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Efficient protein selection based on ribosome display system with purified components

Hiroyuki Ohashi [a], Yoshihiro Shimizu [a,*], Bei-Wen Ying [a,b], Takuya Ueda [a]

[a] *The Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, FSB-401, 5-1-5, Kashiwanoha, Kashiwa, Chiba 277-8562, Japan*

[b] *ICSN–CNRS, 1 avenue de la terrasse, 91190 Gif-sur-Yvette, France*

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Abstract

Using the PURE (Protein synthesis Using Recombinant Elements) system, we developed an efficient and highly controllable ribosome display method for selection of functional protein. The PURE system is composed of purified factors and enzymes that are responsible for gene expression in *Escherichia coli*. We performed the detailed analyses and optimization of the ribosome display system and demonstrated the formation of stable mRNA/ribosome/polypeptide ternary complexes. As complex formation is fundamental to successful ribosome display, these improvements resulted in a dramatic increase in the mRNA recovery rate. As a result, a ~12,000-fold enrichment of single-chain antibody (scFv) cDNA was achieved in a single round of selection. Specific selection of scFv mRNA from a $1{:}10^{10}$ dilution in competitor mRNA was achieved with only three rounds of affinity selection. These findings, together with the results in the accompanying paper [T. Matsuura, H. Yanagida, J. Ushioda, I. Urabe, T. Yomo, Nascent chain, RNA, and ribosome complexes generated by pure translation system (see the accompanying paper).], demonstrate that the PURE system can provide a basis for reliable and reproducible ribosome display.

Keywords: Ribosome display; Cell-free protein synthesis system; *In vitro* translation; Translation; Protein engineering; *In vitro* selection; Molecular evolution; Antibody; scFv; PURE system

The ribosome display technology is based on the formation of an mRNA/ribosome/polypeptide ternary complex in the cell-free protein synthesis system, providing a physical linkage between phenotype and genotype [2]. The sequence information for proteins or peptides of interest can be selected by affinity purification of this complex. In recent years, this technology is becoming one of the most widely used tools for the *in vitro* selection of functional proteins or peptides, and now being applied to the field of drug discovery [2,3].

At present, crude cell extract-based cell-free protein synthesis system is the only format for the *in vitro* selection. However, such system is often a "black box" system where the controllability is limited due to our incomplete knowledge of the system components. Furthermore, the presence of intrinsic components such as proteinases and nucleases inevitably reduces a stability of the mRNA/ribosome/polypeptide complex by degrading mRNAs or nascent peptides, which would prevent attainment of reliable results for users. Thus, as long as the cell extract is used for the cell-free protein synthesis system, it is unlikely that these problems would be overcome.

We have previously reported the development of the PURE (Protein synthesis Using Recombinant Elements) system, a highly controllable cell-free protein synthesis system that is composed of individually prepared components required for gene expression in *Escherichia coli* [4]. Using this system, we have performed a novel screening system for mRNA aptamers that contain upstream mutations conferring the ability to bind downstream translation products [5]. In this study, we developed a new strategy termed

* Corresponding author. Fax: + 814 7136 3648.
E-mail address: shimizu@k.u-tokyo.ac.jp (Y. Shimizu).

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"pure ribosome display (PRD)", which allows preparation of mRNA/ribosome/polypeptide ternary complexes that are more stable than those obtained through the conventional ribosome display. We refined the system for efficient selection of functional proteins, especially for single chain Fv (scFv), overcoming the experimental limitations of conventional ribosome display and increasing enrichment of scFv cDNA ~12,000-fold in a single round of selection. We also present specific selection using a $1:10^{10}$ dilution of scFv to competitor mRNAs, with several rounds of selection.

