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Tandem phage-display for the identification of non-overlapping binding pairs of recombinant affinity reagents

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ABSTRACT

The 'sandwich' binding format, which uses two reagents that can bind simultaneously to a given analyte, is the gold standard in diagnostics and many biochemical techniques. One of the bottlenecks in creating a sandwich assay is identifying pairs of reagents that bind non-competitively to the target. To bridge this gap, we invented Megaprimer Shuffling for Tandem Affinity Reagents (MegaSTAR) to identify non-competitive binding pairs of recombinant affinity reagents through phage-display. The key innovation in MegaSTAR is the construction of a tandem library, in which two reagents are randomly-displayed on the phage surface. This is accomplished by using a pool of 300-nucleotide long 'megaprimers', which code for previously-selected reagents, to prime second strand synthesis of a single-stranded DNA template and generate millions of pair-wise combinations. The tandem library is then affinity selected to isolate pairs that both reagents contribute to binding the target. As a proof-of-concept, we used MegaSTAR to identify pairs of fibronectin type III monobodies for three human proteins. For each target, we could identify between five and fifteen unique pairs and successfully used a single pair in a sandwich assay. MegaSTAR is a versatile tool for generating sandwich ELISA-grade and bispecific reagents.

INTRODUCTION

One of the most versatile binding formats for biomarker detection and diagnostics is the 'sandwich' assay. In this assay format, one antibody is typically immobilized and used to capture the antigen from a complex mixture (i.e. serum, urine etc.), while another antibody is used for detection of the antigen. Thus, two independent, simultaneous binding events must occur to obtain a signal, making it an extremely specific and, therefore, powerful assay format. In fact, the sandwich format is the foundation for many research techniques, including AlphaScreen (1), time-resolved fluorescence energy transfer (FRET) (2) and the proximity ligation assay (3). In addition, the sandwich assay is considered the 'gold standard' in diagnostics, with the most familiar application of the sandwich binding format being the lateral flow assay. This point-of-care diagnostic format is used in at-home pregnancy tests, which detect human chorionic gonadotropin, a biomarker of pregnancy (4), and is also used to identify the presence of cardiac troponins T or I in the blood of patients who are suspected of heart attack (5). The sandwich format has also been used to detect biomarkers of Ebola (6), cancer (7,8), HIV (9,10) and *Streptococcal pharyngitis* (11), commonly referred to as 'strep throat'. Due to its versatility and specificity, it is also used to generate assays that can detect/monitor new and emerging biological threats, such as Zika virus (12). Despite its extensive role in clinical and basic research, generating reagents that can work in the sandwich assay format is accomplished via trial and error, which is time-consuming and costly.

Currently, there are multiple avenues for generating sandwich assays. The first might be to purchase or produce 10 monoclonal antibodies, for example, and test all 45 pairwise combinations to determine if a sandwich pair exists. There are many inherent limitations to this approach: one is limited by what is commercially-available, the reagents are difficult to customize, as they are produced in animals, and a huge percentage of commercially-available antibodies are promiscuous (13). Alternatively, one might use phage-display to produce recombinant affinity reagents and then identify pairs of clones that will work in a sandwich format. While this approach overcomes the inherent limitations of using animal-derived monoclonal and polyclonal antibodies (14,15), the pairs themselves are still identified in a time-intensive, trial-and-error manner. Therefore, a method that directly yields potential sandwich pairs—as part of the phage-display affinity selection process—will fill an important technology gap.

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Table 1. Selection target information

Selection target	Uniprot ID	Abbreviation	Biological process	Region used for selection (amino acids)
Cop9 signalosome subunit 5	Q92905	COPS5	Ubiquitination	9–309
p21-associated kinase 1	Q13153	PAK1	Proliferation, apoptosis, migration	270–521
Rho GTPase-activating protein 32	A7KAX9	RICS	Signal transduction	367–577

We have developed a method termed 'Megaprimer Shuffled Tandem Affinity Reagents' (MegaSTAR) in which we construct tandem display libraries from combinatorially-combined coding regions of previously-selected affinity reagents. The scaffold protein we used in this work is the fibronectin type III domain (FN3), often referred to as the 'monobody'. This scaffold is small (i.e. ∼10 kDa), thermal stable (i.e. T_m = 90°C), overexpressed well in *Escherichia coli* (i.e. 60 mg/l), and amenable to phage-display and protein engineering (16,17). Using MegaSTAR, one can create and test millions of different pair-wise combinations simultaneously and identify pairs that recognize distinct areas on the selection target (i.e. pairs that could be used in the sandwich assay). For proof-of-concept, we constructed MegaSTAR libraries for monobodies affinity-selected to three human protein targets: Cop9 signalosome subunit 5 (COPS5), p21-associated kinase 1 (PAK1), and Rho GTPase activating protein 32 (RICS) (Table 1). With the tandem display libraries, we identified between five and fifteen unique binding pairs of monobodies for each target. We also showed with sandwich ELISA that matched pairs recognized non-overlapping epitopes for all three targets. The three selected pairs, fused by the linker, worked as tandem dimers and exhibited affinities of 1.5–22 nM to their targets, demonstrating the second utility of the pairing reagents as potent bispecific reagents.

