

Tapping diversity lost in transformations—*in vitro* amplification of ligation reactions

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ABSTRACT

Molecular evolution is a powerful means of engineering proteins. It usually requires the generation of a large recombinant DNA library of variants for cloning into a phage or plasmid vector, and the transformation of a host organism for expression and screening of the variant proteins. However, library size is often limited by the low yields of circular DNA and the poor transformation efficiencies of linear DNA. Here we have overcome this limitation by amplification of recombinant circular DNA molecules directly from ligation reactions. The amplification by bacteriophage Phi29 polymerase increased the number of transformants; thus from a nanogram-scale ligation of DNA fragments comprising two sub-libraries of variant antibody domains, we succeeded in amplifying a highly diverse and large combinatorial phage antibody library (>10⁹ transformants in *Escherichia coli* and 10⁵-fold more transformants than without amplification). From the amplified library, but not from the smaller un-amplified library, we could isolate several antibody fragments against a target antigen. It appears that amplification of ligations with Phi29 polymerase can help recover clones and molecular diversity otherwise lost in the transformation step. A further feature of the method is the option of using PCR-amplified vectors for ligations.

INTRODUCTION

The assembly of self-replicating, circular DNA molecules and their transformation into bacteria as first reported by Berg and co-workers in 1972 (1) lies at heart of recombinant DNA technology. However the assembly (by ligation) of circular DNA molecules from cohesive linear fragments is inefficient especially with multiple fragments (2), as is the transformation of bacteria (3). Less than 1 in 10² molecules of circular supercoiled plasmid can be successfully transformed (4), and for

linear molecules the efficiency is even lower (<1 in 10⁵ molecules) (5). To obtain large numbers of recombinant clones, it is therefore usually necessary to use large amounts of DNA for the ligations, and to undertake multiple transformations. Here we have investigated an alternative strategy; to amplify circular DNA by the use of bacteriophage Phi29 polymerase. As the enzyme specifically amplifies DNA circles (6–8) at the expense of short linear DNA molecules, we realized that it might be suited for the amplification of circular (and transformable DNA) directly from ligation reactions.

Phi29 polymerase has the unique property of catalyzing strand displacement synthesis with high processivity (9) and low error rate (10), and unlike PCR introduces little bias into the amplified population, as shown for whole-genome amplifications (11). Several applications of Phi29 polymerase for the amplification of DNA molecules have been reported (6,12–17). These include the amplification of DNA circles such as plasmid and phage genomes (6), as well as extended linear molecules such as human chromosomes (17). Most approaches are based on the work of Lasken and co-workers (6), and rely on random priming using high concentrations of short synthetic oligonucleotides. The amplification is based on a rolling-circle mechanism and leads to linear concatamers containing multiple template repeats (6). This process is facilitated by the strongly strand displacing properties of the polymerase and high primer concentrations. The tandem repeats are subsequently used as templates for further amplification, thereby producing extensive amounts of linear DNA suitable for sequencing and hybridization.

MATERIALS AND METHODS

Model amplifications

Regions of the pUC19 plasmid (18) were amplified by PCR using primers 5'-GAAATTGCGGCCGCATTTTAAATTTAAAGGATCTAGGTG-3' and 5'-TGCATTCTCGAGCATT-TCCCCGAAAAGTGCCACCTG-3'. A fragment encoding the β -lactamase (*bla*) gene (ampicillin resistance marker) was amplified from pUC 19 using primers 5'-ACTATTGCG-GCCGCGAAGTTTTAAATCAATCTAAAG-3' and 5'-CC-AAGTCTCGAGTTACTTACTTATGTGCGCGGAACCCC-TATTTG-3', while the chloramphenicol-acetyl-transferase

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(*cat*) gene (chloramphenicol resistance marker) was amplified from plasmid pBCSK⁺(Invitrogen) using primers 5'-AGGTCACCTCGAGTAAAGTAAGGCACGTAAGAGGTTCCAAC-3' and 5'-GTAGCAGCGGCCCGCAATTTCTGCCATTCATC-3'. All PCR amplifications were performed using Expand HighFidelity polymerase (Roche). The amplified fragments were digested with XhoI and NotI (New England Biolabs) (Phosphatase treatment of DNA should be avoided, as the presence of gaps in the circular product will prevent rolling-circle amplification) and purified by gel electrophoresis. DNA concentrations were determined by measuring absorbance at 260 nm. After purification, ligations were performed over night at 16°C at a concentration of 40 ng/μl vector and 40 ng/μl insert using 16 U/μl of T4 DNA ligase in ligase buffer (New England Biolabs). Ligation reactions were purified by phenol/chloroform extraction and diafiltration. Dilutions were performed by serial dilution of *bla* ligations into *cat* ligations (5-fold dilution steps were used).