Materials and methods

Construction of templates. The gene for scFv-HyHEL10 was amplified from pCANTAB5E-HyHEL-10 [6], kindly provided by Dr. Hiroshi Ueda, and then sub-cloned into pET20b (Novagen) downstream of the Shine-Dalgarno (SD) sequence. Dihydrofolate reductase (DHFR) gene was subcloned into pET17b (Novagen) [4]. The partial gene for M13 phage gene III (amino acid residues 220–326) was amplified from pCANTAB-5E (GE Healthcare), and then cloned downstream of the ORFs for scFv-HyHEL10 and DHFR. Resultant plamids were used as templates for PCR amplification with primers T7p (5′-AATTAATACGACTCACTATAGG GAGACCACAACGGTTTCCCTCTAG-3′) and Gene III-SecMp (5′-A GTTAAACGTTGAGGACCAGCACGAATACCTTGAGCTTGAGAAA TCCAAACAGGAGTAGAAAATTTGGCGCCGGAAACGTCACCAAT GAAACCATCGATAGCAGC-3′). PCR products were purified by gel electrophoresis and used as templates for the second step PCR using primers T7p and SecMp (5′-CTAAGTTAAACGTTGAGGACCAGCACGAA TACC-3′). Products were purified by gel electrophoresis and used as templates for subsequent *in vitro* transcription. Transcription reactions (⩽40 μL) were performed using the CUGA 7 *in vitro* Transcription Kit (Nippongene) with up to 2 pmol DNA template, and incubated at 37 °C for 2 h. Synthesized mRNAs were purified using ISOGEN-LS (Nippongene), precipitated by ethanol, and dissolved in water for the use in translation experiments.

Ribosome preparation. Escherichia coli A19 was grown at 37 °C in LB broth to mid log-phase. A cell pellet was harvested by centrifugation and stored at −80 °C. Cells were resuspended in an equal volume of suspension buffer (10 mM Hepes–KOH, pH 7.6, 50 mM KCl, 10 mM $Mg(OAc)_2$, 7 mM β-mercaptoethanol) and disrupted by French press at 7000–10,000 psi. Cell debris was removed by centrifugation at 20,000*g* for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 1.5 M, the precipitated fraction was removed by centrifugation at 20,000*g* for 30 min and the supernatant was filtered through a 0.45 μm membrane. Supernatant (1000 U at OD_{260}) was loaded onto a 10 mL HiTrap Butyl FF column (GE Healthcare) that had been equilibrated with buffer A (20 mM Hepes–KOH, pH 7.6, 1.5 M $(NH_4)_2SO_4$, 10 mM $Mg(OAc)_2$, 7 mM β-mercaptoethanol). Lower concentrations of ammonium sulfate buffer were prepared by diluting buffer A with buffer B (20 mM Hepes–KOH, pH 7.6, 10 mM $Mg(OAc)_2$, 7 mM β-mercaptoethanol). The column was washed and the ribosome fraction was eluted with 1.2 and 0.75 M ammonium sulfate buffers, respectively. The eluate was overlaid onto an equal volume of cushion buffer (20 mM Hepes–KOH, pH 7.6, 30 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 30% sucrose, 7 mM β-mercaptoethanol) and ultracentrifuged at 36,000 rpm for 16 h in a Beckman 70Ti rotor. The resulting clear pellet contained 70S ribosomes, which were dissolved in ribosome buffer (20 mM Hepes–KOH, pH 7.6, 30 mM KCl, 6 mM $Mg(OAc)_2$, 7 mM β-mercaptoethanol) and stored at −80 °C for later use.

In vitro translation using the PURE system. The standard PURE translation mixture (30 μL) was prepared as described previously [7], with a modification to the buffer system, which contained 1 mM oxidized glutathione, 0.1 mM reduced glutathione, 1 μM protein disulfide isomerase (PDI) and 0.6 U RNasin (Promega). Release factors (RF1, RF3, and RRF) and dithiothreitol were not contained in the present study. Reactions were pre-incubated at 37 °C for 5 min, and then mRNA was added and the translation reaction was performed at 37 °C for another 20 min. The reaction was stopped using 120 μL ice-cold WBTH (50 mM Tris–acetate, pH 7.5, 150 mM NaCl, 50 mM $Mg(OAc)_2$, 0.5% Tween 20, 2.5 mg/mL heparin sodium), and then centrifuged at 14,000*g* for 10 min to remove insoluble components. For the subsequent affinity selection, 50 μL WBT (50 mM Tris–acetate, pH 7.5, 150 mM NaCl, 50 mM $Mg(OAc)_2$, 0.5% Tween 20, 5% BSA (Takara Bio) and 4% Block Ace (Dainippon Sumitomo Pharma)) were added to the supernatant.

