	None	90
HedrinpA90	Polyhedrin	luciferase	None	90
QpA90	Ω	luciferase	None	90
TEVpA90	TEV	luciferase	None	90
TbmpA90	CfTbm	luciferase	None	90
QpA25	Ω	luciferase	None	25
QpA50	Ω	luciferase	None	50
QpA170	Ω	luciferase	None	170
ΩFBAL	Ω	luciferase	FBA1 1465nt	0
ΩFBAS	Ω	luciferase	FBA1 659nt	0
ΩTMV13U200	Ω	luciferase	TMV13U 200nt	0
ΩTMV13U400	Ω	luciferase	TMV13U 400nt	0
ΩTMV13U700	Ω	luciferase	TMV13U 700nt	0
ΩTMV23U	Ω	luciferase	TMV23U	0
ΩN3U	Ω	luciferase	None	0
N5UpA90	None	luciferase	None	90
QpA	Ω	luciferase	None	0
Luc	None	luciferase	None	0

Figure 1. Expression template structures for cap-independent yeast CFPS used in this study. 5′-UTR, 5′-untranslated region; ORF, open-reading frame; 3′-UTR, 3′-untranslated region; PolyA, the poly(A) tail. All templates contain a T7 promoter in front of 5′ UTRs for transcription. “None” indicates there is no corresponding component in this template. The number in “PolyA” column indicates the length of the poly(A) tail. The details of cloning and construction are described in the Section 2. The template N5UpA90 and Luc contain a 60 nt sequence from pET23c vector including an *E. coli* ribosome-binding site. These two templates are used as negative control of 5′-UTR components, noting that N5UpA90 has a polyA90 tail while Luc does not.

HAP270-r, and YAP1-f/YAP1-r primer pairs respectively, and inserted into plasmid pET23LucA between the T7 promoter and luciferase with *NdeI* and *XbaI*. These three plasmids are identified as pET23TFIIDLucA, pET23HAP270LucA, and pET23YAP1LucA. The 5'-UTR of p150 gene [28] was amplified from yeast genomic DNA using primers P150-f/P150-r flanking with *XbaI* and *BamHI* sites, and inserted into pET23LucA plasmid where *NdeI* had been replaced with *BamHI*, since the insert fragment contains an *NdeI* site.

The Ω sequence (65 nt) from TMV was introduced into pET23LucA upstream of luciferase with primers Sf-f/Omega-r (underlined segment). The 5'-UTR of polyhedrin gene (44 nt) was introduced into pET23LucA with primers Sf-f/Polyhedrin-r (underlined segment). A 5-end poly(A)₆₄ sequence was introduced into pET23LucA with primers Sf-f/PolyA64-r. A 5'-UTR fragments (143 nt) from tobacco etch virus (TEV) genome (Accession number: NC_001555) was cloned into pET23LucA upstream of the luciferase gene by oligo TEV-r (underlined segment); another plant viral 5'-UTR fragments (65 nt) from Crucifer tobamovirus (CfTbm) genome (Accession number: NC_003355.1) was inserted into pET23LucA upstream of luciferase gene using oligo CfTbm-r (underlined segment). An IRES sequence of the cricket paralysis virus (CrPV) intergenic region (IGR) was amplified from the plasmid pSal-IGR [29] using primers IGR-f/IGR-r.

Two fragments were cloned from the 3'-UTR of yeast *FBA1* gene (Accession number: NM_001179626) that encodes fructose-1,6-bisphosphate aldolase. The longer fragment was amplified by primers FBA3UTR-f/FBA3UTR1-r containing 1465 nt from the first nucleotide after stop codon. The shorter fragment was amplified by primers FBA3UTR-f/FBA3UTR2-r with the length of 659 nt from the first nucleotide after stop codon. The two 3'-UTR fragments were then placed after the stop codon of luciferase with *XhoI* and *SacI* sites. Two DNA fragments of TMV genome were synthesized (GenScript, Piscataway, NJ). The first fragment, TMV1, ranges from 4920 to 5711 of genome (792 nt in length) containing the sequence between two open-reading frame TMVgp1 and TMVgp6; the second fragment, TMV2, ranges from 6192 to 6395 genome (204 nt in length). Three fragments, TMV13U200, TMV13U400, and TMV13U700, were amplified from TMV1 with the length of 200, 400, and 700 nt, respectively using primers: TMV13U-f/TMV13U200-r, TMV13U-f/TMV13U400-r, and TMV13U-f/TMV13U700-r. One fragment was amplified from TMV2 with the length of 204 nt by using TMV23U-f/TMV23U-r. All four fragments amplified from TMV1 and TMV2 were placed after the stop codon of the luciferase-coding frame with *XhoI* and *SacI* sites as 3'-UTRs.