MATERIALS AND METHODS

Construction of tandem phagemid vector (pKG25) and generation of uracilated template DNA

Phagemid DNA, encoding a single fibronectin domain (FN3) coding region and an N-terminal Flag epitope (DYKDDDDK) (18), was linearized with AatII and agarose gel-purified using a Zymoclean Gel DNA recovery kit (Zymo Research). A double-stranded DNA (gBlock™, Integrated DNA Technologies), coding for the Myc epitope (EQKLISEEDL), a second FN3 coding region, and a linker peptide sequence (5 × GGGGS), was synthesized. The coding sequence was codon-optimized for *E. coli*. This double-stranded DNA was introduced into the linearized vector upstream of the existing FN3 sequence through homologous recombination (19). The tandem phagemid (referred to as pKG25 from here on) was then electroporated into *E. coli* strain CJ236 (New England BioLabs), which yielded uracilated, single-stranded tandem phagemid template DNA for mutagenesis with previously published methods (18,20). Briefly, the CJ236 cells were grown in 2 × YT medium (per liter 16 g tryptone, 10 g yeast extract, 5 g NaCl) plus carbenicillin (50 μg/ml) to mid-log stage at 37°C and infected with M13-KO7. Infected cells were supplemented with kanamycin (50 μg/ml) and uridine (0.25 μg/ml) and incubated at 25°C and 200-250 RPM for 20-22 hours of phage amplification. The next day, the culture was centrifuged at top speed to remove the bacterial cells. The supernatant was mixed with PEG-8000 (Final 5% v/v) plus 300 mM NaCl (Final 50 mM) for 15 min incubation on ice, which was then centrifuged at top speed to pellet the phage particles. Phage pellet was suspended in PBS and processed with the QIAprep Spin M13 kit (Qiagen) for purification of uracilated single-stranded DNA.

MegaSTAR to generate tandem, phage-display libraries

Soluble protein for COPS5 and RICS was provided by Dr Susanne Gräslund's group within the Structural Genomics Consortium. Purification of PAK1 protein and affinity selection of the three targets were performed as described previously (21). DNA from the resulting clone pools was isolated via the Wizard DNA Miniprep Kit (Promega) and used as template in polymerase chain reaction (PCR) to amplify the FN3 coding regions (5′-ATGGCCGTTTCTGATGTTCCG-3′; 5′-GCTGGTACGGTAGTTAATCGAGATTGG-3′). These double-stranded segments of DNA were subsequently gel-purified and phosphorylated using T4 polynucleotide kinase (New England BioLabs). The phosphorylated 'megaprimers' were heat-denatured to separate the strands and then annealed to the single-stranded, uracilated tandem display phagemid template at a 10:1 molar ratio. The heteroduplex DNA was synthesized in the presence of T4 ligase and T7 DNA polymerase (New England Biolabs) and then was purified with the QIAquick PCR purification kit (Qiagen). The purified DNA was electroporated into the *E. coli* strain TG1 (18,20) electrocompetent cells from Lucigen. The transformed cells were diluted and plated onto 2 × YT medium supplemented with carbenicillin (50 μg/ml). The number of transformants in each library was estimated through serial dilutions. An enzyme linked immunosorbent assay (ELISA) was used to identify phage clones with open reading frames and thereby determine the recombination rate of the library (21). Phage libraries were amplified as previously described (18), titered, flash-frozen in liquid nitrogen and stored at –80°C.

Affinity selection using tandem libraries

Affinity selection was performed with a Kingfisher mL magnetic bead particle processor (Thermo Fisher). In the first round of selection, the biotinylated target (500 nmol) was added to streptavidin-coated paramagnetic beads (Promega) for 15 min, and then the non-specific binding sites on the beads were blocked with excess casein (Thermo Fisher Scientific) for 1 h. Next, ∼5 × 10^{12} phage particles were diluted in PBS and mixed with the target-coated beads

for 1 h, after which the beads were washed three times to remove any non- or weak-binding phage. Bound virions were eluted with 50 mM glycine (pH 2.0) and then transferred from the Kingfisher test strip to a 1.5 ml microfuge tube containing neutralization solution (Tris–HCl, pH 10). Recovered virions were then used to infect TG1 cells at mid-logarithmic phase (optical density of 0.4 at 600 nm wavelength) growth. Infection lasted 1 h in a shaking incubator at 37°C and 150 RPM. The infected cells were then spread onto 2 × YT agar plates and incubated at 30°C overnight.