In vitro selection. Biotinylated lysozyme was prepared as follows: 10 mg lysozyme (Egg White, Seikagaku Corporations) was dissolved in 1 mL PBS and incubated with 10 mM Ez-Link Sulfo-NHS-LC-LC-Biotin (840 μL, PIERCE) at room temperature for 30 min. Unreacted reagent was removed by dialysis against PBS. In order to select scFv-HyHEL10 mRNA, biotinylated lysozyme (3 pmol) was added to the supernatant and the mixture was incubated at room temperature for 1 h. Subsequently, 30 μL streptavidin-coated magnetic beads (M270 streptavidin, DYNAL) were added and the mixture was incubated at room temperature for further 30 min with gently shaking. The Beads were recovered and washed 10 times with 1 mL WBT. The complex was removed from the beads by incubation in 60 μL elution buffer A (50 mM Tris–acetate, pH 7.5, 150 mM NaCl, 50 mM EDTA, 10 μg/mL *Saccharomyces cerevisiae* total RNA (Sigma)) for 30 min at room temperature with gently shaking. Eluted mRNA was purified using ISOGEN-LS (Nippongene), then precipitated with ethanol and dissolved in water. Specific selection of DHFR mRNA was essentially the same as for scFv-HyHEL10 mRNA, except for the use of 10 μL methotrexate (MTX)–agarose beads (Sigma) and elution buffer B (50 mM Tris–acetate, pH 7.5, 150 mM NaCl, 50 mM $Mg(OAc)_2$, 0.03 mM methotrexate (Wako), 10 μg/mL *S. cerevisiae* total RNA). Purified mRNAs were subjected to reverse transcription and PCR (RT-PCR) using the T7p and SecMp primers with the SuperScript III One-Step RT-PCR System and Platinum *Taq* DNA Polymerase (Invitrogen).

Real-time RT-PCR. Real-time RT-PCRs were performed in a Smart Cycler II System (Cepheid). HyHEL10s-F (5′-TGCAAACTGGGA CGGTGATTA-3′), HyHEL10s-R (5′-CTGGAGACTGGGTCAGCACA A-3′), DHFR-F (5′-CTGACGCATATCGACGCAGAA-3′), and DHFR-R (5′-CCGCTCCAGAATCTCAAAGCA-3′) were used. ExScript RT reagent Kit (Takara-Bio) and SYBR Premix Ex *Taq* (Takara-Bio) were used for Reverse transcription and PCR, respectively.

Results and discussion

Improvements in ribosome preparation reduce nuclease activity in the cell-free protein synthesis system

In order to construct an efficient protein or peptide screening system using ribosome display, it is desirable to use a nuclease-free cell-free protein synthesis system. In this respect, the PURE system may be considered as an ideal tool, as it is a cell-free protein synthesis system that has been reconstituted from essential elements [4]. Although the nuclease activities in the PURE system prototype were reduced substantially when compared to the conventional S30 system, we identified a small amount of contaminating nuclease activity. Further analysis indicated that these activities derived mainly from the ribosome preparation.

