Simple method for production of randomized human tenth fibronectin domain III libraries for use in combinatorial screening procedures

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Challenges such as the rapid development of detection reagents for emerging or engineered pathogens, the goal of identifying probes for every protein in the human proteome, and the development of therapeutic molecules require systems for development of epitope binding molecules that are faster and cheaper than conventional antibody development. To be practical and effective, antibody mimics must be small, stable molecules that contain exposed loops or surfaces that can be randomized and screened using selective combinatorial assays. The tenth human fibronectin type III domain (10Fn3) fits these requirements and has recently been developed as an antibody mimic for use in detection and therapeutic platforms. Previously described systems for working with 10Fn3 used PCR-based approaches to anneal multiple oligonucleotides to generate randomized 10Fn3 libraries. Here we describe a simplified approach for creating randomized 10Fn3 libraries and report the first use of a T7-based phage display system for screening these libraries.

Antibodies are by far the most commonly used molecules for detection of physical epitopes in biological research. They are used as research reagents, diagnostics, and therapeutics. However, because antibodies are produced in animals, their production is time consuming and expensive. Further, immunization of animals does not always assure production of a useful antiserum, especially if the antigen is toxic or nonimmunogenic. These limitations have driven the search for in vitro systems for generating synthetic antibodies and antibody mimics in the hope of developing a rapid, cost-effective, and efficient system for developing effective detection reagents. Indeed, alternative technologies are required for ambitious goals such as the ability to rapidly develop detection reagents for emerging or engineered pathogens and the proposal to develop detection reagents for every protein in the human proteome (1). In vitro-selected synthetic antibodies, including recombinant versions of native antibodies, Fab fragments, diabodies, and single-chain Fv domains have been extensively explored with some notable successes (2). However, the large and complex (multichain, glycosylated, disulfide-bonded) nature of antibody molecules complicates their use. Initially, short peptides (7- to 12-mers) isolated via phage display were found to have useful binding properties (3). While these systems are simple and effective, the short peptides selected are often limited by low binding affinities that are probably the result of limited contact area. More recently, small protein scaffolds have been developed to mimic antibodies in terms of binding specificity and affinity. Most of these protein scaffolds are based on small, stable, single-chain proteins that lack disulfide bonds to facilitate bacterial expression and purification. Furthermore, these protein scaffolds also contain exposed loops or surfaces that can be randomized without compromising the integrity of the scaffold.

The tenth human fibronectin type III domain (10Fn3) is one protein that is being used as a scaffold to engineer binding proteins (4–6). The 10Fn3 protein is relatively small (10 kDa), soluble, free of disulfide bonds, easily expressed in Escherichia coli, and very stable [melting temperature (Tm) = 82°C] (5–7). Although 10Fn3 is not homologous to immunoglobulins, it has an immunoglobulin-like fold with three solvent-exposed loops that are reminiscent of complementarity-determining regions (CDR loops) of antibodies (5,8). Most importantly, the 10Fn3 scaffold has been shown to tolerate randomization of these surface-exposed loops (4,5). Accordingly, these loops have been randomized to produce 10Fn3 protein libraries that can be screened for binders to macromolecules of interest. To date, 10Fn3 antibody mimics have been isolated via M13 phage display (5,9,10), yeast two-hybrid (11), yeast display (12), and messenger RNA (mRNA) display (8,13). Using these strategies, 10Fn3 binders have been identified for proteins of interest including human αβ3 integrin, vascular endothelial growth factor, the SH3 domain of human C-Src, and others (6,8–10,12,13). Following the isolation of specific 10Fn3 binding proteins, affinity maturation strategies have led to the development of 10Fn3 binding proteins with submicromolar to picomolar affinities (9–13).

The general strategy for producing randomized 10Fn3 libraries has been to piece together several DNA oligonucleotides that correspond to the 10Fn3 gene (5,8,9,12). Typically, amino acids within the BC and FG loops are randomized by replacing codons within these loops with NNNK or NNNS, where N is an equimolar mixture of alanine or histidine, K is an equimolar mixture of G and C, G and T, and S is an equimolar mixture of G and C. This allows any one of 20 amino acids to replace selected amino acids and reduces the frequency of stop codons to ∼3%. Previously described strategies synthesize 10Fn3 randomized libraries by annealing up to four pairs of overlapping oligonucleotides followed by PCR amplification of the full-length product. While this strategy has been used successfully (5,8,9,12), it requires several iterative steps of oligonucleotide stitching to build up the full-length 10Fn3 open reading frame (ORF), which can be cumbersome, especially if any optimization is required for the individual stitching steps.
Here, we present a novel simplified single-step method for synthesizing a recombinant \(^{10}\)Fn3 library, in which 14 amino acid residues are randomized between two loop regions. This method uses only two oligonucleotides and a single DNA synthesis reaction and is therefore faster, simpler, and more reliable than protocols requiring multiple rounds of PCR stitching. We also report the first use of a T7-based screen of a \(^{10}\)Fn3 library to identify binders to streptavidin. Our results show that this strategy is a faster and simpler alternative to previously described library construction and selection strategies for the \(^{10}\)Fn3 scaffold.