Phi29 amplifications were performed in a 50 μl volume using 10 ng of purified ligation reaction as a template and 10 U of Phi29 polymerase (New England Biolabs). Reaction conditions were as follows: 1 mM dNTPs, 50 μM random hexamer primer, 0.1 mg/ml BSA, 50 mM Tris-HCl pH 7.4, 10 mM (NH₄)₂SO₄, 10 mM MgCl₂, 4 mM DTT. The reaction was incubated over night at 30°C and purified by phenol/chloroform extraction and diafiltration. The amplified concatemer was digested over night with NotI and the restriction digest purified using a PCR purification kit (Qiagen). Plasmids were re-circularized by self-ligation at dilute DNA concentrations (<1 ng/μl) using 4 U/μl of T4 DNA ligase in ligase buffer. After 2 h at room temperature the ligase was inactivated by phenol/chloroform extraction and the reaction concentrated by diafiltration. The reaction was transformed into *E.coli* TG1 (19) by electroporation and plated on agar plates containing 4% glucose and either 100 μg/ml ampicillin or 15 μg/ml chloramphenicol. Plates were incubated at 37°C over night.

Library construction

As a template we used a previously reported synthetic domain antibody repertoire, 'library 1' (20), based on the human DP47 heavy chain framework. The library incorporates diversity in all three CDR regions and is cloned in a phage format. The repertoire was pre-selected to enrich for antibodies that resisted aggregation upon heating (data not shown).

Regions of the library comprising to CDRs1/2 and CDR 3 (all with attached framework regions) were amplified by PCR from a plasmid preparation. Primer pairs 5'-ACGTC-AGAAGACATCAGGTGCAGCTGTTGGAGTC-3', 5'-TG-GACTGAAGACAGTCACGGAGTCTGCGTAGTATGTG-3' (CDR1/2) and 5'-GTAAGTGAAGACTAGTGAAGGG-CCGTTCCACCATC-3', 5'-TCAGTTGAAGACCTCGAATTCAGATCCTTCTGAGATG-3' (CDR3) were used in the amplification. Phagemid pR2 was amplified using the primer pair 5'-ATAGTGAAGACATTTCCGGCCGCACATTATACAGACATGAGATGAAC-3' and 5'-GATTACGAAGACACCTCGGCCATCGGCTGGGCCGCATAG-3'. The phagemid is derived from pHEN1 (21) and encodes a VSV tag upstream of the phage gene III. Amplifications

were performed using Expand HighFidelity polymerase (Roche). DNA concentrations were determined by measuring absorbance at 260 nm. The amplified fragments were digested with BbsI (New England Biolabs), gel purified and ligated at 16°C for 6 h in a three-way ligation using 40 U/μl of T4 DNA ligase in ligase buffer (New England Biolabs). Vector concentrations of 40 ng/μl and insert concentrations of 22 ng/μl (CDR1/2) and 33 ng/μl (CDR3) were used in the ligation. After heat inactivation (5 min at 70°C) and diafiltration, 125 ng of the reaction was either used as template for Phi29 amplification or directly transformed into *E.coli* XL-1 Blue (Stratagene) by electroporation and plated on agar plates containing 4% glucose and 100 μg/ml ampicillin and incubated at 37°C over night.

Phi29 amplification of antibody library

Amplifications were performed using a 500 μl starting volume and 125 ng of purified ligation reaction as a template (reaction conditions as above). Due to the increased reaction volume the concentration of Phi29 polymerase was reduced to 0.025 U/μl in order to minimize the amount of enzyme required (as was the concentration of ligase, as below). The reaction was heated at 70°C for 5 min before adding the polymerase and incubated at 30°C. The reaction volume was doubled every 3 h by adding reaction mixture and polymerase (no additional template was added) up to a volume of 8 ml. After an additional 3 h at 30°C the reaction was stopped by heating to 70°C for 20 min.