The conventional method for ribosome preparation separates 70S ribosomes from the crude ribosome extract using sucrose density gradient (SDG) centrifugation, which results in the formation of a pellet (Fig. 1A). Since this method is dependent upon the high molecular weight of the ribosome, other high molecular weight complexes such

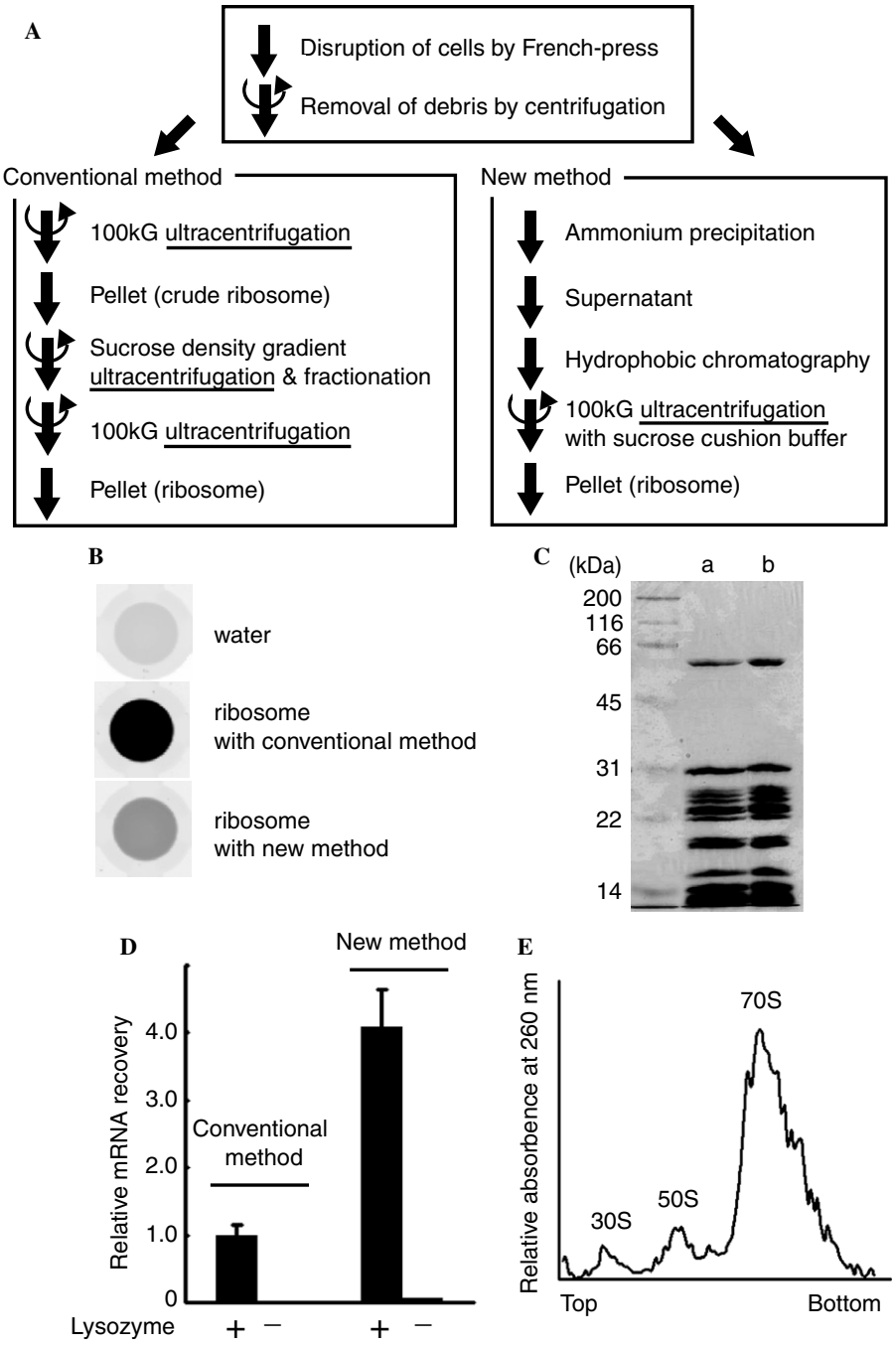

Fig. 1. Improvements in ribosome preparation. (A) Comparison between the conventional and new methods for ribosome preparation. (B) Relative activities of intrinsic nucleases contained in each preparation of the ribosome. Ribosomes (60 pmol) were added to 50 μL RNase Alert (Ambion) and incubated at 37 °C for 1 h in a 96-well microplate. The plate was scanned by a Typhoon Variable Scanner (GE Healthcare) using an excitation laser at 532 nm and emission filter at 526 nm. (C) SDS–PAGE analysis of ribosomes. Ribosomes (40 pmol) were prepared by the conventional (lane a) and new (lane b) methods, then separated by 12% SDS–PAGE and stained with Coomassie brilliant blue. (D) The effect of ribosome preparation on mRNA recovery from the PURE ribosome display system. The scFv-HyHEL10 mRNA was translated in the PURE system and the affinity purification of the mRNA/ribosome/polypeptide ternary complex was performed in the presence or the absence of lysozyme. After single round of affinity selection, mRNA was isolated and subjected to real-time RT-PCR. The relative mRNA recovery from the PURE system supplied with the ribosome prepared by the improved or the conventional method is shown. (E) SDG analysis of ribosomes prepared by the new method. A 6–38% SDG was used for sedimentation of ribosomes and the peaks representing the 30S and 50S subunits, as well as the 70S ribosome, are indicated.

as the degradosome complex or membrane-associated nucleases are difficult to be removed completely and become incorporated into the final ribosome preparation. In order to eliminate such activities, we added ammonium sulfate to the turbid S30 extract and obtained a clear supernatant, suggesting that large parts of the membrane fraction were eliminated. We then followed a method described by Ramakrishnan et al. [8], who suggested that