The conversion of two single-stranded oligonucleotides into a full-length double-stranded \(^{10}\)Fn3 gene fragment is shown in Figure 1. Oligonucleotides (Gene Link, Hawthorne, NY, USA; see legend to Figure 1) contained seven randomized (NNS) codons in the BC and the FG loops, and the randomized \(^{10}\)Fn3 gene was generated by annealing and extending the two oligonucleotides, which overlap by 20 nucleotides. This conversion was monitored after each annealing and extension cycle and found to be nearly complete after three cycles. Higher mass nontarget products accumulated significantly when more than three fill-in cycles were performed. Using this method, efficient production of \(^{10}\)Fn3 DNA for cloning could be accomplished in a single reaction tube in approximately 20 min.

Full-length fibronectin products were purified from 2.0% (w/v) agarose gels, digested with EcoR1 and HindIII, and then purified using PCR purification columns (Qiagen, Valencia, CA, USA) and ligated to EcoR1/HindIII-digested T7 DNA (Novagen, Madison, WI, USA) according to the manufacturer’s recommendation. The ligation reaction included 0.02–0.06 pmol insert DNA and 0.02 pmol T7 select vector and was allowed to incubate at 16°C for 10–16 h. The ligation reaction was packaged using T7 packaging extracts and titered according to the manufacturer’s recommendation.

Library construction was performed five times, and library diversities of \(3 \times 10^6\) to \(1 \times 10^7\) independent sequences were routinely produced. Following amplification, library titers were typically \(10^{12}\) plaque-forming units (pfu)/mL.

Individual plaques were picked with a sterile pipet tip and dispersed into 100 \(\mu\)L 10 mM EDTA, pH 8.0, for sequencing. The tubes were vortex-mixed and incubated at 65°C for 10 min, and then centrifuged at 14,000x g for 3 min to clarify. PCR amplification of \(^{10}\)Fn3 DNA from individual phage particles was carried out in 50-\(\mu\)L reactions containing 2 \(\mu\)L phage lysate and 5 pmol each of T7 select DOWN and T7 select UP primers (both from Novagen). PCR products were directly sequenced using BigDye Terminator (Applied Biosystems, Foster City, CA, USA) after treatment with ExoSAP-It (USB, Cleveland, OH, USA) according to the manufacturer’s instructions. Sequencing reactions were run on an ABI PRISM 3100 automated sequencer (from Applied Biosystems) at the New Mexico State University (NMSU).
Results of enzyme-linked immunosorbent assay (ELISA) for individually amplified \( ^{10} \text{Fn3-T7} \) phage clones eluted from streptavidin. The negative control is a randomly selected phage clone taken from the unselected library. \( ^{10} \text{Fn3} \), tenth human fibronectin type III domain; BSA, bovine serum albumin; A450, absorbance at 450 nm.

Figure 2. Results of enzyme-linked immunosorbent assay (ELISA) for individually amplified \( ^{10} \text{Fn3-T7} \) phage clones eluted from streptavidin. The negative control is a randomly selected phage clone taken from the unselected library. \( ^{10} \text{Fn3} \), tenth human fibronectin type III domain; BSA, bovine serum albumin; A450, absorbance at 450 nm.

Molecular Biology Core Facility. Sequences of 48 randomly selected plaques showed that 96% of the phage clones had the \( ^{10} \text{Fn3} \) insert, that each clone had unique FG and BC sequences, and that 40% of clones conformed to library design.

For comparison, we constructed \( ^{10} \text{Fn3} \) libraries using eight oligonucleotides as described by Lipovsek et al. (12). This method required optimization for all seven of the annealing/extension steps, making this procedure much longer and more complicated than the method we describe here. Comparison of these libraries showed that both methods produced similar diversities (~5 × 10⁶) and equal numbers of clones that conformed to library design (~40%). These results are comparable with those described in the report by Lipovsek et al. (12).