The amplified concatemer was cleaved by digestion with HindIII (New England Biolabs), the restriction endonuclease was heat inactivated and the reaction concentrated by diafiltration. Excess random hexamer primers were removed by gel filtration on a Sephacryl S300 column (Amersham). Plasmids were re-circularized by self-ligation at dilute DNA concentrations (<3 ng/μl) using 0.7 U/μl of T4 DNA ligase in ligase buffer (New England Biolabs). After incubation for 2 h at room temperature the ligase was heat inactivated (20 min at 70°C) and the reaction concentrated by diafiltration. The reaction was transformed into *E.coli* XL-1 Blue (Stratagene) by electroporation of 1 μl in 20 separate transformations, plated on agar plates containing 4% glucose and 100 μg/ml ampicillin and incubated at 37°C over night.

For analyses of transformation efficiencies, 125 ng portions were transformed in five separate reactions into *E.coli* XL-1 Blue bacteria by electroporation, plated on agar plates containing 4% glucose and 100 μg/ml ampicillin and incubated at 37°C over night (a single batch of competent cells was used).

Selection of binders

Libraries were re-transformed into *E.coli* TG1 (19) for selection. Phages were produced by co-infection with helper phage KM13 (22). Binding selections were performed directly from culture supernatant using streptavidin-Sepharose (Amersham) and biotinylated β-galactosidase (Sigma-Aldrich). Marvel milk powder and Tween-20 were present during the binding steps [5% (w/w) and 2% (v/v), respectively]. Washes were performed with phosphate buffered saline supplemented with 0.05% (v/v) Tween-20. Phages were eluted with 10 μg/ml trypsin and used to infect TG1 bacteria. For the

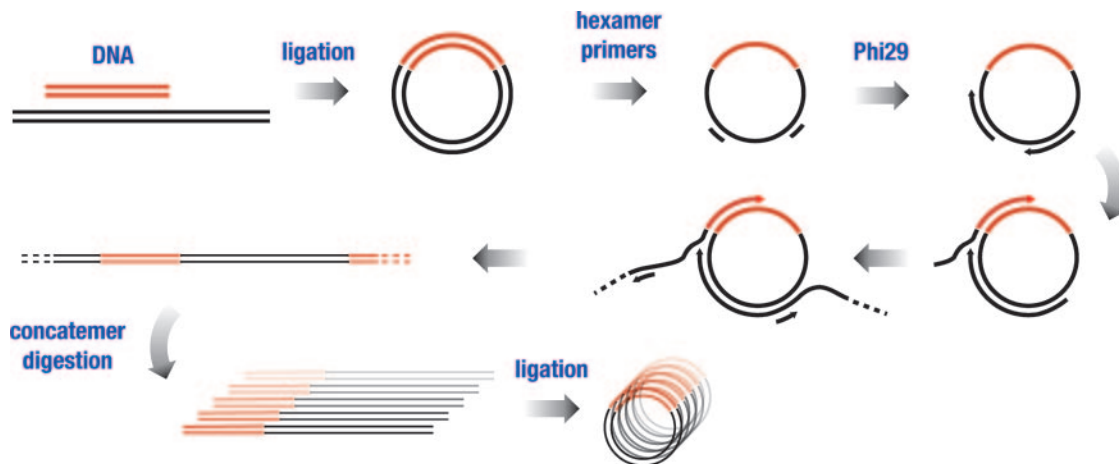


Figure 1. *In vitro* amplification of ligation reactions. Linear fragments were joined into recombinant, circular units by treatment with DNA ligase. Hexamer primers were annealed and Phi29 polymerase added. This causes extensive amplification of circular species through rolling-circle replication and the formation of extended linear concatemers. The concatemers were cleaved by restriction digestion and re-circularized using DNA ligase.

second round of selection β -galactosidase (Sigma-Aldrich) was directly immobilized on MaxiSorb plates (Nunc).