The next day, the lawn of bacteria was scraped in 2 × YT media supplemented with glycerol (16%, v/v) and carbenicillin (50 μg/ml). A new culture was started from an aliquot of cells and grown at 37°C until the cultured reached mid-logarithmic phase. At this point, the cells were infected with M13-KO7 helper phage (New England BioLabs) for 1 h at 37°C and 150 RPM. After infection, the cells were spun and resuspended in fresh 2 × YT medium, which was supplemented with carbenicillin (50 μg/ml) and kanamycin (50 μg/ml). The infected culture was placed in shaking incubator (200 RPM) for 22 h at 25°C for phage amplification. For the second round of selection, the amount of target was reduced (500 nmol → 50 nmol) and additional wash steps were implemented (3 → 9 times). Virions were recovered, as described above, and used to infect TG1 cells, which were spread onto petri plates containing 2 × YT medium supplemented with carbenicillin (50 μg/ml) and 1.5% agar.

Characterizing selection output: monoclonal ELISA and sequencing

Ninety-five output clones were picked from each library and screened by ELISA (22) for their ability to bind the selection target. DNA from binding clones was isolated via the Wizard DNA Miniprep Kit (Promega) and sequenced using the following primer, 5′-CGCTGGCTGGTTTAGTTTTAGCGT-3′. The clones were sorted based on the amino acid sequences of the variable regions of the displayed monobodies.

Cloning and purification of soluble monomers from tandem dimer

To generate soluble forms of monobodies, a set of oligonucleotides (see Supplementary Data for sequences) was used to amplify the FN3 coding regions from pKG25 and to add regions of homology flanking the cloning sites on the destination vector. The insert was gel-purified and cloned into the pET14B-SUMO expression vector (21) via SLiCE (19). The SLiCE reaction was electroporated into BL21 *E. coli* cells.

The Overnight Express™ Autoinduction System (Millipore) was used to induce expression of recombinant His6-SUMO-FN3 in transformed BL21 cells. After 24 h of induction, the cells were lysed via sonication, and the fusion proteins were purified to >95% homogeneity via immobilized metal affinity chromatography (IMAC) using His60 Ni Superflow resin (Clontech). The soluble protein was eluted into 300 mM imidazole, 300 mM NaCl and 50 mM sodium phosphate (pH 7.4). The purity of the overexpressed monobodies was analysed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the samples were subjected to thermofluor analysis to confirm their proper folding (23). Protein samples were flash frozen in liquid nitrogen and stored at –80°C.

Competition ELISA to estimate the affinities of tandem dimers and constituent monomers

A Nunc-Maxisorp® plate was coated with NeutrAvidin (5 ng/μl) overnight at 4°C. Meanwhile, phage particles displaying tandem dimers were amplified from *E. coli* as previously described and mixed with varying amounts of non-biotinylated selection target (i.e. competitor). The microtiter plate was blocked and subsequently coated with biotinylated selection target (5 ng/μl). The phage/competitor mixture was added to the plate and allowed to incubate for 1 h. The plate was then washed with PBS plus 0.1% Tween-20 (PBST), followed by detection of bound virions via anti-M13-phage antibody that is conjugated to horseradish peroxidase (HRP) (1:5000 dilution; GE Healthcare). The ELISA signal (405 nm) was recorded 30 min after addition of the substrate. The raw signals were used to calculate the '%-binding' values at each competitor concentration. These values were plotted using Origin software to obtain a curve, from which the half maximal inhibitory concentration (IC_{50}) for each tandem dimer/monomer was interpolated. To estimate the affinities for the COPS5 binding monomers, the monobody coding regions were cloned into the pKP300 phagemid via SLiCE (19), and the competition ELISA was repeated as described above.

Phage-protein sandwich assay

Capture monobody proteins were coated onto Nunc-Maxisorp® 96-well plates overnight at 4°C. The next day, the plates were blocked with casein followed by incubation with the protein target for 1 h. Phage particles displaying the detection monobody were then added to the plate for 1 h. The plates were washed with three times with PBST, followed by incubation with the anti-M13-HRP antibody (1:5000; GE Healthcare) for 1 h. After the plates were washed three times with PBST, H_2O_2 and 2′,2′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid were added to the wells. The optical absorbance of wells (405 nm) was measured 30 min after addition of chromogenic substrate. The concentrations of capture monobody and target were optimized to maximize the signal-to-noise ratios.

