A high-throughput, restriction-free cloning and screening strategy based on ccdB-gene replacement

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Abstract

Background: In high-throughput demanding fields, such as biotechnology and structural biology, molecular cloning is an essential tool in obtaining high yields of recombinant protein. Here, we address recently developed restriction-free methods in cloning, and present a more cost-efficient protocol that has been optimized to improve both cloning and clone screening.

Results: In our case study, three homologous β-lactamase genes were successfully cloned using these restriction-free protocols. To clone the genes, we chose a gene replacement strategy, where the recombinant genes contained overhangs that targeted a region of the expression vector including a cytotoxin-encoding ccdB-gene.

Conclusion: We provide further evidence that gene replacement can be applied with high-throughput cloning protocols. Targeting a replacement of the ccdB-gene was found to be very successful for counterselection using these protocols. This eliminated the need for treatment with the restriction enzyme DpnI that has so far been the preferred clone selection approach. We thus present an optimized cloning protocol using a restriction-free ccdB-gene replacement strategy, which allows for parallel cloning at a high-throughput level.

Keywords: Restriction-free cloning, Exponential megapriming PCR, High-throughput, Counterselection, Recombinant DNA technology, Parallel cloning

Background

To meet the demands in biotechnology and structural biology, high-throughput molecular cloning methods have been developed to obtaining sufficient amounts of recombinant protein in a parallel manner. For this purpose, restriction-free (RF) cloning is a simple PCR-based approach for inserting any DNA fragment into any position of a vector, independently of restriction-sites, ligation, without elaborate pretreatments of the vector and without sequence constraints [1-3]. Today’s high fidelity polymerases used for cloning have extraordinary low error rates. Therefore, PCR amplification of large DNA regions, such as entire plasmids, is no longer challenging. The RF-method relies on site-specific insertion of the gene of interest to the vector by linear plasmid amplification [2,3].

Briefly, the insertion requires that the gene of interest is flanked by sequences complimentary to the insertion site of the vector. These overhang regions are generated during a standard PCR amplification from the relevant template using primers containing both a gene-specific region and an adapter region. Finally, the amplified gene with overhangs is used as a megaprimer in the second PCR, where the insertion takes place during linear plasmid amplification. The complementary DNA products form a plasmid containing two single-stranded nicks, which is ready for transformation and clone screening. To shorten the protocol, the gene-specific and the linear plasmid amplification can be combined in a single PCR reaction. Exponential megapriming PCR (EMP) cloning addresses the major shortcoming of the published RF-cloning protocol, the product- and size-limiting linear plasmid amplification, by introducing a reverse vector-specific primer making the amplification exponential [4]. This protocol, however, requires two additional, successive steps of phosphorylation and ligation to close the plasmid. The fact that these
cloning regimes requires absolutely no pretreatments of the vector, in great contrast to other high-throughput cloning methods, such as TA-cloning and ligation-independent cloning [5-7]. This makes these protocols beneficial for many high-throughput applications, such as structural genomics and biotechnology.

The RF- and EMP-protocols rely on a screening process to select positive clones from background clones. This screening includes treating the product mix from the second PCR with the restriction enzyme *Dpn*I [8]. The enzyme will remove the methylated parental DNA before transformation, such as established for the mutagenesis protocol [9,10]. Typically, *Dpn*I is incubated with the substrate at 37°C for an hour. The efficiency is improved by longer incubations, up to several hours, with the risk of over-digestion. Also, efficiency of *Dpn*I is dependent on buffer and the methylation state of the template DNA, and requires vector propagation in Dam+ strains [11].

In our case study, we have investigated using a gene replacement principle [2] in combination with negative ccdB selection to improve the clone screening process of RF- and EMP-cloning. In this study, three homologous bla_{OXA-48}-genes, encoding the oxacillinase (OXA) β-lactamases OXA-48, 181 and 245 [19-21], were successfully cloned downstream of the T7 promoter. We carried out the cloning by replacing a 1.7 kb region of the commercial Gateway® vector, pDEST17, with our genes of interest, not by traditional recombination, but by using the newly invented RF- and EMP-cloning protocols. We show that RF- and EMP-cloning protocols can successfully be used in gene-replacement applications. Such applications increase the potential of this method in the manipulations of plasmids. Also, we have found that ccdB-selection was a successful approach for clone selection for the newly invented cloning regime, which eliminates the need for *Dpn*I removal of parental DNA. Taken together, we present an improved, cost-efficient and less labour-intensive cloning protocol, which allows for cloning and screening in a parallel fashion, using ccdB-gene replacement. Our data increase the potential of the cloning method in high-throughput technologies.

