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Journal of Molecular Catalysis B: Enzymatic 28 (2004) 223–228
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Rapid screening for affinity-improved scFvs by means of single-molecule-PCR-linked in vitro expression

Suang Rungpragayphan, Megumi Haba, Hideo Nakano*, Tsuneo Yamane

Laboratory of Molecular Biotechnology, Graduate School of Biological and Agricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

Received 29 September 2003; received in revised form 13 January 2004; accepted 24 January 2004

Abstract

We have applied a combination of in vitro saturation mutagenesis and the single-molecule-PCR-linked in vitro expression (SIMPLEX) for rapid generation and screening of antibody mutants with improved affinity. A pool of anti-human serum albumin scFv mutant genes was created by in vitro saturation mutagenesis of a parent's CDR-H3. Individual mutant genes were amplified by single-molecule PCR, and the PCR products were used directly as templates for in vitro coupled transcription/translation. Thereafter, all expressed scFv mutants were screened by competition ELISA. Two mutants showing improved affinities were selected and kinetically characterized. Because the single-molecule PCR, in vitro expression, and selection of distinct mutants were carried out parallelly in an array format, the process was very speedy and perfectly suited automation.
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Keywords: Single-molecule PCR; In vitro coupled transcription/translation; Combinatorial protein library; High-throughput screening; Complementarity determining region

1. Introduction

Antibody has become a very valuable biomolecule for both biological research and clinical therapy [1]. High-affinity antigen recognition is very critical to its applications. In vitro mutagenesis is one of powerful tools for improvement of the antibody binding affinity [2]. Since lack of structural knowledge on antigen–antibody complex limits a precise rational design, random mutagenesis has become a general method for diversification of the sequence. Within the antibody encoding gene, complementarity determining regions (CDR) are highly diverse among antibodies, and consequently targeted for mutations [3]. The advance of cell-based peptide display technologies, such as phage display, yeast cell surface display, in the past decade dramatically empowers vitro affinity maturation of antibody [4,5]. Besides, in vitro transcription/translation-based systems such as ribosome display, mRNA–protein fusions, and in vitro compartmentalized system, have also been progressively developed as alternative selection technologies [6].

Recently, we have developed a technology termed single-molecule-PCR-linked in vitro expression (SIMPLEX), which is a sequential combination of single-molecule DNA amplification (SM-PCR) and in vitro coupled transcription/translation, for high-throughput generation of a protein library [7–9] (Fig. 1). It enables construction and screening of proteins devoid of time-consuming manipulations, such as cloning, cultivation and rounds of biopanning and is compatible with various screening methods. In this work, we applied saturation mutagenesis and the SIMPLEX for construction and screening an anti-human serum albumin scFv (anti-HSA-scFv) library. All amino acid residues in the CDR-H3 of the wide type anti-HSA-scFv were mutagenized individually to create a mutant pool. Subsequently, the mutant pool was converted to a scFv mutant library by SIMPLEX technology, and screened by competition ELISA

Abbreviations: SM-PCR, single-molecule polymerase chain reaction; SIMPLEX, single-molecule-PCR-linked in vitro expression; HSA, human serum albumin; anti-HSA-scFv, anti-human serum albumin single chain variable fragment; CDR, complementarity determining region; RBS, ribosome binding site; T7P, T7 promoter; T7T, T7 terminator

* Corresponding author. Tel.: +81-52-789-4143; fax: +81-52-789-4145.
E-mail address: hnakano@agr.nagoya-u.ac.jp (H. Nakano).

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doi:10.1016/j.molcatb.2004.01.019

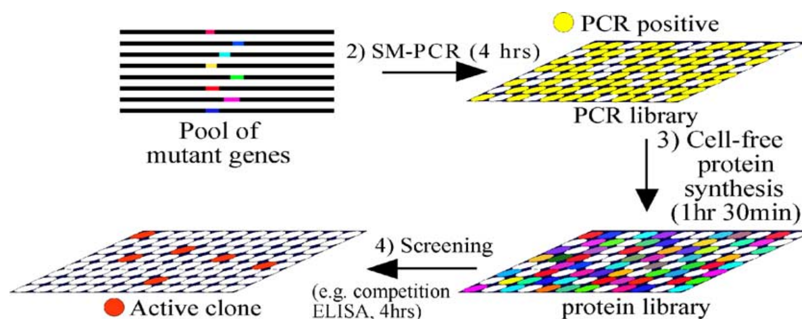

Fig. 1. Schematic representation of high-throughput protein library construction by SIMPLEX and screening. (1) DNA templates in the mutant gene pool are extensively diluted to one molecule per well and amplified by SM-PCR yielding a PCR library (on 384-well plates in this study). (2) The PCR library is converted to a protein library by means of cell-free protein synthesis. (3) The protein library is screened for clones with desired properties (e.g. by competition ELISA for mutants with improved affinity in the present work). Times consumed for SM-PCR, cell-free protein synthesis, and screening by competition ELISA are about 4 h, 1 h 30 min, and 4 h, respectively.