Analysis of selected antibodies

Binders were detected after two rounds of selection by ELSA using β -galactosidase coated MaxiSorb plates (as above), anti-M13 HRP-conjugate (Amersham) and 3,3',5,5'-tetramethylbenzidine as substrate. For expression, phage-mids were transferred into *E.coli* HB2151 by phage infection. Proteins were purified using a Streamline rProteinA column (Amersham), followed by acid elution and gel filtration on a Sephadex HR75 column (Amersham). Surface plasmon resonance measurements were performed on a Biacore 2000 instrument using biotinylated β -galactosidase (Sigma-Aldrich), streptavidin (SA) chips and HBS-EP running buffer (Biacore).

RESULTS

Amplification of ligation reactions

In a first step we tested whether our approach would amplify circular DNA from ligation reactions. As a model system we ligated an antibiotic resistance marker into a bacterial plasmid; we used PCR-amplified fragments comprising the origin of replication of the *Escherichia coli* plasmid pUC19 and the ampicillin resistance marker from the same plasmid. After restriction digestion the resulting PCR products were joined by ligation.

A dilution series was prepared from the ligation reaction and used as a template for Phi29 amplification. Hexamer primers and Phi29 polymerase were added and the reactions incubated isothermally at 30°C over night, allowing circular ligation products to be amplified through a rolling-circle mechanism (Figure 1). After amplification the resulting linear concatemers were cut with restriction endonuclease, and the plasmids were re-circularised by self-ligation at low DNA concentrations. Both amplified and un-amplified reactions

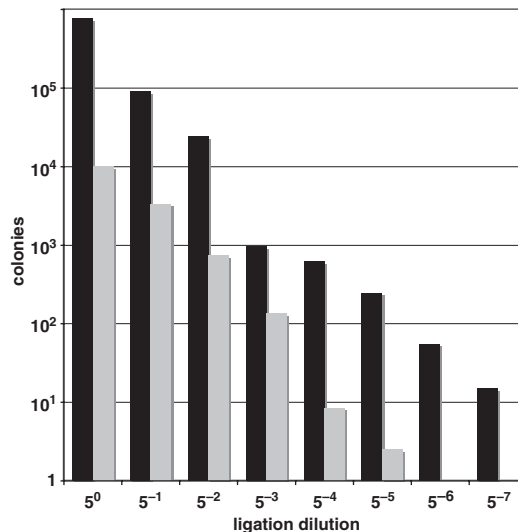


Figure 2. Amplification of dilutions of a model ligation reaction. Products from ligation reactions of ampicillin resistance gene into vector, were diluted, amplified, and used to transform *E.coli*, plating in the presence of ampicillin. The number of colonies (before and after amplification, gray and black, respectively) was plotted as a function of the dilution of the ligation reaction.

were electroporated into *E.coli* bacteria and plated on agar plates containing ampicillin.

Phi29 amplification significantly increased the number of transformants (~50-fold), with the number of colonies directly proportional to the amount of template DNA used (Figure 2). We were able to obtain colonies from the amplified ligations with template dilutions that yielded no colonies from the un-amplified ligations (Figure 2; 5⁻⁶ and 5⁻⁷ dilution steps).

Amplification of mixtures

We then analyzed whether our method was also capable of amplifying mixtures of DNA molecules without detectable

bias. For this purpose, the template dilution series (as above) had been ‘spiked’ with a constant amount of a ligation reaction incorporating a different, selectable resistance marker (a PCR-amplified chloramphenicol gene ligated into the pUC19 vector backbone). From the ratio of the colonies before and after amplification (by counting colonies on ampicillin or chloramphenicol plates), it appears that these two constructs are amplified to a similar extent (Figure 3), despite the differences in sequences and lengths of the DNAs encoding the two resistance markers.

