

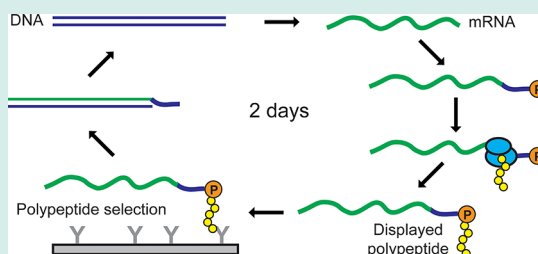
Streamlined Protocol for mRNA Display

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ABSTRACT: mRNA display is a powerful method for in vitro directed evolution of polypeptides, but its time-consuming, technically demanding nature has hindered its widespread use. We present a streamlined protocol in which lengthy mRNA purification steps are replaced with faster precipitation and ultrafiltration alternatives; additionally, other purification steps are entirely eliminated by using a reconstituted translation system and by performing reverse transcription after selection, which also protects input polypeptides from thermal denaturation. We tested this procedure by performing affinity selection against Her2 using binary libraries containing a nonspecific designed ankyrin repeat protein (DARPin) doped with a Her2-binding DARPin (dopant fraction ranging from 1:10 to 1:10 000). The Her2-binding DARPin was recovered in all cases, with an enrichment factor of up to 2 orders of magnitude per selection round. The time required for 1 round is reduced from ~4–7 days to 2 days with our protocol, thus simplifying and accelerating mRNA display experiments.

KEYWORDS: mRNA display, directed evolution, purification, in vitro translation, binary library



In vitro directed evolution techniques, such as ribosome display¹ and mRNA display,² are powerful tools for protein engineering, capable of handling libraries containing up to $\sim 10^{14}$ members. Ribosome display involves in vitro translation of mRNA sequences lacking stop codons to generate ternary complexes in which each mRNA and its corresponding protein are noncovalently associated via a stalled ribosome. This requires that any selections carried out with these ternary complexes be performed under conditions that maintain the integrity of the ribosome.³ By contrast, mRNA display uses an mRNA-DNA-puromycin fusion as its template for in vitro translation, which results in a covalent puromycin linkage between the mRNA and the corresponding nascent peptide.⁴ This creates a highly stable selection particle, which is particularly useful for performing in vitro selections in harsh environments that are not compatible with ribosome display. mRNA display has previously been used for the investigation of protein-protein interactions⁵ and the development of peptides,⁶ enzymes,⁷ scFvs,⁸ and novel binders based on alternative scaffolds.⁹ Despite the potential utility of mRNA display in a plethora of protein engineering applications, the technically demanding nature of this method has precluded its widespread use. We therefore sought to simplify and streamline the mRNA display procedure to make it more broadly accessible to the scientific community.

A traditional mRNA display schematic is given in Figure 1,^{2,4,10} with our modifications highlighted in red. First, we redesigned the DNA construct with a different 5' end (Figure 1a). The 5' untranslated region (5' UTR), taken from the modified ribosome display vector pRDV2,¹¹ contains a T7 promoter and a Shine-Dalgarno ribosome binding site (RBS). This 5' UTR drives strong transcription by T7 RNA

polymerase, as well as efficient translation by *Escherichia coli* ribosomes, and has been shown to work in both crude extract¹ and a cell-free reconstituted system.¹² Since the minimal translation system used in this work (discussed below) contains reconstituted components from the *E. coli* translational machinery, it is necessary to replace the tobacco mosaic virus translational enhancer commonly used with the eukaryotic rabbit reticulocyte lysate in mRNA display with the Shine-Dalgarno RBS to allow efficient translation with the prokaryotic *E. coli* components.^{13,14}

After in vitro transcription, mRNA is traditionally purified using denaturing PAGE (Figure 1b).^{2,4} While effective at separating full-length RNA transcripts from truncated RNA and PCR primers, this method requires lengthy steps and manual excision from the gel. Instead, we use lithium chloride (LiCl) precipitation to isolate transcribed mRNA, a technique that has been used to purify mRNA prior to in vitro translation.³ LiCl selectively precipitates RNA from unwanted DNA,¹⁵ smaller RNA, unincorporated NTPs, and proteins,¹⁶ thus enabling rapid purification while minimizing the number of processing steps during which detrimental nucleases and proteases could be introduced.