To assemble linear expression templates of luciferase, green fluorescence protein (GFP), and chloramphenicol acetyl transferase (CAT), the coding region of the three enzymes were amplified with primers OEluc-f/OEluc-r,

OEGFP-f/OEGFP-r, and OECAT-f/OECAT-r, respectively. Therefore, T7 promoter, Ω sequence, and poly(A)₅₀ tail were overlapped to the coding region by OET7Ome-f/PolyA50-r, respectively.

2.4 In vitro transcription

Capping in vitro transcription was performed with the Ambion mMessage mMachine[®] Kit (Life Technologies, Grand Island, NY). The capped mRNA was purified following a phenol-chloroform extraction and desalted using a Micro Bio-Spin[®] 6 chromatography column (Biorad, Hercules, CA). Non-capping RNA was prepared according to Mureev et al. [7].

2.5 Yeast cell-free translation-only reactions using mRNA template

Yeast cell-free translation was prepared as described by Iizuka and Sarnow [10] with some modifications. CFPS reactions were primed with 20 nM mRNA in 15 μ L reactions. The cell-free reaction mixture was assembled on ice from stock solutions to the following working concentrations: 25 mM HEPES-KOH pH 7.4, 120 mM potassium glutamate, 1 mM magnesium glutamate, 1.5 mM adenosine triphosphate (ATP), 0.2 mM guanosine triphosphate (GTP), 0.1 mM of each of 20 amino acids, 25 mM creatine phosphate, 1 mM DTT, 0.27 mg mL⁻¹ creatine phosphokinase (C3755-1KU, Sigma), 200 U mL⁻¹ RNase Inhibitor (Qiagen), and 50% v/v S60 yeast extract.

2.6 Combined transcription-translation (Tx/Tl) cell-free protein synthesis

Combined cell-free Tx/Tl reactions were carried out in 1.5-mL Eppendorf tubes in 15 μ L reactions. The reaction was primed with 3.2 nM PCR product. The cell-free reaction mixture was prepared on ice from stock solutions to the following working concentrations for translation only reactions: 25 mM HEPES-KOH pH 7.4, 120 mM potassium glutamate, 6 mM magnesium glutamate, 1.5 mM ATP, 2 mM of each GTP, CTP and UTP, 0.1 mM of each of 20 amino acids, 25 mM creatine phosphate, 2 mM DTT, 0.27 mg mL⁻¹ creatine phosphokinase (C3755-1KU, Sigma), 200 U/mL RNase Inhibitor (Qiagen), 27 μ g mL⁻¹ T7 RNA Polymerase, and 50% v/v S60 yeast extract. All combined cell-free Tx/Tl reactions were performed using above conditions unless specified otherwise. For the analysis of [³⁵S]-methionine-labeled protein products, combined Tx/Tl CFPS was performed as described above except that [³⁵S]-methionine was supplemented with the final concentration of 0.58 μ M. The protein products were resolved by NuPAGE[®] Novex[®] 4–12% Bis-Tris Gels (Invitrogen, Grand Island, NY).

2.7 Luciferase activity assay

The amount of active firefly luciferase was determined by ONE-GLO™ Luciferase Assay System (Promega), in a white 96-well plate. Five μL of CFPS sample was added to 30 μL of Luciferase Assay Buffer. Luminescence was read every 2 min over a 20 min period using a BioTek Synergy 2 plate reader (Winooski, VT).

2.8 Chloramphenicol acetyl transferase assay

Active CAT was measured as previously described [30].