straightforwardly. Mutants with improved affinity were selected and further characterized. Merits of the SIMPLEX technology and its application on enzyme screening are also discussed.

2. Materials and methods

2.1. Preparation of DNA templates with random mutation

All codon of the CDR-H3 were individually randomized by combination of conventional PCR and overlapping PCR as shown in Fig. 2b. The original anti-HSA-scFv was from the pRSETa-scFv. Mutation primers for position 99–105 were SCA-R95: 5′-CCCCAGTAAGCCCCGTTCCATCC-**SNN**CCTTACACAATAATACATGGCTGT-3′, SCA-R96: 5′-CCCCAGTAAGCCCCGTTCCA**SNN**CTCTCTTACAC-AATAATACATGGC-3′, SCA-R97: 5′-CCTTGGCCCCAG-TAAGCCCCGTT**SNN**GCCTTCTCTTACACAATAATAC-ATGG-3′, SCA-R98: 5′-CCTTGGCCCCAGTAAGCCCC-**SNN**CCAGCCTTCTCTTACACAATAATACATGG-3′, SC-A-R99: 5′-ACCGTGGTCCCTTGGCCCCAGTAAGC**SNN**-ATTCCATCCTTCTCTTACACAATA-3′, SCA-R101: 5′-ACCGTGGTCCCTTGGCCCCAGTA**SNN**ACCGTTCCA-TCCTTCTCTTAC-3′, and SCA-R102: 5′-ACCGTGGTCC-CTTGGCCCCA**SNN**CGCCCCGTTCCATCCTTCT-3′, respectively. The overlapping primer for mutation at position 99 and 100 was F3: 5′-TGGAACGGGGCTTACTGGGG-3′, for position 101 and 102 was F4: 5′-GGGGCTTACTGGGG-CCAAG-3′, and for position 103, 104, and 105 was F5: 5′-TGGGGCCAAGGGACCACGG-3′. The primers In-FS2: 5′-ACACGACGTGAACGATAGGAATTGACCAATACG-CAAACCGCCTCT-3′ and In-RS2: 5′-ACACGACGTGAA-CGATAGGAATTGATACAGGGCGCGTCCCATTCG-3′ were used to amplify full-length mutant genes. The DNA templates with random mutation at each position were purified and their concentrations were determined by measuring absorbance at 260 nm. Equal amount of each was mixed to make a gene pool.

2.2. Single-molecule PCR

The DNA templates were typically diluted with 0.1% (w/v) Blue Dextran 2000 (Amersham-Pharmacia) in TE buffer to one molecule per well (1.28×10^{-9} ng of the DNA template was estimated as one molecule). The amplification was carried out in a total volume of 7 μl with 0.25 U of *Pfu Turbo*™ DNA polymerase (Stratagene),