**Results and discussion**

RF-cloning, including the optimized EMP-protocol, is a simple method for inserting any gene into a vector, independently of restriction-sites [2-4]. The benefit of the method is that it has no requirements for pretreatments of vector. The method holds a great potential in biotechnology, structural biology and other high-throughput demanding fields.

In the process of selecting positive clones, *Dpn*I treatment is used for the removal of template DNA in the published RF- and EMP-cloning protocols [2-4]. This
procedure is, however, time-consuming and may lead to over-digestion of positive clones. Furthermore, efficiency of \textit{DpnI} depends on buffer composition and the methylation state of the template DNA, and requires that template DNA has been propagated in Dam$^+$ strains. In this report, we have investigated using the gene replacement principle [2] in combination with negative \textit{ccdB} selection [12-15] to improve the clone screening process of RF- and EMP-cloning protocols (Figure 1). The product generated in the second PCR during plasmid amplification can thus be transformed directly to competent cells (Figure 1) without the need of \textit{DpnI} pre-treatment. Positive clones were identified by standard colony PCR screening using vector-specific primers flanking the insertion site (Figure 2C), and confirmed by Sanger sequencing using the same primers. Generally, cloning was successful independently of linear or exponential amplification, judged from the ratio of positive clones to total screened clones identified by colony PCR (Table 1). Furthermore, induction of recombinant OXA-proteins for heterologous expression results, activity data confirm that the gene replacement strategy was successful for obtaining recombinant clones of \textit{bla$^{OXA-48}$}.

The replaced 1.7 kb region of the pDEST17 vector containing the \textit{ccdB}-gene is designed to increase the efficiency of clone selection in recombinational cloning [15]. We explored the vectors containing the \textit{ccdB}-gene for selection using other cloning methods more applicable for high-throughput cloning, such as the recently developed RF- and EMP-cloning protocols. These cloning protocols have not, previously, been reported in combination with negative \textit{ccdB} selection. We have also examined \textit{ccdB}-selection in combination with \textit{DpnI} restriction using the EMP cloning protocols. Parental pDEST17-vectors with frame shifts within the \textit{ccdB}-gene that would not kill the cell would thereby be removed. As an example, when the \textit{bla$^{OXA-181}$} gene was inserted to pDEST17 by our \textit{ccdB}-gene replacement strategy using the EMP-cloning protocol, thirteen of twenty-nine colonies were confirmed positive by colony PCR screening using vector-specific primers flanking the insertion site (Figure 2C, Table 1). Background clone numbers did not dramatically improve by additional \textit{DpnI} treatment. Although the success of \textit{ccdB} selection has been reported before [15], we could confirm that the \textit{ccdB} counterselection itself was sufficient for positive clone selection. Similar results were found when we cloned homologous \textit{bla$^{OXA-48}$} genes using the

\begin{figure}
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\caption{**Principle of \textit{ccdB}-gene replacement strategy.** The schematic representation of the \textit{ccdB}-gene replacement strategy follows three simple steps, two PCR reactions and a direct transformation of the second PCR product. Primers F1 and R1 are used in a gene-specific amplification in the first PCR round. Gene-specific regions of primers are indicated by thin, green arrows, whereas the introduced overhang regions are coloured in orange. In the second PCR, the product from the first PCR is used as a megaprimer in linear plasmid amplification. The overhang regions of the product bind to the desired insertion sites in the vector that flank the \textit{ccdB} gene. \textit{ccdB}-gene for negative selection becomes replaced during amplification of the plasmid. The R2 primer can be used for exponential amplification. Complementary primer-binding regions in the vector are marked in the same colours as primers. Finally, the parental vector and the product from the second PCR can be transformed directly to competent cells. Those cells that take up the paternal vector will be subjected to un-repairable chromosomal damage caused by a toxin encoded by the \textit{ccdB}-gene, and will ultimately die. Positive clones, however, will survive since the negative selection marker gene has been replaced with the gene of interest. Generally, input DNA, such as genomic DNA and vector, is coloured in grey to differentiate it from the black-coloured product DNA. Thin and fat arrows differentiate primers and open reading frames, respectively.}
\end{figure}
EMP-cloning protocol (Table 1). Judging from the number of positive clones to total number of clones tested some clones seem to fail in the insert replacement, but still escape the ccdB-determined toxicity as determined by negative PCR colony screen. This can be explained by primer-dimer products from the first PCR being inserted to the vector in the second PCR, or simply due to failed colony inoculation to PCR screening reaction. In our study, primer-dimer formations may have been relevant for the gene-specific amplification of blaOXA-181, leading to false positives in the plasmid amplification and thus a low number of positive clones to total screened clones. Optimizations, such as adding dimethyl sulfoxide (DMSO), could be carried out to reduce the primer-dimer formation during the gene-specific amplification. In cases where DpnI treatment was not performed, frameshifts or deletion of the ccdB gene may also have caused false positives.