We have also replaced another PAGE purification step after ligating mRNA to the DNA-puromycin linker (Figure 1c). Instead, we utilize the repertoire of commercially available ultrafiltration devices to quickly purify the mRNA-DNA-puromycin molecules from the DNA splint and unligated DNA-puromycin linkers, which can interfere with the

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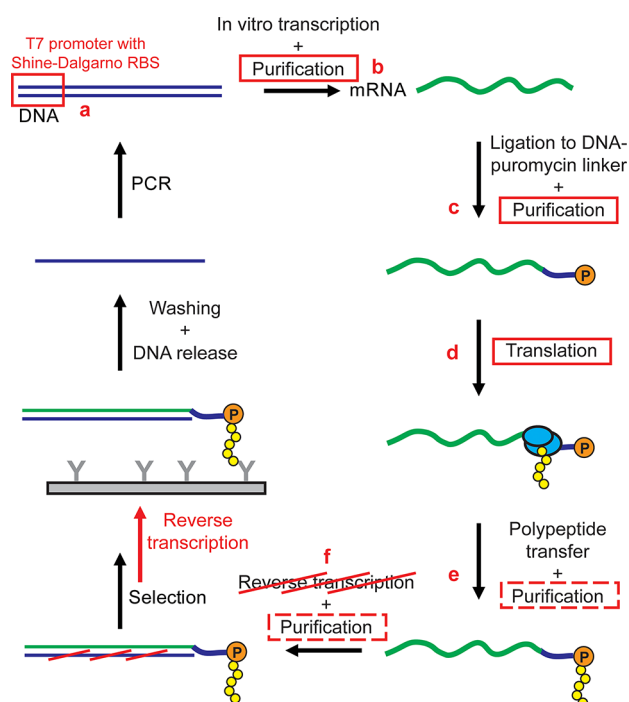


Figure 1. Changes in the streamlined mRNA display protocol. A traditional mRNA display protocol is shown,^{2,4,10} with our modifications highlighted in red: (a) 5' UTR with T7 promoter and Shine-Dalgarno RBS; (b) mRNA purification with LiCl precipitation instead of lengthy PAGE purification; (c) mRNA-DNA-puromycin purification with ultrafiltration instead of lengthy PAGE purification; (d) in vitro translation with minimal, reconstituted translation system (PURExpress) instead of crude lysate to achieve higher library diversity with less nuclease and protease activity, which, in turn, can (e) eliminate need for purification of selection particles; (f) postpone reverse transcription, thus eliminating an additional purification step as well as avoiding a high-temperature incubation step of the displayed polypeptide prior to selection.

subsequent translation reaction if not removed.⁴ Although ultrafiltration cannot separate mRNA-DNA-puromycin molecules from unligated mRNA molecules, this limitation is also encountered in PAGE purification with templates longer than 500 nucleotides.⁴ Furthermore, any mRNA molecules lacking puromycin will not be able to covalently attach to their corresponding polypeptide sequences and therefore will not be erroneously enriched during the selection step. We have successfully purified the desired product using Amicon Ultra-0.5 mL (Millipore) or Vivaspin 500 (Sartorius) ultrafiltration columns, which are available in a range of molecular weight cutoffs (30–300 kDa) that enable their use across a broad range of construct sizes. In the current proof-of-principle studies, a 100 kDa ultrafiltration device can effectively remove the splint and unligated linkers (9.5 kDa), while retaining the mRNA-DNA-puromycin molecules (170–210 kDa) with a yield of 85–90%.

In vitro translation reactions for mRNA display and ribosome display have typically relied on crude cell lysates for the necessary translational machinery. However, cell lysates are not ideal for reproducibly achieving high-complexity libraries because they contain endogenous RNases and proteases and time-intensive optimization procedures must be applied to correct for the inherent batch-to-batch variability. These limitations can be overcome by using a minimal *E. coli*-based translation system reconstituted from purified components

(PURExpress, New England Biolabs) (Figure 1d). The fully defined PURExpress system offers better reproducibility, lack of nucleases and proteases, and a high concentration of ribosomes. These characteristics maximize the number of intact selection particles, increasing the likelihood of a successful selection. The resulting mRNA display particles can be used directly in selection experiments without additional purification steps (Figure 1e).

Selection is carried out after translation and followed by reverse transcription (RT). We delay RT until after selection because the displayed proteins may be adversely affected by the elevated temperatures required during the RT process, including initial thermal denaturation of the RNA template and high-temperature incubation with the reverse transcriptase (Figure 1f). Postponing RT also eliminates an additional purification step, as RT products can be used directly in subsequent PCR amplification but require a gel filtration step if RT is done prior to selection, further streamlining the procedure. However, since it is possible that delaying the RT step may allow for enrichment of RNA aptamers in addition to polypeptide binders in some affinity selections, an additional selection round can be performed with RT prior to selection to specifically isolate the polypeptide binders. Since the polypeptide binders should be substantially enriched at this point, the adverse effects of RT can be better tolerated than in initial rounds in which binders are scarce. Alternatively, DNA primers that are complementary to conserved regions of the mRNA can be added prior to selection to destabilize any RNA aptamers in the pool.