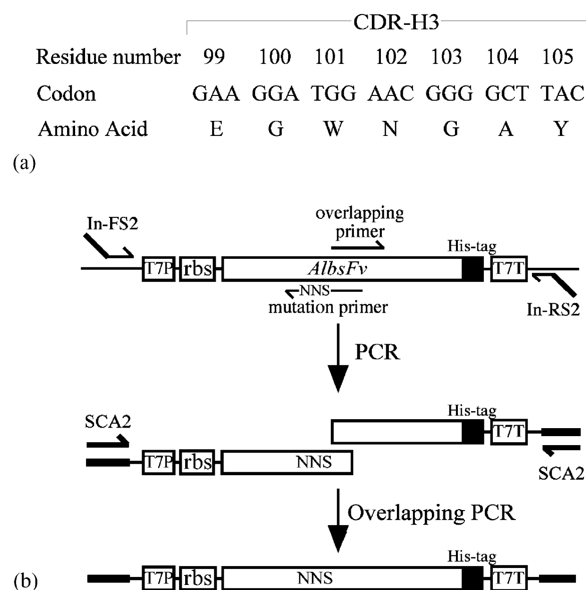

Fig. 2. (a) The nucleotide and amino acid sequence of CDR-H3 of the parent anti-HSA-scFv. (b) A schematic diagram showing preparation of the anti-HSA-scFv mutant templates for the SIMPLEX. The plasmid pRSETA-scFv containing T7 promoter (T7P), a ribosome binding site (RBS), anti-HSA-scFv encoding gene (*AlbsFv*), histidine tag, and T7 terminator (T7T) sequence successively was amplified and simultaneously mutagenized using two pairs of primers (In-FS2/mutation primer) and (overlapping primer/In-RS2), yielding two DNA fragments. The mutation primer contains a randomizing NNS codon (N = A, T, C, or G and S = C or G). Thereafter, two DNA fragments were joined by overlapping PCR using a single primer named SCA2.

0.125 mM of each dNTP, and 0.5 μM of a primer (SCA2: 5′-ACACGACGTGAACGATAGGAATTGA-3′), in cloned *Pfu* buffer. The reaction mixtures were preheated at 94 °C for 5 min, followed by 65 cycles of: 5 s at 96 °C; 10 s at 60 °C; 1 min at 72 °C. The amplifications were carried out in two plates (384 wells per plate, PE Applied Biosystems) by GeneAmp® PCR system 9700 (PE Applied Biosystems).

2.3. In vitro coupled transcription/translation

The in vitro coupled transcription/translation of all PCR products were carried out in 20 μl reactions with components as previously reported [9], also in 384-well polystyrene plates (Nunc™). The reactions were incubated at 30 °C for 60 min, subsequently put on ice to stop the reaction. Negative control reactions were performed under identical conditions without DNA template. In vitro coupled transcription/translation of the wild type anti-HSA-scFv with His-tag was also performed under identical conditions to serve as an internal standard. Wild type with cMyc-tag which was used as a competitor in the competition ELISA was also expressed in vitro.

2.4. Library screening by competition ELISA

The scFv library was screened by means of competition ELISA. Briefly, 384-well plates (Nunc-Immuno™) were coated with human serum albumin (Nakarai, Japan), and blocked by BlockAce (Dainihon pharmaceutical, Japan). Each cell-free reaction mixture in the protein library and equal volume of cell-free reaction mixture of the wild type with cMyc-tag, the competitor, was mixed and diluted by PBS buffer and added into the pre-coated plate. Penta-His HRP conjugate (Qiagen) was used as a secondary antibody interacting with the histidine-tag of the anti-HSA-scFv. Finally, orthophenylenediamine (Wako Pure Chemical Industries, Japan) solution, a substrate for the enzyme HRP, was added to each well. The enzymatic reaction was terminated by 2 M H_2SO_4 and ELISA signals were determined by measuring the absorbance at 492 nm by the Fusion™ (Packard BioScience).

2.5. Sequencing

The selected PCR products were treated with PCR pre-sequencing kit, ExoSAP-IT (Amersham Biosciences) and subjected to the cycle sequencing reaction using Thermo Sequenase™ II dye terminator cycle sequencing kit (Amersham Biosciences) with a protocol recommended by the supplier. All sequences were analyzed using an ABI PRISM™ 310 Genetic Analyzer (PE Applied Biosystems).

2.6. In vivo expression and purification of anti-HSA-scFvs

Selected mutant genes from library were cloned into a plasmid vector pRSTEB (Invitrogen) via *Nde*I and *Hin*dIII sites. Transformed *E. coli* BL21(DE3) cells were incubated in LB medium at 37 °C, and expressions of anti-HSA-scFvs were induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation at 3500 × *g* and suspended in PBS buffer (pH 7.4). After sonication and centrifugation at 5800 × *g*, pellet was washed several times by PBS buffer (pH 7.4) with 4% Triton X 100, and finally washed by water to remove Triton X 100. The pellet was solubilized in solution of PBS buffer (pH 7.4) with 6 M GdnHCl, and centrifuged at 10,000 × *g*. The solubilized scFv was refined by histidine-tag affinity using a metal-chelate chromatography column (HiTrap™ Chelating HP, Amersham Pharmacia Biotech AB).