In one EMP-cloning experiment, eight hundred colonies were obtained for the DpnI-treated sample, whereas more than fifteen hundred colonies were obtained for the untreated sample (Table 1). When selectivity was determined by colony PCR, the DpnI treated sample did not give higher score for positive clone selection. Hence, we hypothesize that DpnI treatment may lead to over-digestion of DNA, thereby reducing the probability of positive clones. This hypothesis was, however, not further investigated.

We chose to explore the commercially available Gateway® vectors encoding a ccdB-gene. For labs that have already implemented recombinational cloning, our
optimized protocol allows for parallel cloning of PCR products into Gateway® expression vectors harbouring different fusion-tags based on conserved regions shared among the vectors. Other vector series encoding the ccdB-gene are, however, academically available [such as described in 12,16,23].

Conclusions

In our report, we have provided further evidence that gene replacement can be applied with restriction-free cloning protocols, thus, increasing the potential of these methods in the manipulations of plasmids. Also, we have found that ccdB-selection was a successful method for clone selection for the newly invented cloning regimes. Our optimized procedure represents a more efficient, cost reducing and less labour-intensive approach for molecular cloning and clone screening. Together with previous reports, our data confirms and improves the potential of the RF- and EMP-cloning protocols in high-throughput technologies.

Methods

Cloning

Primers were designed to replace the ccdB-gene with the blaOXA-48-genes encoding OXA β-lactamases, OXA-48, −181 and −245 [GenBank: KC757416, JN205800 and JX43800, respectively]. The RF-cloning.org webservice [24] was used to design primers F1 (5′-ATAATTTTGTTAACTTTAAGAAGGAGATATACAT-′3′), where italic represents blaOXA-gene-specific region) and R1 (5′-GGCTTTGTTAGCAGCCATCTCGTAGTAGTTACATATGCGGTATTA-′3′), by 25 cycles of 8s denaturation at 98°C, 20s annealing at 56°C and 15s elongation at 72°C, and the reaction was terminated by a final 5 minutes elongation step at 72°C. PCR products were purified by the Nucleo-Spin gel and PCR cleanup kit (Macherey-Nagel). PCR products were analyzed by gel electrophoresis (1% agarose).

RF-cloning was performed as described in previous reports [2,3]. The linear amplifications took place in 1x HF Phusion buffer, using 100 ng megaprimer, 200 μM dNTP, 1% DMSO (NEB), 25 ng Gateway® pDEST17 vector (Invitrogen) and 1U Phusion DNA polymerase. The mixture was heated to 98°C for 30s denaturation, followed by 30 cycles of 8s denaturation at 98°C, 20s annealing at 54°C and 107s elongation at 72°C, terminated by a final 5 minutes elongation step.

Our EMP protocol was performed as described previously [4], with minor modifications. Exponential amplifications of the plasmids were performed with 25 ng megaprimer, and 1x HF Phusion buffer, 200 μM of dNTP, 0.5 mM of each primer F1 and R2, 25 ng pDEST17 and 1U Phusion DNA polymerase. Both the F1- and R1-primers described above carry overhangs complementary to the insertion site of the vector, in contrast to the reported EMP-cloning, where F1-primer is designed without an overhang [4]. Consequently, the R2-primer (5′-TTCTAGAGGGAAACCGTTGTGGTCT) was designed complementary to the vector region 5′ to the F1-primer to ensure an exponential amplification.

The samples were denatured at 98°C for 30s, followed by 25 cycles of 10s denaturation at 98°C, 30s annealing at 56°C and 107s elongation at 72°C.

The EMP products were first purified as described above, then phosphorylated and ligated by incubating 16.5 μL PCR-product in 1.7 μL 10xT4 DNA ligase buffer (NEB) with 5U T4 polynucleotide kinase (NEB) for 30 minutes at 37°C, followed by the addition of 6U T4 DNA ligase (NEB) and overnight incubation at 4°C.

For DpnI treatment, 20U enzyme (NEB) was added directly to half of the PCR product and incubated at 37°C for 30 minutes. 2 μL reaction products, DpnI treated or not, was then transformed by a conventional heat-shock protocol to competent E. coli XL1Blue cells (Stratagene). The mix of transformed bacteria was spread on an LB-agar plate supplemented with 100 μg/ml ampicillin (Sigma).
Screening was performed by colony PCR using the vector-specific T7 promoter primer (5′-TAATACGACTCACTATAGGG) and T7 terminator primer (5′-GCTAGTTATGCTACGGCGG) in 1x Ampliton Taq DNA polymerase master mix (Ampliqon). Sequences were verified by BigDye 3.1 sequencing (Applied Biosystems).