Finally, we performed a proof-of-principle selection to test our new streamlined mRNA display procedure. Two designed ankyrin repeat proteins (DARPin), Off7,¹⁷ a maltose-binding protein (MBP) binder, and H10-2-G3,¹⁸ a Her2 binder, were assembled in mRNA display format and their mRNAs were combined at 4 different molar ratios (1:10 to 1:10 000 H10-2-G3:Off7). These binary libraries were subjected to selection against immobilized Her2. After 2–3 rounds of our streamlined mRNA display protocol, the Her2-binding DARPin was selectively enriched, as evidenced by agarose gel electrophoresis visualization of the RT-PCR products (Figure 2). Since RT-

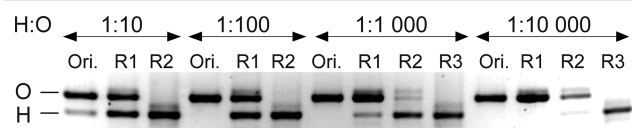


Figure 2. Model DARPin selections using our streamlined mRNA display protocol. Four binary libraries containing mRNAs encoding H10-2-G3 (H = a Her2 binder) and Off7 (O = an MBP binder) at different molar ratios (1:10, 1:100, 1:1 000, and 1:10 000) were subjected to 2–3 rounds of selections against immobilized Her2. The original library (Ori.) and selective enrichment of H10-2-G3 in the RT-PCR product after each round of mRNA display (R1, R2, and R3) is visualized by agarose gel electrophoresis. Each selection round took 2 days in the laboratory.

PCR of the original 1:100 mRNA mixture shows no discernible signal for H10-2-G3 but the original 1:10 mixture shows a faint yet clear lower band, we infer that the enrichment factor in these selection experiments is as high as 2 orders of magnitude. On the basis of the gel, we estimate an average enrichment of approximately 50- to 100-fold per selection cycle.

In summary, we have developed a simplified, streamlined protocol for mRNA display that reduces the time for a single

Table 1. Step-by-Step Comparison of 2 Traditional mRNA Display Procedures and Our Proposed Streamlined Method

Keefe procedure ^a	Cotton et al. procedure ^b	proposed procedure
in vitro transcription (3 h)	in vitro transcription (7 h to overnight)	in vitro transcription (3 h)
denaturing PAGE purification of mRNA (overnight + 3 h)	DNase digestion to remove cDNA from mRNA (3.5 h)	LiCl-based purification of mRNA ^{c,d} (2.5 h)
phosphorylation of DNA-puromycin linker (2.5 h)	conjugation of mRNA with DNA-puromycin linker (1 h)	phosphorylation of DNA-puromycin linker (2.5 h)
splinted ligation of DNA-puromycin linker and mRNA (1 h)		splinted ligation of DNA-puromycin linker and mRNA (1 h)
denaturing PAGE purification of ligated mRNA (overnight + 3 h)	LiCl-based purification of ligated mRNA (4 h)	ultrafiltration of ligated mRNA ^e (1.5 h)
in vitro translation with rabbit reticulocyte lysate (1.5 h)	in vitro translation with rabbit reticulocyte lysate (1 day)	in vitro translation with PURExpress system (1 h)
oligo(dT) purification (1.5 h)	oligo(dT) purification (3 h)	
Ni-NTA purification (3 h)	reverse transcription (RT) (1 h)	
reverse transcription (RT) (1 h)	buffer exchange after RT (0.5 h)	
buffer exchange after RT (0.5 h)	anti-FLAG purification (4.5 h)	
selection (varies)	selection (varies)	selection (varies)
		reverse transcription (RT) (1.5 h)
PCR amplification (3 h)	PCR amplification (3 h)	PCR amplification ^f (3 h)
~4 days	~7 days	~2 days

^aRef 4. ^bRef 10. ^cAn RNA purification kit could be used to further shorten the time required for this step, but the LiCl precipitation procedure is significantly cheaper and also lends greater flexibility to the protocol by providing an additional potential stopping point that does not unnecessarily introduce an extra freeze-thaw cycle for the mRNA. ^dPotential stopping point: storage of mRNA at $-20\text{ }^{\circ}\text{C}$ at an intermediate step during LiCl-based purification. ^ePotential stopping point: storage at $-80\text{ }^{\circ}\text{C}$ after ultrafiltration of ligated mRNA. ^fPotential stopping point: storage of PCR product at $4\text{ }^{\circ}\text{C}$ or $-20\text{ }^{\circ}\text{C}$ after PCR amplification.