3 Results

3.1 Optimization of cap-independent translation

To evaluate cap-independent translation initiation, we carried out 15 μL batch cell-free translation only reactions for 1 h at 24°C. These reactions were charged with 0.3 pmol purified in vitro transcribed luciferase mRNA having a 90-mer poly(A) tail. Initially, we tested three yeast native IRES sequences, the 5'-UTR of TFIID, HAP270, and YAP1 genes [27], as well as the 5'-UTR of the gene TIF4631, the yeast homolog of the mammalian translation initiation factor eIF4G (the mRNA is called p150) [31, 32]. As a control, non-capped mRNAs harboring these different cap-independent translation leader sequences placed upstream of luciferase gene were compared to capped mRNA. Compared to capped luciferase mRNA, 5'-UTRs of HAP4 and TFIID showed low activities, while YAP1 and p150 did not direct translation (Table 1). We next considered non-native, viral cap-independent sequences that had been successfully applied to eukaryotic CFPS as well as an artificial species-independent CFPS [7, 21, 22, 24]. The Ω sequence showed surprisingly high activity among all tested cap-independent translation sequences, outperforming the capped mRNA by almost

two-fold (Table 1). As the next best leader sequence, the polyhedrin 5'-UTR sequence was $\sim 17\%$ as efficient in initiating translation as capped mRNA (Table 1). The species-independent translational mRNA sequence A64pA90 showed a low efficiency of translation initiation. Finally, we looked at the intergenic region (IGR) IRES from CrPV, which initiates translation in yeast cells without initiation factors [29]. This was further selected because Kubick and colleagues recently demonstrated its use for combined Tx/Tl in CHO cell based CFPS [8, 16]. Unfortunately, when compared to capped mRNA and mRNA harboring the Ω leader, the CrPV IRES showed little activity in our assay (Table 1). Based on our cell-free translation results, we selected to move forward with the Ω leader sequence for initiating combined Tx/Tl.

3.2 Optimization of physiological solutes in combined transcription-translation

We next sought to demonstrate, and improve, combined Tx/Tl from plasmid vectors equipped with the Ω leader sequence. Specifically, we carried out a series of optimization experiments to explore the effect of temperature, DTT concentration, plasmid concentration, magnesium concentration, and NTP concentrations on batch Tx/Tl reactions. Excepting temperature, these variables were selected because they were newly required for our combined Tx/Tl system, as opposed to the translation only reactions described above. Notably, these variables are also interdependent, as has been observed before in the development of crude extract based CFPS systems [33]. Here, we discuss and show trends for the aforementioned optimizations with only a single variable deviating from the finalized solute concentrations reported in Section 2.

Figure 2A shows active luciferase yield throughout the duration of a batch combined Tx/Tl reaction, monitored by samples taken at 0, 0.5, 1, 1.5, and 2 h. We observed that the synthesis of luciferase shows slight lag during the first 0.5 h and then progresses linearly from 0.5 to 1.5 h, with reaction termination occurring by 2 h. The lag in the first 30 min is possibly associated to a delay resulting from combining Tx/Tl, and has been previously observed [15]. The temperature optimum was observed to be approximately 24°C (Fig. 2B). The combined Tx/Tl system was insensitive to template concentration above 3.2 nM, reaching saturation by ~ 5.3 nM (Fig. 2C). The system was also insensitive to DTT concentration over a range of 2–7 mM (Fig. 2D), suggesting that the S60 extract has a comparatively low potential of oxidation. This is important because the T7 RNA polymerase used to drive transcription requires a reducing environment for maximal activity.

The most interesting data came from the magnesium and NTP concentration optimization. The four nucleoside triphosphates play a role in both Tx/Tl, yet CTP and UTP were not present in our initial translation only reactions.

Table 1. The efficiency of cap-independent and IRES-mediated yeast cell-free translation as compared to capped mRNA^{a)}

Template	Efficiency (%)
CappA90	100 \pm 20
Ω pA90	187 \pm 13
HedrinpA90	17 \pm 4
A64pA90	1.5 \pm 0.2
IGRpA90	<1 \pm 0.02
TFIIDpA90	<1 \pm 0.07
YAP1pA90	<1 \pm 0.03
p150pA90	<1 \pm 0.08
N5UpA90	2 \pm 0.3

a) Cap, capped message. All other abbreviations described in the text. All templates have a poly(A) tail of 90 nucleotides (pA90) and are normalized to capped message.