2.7. In vitro refolding of scFvs by stepwise dialysis

The in vitro refolding was carried out as reported by Umetsu et al. [10] with modification. Briefly, the refined unfolded scFvs were diluted to 7.5 μM in the PBS buffer (pH 7.4) with 6 M GdnHCl, and 1 mM EDTA and reduced by 2-mercaptoethanol (β-ME). Afterward, β-ME was removed by dialysis against the same buffer without β-ME. The scFv was slowly refolded in stepwise dialysis by gradual removal of GdnHCl from 6 to 0 M through 3, 2, 1, 0.5 M. To help refolding, in 1 and 0.5 M GdnHCl stages, 375 μM GSSG and 0.4 M L-arginine were added.

2.8. Kinetic parameter determination of scFv by ELISA

The purified scFv were incubated with increasing concentrations of HSA ranging between 1 and 100 nM in parallel tubes. After binding equilibrium was established, the fraction of unbounded scFv was captured on an HSA-coated plate and ELISA signals were developed. Dissociation constants (K_ds) of scFvs were determined by Klotz plot [11].

3. Result

3.1. Saturation mutagenesis and gene pool preparation

To mimick affinity maturation, a mutagenesis scheme that creates plenty of variation is preferred. However, the achievable size of the library constructed by SIMPLEX intrinsically depends largely on capacity of a thermocycler [9,12]. One strategy to keep the library size within a practically searchable scale is to focus on a major site that influences the interaction with antigen. Therefore, we targeted the CDR3 of the heavy chain (CDR-H3), which is the most diverse region likely providing a major contact surface to an antigen [3], and an attempt to change specificity or improve affinity by introducing alterations into only CDR-H3 has been successfully demonstrated [13]. CDR-H3 of the parent anti-HSA-scFv is seven amino acids long, extending from position 99 to 105 of the heavy chain (Fig. 2a). Every

codon in the CDR-H3 of an anti-HSA-scFv was randomized individually (NNS; N = A or T or C or G, S = C or G) by means of PCR (Fig. 2b) [9] to allow saturation mutagenesis at each amino acid. In addition, a silent mutation was also introduced neighboring the random site to make a recognizable mark for clones from the pool. In order to enable PCR-directed in vitro expression by *E. coli* S30 system, T7 promoter, ribosome binding site (RBS), and T7 terminator were positionally incorporated, and a well-designed DNA tag was joined to both ends of the DNA serving as annealing sites of a single primer (Fig. 2b). At the end of the scFv encoding gene, a histidine tag was also placed enabling detection by ELISA in the screening. Although each small gene pool resulted from random mutagenesis of individual codons (NNS; $4 \times 4 \times 2$) could be subjected to SIMPLEX and evaluation separately, it is reasonable to carry out at the same time to control any unknown variations which might occur among batches of PCR and in vitro coupled transcription/translation. Furthermore, it would give higher throughput. Therefore, an equal amount of seven single-codon randomized genes was mixed to make a pool of 224 gene variants (seven codons with NNS mutation; $4 \times 4 \times 2 \times 7$).

3.2. Generation of scFv mutants by SIMPLEX and screening

The gene pool was transformed to an scFv mutant library by SIMPLEX as shown in Fig. 1. In principle of the SM-PCR, although the template concentration is precise and amplification of one molecule is 100% efficient, only 63% of all reactions will be successfully amplified and only 37% would be of single molecule template according to the Poisson distribution [9]. Therefore, in order to certainly cover all variants in the pool, 768 reactions of SM-PCR and following in vitro coupled transcription/translation were carried out, on two of 384-well plates. Along with all mutants in the library, the wild type gene with His-tag was also amplified and in vitro expressed to serve as an internal standard. Then, scFv mutants were directly screened by competition ELISA, which was supposed to assist discriminating between clones with modestly different affinities. The wild type with cMyc-tag was used as a competitor to the mutants which possessed histidine tag and anti-His tag with conjugated HRP was used as a secondary antibody. From the screening, 26 promising clones that showed a higher ELISA signal than the wild type internal standard were picked for reassessment. Genes encoding the selected mutants were re-amplified by PCR and the PCR products were purified. An equal amount of each gene was used as DNA template for in vitro coupled transcription/translation, and subsequently antigen binding affinity of produced mutants were assessed by the competition ELISA.

Eventually seven clones showing improved affinity over the wild type were picked and their sequences were determined as shown in Table 1. Five clones that gave the highest ELISA signals had mutation at N102 to various amino acids, and clone 2-14-M had mutation at A105 to methionine. The sequence of clone 1-20-D was unidentifiable because it was apparently originated from more than one gene. We also analyzed sequences of some clones that showed ELISA signals lower than that of the wild type and found that each clone had various distinct mutations apart from the N102 or A105 (data not shown). The results indicated that the scFv mutant pool was practically generated by our strategy, and the significant improvement in affinity was caused mainly by mutation at the position N102 of the CDR-H3.