selection round from ~4–7 days to 2 days (Table 1). In addition to a number of simplifications in purification steps, the procedure postpones the RT step until after selection, which has the extra benefit of protecting displayed polypeptides from thermal denaturation. Wherever possible, complex or poorly regulated steps in the procedure have been replaced with simple, robust alternatives. Importantly, in applying these procedural simplifications, we have not compromised the achievable library size ($\sim 10^{14}$) or the enrichment capability of mRNA display. Furthermore, any of our streamlined steps can be readily reverted to the older procedures if there are additional constraints specific to a given experiment. Whether the entire protocol or only selected individual steps are adopted, this new protocol should enable faster and easier in vitro directed evolution experiments.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were purchased from Integrated DNA Technologies (IDT; Coralville, IA) unless otherwise specified. DNA purification was performed using agarose gel electrophoresis with SYBR Safe (Invitrogen, Carlsbad, CA) and the QIAquick gel extraction kit (Qiagen, Valencia, CA). Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase (used for all ligations), and Phusion DNA polymerase (used for all PCRs) were purchased from New England Biolabs (Ipswich, MA) and used as recommended by the manufacturer. DNA and RNA concentrations were determined by absorbance readings at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA).

Plasmid Construction. To create DNA constructs suitable for in vitro transcription, the genes encoding H10-2-G3 and Off7 were first cloned into pRDV2.¹¹ The cDNA encoding H10-2-G3¹⁸ was synthesized by IDT and PCR amplified with H10_BamHI_f (5'-GAC AAA GGA TCC GAC CTG GGT AAA AAA CTG CTG-3') and H10_HindIII_r (5'-AGA CCC AAG CTT TTG CAG GAT TTC AGC CAG-3'). The

resulting product was digested with *Bam*HI and *Hind*III, and ligated into similarly digested pRDV2.¹¹ The ligation mixture was transformed into chemically competent *E. coli* XL1-Blue.¹⁹ Individual colonies were selected for standard liquid culturing and plasmid extraction procedures using a Qiagen miniprep kit. The purified plasmid, pRDV2-H10-2-G3, was sequence verified. The cDNA encoding Off7¹⁷ was modified with silent mutations to eliminate repeating nucleotides in order to minimize nonspecific amplification products in PCR. This gene was synthesized by IDT and PCR amplified with pRDVFLAG_BamHI_f (5'-GAT GAC GAT GAC AAA GGA TCC-3') and pRDV_BbsI_r (5'-GGC CAC CGG AAG ACC CAA GC-3'). The resulting product was *Bam*HI/*Hind*III digested and ligated into pRDV2 as described above to obtain pRDV2-Off7, which was also purified by miniprep and sequence verified.

In Vitro Transcription. Primers T7_no_BsaI (5'-ATA CGA AAT TAA TAC GAC TCA CTA TAG GGA CAC CAC AAC GG-3') and pRDV_BbsI_r were used to amplify plasmids pRDV2-H10-2-G3 and pRDV2-Off7. The products were gel purified and used directly for transcription, as described previously.²⁰

Ligation of mRNA to DNA-Puromycin Linker. A DNA-puromycin linker⁴ (5'-AAA AAA AAA AAA AAA AAA AAA AAA AAA CC-3'-puromycin) was purchased from Biosearch Technologies (Novato, CA) and an oligonucleotide splint (GS_splint, 5'-TTT TTT TTT TGG CCA CCG GAA-3') was also used. The DNA-puromycin linker was phosphorylated according to Keefe et al.⁴ and desalted using Illustra ProbeQuant G-50 Micro Columns (GE Healthcare, Piscataway, NJ). The ligation was adapted from Keefe et al.⁴ to include: 1 nmol mRNA, 1 nmol 5' phosphate DNA linker, 1 nmol GS_splint, water up to 86 μL , and 2 μL RNasin Plus RNase inhibitor (Promega, Madison, WI). These components were heated at $95\text{ }^{\circ}\text{C}$ for 2 min. Then, 10 μL 10 \times T4 DNA ligase buffer was added. The reaction was vortexed and then cooled on ice for 10 min. The tubes were removed from ice for 5 min,

at which time 4 μL high-concentration T4 DNA ligase (2000 U/ μL) was added. The reaction was incubated at room temperature for 50 min, and then 400 μL 7 M urea was added and the tube was lightly vortexed.