RESULTS

MegaSTAR is used to generate large tandem phage-display libraries

The first step toward implementing MegaSTAR was to construct a vector, pKG25, for tandem display on the surface of bacteriophage M13. The phagemid vector (Figure 1A) was designed to contain the following features: N-terminal c-Myc tag, the first fibronectin type III (FN3 I) coding region, a linker region (5 × GGGGS), a Flag-tag and a second FN3 coding region (FN3 II), fused to the truncated minor coat protein III (P3) of M13 phage. Two stop codons were

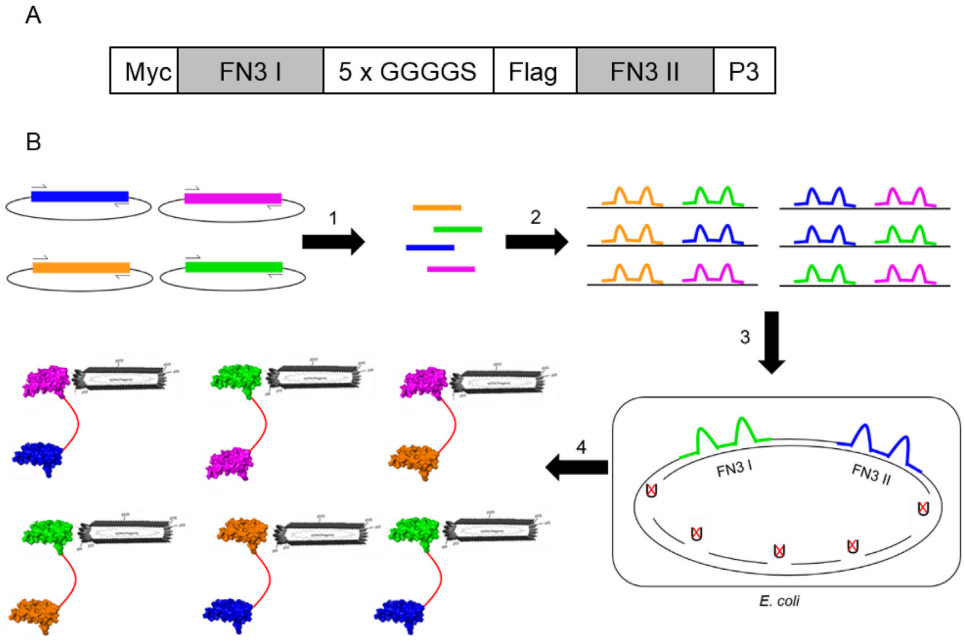


Figure 1. Megaprimer shuffling for tandem affinity reagents (MegaSTAR). (**A**) The design of the pKG25 phagemid vector for displaying the tandem FN3 dimer. (**B**) MegaSTAR workflow: (1) Coding regions from a pre-selected clone pool are amplified via PCR to generate megaprimers. (2) Megaprimers are heat-denatured into two strands, allowing the anti-sense strand to anneal to the single-stranded, uracilated tandem phagemid vector. The template strand contains stop codons within the variable regions, which prevent the translation of non-recombinants. (3) Kunkel mutagenesis is used to generate heteroduplex DNA, which is electroporated into *E. coli* to facilitate *in vivo* degradation of template strand. (4) Virions displaying randomly linked tandem dimers (linker region shown in red) are amplified from the cells to create a tandem phage-display library.

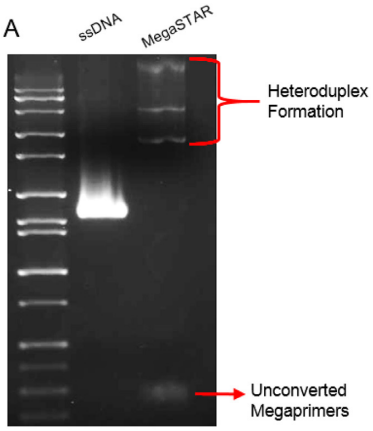
positioned within each FN3 coding region to prevent translation of non-recombinants. To promote flexibility between each monobody, we designed a linker that contains five repeats of a four-glycine-one-serine sequence (GGGGS).

Once the uracilated, single-stranded DNA template was purified, the next step was to identify targets for the proof-of-concept experiment. Previously, we used a first generation (G1) FN3 library to isolate binding clones to various targets (21); from this set, we chose output clone pools for Cop9 signalosome subunit 5 (COPS5) and p21-associated kinase (PAK1) because multiple binding motifs were present in both pools (unpublished results). In addition to these two targets, we used a clone pool of second generation (G2) FN3 library (unpublished results) that had been affinity selected with Rho-GTPase activating protein 32 (RICS). The G2 library contains a third randomized region, the DE loop, and the length of all three loops (BC, DE and FG) is varied between 3 and 13 amino acids.

With the template and clone pools in hand, the next step was to perform MegaSTAR (Figure 1B). First, the coding regions present in the pool of clones, recovered after the second round of affinity selection for each target, were amplified by PCR. Next, the double-stranded 'megaprimers' were heat-denatured and annealed to the single-stranded, uracilated tandem display phagemid template (pKG25). During this step, millions of different pair-wise combinations were generated simultaneously. After annealing, the primed DNA strands were extended through *in vitro* DNA synthesis, and the reaction products were examined by agarose gel electrophoresis (Figure 2A) and electroporated into *E. coli* cells. After mutagenesis was completed, we proceeded with characterizing and amplifying the tandem libraries; the size for each library ranged from 2.1×10^7 to 5.3×10^8 (Figure 2B) members, which ensured that many different pair-wise combinations were present in each library.