hydrophobic interaction chromatography was effective for preparation of 70S ribosomes. The cleared supernatant was separated by hydrophobic interaction chromatography. In the final step, a pellet containing the eluted ribosomes was formed through ultracentrifugation in a 30% sucrose cushion, which eliminated any low molecular weight soluble factors that remained. The improved method is presented in Fig. 1A.

Examination of the ribosome fraction indicated that nuclease contamination was reduced significantly in comparison to the conventional preparation (Fig. 1B). SDS–PAGE analysis demonstrated a high degree of homogeneity in the ribosome purification (Fig. 1C). The mRNA recovery from PURE ribosome display system (see below) supplied with the ribosome prepared by the improved method was increased 4-fold compared to that by the conventional method (Fig. 1D). These results indicate that the improved method is suitable for facilitating the PURE ribosome display system due to the reduced nuclease activities in the final ribosome preparation. Exactly, the modified version of the PURE system comprises exceptionally low levels of the nuclease activities in comparison to the standard S30 system (data not shown).

SDG centrifugation was used to analyze the ribosome fraction purified by the improved method, and this indicated that the major ribosomal components were tightly coupled in the 70S ribosome (Fig. 1E). Thus, ribosomal activity in the PURE system was similar to that of conventionally purified ribosomes. In addition, the new method requires only one over-night ultracentrifugation step to obtain a highly purified and active ribosome preparation, whereas the conventional method required at least two over-night ultracentrifugation steps. Thus, the new preparation method enables us to obtain ribosomes from an *E. coli* cell pellet in a single day.

Affinity purification of the mRNA/ribosome/polypeptide ternary complex using the PURE system

To examine the applicability of the PURE system to the ribosome display, we constructed the DNA sequence shown in Fig. 2A. We introduced the genes encoding