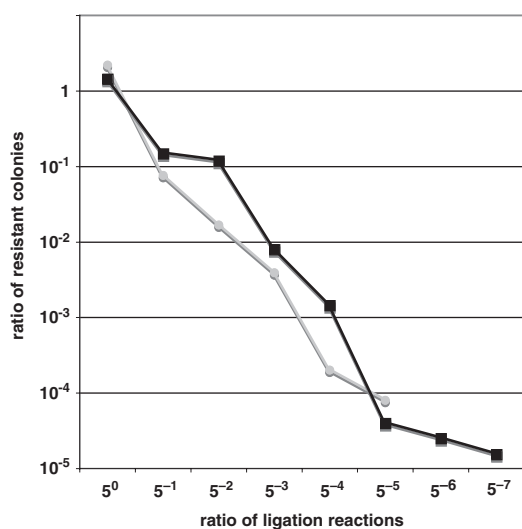


Figure 3. Amplification of mixtures of two model ligation reactions. Products from ligation reactions of ampicillin and chloramphenicol resistance genes into vector were mixed in different ratios, amplified and used to transform *E. coli* in the presence of ampicillin or chloramphenicol. The ratio of the number of colonies (ampicillin/chloramphenicol) growing under the two different antibiotic selections (before and after amplification, gray and black, respectively) was plotted as a function of the ratios of the original mixtures (ampicillin/chloramphenicol).

Generation of a large antibody repertoire from nanogram-scale ligation reactions

We generated a repertoire of antibody variable heavy chains by recombining gene segments from a library (20) with diversity in all three complementary determining regions (CDRs) (Figure 4). Thus, we PCR-amplified a segment comprising CDRs 1 and 2, and also a segment comprising CDR 3 from the library. The two segments of amplified DNAs were cut with restriction endonuclease and recombined by ligation into a phagemid vector, itself generated by PCR, in order to create a combinatorial library. As three-way ligations are inefficient (2), we tried to optimize the process. For this purpose restriction sites cleavable by the type II restriction endonuclease BbsI were introduced into the PCR primers, enabling ‘sticky’ but non-palindromic overhangs and non-ambiguous assembly. Nevertheless when a portion of the ligation reaction (comprising 125 ng vector backbone, $>10^{11}$ DNA molecules) was directly transformed into *E. coli* bacteria, only 2×10^4 colonies were obtained (and 10^4 fold less than with the same amounts of circular supercoiled plasmid).

An identical portion of the ligation was then used as a template for Phi29 amplification. This polymerase exhibits a strong exonuclease activity and primers incorporating phosphothioate linkages (that are resistant to such exonucleolytic attack) have been reported to substantially increase DNA yields (6). However, we had noted that the incorporation of phosphothioates also reduced transformation efficiencies (data not shown). We therefore adopted a different strategy; we added primer at regular intervals during the amplification in an attempt to maintain the primer concentration (see Material and Methods for details). In addition, the final reaction volume was significantly increased (160-fold) compared to the model amplifications (as above). The amplified concatemers were then purified by gel filtration chromatography, cleaved by restriction digestion and the plasmids recircularized by self-ligation. The process yielded ~ 0.1 mg of amplified DNA (corresponding to a 10^3 -fold amplification of the template).

We compared transformation efficiencies of the amplified and un-amplified DNA by transforming multiple portions of each into *E. coli* bacteria. This revealed that the transformation efficiency of the amplified DNA was significantly higher than that of the un-amplified DNA. For example, in a typical

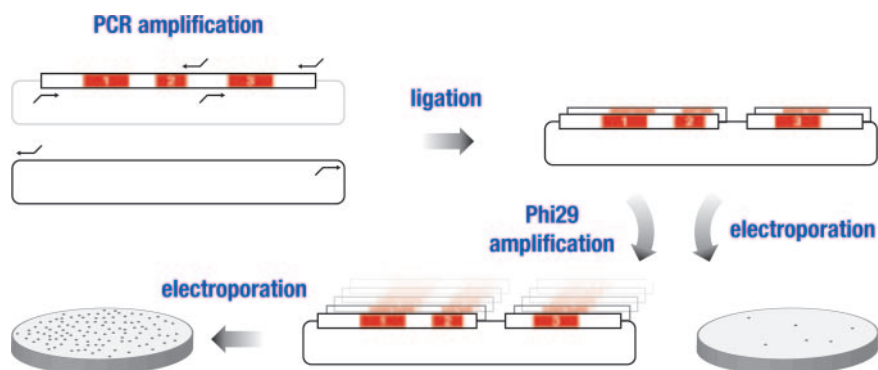


Figure 4. Antibody repertoire by combinatorial ligation. Regions corresponding to CDRs 1/2 and CDR 3 were PCR-amplified and recombined by ligation into a PCR-amplified phagemid vector backbone. The ligation reactions were either directly electroporated or amplified using Phi29 polymerase followed by electroporation into *E. coli* bacteria.