Selection of tandem libraries identifies many potential unique pairs

We hypothesized that phage-displayed tandem dimers would bind more tightly if both displayed monobodies could bind simultaneously to the target. Therefore, we relied on the increased stringency of the two round affinity selections to identify such clones. After selection, we picked 95 random clones from each pool and evaluated their ability to recognize the selection target. We confirmed with ELISA that >92% of the output clones bound their cognate target. We then sequenced the coding regions of positive binders from each selection output and sorted them by their amino acid sequences of the variable regions (Figure 3A). The first property we noticed was the low incidence (i.e. 5–7%) of homodimers in each pool. We then examined the sequencing data to identify potential unique binding pairs; our criterion for selecting a tandem heterodimer as a potential bivalent binder is that each monomer of a given heterodimer must appear more than once in the pool of sequenced clones. For COPS5, PAK1 and RICS, the data suggested 8, 5 and 15 unique binding pairs, respectively (paired FN3 sequences for RICS are shown in Figure 3B; for COPS5 and PAK1, sequences are provided in Supplementary Figure S1).

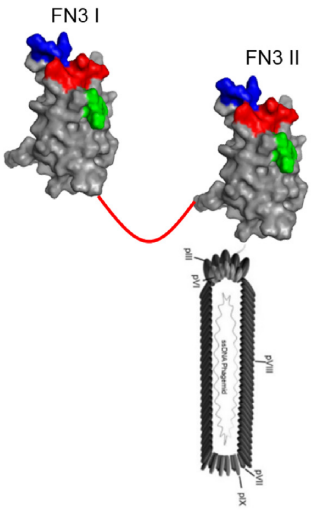


B

Selection Target	Recombination Rate	# of Transformants	Estimated Size of Tandem Library
COPS5	34.4%	1.5 X 10^9	5.3 X 10^8
PAK1	4.0%	5.3 x 10^8	2.1 X 10^7
RICS	25.0%	7.5 X 10^8	1.9 x 10^8

Figure 2. Tandem library synthesis via MegaSTAR. A) Representative agarose gel image with MegaSTAR product. Template (ssDNA) is included for comparison. Heteroduplex formation and unconverted megaprimers are denoted by red bracket and arrow, respectively. B) Estimation of tandem library size for each target. Recombination rate is the percentage of recombinant transformant in each library.

A



B

Pair	BC Loop	DE Loop	FG Loop	BC Loop	DE Loop	FG Loop	Freq.
1	WAEYYFPS	GYYY	PHYYYHYSTS	KVSSYNYT	WSYYYSSY	DYGQYSYTWS	1
2	YYDYYGE	YYSS	DYYNRAGDYG	GGWYEY	GYYGGGG	YFYSRSSQ	11
3	KVSSYNYT	WSYYYSSY	DYGQYSYTWS	YYDYYGE	YYSS	DYYNRAGDYG	9
4	WYKYW	GSKS	ALLSK	YYDYYGE	YYSS	DYYNRAGDYG	6
5	YGSYEYND	YYSS	DYSYQYGAGPGY	KVSSYNYT	WSYYYSSY	DYGQYSYTWS	2
6	YYDYYGE	YYSS	DYYNRAGDYG	GYYVYYQG	GYYG	DYGYSLYYDS	2
7	YGSYEYND	YYSS	DYSYQYGAGPGY	YYDYYGE	YYSS	DYYNRAGDYG	1
8	YNFYWFPE	GYYW	GSYSFYPPVY	KVSSYNYT	WSYYYSSY	DYGQYSYTWS	2
9	NYTWYYD	WYGG	YGGSSYA	GGWYEY	GYYGGGG	YFYSRSSQ	1
10	IYFDNSI	YYGG	FYGYGYSDSYGY	KVSSYNYT	WSYYYSSY	DYGQYSYTWS	3
11	NYTWYYD	WYGG	YGGSSYA	KVSSYNYT	WSYYYSSY	DYGQYSYTWS	1
12	IYFDNSI	YYGG	FYGYGYSDSYGY	YYDYYGE	YYSS	DYYNRAGDYG	1
13	IYFDNSI	YYGG	FYGYGYSDSYGY	GGWYEY	GYYGGGG	YFYSRSSQ	1
14	WYKYW	GSKS	ALLSK	KVSSYNYT	WSYYYSSYWS	DYGQYSYTWS	1
15	WAEYYFPS	GYYY	PHYYYHYSTS	GGWYEY	GYYGGGG	YFYSRSSQ	1

Figure 3. Sequencing results after affinity selection of the tandem library with RICS. (**A**) Cartoon representation of phage-displayed tandem dimer. Variable regions on each fibronectin domain are coloured (i.e. red = BC loop, green = DE loop, blue = FG loop). (**B**) Amino acid sequences for variable regions of each unique pair identified in selection against RICS. Motifs are differentiated by fill color while unique pairs are numbered. The frequency at which a given pair occurs among the sequenced clones is also shown.