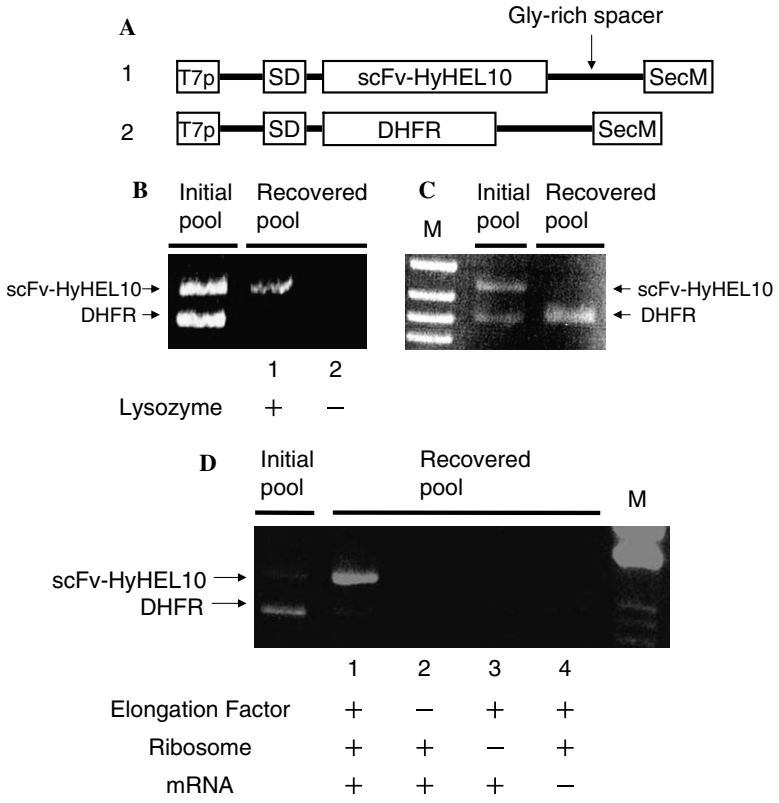

Fig. 2. Construction of the pure ribosome display (PRD) system. (A) DNA constructs used for the PRD. Genes encoding scFv-HyHEL10 or DHFR were inserted downstream of the T7 promoter and Shine-Dalgarno sequence. A Gly-rich linker (gene III) is located between the gene encoding the target protein and SecM. (B,C) Affinity selection for lysozyme (B) and MTX (C). The initial pool was a 1:1 mixture of scFv-HyHEL10 and DHFR mRNAs, which were translated and selected with lysozyme and MTX, respectively. The selection of scFv was performed in the presence (+) or absence (−) of lysozyme. After a single round of affinity selection, mRNA was isolated and subjected to RT-PCR, followed by agarose gel electrophoresis. (D) Translation-dependent formation of the mRNA-ribosome-polypeptide ternary complex. Affinity selection was performed in the presence of all essential components, as well as in the absence of individual components, i.e., elongation factors, ribosomes, and mRNA. The initial mRNA pool contained a 1:30 mixture of scFv-HyHEL10 and DHFR mRNAs. The products of a single round affinity selection with lysozyme were separated by agarose gel electrophoresis.

scFv-HyHEL10 and DHFR downstream of the T7 promoter and SD sequence. We included a spacer sequence (gene III) downstream of these ORFs, in order to prevent steric hindrance between the nascent protein and the ribosome by providing sufficient room for correct folding of the polypeptide. In addition, the SecM elongation arrest sequence (amino acid residues 148–170) was positioned downstream of gene III, in order to stabilize the mRNA/ribosome/polypeptide ternary complex. This elongation arrest sequence has been shown to interact tightly with the ribosomal polypeptide exit tunnel [9], and has been demonstrated to arrest ribosome elongation efficiently in the PURE system [1,10]. However, it seems that such stalling has no considerable change on selection efficiency (see the accompanying paper; [1]), although we have no good explanation for this. More detailed analysis of the PURE system itself might be necessary to elucidate this point.

We performed affinity purification of the mRNA/ribosome/polypeptide ternary complex from the PURE system, in which the equal amounts of the scFv-HyHEL10 and DHFR mRNAs have been translated (Fig. 2). The ternary complex could be affinity purified using target-specific ligands, i.e., biotinylated hen egg lysozyme with the streptavidin-coated magnet beads or MTX-coated agarose beads, respectively. mRNAs recovered from each affinity selection were subjected to RT-PCR using mRNA-specific primers and the only cDNAs, which encoded proteins specific to each ligand, were selected (Fig. 2B and C). The amplification of non-specific cDNAs was not observed under these conditions. Moreover, in the absence of specific ligands, neither cDNA was amplified, demonstrating that the purification procedure was highly specific.