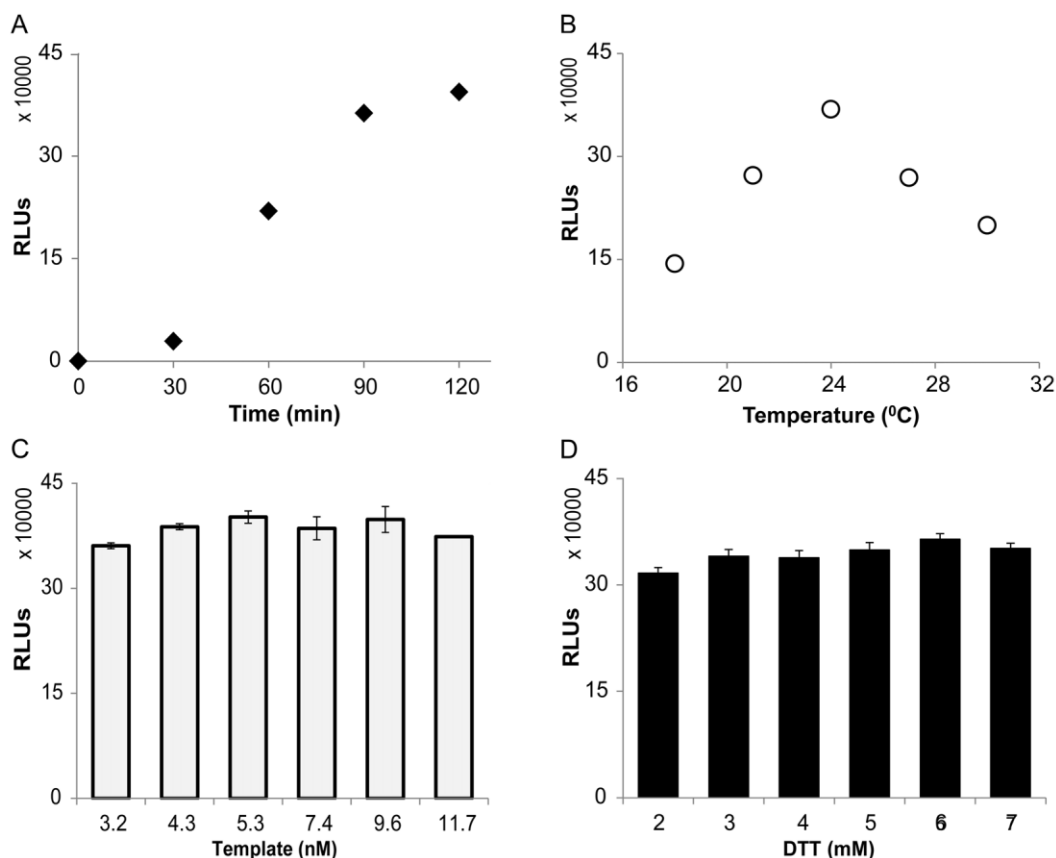


Figure 2. Combined transcription and translation is activated in yeast CFPS from the Ω leader sequence. (A) Time course. Active luciferase synthesis is shown over the course of a standard batch reaction. (B–D) CFPS reaction optimization. The physicochemical environment of the CFPS reaction was optimized by altering (B) temperature, (C) DNA template amount, and (D) DTT concentration. Values show means with error bars representing standard deviations (SD) of at least three independent experiments. Luciferase data are presented in RLUs, or relative light units.

Since ATP is maintained through the creatine phosphate secondary energy system [15], the concentration of the other three types of nucleoside triphosphates, GTP, UTP, and CTP (abbreviated GUC) [34] were adjusted. Importantly, this required a change in magnesium concentration. It is well known that NTP concentration has a strong buffering effect on magnesium concentration and that optimal magnesium concentration is necessary for highly active CFPS [14]. Thus, not unexpectedly, increasing the total nucleotide pool from 1.7 mM total (ATP, GTP only) to 7.5 mM total (ATP, GTP, CTP, UTP) when shifting from cell-free translation only reactions (our initial experiments described above) to combined Tx/Tl reactions, also required higher concentrations of magnesium (an increase of 1 to 6 mM). We simultaneously explored the optimal concentration of GUC with different magnesium concentrations (Table 2). The maximum protein synthesis yield occurred when using 12 mM magnesium and 3.5 mM GUC. Notably, the highest yielding samples occurred along the diagonal of Table 2, indicating that unbalanced concentrations of magnesium and GUC significantly reduced the protein yield. With an eye towards ultimate-