Tandem dimers bind tighter than constituent monomers

From the sequencing data alone, it was unclear if the constituent monomers of a given tandem dimer bound distinct epitopes on the target. To address this question, we focused on a prevalent COPS5 binding tandem clone, pair 5 (Supplemental Figure S1B), which was present 18 times in the sequenced clones, and compared its apparent affinity with that of its constituent monomers. As seen in Figure 4, the estimated affinity of the tandem dimer is approximately 5- to 10-fold better than the two monomers. The tighter binding of pair 5 compared to its two component monobodies for COPS5 suggests that the apparent improved affinity of the tandem clone is due to avidity. As the affinity increase from two monomers to the linked dimer is modest, we suspect that either the two monomers have partially overlapping epitopes or the current linker does not permit optimal simultaneous binding of these two monomers to the target.

For the two tandem dimers isolated most frequently from the PAK1 (pair 1, upper FG sequence, Supplemental Figure S1A) and RICS (pair 2, Figure 3B) libraries, the estimated affinities of each were 1.5 nM (data not shown) and 22 nM (Figure 5), respectively. The affinities of the two monomers consisting of the PAK1 dimer were also measured. One had an estimated affinity of 240 nM (data not shown), while the other monomer had too weak binding to generate a binding curve. The 160-fold affinity increase in apparent affinity suggests that for these two monomers, the linker promotes

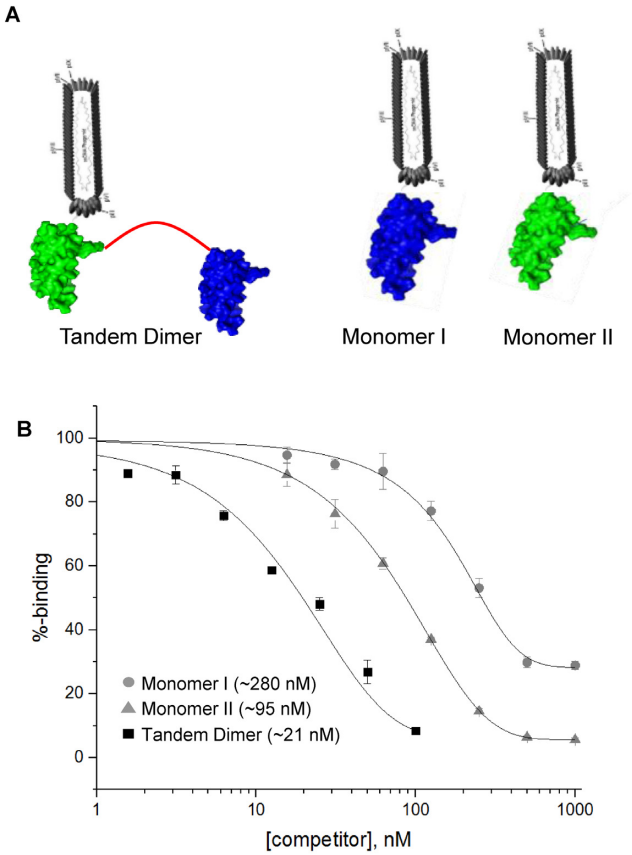


Figure 4. Comparing estimated affinities of a tandem dimer and its constituent monomers. (**A**) Cartoon representations of the reagents. The tandem dimer was selected against the target COPS5. (**B**) Competition phage-ELISA was used to estimate the affinity for each construct. Estimated affinities for each monomer and the tandem dimer are noted.

much optimized simultaneous binding of both domains to the target, thereby enhancing the avidity effect.

In some cases, the orientation of the monomers does not affect apparent affinity

Another characteristic of the selection output was the propensity for a given pair of monobodies to appear in both orientations (i.e. A–B and B–A) within a given pool. This finding suggested that for some pairs of monobodies the two orientations bind equally well. We estimated the affinities for two tandem dimers in the RICS binding pool, which were composed of monobodies in A–B and B–A orientations (Figure 5A). Interestingly, these clones had nearly identical estimated affinities (21 and 22 nM; Figure 5B). Thus, it appears in this instance, orientation of the monomers does not affect binding strength. We observed other clones with opposite orientations in both the PAK1 and COPS5 pools as well, indicating that MegaSTAR is very efficient and inclusive at sampling many pair-wise combinations of monobodies.