Table 1. Selection of antibody repertoire

Antibody repertoire	Size	Antigen-binding clones	Unique antigen-binding clones
Un-amplified	2×10^4	0/18	0
Amplified	3×10^9	7/18	4

Beta-galactosidase was used as an antigen in phage display selections. Antigen-binding clones were identified after two selection rounds by phage ELISA and their DNA sequences were determined.

experiment, transformation with 125 ng of amplified or un-amplified DNA yielded 3×10^6 and 10^4 colonies, respectively. The colony numbers differed by an average factor of 300-fold over five experiments. Clones from the transformed repertoires were analyzed by DNA sequencing; of these 31/36 (amplified) and 36/46 (un-amplified) were free of mutations (outside the CDR regions) and encoded full-length antibody domains. Thus, no detectable increase of point mutations or rearrangements was observed for the amplified repertoire.

After these preliminary experiments we generated a large repertoire in bacteria by transforming 1 μ g portions of the amplified DNA into *E.coli*. Each portion yielded $\sim 1.5 \times 10^8$ colonies; 20 portions were pooled, providing a library of 3×10^9 transformants. The Phi29 amplification had therefore increased the number of transformants by more than 10^5 -fold, due to both the greater amounts of transformed DNA (~ 160 -fold) and its higher transformation efficiency (~ 900 -fold).

Analysis of library and selection of binders

To characterize the libraries we selected binders from both the amplified and un-amplified antibody library (encompassing 3×10^9 and 2×10^4 transformants, respectively) by phage display. We used β -galactosidase as the antigen, performed two rounds of binding selections and then screened 18 clones of each repertoire by phage ELISA (23). Among these, seven phage clones from the amplified repertoire bound to β -galactosidase as shown by ELISA (but did not bind to BSA), and were found to comprise four unique antibody domain sequences (Supplementary Data). No binders were observed for the un-amplified repertoire (Table 1). The four domains were expressed in *E.coli* by secretion and purified by affinity chromatography using protein A-Sepharose. All bound to biotinylated β -galactosidase as determined by surface plasmon resonance (but not to the streptavidin-coated capture surface), and we observed dissociation rates in the range of 10^{-1} – 10^{-2} s $^{-1}$ for three of the four proteins (clone 1: 5×10^{-2} s $^{-1}$, clone 2: 1×10^{-2} s $^{-1}$, clone 3: 4×10^{-2} s $^{-1}$). The off-rates for these single antibody domains are therefore comparable to those reported for single chain Fv fragments isolated from a large repertoire ($>10^{10}$ clones) (24).

CONCLUSIONS

Our results demonstrate that Phi29 polymerase can amplify ligation reactions *in vitro* without detectable biases, yielding sub-milligram quantities of DNA and potentially increasing the number of transformants in excess of 10^6 -fold. This

enabled us to rapidly generate a library of antibody heavy chains from which binders could be readily selected. We did not observe any binders in the un-amplified repertoire, strongly indicating that the amplification process had allowed us to tap molecular diversity otherwise lost in the transformation process. The methodology should also be suitable for the generation of large amounts of non-methylated DNA for the transformation of eukaryotic species. While for many host organisms re-circularization of the amplified concatemer will increase transformation efficiencies, other organisms (such as yeast) may be directly transformable with linear DNA.

Our method requires only minute quantities of DNA as a starting template. This allowed us to assemble transformable constructs in a single step from PCR products, removing the need for plasmid preparations or stepwise cloning. It may also facilitate the cloning of rare DNAs from fossil, environmental or forensic samples, that are frequently not accessible by direct cloning or amplification. Although we have so far exclusively used the method for the amplification of ligation reactions, it should also be suitable for other applications in which only limited amounts of circular DNA are available, such as the amplification of BAC libraries. We suggest that amplification of circular DNA by Phi29 polymerase *in vitro* has the potential to improve a wide range of cloning techniques that are currently limited by the transformation efficiency of the host organism.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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