In order to investigate whether affinity purification and subsequent amplification of the cDNA is dependent upon ternary complex formation in the PURE system, the same experiments were executed in the absence of components that are essential for polypeptide synthesis. The omission of ribosomes, three elongation factors, or mRNA resulted in the complete abolition of cDNA amplification (Fig. 2D). These results demonstrate that the ribosome display in the PURE system is strictly dependent upon the protein synthesis reaction and suggests that the physical linkage between genotype and phenotype on the ribosome is essential for the selection.

The effect of the ribosome concentration on mRNA recovery was also evaluated (Fig. 3). Our results demonstrated that maximum mRNA recovery was achieved with 10 pmol ribosomes in 30 μL reactions, a concentration 10-fold higher than used previously for scFv-HyHEL10 mRNA (1 pmol in 30 μL reactions). The optimized conditions achieved a 2.5% recovery, representing an order of magnitude increase over conventional ribosome display using the S30 system [11]. This value indicates that one molecule of mRNA can be recovered from 40 molecules of mRNA, and indicates that in a 30 μL PURE system, ligand-specific selection of peptides or proteins can be readily achieved from a 10^{10} genetic library.

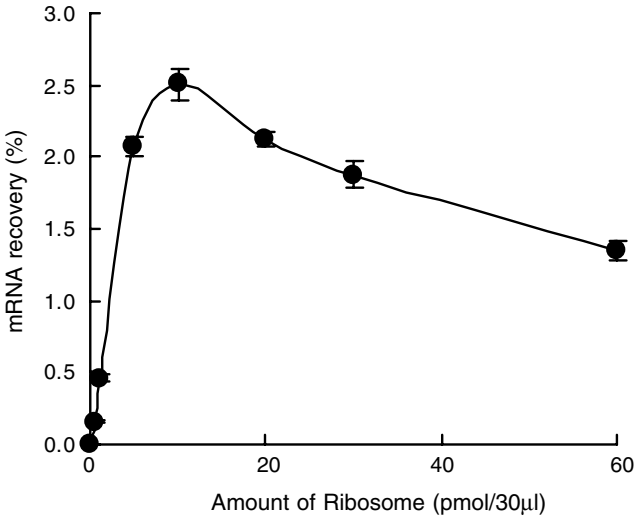

Fig. 3. The effect of ribosome concentration on mRNA recovery rate. scFv-HyHEL10 mRNA (1 pmol) was added to 30 μL reaction mixtures containing 0–60 pmol ribosomes with the ligand. The vertical axis represents the recovery rate of mRNA after a single round of selection. Selected mRNAs were subjected to real-time RT-PCR and quantified according to a standard curve. Error bars indicate the SD of five independent measurements.

Significant enrichment of scFv-HyHEL10 from DNA pools of various scales

Enrichment following a single round of selection in the PRD system was examined using scFv-HyHEL10 mRNA as a selection target and DHFR mRNA as the competitor. scFv-HyHEL10 and DHFR mRNAs were mixed in 1:10^3, 1:10^4, and 1:10^5 ratios and then subjected to a single round of lysozyme selection (Fig. 4A and Table 1). The enrichment efficiencies were quantified by real-time RT-PCR. With respect to the 1:10^5 ratio, a ~12,000-fold enrichment was achieved (Table 1), an order of magnitude higher than previously reported (<~1000-fold; [2,11–15]). It is likely that this high efficiency resulted from the increased mRNA recovery ratio in the PRD system (Fig. 2D).

Since recovery of scFv-HyHEL10 mRNA could not be detected at ratios greater than 1:10^6, we performed several rounds of selection using ratios from 1:10^8 and 1:10^{10} of scFv-HyHEL10 to DHFR mRNAs. After two and three rounds of selection, scFv-HyHEL10 cDNA was visible from the 1:10^8 (Fig. 4B) and 1:10^{10} (Fig. 4C) dilutions, respectively. After three and four rounds of selection, cDNA of the competitor (DHFR) had diminished completely. These observations indicate the high efficiency of the PRD system compared to conventional ribosome display using *E. coli* S30 extract, in which selection of scFv from a 1:10^8 dilution required five rounds [11].