ly developing a cost-effective CFPS system, the ~25% increase in yield with 150% additional NTPs was insufficient motivation to keep the higher NTP concentrations, given the cost increase. As compared to cell-free translation alone, the newly designed combined Tx/Tl system improved overall protein synthesis yields more than two-fold (up to 7 $\mu\text{g mL}^{-1}$). More importantly, it eliminated inconsistency issues with the capping reaction, a known issue with eukaryotic CFPS [35] and further removed the dependence of the reaction on the costly and potentially inhibitory $m^7\text{GpppG}$ RNA cap structure analog.

3.3 Optimization of functional elements of the DNA template

With our new combined Tx/Tl system at hand, we next asked whether or not luciferase synthesis yields could be improved by modifying the expression template. Thus, we explored additional 5'-UTR sequences, the length of the poly(A) tail, and 3'-UTR sequences (Fig. 3). First, we tested 5'-UTRs from TEV and Crucifer-infecting tobamovirus (Tbm), which have shown high activity in

Table 2. Optimization of [Mg]–[GUC]^{a)}

GUC (mM/each)	Percent yield (%)				
	6	8	10	12	14
2	100 ± 13.5	120 ± 0.5	105 ± 4.4	81 ± 10.3	55 ± 0.1
3	48 ± 5.8	121 ± 5.8	126 ± 8.2	100 ± 1.1	82 ± 4.4
3.5	3 ± 0.0	93 ± 2.1	119 ± 15.8	128 ± 3.1	102 ± 4.5
4	0 ± 0.0	49 ± 6.3	92 ± 31.2	115 ± 22.4	110 ± 4.5

a) All reactions were performed using standard combined cell-free transcription-translation conditions except magnesium glutamate (Mg) and GTP/UTP/CTP (GUC) concentrations were varied. The luminescence value of standard reaction (6 mM Mg, 2 mM/each GUC) was counted as 100 (bold and italic value). The luminescence values of all other samples were represented as the ratio to standard reaction. All values are the average of three individual reactions with standard deviations shown. The concentrations of Mg do not include Mg from S60 extract (see Section 2). The values (bold font) highlight the comparatively high-yield samples among different concentrations of Mg and GUC.

translation initiation [34, 36, 37]. TEV 5'-UTR showed ~5% lower activity than that of Ω sequence; the activity of Tbm 5'-UTR is half of Ω sequence (Fig. 3A). The length of poly(A) tail was also optimized; 50 and 170 nt showed similar activities, while those of 90 and 25 nt showed 1.5- to 2-fold decrease (Fig. 3B). Notably, the poly(A) tail is essential for yeast CFPS. Without the poly(A) tail, luciferase

synthesis is decreased to 8.3% of the complete template (Fig. 3C). These data are consistent with Sarnow's previous reports, but contrast to WGE based CFPS [27, 38]. After deletion of Ω , the protein yield decreased to 1.7%, with the protein yield decreased to 0.1% when missing both Ω and poly(A)90 tail (Fig. 3C). Finally, we also investigated the contribution of various 3'-UTRs in combined