3.3. Characterization of selected mutants

Two clones that gave the highest ELISA signal, 1-2-M and 2-16-C, were cloned into a plasmid vector pRSETB and expressed in *E. coli* BL21(DE3). The scFvs, which were produced mainly as inclusion bodies, were solubilized, purified by histidine-tag affinity chromatography, and then dialyzed for in vitro refolding. Using an ELISA method, dissociation constants (K_d) of purified scFvs were determined from the slopes of the Klotz plots (Fig. 3) and results were summarized in Table 2. K_d of the mutant 1-2-M, the mutant 2-16-C, and the wild type were 5.2 nM, 8.0 nM and 25.2 nM, respectively, which suggested that affinities to HSA of the mutant 1-2-M and the mutant 2-16-C were improved 4.8- and 3.1-folds, respectively.

Table 1
Nucletide sequences and mutations of clones showing increased ELISA signals

Clone	Nucleotide sequence at CDR-H3							Mutation	ELISA signal
	99	100	101	102	103	104	105		
1-2-M	GAA	GG*C*	TGG	<u>TTC</u>	GGG	GCT	TAC	N102F	0.872
2-16-C	GAA	GG*C*	TGG	<u>CTG</u>	GGG	GCT	TAC	N102L	0.833
1-2-C	GAA	GG*C*	TGG	<u>ACG</u>	GGG	GCT	TAC	N102T	0.772
1-3-K	GAA	GG*C*	TGG	<u>TCC</u>	GGG	GCT	TAC	N102S	0.560
1-21-G	GAA	GG*C*	TGG	<u>TGC</u>	GGG	GCT	TAC	N102C	0.500
1-20-D				nd[a]				nd	0.480
2-14-M	GAA	GGA	TGG	AAC	GGG	GC*G*	<u>ATG</u>	Y105M	0.462
Wild type	GAA	GGA	TGG	AAC	GGG	GCT	TAC	–	0.324

Mutated codons are underlined and silent mutations, which were introduced to mark clones from library, are italicized.
[a] Not determined because it was apparently amplified from multiple origins.

Table 2
Affinities of selected anti-HSA-scFvs

Clone	K_d (M)	Relative affinities
Wild type	2.5 (± 0.1) × 10^{-8}	1
1-2-M	5.2 (± 0.2) × 10^{-9}	4.8
2-16-C	8.0 (± 0.3) × 10^{-9}	3.1

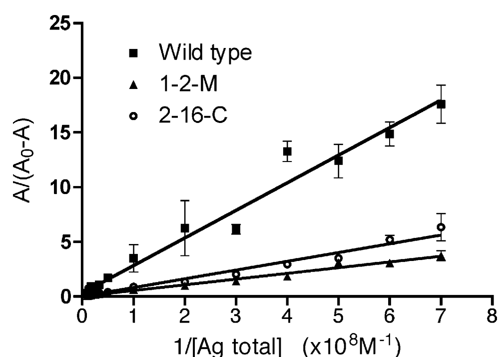

Fig. 3. Klotz plot of Ag competition ELISA data. A certain amount of a purified scFv was mixed with various amounts of antigen, HSA. After binding equilibrium was established, the fraction of unbounded scFv was captured on an HSA-coated plate and ELISA signals were developed. K_d of an scFv is represented by the slope of the plot between $A/(A_0 - A)$ and 1/[Ag total], where A is a developed ELISA signal, A_0 is a developed ELISA signal when no antigen was mixed with the scFv, and [Ag total] is the concentration of mixed HSA.

4. Discussion

We have developed the SIMPLEX technology, which is a sequential combination of single-molecule DNA amplification by PCR (SM-PCR) and in vitro coupled transcription/translation, for high throughput construction of protein libraries. By using a high fidelity DNA polymerase, the SM-PCR, which is performed for several cycles (e.g. 75 cycles), produced consistent amount of products with very low simultaneous mutation rate [8,9]. Amounts of proteins synthesized by in vitro coupled transcription/translation of SM-PCR products were markedly uniform with relative standard deviation of ∼8%, which was much lower than that of classical in vivo expression (∼24%) [9]. In this work, we combined in vitro saturation mutagenesis with SIMPLEX for rapid generation and screening of scFv mutants with improvement in affinity. The CDR-H3 of an anti-HSA-scFv was targeted for alterations to make a mutant pool. Despite the fact that simultaneously randomizing all amino acid residues in the CDR-H3 will enable entire amino acid combinations, the number of variants will exceed capacity of the SIMPLEX. Although the SIMPLEX can theoretically generate a library of unlimited size if one can perform PCR and in vitro expression, in practice, the library size is currently constrained due to limited capacity of thermocyclers, and requirement of large amount of reagents [12]. Interestingly, mutations at some hot spots in the antibody encoding gene have been reported to be successful in affinity improvement [14]. Our effort on some hot spots in the CDR-L3 of the same anti-HSA-scFv, however, has been fruitless [9]. Therefore, we opted to randomize all amino acid in the CDR-H3 instead of any specific residues.