Purification of mRNA-DNA-Puromycin. Each ligation reaction was loaded onto a 100 kDa cutoff Amicon Ultra-0.5 mL centrifugal filter unit (Millipore, Billerica, MA) and ultrafiltration was performed at 2000g for 15 min. After addition of 7 M urea (200 μL), the device was centrifuged again. RNase-free water (500 μL) was then added, followed by an additional spin. This washing step with water was repeated twice more to remove essentially all of the urea.

PURExpress Reaction. The PURExpress in vitro protein synthesis kit (New England Biolabs) was used for translation reactions. The master mix included 10 μL Solution A, 7.5 μL Solution B, 0.5 μL RNasin Plus, and 5 μL RNase-free water. The master mix was then split into 4 reaction tubes with 5.5 μL each, and 60 pmol total mRNA was added to each reaction (ribosome content of PURExpress is 15 pmol per reaction). In the first round, the input in the 4 reactions contained the mRNAs encoding H10-2-G3 and Off7 in molar ratios of 1:10, 1:100, 1:1 000, and 1:10 000, with the mRNA encoding Off7 in molar excess in all cases. In each subsequent round, the input DNA from the previous round was transcribed and \sim 60 pmol total mRNA was added to each translation reaction. Translation was performed for 30 min at 37 $^{\circ}\text{C}$ and then the reaction was kept at room temperature for another 10 min. In the first round, 1 μL of this translation reaction was saved and kept on ice for direct RT-PCR. Then, 100 μL ice-cold TBSC [TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl)³ with 0.5% casein (Sigma-Aldrich, St. Louis, MO)] was added to the remaining translation reaction and the solution was kept on ice.

Affinity Selection for Her2. Affinity selection for Her2 was performed on NUNC Maxisorp plates (Thermo Fisher Scientific, Rochester, NY). Each well was coated with 1.4×10^{13} Her2 receptors (Sino Biological, Beijing, China) in 100 μL TBS for at least 16 h at 4 $^{\circ}\text{C}$, washed 3 times with 300 μL TBS, blocked with casein (300 μL TBSC added) at room temperature for 1 h with shaking, and then washed 3 times with TBS and once with TBSC. The translation reaction in TBSC was added to each well and incubated for 1 h at 4 $^{\circ}\text{C}$ with shaking to allow binding. The wells were subjected to 3 quick washes with TBSC and 3 additional 5-min washes prior to reverse transcription.

Reverse Transcription. Reverse transcription was performed in situ using AffinityScript reverse transcriptase (Agilent Technologies, Santa Clara, CA) and reverse primer pRDV_BbsI_r. A reverse transcription protocol adapted from He and Taussig²¹ was followed. Briefly, Solution 1 (12.75 μL water, 0.25 μL 100 μM pRDV_BbsI_rev, and 0.5 μL RNasin Plus) was pipetted into the wells (or into 1 μL saved translation reaction in the first round), incubated at 70 $^{\circ}\text{C}$ for 5 min, and allowed to cool at room temperature for 10 min. Solution 2 [2 μL 10 \times AffinityScript buffer, 2 μL 0.1 M dithiothreitol, 2 μL dNTPs (5 mM each), and 0.5 μL AffinityScript reverse transcriptase] was then added and the reaction was incubated at 25 $^{\circ}\text{C}$ for 10 min, 50 $^{\circ}\text{C}$ for 1 h, and then heat-inactivated at 70 $^{\circ}\text{C}$ for 15 min. Although not necessary for highly oversampled libraries such as the ones used in this study, this reaction could be scaled up to recover rare sequences that may be immobilized higher on the well walls after selection.

PCR to Verify Enrichment. The products of reverse transcription (2 μL) were amplified by PCR with T7B_no_

BsaI and pRDV_BbsI_r for 35 cycles in a 50 μL reaction (30 cycles for saved translation reaction without selection in the first round). Each PCR product was subjected to gel purification by excising a rectangle encompassing both full-length DARPIn product bands and this purified DNA was then reamplified with T7_no_BsaI and pRDV_BbsI_r to obtain enough product for transcription for the next round. For analytical gel electrophoresis, PCR products were visualized in agarose gels with ethidium bromide staining and imaged using an ultraviolet transilluminator and CCD camera.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

5' UTR, 5' untranslated region; RBS, ribosome binding site; NTP, nucleoside triphosphate; RT, reverse transcription; MBP, maltose-binding protein; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris-buffered saline; TBSC, TBS with 0.5% casein

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