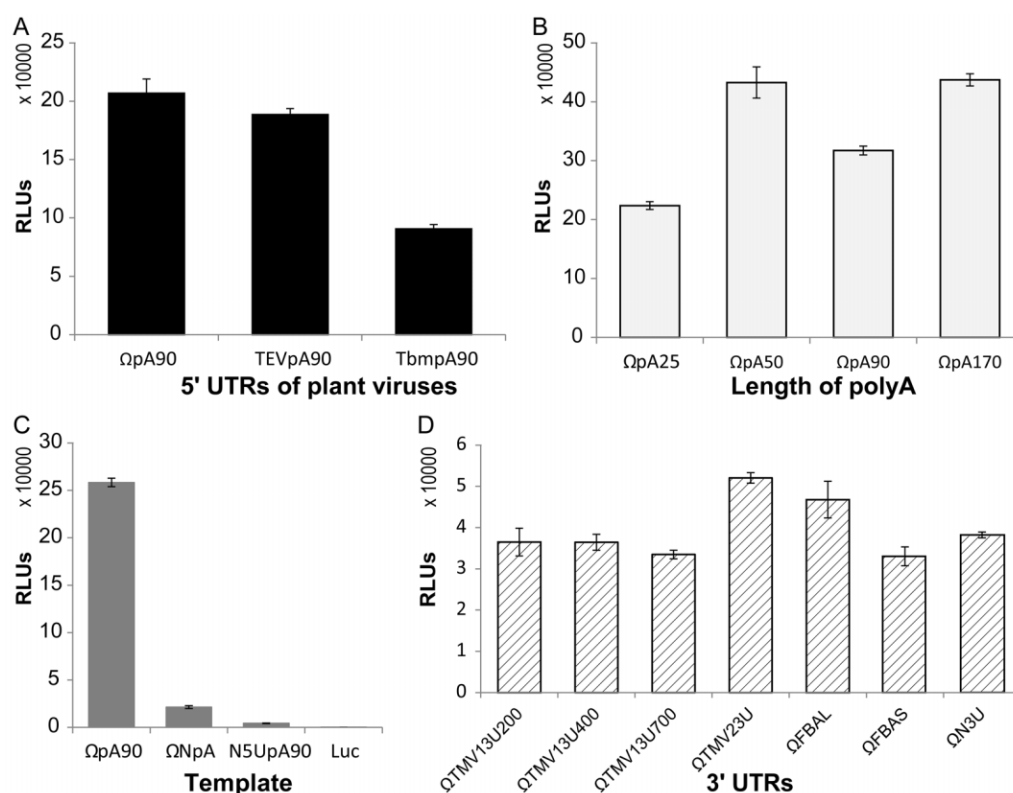


Figure 3. Optimization of functional elements of the DNA template for combined transcription and translation. (A) Three 5'-UTRs from tobacco mosaic virus (Ω), tobacco etch virus (TEV), and tobamovirus (Tbm) were tested for the ability to enable translation initiation in combined yeast CFPS. (B) Different length of Poly(A) tails, 25, 50, 90, and 170 nt were tested for the ability to enable translation initiation in combined yeast CFPS. (C) The effect of 5' Ω sequence and 3' poly(A) tail in cap-independent translation initiation of yeast CFPS demonstrates that both the leader sequence and poly(A) tail are required for efficient translation with Ω . The structures of the expression templates are shown in Figure 1. (D) The effect of various 3'-UTRs in combined yeast CFPS. Values show means with error bars representing standard deviations (SD) of at least three independent experiments. Luciferase data are presented in RLUs, or relative light units.

Tx/Tl, as a few are also found in the 3'-UTRs and intergenic regions important for IRES function [17]. As compared to the no 3'-UTR control, the protein yields of various 3'-UTRs changed slightly (86–136%) (Fig. 3D). Notably, the overall system performance decreases in these experiments because the templates shown in Fig. 3D do not have poly(A) tails. Our results suggest that 3'-UTRs in yeast CFPS have less functional importance than in wheat germ CFPS.

3.4 Rapid and robust preparation of linear DNA templates for high-throughput yeast CFPS

Having demonstrated improved combined Tx/Tl as a result of the shorter poly(A) tail, we then set out to demonstrate the potential for using linear DNA templates for yeast CFPS. High-throughput protein expression has become a key technology for systems and synthetic biology [1, 3, 39, 40]. Using linear DNA molecules, i.e. PCR products, expedites the process since there are no laborious cloning steps. We therefore developed a two-step PCR method that enabled direct synthesis of linear DNA templates for yeast CFPS (Fig. 4). In the first step, a target region within the coding sequence is amplified using two gene-specific forward and reverse primers, which contain linker sequences and the terminal sequences of the target region (see linkers designated by different shapes in Fig. 4). The second PCR step appends the first PCR product with the N- and C-terminal double-stranded fragments. The 5' fragment comprises the linker sequence, the Ω leader sequence for the initiation, and the T7 promoter for transcription initiation. The 3' fragment comprises the linker sequence and the poly(A)50 tail. Following construction, the entire linear template can be amplified using a universal T7 primer. After construction of linear templates, we carried out CFPS using three different