Constituent monomers from tandem clones can be used in sandwich ELISA

To confirm that the constituent monomers of the selected dimers can bind distinct epitopes on the target simultaneously, we evaluated them in a sandwich ELISA format. In our experiment, a soluble form of one FN3 was passively immobilized on the surface of a microtiter plate well, and the second FN3 was displayed on the surface of bacteriophage (Figure 6A). This assay format (24) is efficient for rapidly testing matched pairs, as it reduces the need to overexpress and purify both FN3 monobodies of a dimer. As seen in Figure 6B, all three pairs (pair 5 for COPS5, pair 1 for PAK1 and pair 2 for RICS) recognized their cognate targets in the sandwich ELISA, indicating that the tandem dimers are bispecific and recognize non-overlapping epitopes on the targets. The three pairs were also selective in their binding, as they did not react with an unrelated human protein, centaurin-γ 3 (CENTG3), nor did they pair with an unrelated FN3 (Supplementary Figure S2). The second most frequent pair isolated from the RICS tandem library (pair 3), was also separated into its constituent monomers and successfully used in sandwich ELISA (data not shown).

DISCUSSION

Identifying pairs of recombinant affinity reagents that can work in the sandwich assay format can be a difficult, costly and time-consuming process as one must identify, express and purify each individual candidate and subsequently test multiple pairs of reagents at random. To increase the throughput of generating such reagents, we developed MegaSTAR. Our method utilizes long oligonucleotides, which code for previously-selected variants, to randomly prime a tandem phagemid containing two identical priming sites. This approach is vastly superior to current methods for several reasons. First, during MegaSTAR, millions of pair-wise combinations are created in a single test tube, thereby eliminating the need to test individual pairs of clones after the traditional affinity selection process has been completed. Second, MegaSTAR is inherently biased for identification of non-overlapping binding pairs. Third, this approach is 'scaffold agnostic,' in that the MegaSTAR library can display different entities, such as FN3, designed ankyrin repeat proteins (DARPins) (25), human single-chain Fragments of variable regions (scFv) (26), or short linear peptides (27,28), and in various combinations. It is also reasonable to assume that MegaSTAR could be used in conjunction with other display technologies, such as ribosome (29) or yeast display (30,31).

Our method has been successfully applied to three human proteins (COPS5, PAK1 and RICS). For all three targets, 5–15 unique pairs were identified. Using RICS as a target, we identified 15 potential sandwich pairs after sampling a relatively small number of clones. This fact suggests that our approach provides extensive epitope coverage. Affinity-selected binders may recognize epitopes on the target that can be masked by post-translational modifications or conformational changes *in vivo*. Thus, our ability to identify many potential pairs essentially ensures that one can identify at least several pairs that can be used to detect the endogenous biological samples.

A

BC Loop 1	DE Loop 1	FG Loop 1	BC Loop 2	DE Loop 2	FG Loop 2
YYDYYGE	YYSS	DYYNRAGDYG	GGWYEY	GYYGGG	YFYSRSSQ
GGWYEY	GYYGGG	YFYSRSSQ	YYDYYGE	YYSS	DYYNRAGDYG

B

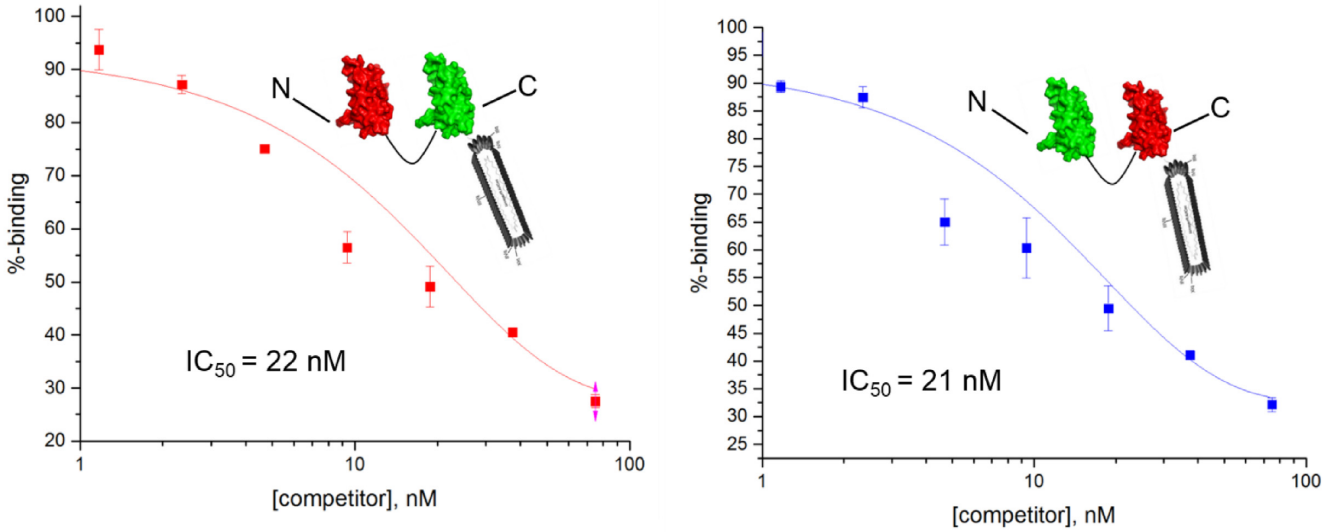


Figure 5. Comparison of estimated affinity of the same pair in opposite orientations. (**A**) RICS pair 2 (listed in Figure 3B) was identified in both orientations during sequencing. (**B**) IC_{50} curves for both tandem clones were generated via phage competition ELISA, with estimated IC_{50} values shown in the figure.