After construction and screening, two clones exhibiting significant improvement in affinity (1-2-M and 2-16-C) were selected for further characterization and the results showed that the binding affinities of mutant 1-2-M and 2-16-C improved 4.8 and 3.1 times, respectively (Table 2). The results shown in Table 1 suggest that the higher affinity phenotype was caused specifically by the N102 mutations. The selected two mutants have substitutions of the hydrophilic asparagine to a hydrophobic phenylalanine or leucine. However, mutants with other hydrophobic substitutions, such as valine and isoleucine, at the N102 were not found positive. There is a possibility that the generation of the library did not cover all possible clones due to the stochastic dilution in which only 33% of SM-PCR products are originated from single molecules [7,9]. By this limited data, the role of the hydrophobic substitutions is still not obvious. In the case of other mutants (N102T, N102S, and N102C), slight improvements in affinity might be resulted from reduction in the size of the hydrophilic side chain of the residue 102, which locates at the turn of the loop according to a predicted three-dimensional structure. Nevertheless, to fully understand the effect of the substitutions, further investigation such as X-ray crystallography is required. Although the clone 1-20-D was amplified from multiple origins, finding of such clones is inherent at a typical dilution employed in the SM-PCR [9].

While other display technologies are capable of handling libraries with much greater size, our strategy provides higher speed, easier miniaturization and automation, and has been proved here to be suitable for fine-tuning of affinity. Without gene cloning and several rounds of biopanning, single genes encoding antibodies with improved affinity were obtained and identified within 24 h (SM-PCR: 4 h, in vitro coupled trnscription/translation: 1 h 30 min, ELISA: 4 h, PCR-direct sequencing: 3 h; totally 12 h 30 min). Significantly, as other reports have shown that combining multiple independently isolated mutants produced additive effect [15], our strategy has a great potential for rapid scanning of all CDRs to find promising mutations before combinatorial mutagenesis. In addition to improvement in affinity, it is also possible to simultaneously attain knowledge about substitutions which reduce or eliminate Ag-binding activity by examining negative mutants in the pool without extensive generation of individual mutants [16]. Since the DNA in the system comprises an expression-ready unit, its straightforward cloning, such as blunt-end cloning, is beneficial for in vivo over-expression. It should also be noted that we were able to distinguish even modest increases in affinity while it was reported that panning by phage display did not easily discriminate between antibodies with modest differences in affinity (two- to three-folds) and the wild type usually dominate the selected population [13]. Size of a library generated by the SIMPLEX could be expanded by several magnitude by

using a modified SIMPLEX-based protocol, which has already been proposed and practically demonstrated [12], and a thermocycler with higher capacity (e.g. 1536 reaction/plate or more).

Besides the high throughput and advantages mentioned above, our system has other attractive features. The genotype–phenotype linkage is well preserved through the position of wells on plates and DNA is more stable than RNA, which is used in other in vitro systems [6]. Particularly, by simple DNA template construction, our strategy is compatible with direct enzymatic activity screening, while other in vitro selections of enzymes require techniques that couple enzymatic activity to selection protocols based on affinity chromatography [17–19]. Selections of lipases with reversed enantioselectivity and manganese peroxidase with improved H_2O_2 stability by the SIMPLEX were already reported [20–22]. Coupling of gene recombination technology such as DNA shuffling with the SIMPLEX will enable the searching for novel catalysts in a high throughput manner. In the near future, the SIMPLEX with a precise liquid handling system and an array technology [23–26] could be a powerful tool for combinatorial protein library screenings.

Acknowledgements

This work was financially supported by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (no. 12450332). We thank Dr. Yoshio Katakura for instruction on K_d measurement by ELISA and Dr. Kouhei Tsumoto for advice on in vitro refolding of antibody.

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