Libraries were screened for binders to streptavidin by incubating the amplified \( ^{10} \text{Fn3-T7} \) library \( \times 10^9 \) pfu/mL; diluted in Tris-buffered saline and Tween-20 (TBST) with 3% nonfat dry milk] on streptavidin-coated 96-well plates (Sigma, St. Louis, MO, USA) for 30 min. After washing five times with TBST, bound phage was eluted with 1% sodium dodecyl sulfate (SDS) for 20 min. Libraries were also screened against streptavidin-coated magnetic beads (Dynal Biotech ASA, Oslo, Norway). Ten microliters bead slurry were washed five times with 1.0 mL TBST, resuspended in 100 μL \( ^{10} \text{Fn3-T7} \) library \( \times 10^9 \) pfu/mL; diluted in TBST with 3% nonfat dry milk), and rotated at room temperature for 30 min. After washing five times with 1.0 mL TBST, bound phage was eluted with 1.0% SDS for 20 min. In each case, eluted phage were amplified, titrated, and used for the next round of screening (\( \times 10^9 \) pfu/mL). After three rounds of biopanning, individual plaques were amplified and used for enzyme-linked immunosorbent assays (ELISAs), which were performed on streptavidin-coated plates. Individually amplified phage clones were diluted 1:10 in TBST containing 3% (w/v) nonfat dry milk. One hundred microliters diluted phage were then added to the streptavidin plates and incubated for 30 min. Plates were washed five times with TBST, and then the T7 tail fiber monovalent antibody (Novagen) was added to each well at a dilution of 1:1000. The plate was incubated at room temperature for 1 h. The plate was then washed five times with TBST, and then rabbit anti-mouse immunoglobulin G (IgG) secondary antibody (Southern Biotech, Birmingham, AL, USA) was added to each well at a concentration of 1:1000. The plate was incubated at room temperature for 1 h and then washed five times with TBST. One hundred microliters 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) were then added to each well and incubated for 15 min at room temperature. The reaction was stopped by adding 100 μL 4.5 M H₂SO₄, and the plate was read at 450 nm in a plate reader (BioTek Synergy HT, BioTek Inc., Winooski, VT).

As shown in Figure 2, about half of the clones recovered after three rounds of panning showed significant binding to streptavidin, whereas binding was not detected in any phage clones picked from a nonselected library. Furthermore, streptavidin binding phage clones did not bind to bovine serum albumin (BSA) or collagen. It should be noted that \( ^{10} \text{Fn3} \) phage clones eluted from streptavidin-coated magnetic beads were also able to bind streptavidin in a plate format. This observation, and the lack of binding to BSA and collagen-coated ELISA plates, indicates that these \( ^{10} \text{Fn3} \) clones bound specifically to streptavidin and not to the solid-state support used during selections (polystyrene for plates and \( \gamma \text{Fe}_2\text{O}_3/\text{Fe}_3\text{O}_4 \) for magnetic beads).

Sequencing of streptavidin binding clones showed that all of these clones contained full-length \( ^{10} \text{Fn3} \) ORFs, that several clones were recovered multiple times, and that many clones contained similar residues in the BC and FG loops (Table 1). For example, many clones contained aromatic residues in the BC loop and positively charged residues in the FG loop. These similarities in the streptavidin binding clones indicate that a common streptavidin binding motif was selected from the randomized library. Clone B6 was consistently the best streptavidin binder in all experiments and was selected for further study.

To verify that the observed binding was due to the displayed \( ^{10} \text{Fn3} \) protein and not the phage on which it was displayed, we overexpressed and purified the \( ^{10} \text{Fn3} \) coding sequence from clone B6. In brief, B6 was amplified using T7-UP and T7-DOWN primers, and the amplified product was digested with EcoR1 and HindIII, gel-purified, and ligated to pET21B in frame with the T7 and His6 tags (Novagen). The resulting plasmid was transformed into BL21 pLYS (Invitrogen, Carlsbad CA, USA). Expression of the B6-\( ^{10} \text{Fn3} \) protein was induced with 1 mM isopropyl-\( \beta \)-thiogalactopyranoside.
Benchmarks

(10) and purified by batch purification using Ni-NTA agarose (Qiagen) according to the manufacturer’s instructions. The B6-Fn3 protein contained a C-terminal His tag and an N-terminal T7 tag for purification and detection, respectively. The purified T7-tagged B6-10Fn3 protein was tested for binding to streptavidin, BSA, and collagen by ELISA as described above, except the primary antibody was replaced by a T7 tag horseradish peroxidase (HRP)-conjugated antibody (Novagen). The ELISA readings from streptavidin-coated wells were ~20 times higher than those from either BSA- or collagen-coated wells, both of which were similar to the uncoated negative control wells (data not shown). The standard deviation between triplicate wells was <2% of the signal for all samples, showing that the results were highly reproducible. This demonstrates that the 10Fn3 protein was responsible for the binding observed in the phage-based ELISA assays and that the selected 10Fn3 protein binds specifically to streptavidin.

By successfully using T7 to display 10Fn3 antibody mimics, we now add T7 to the list of display strategies that have been used to isolate 10Fn3-based binding proteins. T7 has several advantages over M13, including producing plaques within 2 h, being lytic rather than lysogenic, and having the ability to display some molecules that are incompatible with M13, due to interference with tail fiber function. Although the diversity of T7 libraries is lower than that which can be obtained in other systems, such as M13, due to the lower cloning efficiency of T7 systems, we have shown that library sizes attained in these experiments were sufficient to isolate streptavidin binding 10Fn3-T7 clones. These results are also consistent with other reports (12).

This library construction strategy should also be useful for efficiently randomizing other scaffold proteins and compatible with other display technologies (M13, two-hybrid, mRNA display). Furthermore, this library construction method could also be used for simple and rapid generation of secondary libraries used for affinity maturation. Simplified systems for the production of antibody-like binding molecules, such as the system we describe here, will enable advances in high-throughput systems for the efficient generation of target binding proteins and ultimately find broad utility for applications in which antibodies and antibody production systems are not feasible.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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