In addition to being used in sandwich ELISA, tandem clones identified via MegaSTAR could also be used as bispecific reagents: a single molecule that binds two epitopes on its target. This design is very common for inhibitor creation, as two epitopes on the target molecule are bound. In addition, one could use our technology to identify bispecific reagents that bind two unique antigens (32,33). In such an experiment, one can isolate two pools of binders enriched by affinity selections against two different antigens, perform the MegaSTAR, and affinity select the new tandem library against the two antigens simultaneously. Such two-target bispecific reagents have been used to facilitate the degradation of tumours via immune cells (34) and to inhibit epidermal growth factor receptor and insulin-like growth factor-I receptor simultaneously (35). Another potential application for MegaSTAR-derived bispecific reagents could be immunoprecipitation, especially for multi-component protein complexes bound to nucleic acids, such as chromatin (36) and ribonucleoprotein (37). To study these molecules and the nucleic acids contained therein, one requires reagents of extremely high specificity and affinity for challenging experiments such as CHIP-SEQ (36) or other RNA-related studies (38). MegaSTAR could be used to generate bispecific reagents targeting two constituent proteins of the complexes, achieving high specificity and affinity simultaneously and thereby enhancing the recovery of immunoprecipitation.

In the future, we plan to improve MegaSTAR in a few ways. It would be interesting to try linkers of various lengths (up to 50 amino acids) and various compositions (semi-rigid, rigid, helical, etc.), as it is likely that optimal linker length and configuration would vary between targets. We can also add a second or third linker and generate reagents that can target three or four epitopes simultaneously. We also plan to affinity mature monomers from a given pair to test if sensitivity of the sandwich assay improves because of increased binding strength of each component affinity reagent.

MegaSTAR is an innovative method that will change the paradigm for discovery of pairwise affinity reagents. First, it addresses a technology gap by providing renewable recombinant affinity reagents that work as pairs in binding protein targets. As their discovery is part of the affinity selection process, no trial and error is required to find pairs. Second, the ability to interrogate large numbers of epitopes increases the ability to develop effective sandwich assays;

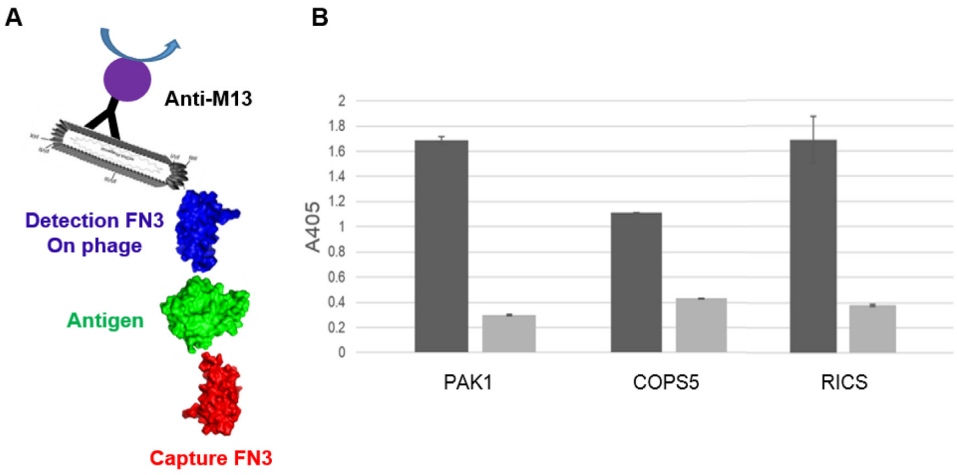


Figure 6. Phage-sandwich ELISA using constituent monomers. (**A**) Protein-phage sandwich ELISA setup. Capture reagent (red) is passively adsorbed directly to the ELISA plate while the detection reagent (blue) is displayed on M13 bacteriophage. Detection of the target (green) is accomplished via anti-M13 antibody (black) conjugated to HRP (purple). (**B**) Phage-sandwich ELISA data for a linked pair from each tandem library. Capture and target concentrations were optimized to maximize signal-to-noise ratio. Dark grey bar represents the signal using the selection target and light gray bar represents the signal using an unrelated protein target (CENTG3).

in fact, assays with three binding partners could potentially be developed with MegaSTAR to produce assays with very low false positive rates. Third, we estimate that using this system, one can develop a robust sandwich assay for 1/10th the cost and time of traditional approaches. Lastly, it can be a powerful tool to engineer bispecific reagents for therapeutic application.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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