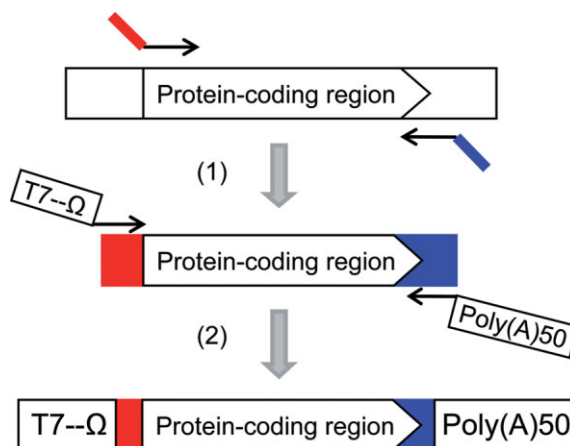


Figure 4. A robust and rapid two-step method for assembly of linear DNA templates for high-throughput protein expression in yeast CFPS. First, protein-coding sequences can be amplified directly from plasmid, genome, cDNA, or other genetic material using gene-specific primers with universal tail sequence. Second, the T7 promoter, Ω sequence, and poly(A)50 are added to protein-coding region by overlapping PCR to the universal tail sequences. Third, assembled linear PCR product can be used directly as template in combined Tx/Tl yeast CFPS.

DNA templates: (i) PCR product amplified directly from plasmid; (ii) assembled linear DNA template produced by the two-step overlap PCR procedure described above; and (iii) circular plasmid. We observed that all three DNA templates can successfully synthesize luciferase, but surprisingly, the linear DNA templates performed approximately 40–60% better than the plasmid (Fig. 5A). To demonstrate utility of our approach, two other proteins, GFP and CAT, were also expressed using DNA templates assembled by our two-step PCR method. By comparison to commercial standard proteins, the yields of active protein are estimated as ~ 7 to $12.5 \mu\text{g mL}^{-1}$ (Table 3). The solubility of

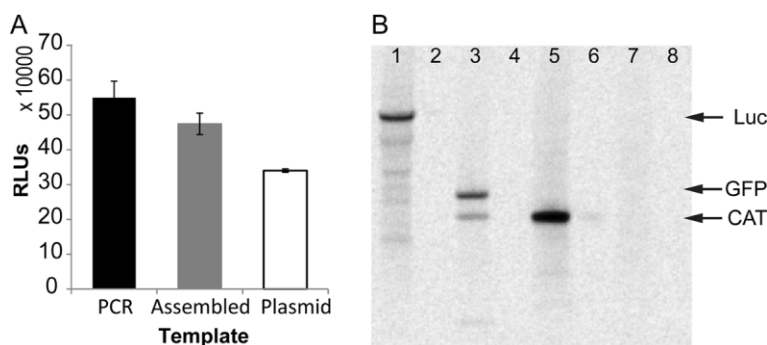


Figure 5. Linear expression templates activate high-level yeast combined transcription and translation. **(A)** Comparison of the efficiency in CFPS of PCR product amplified directly from plasmid, assembled linear DNA template, and plasmid. The concentration of template is 5.3 nM for each sample. These data show that linear templates outperform plasmid DNA. Values show means with error bars representing standard deviations (SD) of at least four independent experiments. Luciferase data are presented in RLUs, or relative light units. **(B)** CFPS of luciferase (Luc), green fluorescence protein (GFP), and chloramphenicol acetyl transferase (CAT) using assembled, linear DNA templates. The [^{35}S]-methionine-labeled products were analyzed in SDS-PAGE and autoradiography. Lane 1 and 2: soluble and insoluble fraction of Luc; Lane 3 and 4: soluble and insoluble fraction of GFP; Lane 5 and 6: soluble and insoluble fraction of CAT; Lane 7 and 8: soluble and insoluble fraction of negative control reaction without template. Notably, nearly the entire product for each protein is soluble. The gel was repeated twice.