Conclusion

The present study focuses on the application of the PURE system to ribosome display and provides several lines of evidence indicating that this system could represent a powerful

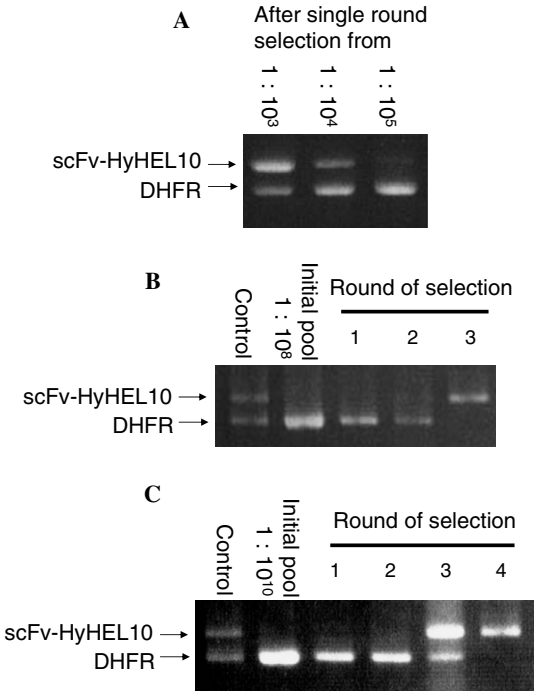

Fig. 4. Specific enrichment of scFv-HyHEL10 mRNA. (A) After a single round of selection. The initial pools were $1{:}10^3$, $1{:}10^4$, and $1{:}10^5$ of scFv-HyHEL10 and DHFR mRNAs. (B,C) Specific enrichment of scFv-HyHEL10 mRNA following multiple rounds of selection. The initial pools were $1{:}10^8$ (B) and $1{:}10^{10}$ (C) mixtures of scFv-HyHEL10 and DHFR mRNAs. After each round of affinity selection, mRNA was isolated, subjected to RT-PCR and analyzed by agarose gel electrophoresis. The isolated mRNA was used for each subsequent selection step.

Table 1
Quantification of enrichment after a single round of selection with lysozyme using the initial mRNA pools described in Fig. 4A

Molar ratio of initial pool HyHEL10:DHFR	Molar ratio of recovered pool HyHEL10:DHFR	Enrichment (fold)
$1{:}10^3$	$1{:}5.5\times10^{-1}$	1818
$1{:}10^4$	1:3.2	3125
$1{:}10^5$	1:8.3	12048

Selected mRNAs were subjected to real-time RT-PCR and quantified according to a standard curve.

platform for selecting polypeptides via affinity purification. Although other *in vitro* selection systems, such as *in vitro* virus or mRNA display [16–18], CIS display [15], and DNA display, which functions by compartmentalizing a single DNA molecule in oil emulsions [19,20], utilize S30 extract-based cell-free protein synthesis, our results suggest that selection would be more efficient if performed in the PURE system.

It is also noteworthy that the latest publication demonstrating that the use of the purchasable PURE system, which had been originally developed in our laboratory, for the ribosome display is highly advantageous against the conventional S30 system [21]. In the report, the mRNA/ribosome/polypeptide complexes are significantly unstable in the S30 system, whereas it is highly stable in the PURE system, which is consistent with the accompanying results of Matsuura et al. [1]. Together with these, we demonstrate here the improved ribosome preparation method for constructing PURE system suitable for the ribosome display, optimized concentration of the ribosome for the maximum mRNA recovery, which cannot be examined as far as using the commercially available PURE system kit. Furthermore, the quantitative evaluation of the enrichment level of specific gene in the PRD system, which was not shown in the previous report [21], is also presented to be an order of magnitude higher than other protein selection system. Thus, we believe that the results demonstrated here assure the usability of the PURE system for the ribosome display and indicate a new direction for the field of protein evolution and the development of pharmaceutical antibodies to treat intractable diseases.

Acknowledgments

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