Table 3. Yield of active proteins from yeast CFPS programmed with linear DNA templates

Protein	Yield ($\mu\text{g mL}^{-1}$ active protein)
Luciferase	7 ± 2
GFP	12.5 ± 2.5
CAT	10 ± 1

each protein is also demonstrated in [^{35}S]-methionine autoradiography (Fig. 5B). Notably, more than 95% of the total protein produced was soluble in all cases. Overall, our high-throughput combined Tx/Tl method enables researchers to go from DNA sequence to protein in under 6 h.

4 Discussion

While used for decades as a foundational research tool for understanding Tx/Tl, recent advances in CFPS have paved the way to exciting new applications [1]. For example, CFPS synthesis platforms are emerging as a foundational technology platform for the high-throughput synthesis of protein libraries for functional genomics [1]. Here, we developed the framework for combined Tx/Tl in yeast that is suitable for such efforts.

First, we identified cap-independent translation sequences that activated combined Tx/Tl in yeast extracts. Compared with capped mRNA, our combined Tx/Tl system not only enhances yield, but also lowers cost [14]. We observed that the Ω sequence from TMV, which is able to enhance translation of genes in plant tissue in vivo and in vitro [20], was the most efficient translation leader sequence. While Endo's group has utilized the Ω sequence to initiate translation in WGE quite successfully [21, 38], it was surprising that the Ω sequence also showed extremely high efficiency of translation initiation in yeast. Moreover, we found that the Ω sequence works differently in yeast CFPS as compared to wheat germ CFPS. In WGE, the Ω sequence initiates translation independently; the poly(A) tail does not significantly contribute to translation initiation while the length of 3'-UTR influences the translation efficiency. In yeast CFPS, however, the presence of poly(A) tail (50–170 nt) enhances translation efficiency significantly. Our observations are consistent with a previous report [27], but the length of 3'-UTR has no obvious effects on translation efficiency. Various mechanisms could explain how the poly(A) enhances CFPS. For example, the poly(A) tail can bind to poly(A)-binding protein (PABP) that cooperates with eIF4F to recruit ribosomes. Alternatively, longer poly(A) tails can resist 3'-end decay of mRNA. While further work is necessary to decipher the mechanisms at play in our system, it is clear that the optimal length of poly(A) tail is likely impacted by a number of factors [41, 42].

Second, after optimizing the physiological solutes (e.g. NTP concentrations) and expression template elements (e.g. poly(A) tail length), we then developed a robust PCR method for high-throughput CFPS. In our method, the yeast extract can be prepared in 2 days including cell cultivation using regular laboratory equipment. Linear DNA templates can be used directly and prepared by two-step PCR from a small amount of sample. Strikingly, we show that linear DNA templates outperform plasmids, obtaining a batch yield of approximately 7–12.5 $\mu\text{g mL}^{-1}$ active protein for several model targets, which is on par with several other commercially available eukaryotic CFPS systems (e.g. rabbit reticulocyte). Thus, tens to thousands of different templates can be handled within 1–2 days. The advantage of our approach is that it avoids labor-consuming cloning work.

In future work, we will seek to improve batch yeast CFPS yields from linear DNA templates. Recently, we have shown that yeast CFPS is limited by a cascade of events resulting from loss of available energy, as well as the accumulation of inhibitory small molecules [15]. Thus, the focus of continued improvements will center on both; (i) enabling long-lived homeostatic energy regeneration; and (ii) generating source strains that upon lysis lead to improved performance. Strain engineering approaches, which were critical in the development of the *E. coli*-based extract platform development [43, 44], will be able to leverage the facile genetic tools that exist for yeast, as well as a wealth of biochemical knowledge in genetic regulation and metabolic pathways.

In summary, continued development in CFPS systems is expected to have a tremendous impact on a range of applications for simple, robust protein production. Recent advances have demonstrated the value of CFPS in the production of proteins with site-specifically incorporated non-standard amino acids [45–47] as well as in microscale to manufacturing scale synthesis of human therapeutics [48]. The demonstration of using linear DNA templates for rapid CFPS positions yeast CFPS as a powerful complement to already established eukaryotic CFPS systems, having utility for high-throughput protein expression and synthetic biology studies.

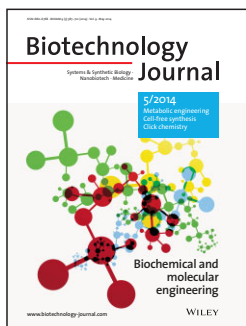
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