**Recombinant expression**
The purified plasmids containing the blaOXA-48 gene were transformed into E. coli strain BL21Star(DE3)pRARE [Invitrogen, Novagen and 25] competent cells by a conventional heat-shock protocol. Cells were used to inoculate 1 L Terrific Broth (TB) media containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Recombinant expression of native OXA-48 was induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside (VWR) at log phase, and the expression was continued at 20°C for 16 hours. OXA-48 was isolated from the periplasm using osmotic shock and lysozyme—treatment [26], and purified through two anionic exchange steps, as described previously [27].

**Kinetics**
The purified OXA-48 enzyme was used to determine the kinetic parameters for hydrolysis of the substrate meropenem (Sigma) using a Synergy H1 spectrophotometer (BioTek) by measuring the change in absorbance at 300 nm. A plate specific extinction coefficient of 0.002733 μM−1 cm−1 was calculated for meropenem concentrations 125-1 μM with 500 nM enzyme in 100 mM Tris-H2SO4 pH 7, 300 mM Na2SO4 and 10 mM NaHCO3 using Gen5 (BioTek). The steady-state parameters were calculated with non-linear regression using Prism 6 (GraphPad Software).

**Abbreviations**
RF: Restriction-free; EMP: Exponential megaprimer PCR; OXA: Oxacillinase; ccdB: Coupled cell division B gene; bla: β-Lactamase.

**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
BAL carried out the molecular cloning, recombinant expression and kinetics experiments, participated in the design of the study and drafted the manuscript, figure and table. HKSL participated in the design of the study and reviewed the manuscript. GEKB conceived of the study, participated in the design of the study, coordinated and drafted the manuscript and illustration. All authors read and approved the final manuscript.

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**References**
1. Chen GJ, Qiu N, Karer C, Caspers P, Page MG. Restriction site-free insertion of PCR products directly into vectors. *Biotechniques* 2000, 28:498–500. 504–495.
2. Unger T, Jacobovitch Y, Dantes A, Bernheim R, Peleg Y: Applications of the Restriction Free (RF) cloning procedure for molecular manipulations and protein expression. *J Struct Biol* 2010, 172:34–44.
3. van den Ent F, Lowe J. RF cloning: a restriction-free method for inserting target genes into plasmids. *J Biochem Biophys Methods* 2006, 67:67–74.
4. Utichi A, Andersen KR, Schwartz TU: Exponential megaprimer PCR (EMP) cloning—seamless DNA insertion into any target plasmid without sequence constraints. *PloS One* 2012, 7:e53360.
5. Holton TA, Graham MW: A simple and efficient method for direct cloning of PCR products using dsdT-tailed vectors. *Nucleic Acids Res* 1991, 19:1156.
6. Gibson DG, Young L, Chuang Ry, Yenter JC, Hutchison CA 3rd, Smith HD: Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 2009, 6:234–345.
7. Aslanidis C, de Jong PJ: A ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res* 1990, 18:6069–6074.
8. Lacks S, Greenberg B: A deoxyribonuclease of Diplococcus pneumoniae specific for methylated DNA. *J Biol Chem* 1975, 250:4060–4066.
9. Weiner MP, Costa GL, Schoettlin W, Cline J, Mathur E, Bauer JC: Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. *Gene* 1994, 151:19–123.
10. Li S, Wilkinson MF: Site-directed mutagenesis: a two-step method using PCR and DpnI. *Biotechniques* 1997, 23:588–590.
11. Li F, Liu SL, Mullins Jr: Site-directed mutagenesis using uracil-containing double-stranded DNA templates and DpnI digestion. *Biotechniques* 1999, 27:734–738.
12. Scholt J, Besir H, Straßer C, Suppmann S: A new method to customize protein expression vectors for fast, efficient and background free parallel cloning. *BMC Biotechnol* 2013, 13:12.
13. Walhurst AJ, Temple GF, Braasch MA, Hartley J, Lonson MA, van den Heuvel S, Vidal M: GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomns. *Methods Enzymol* 2000, 328:575–592.
14. Hartley J, Temple GF, Braasch MA: DNA cloning using in vitro site-specific recombination. *Genome Res* 2000, 10:1789–1795.
15. Bernard P: Positive selection of recombinant DNA by CcdB. *Biotechniques* 1996, 21:320–323.
16. Geertsma ER, Dutzler R: A versatile and efficient high-throughput cloning tool for structural biology. *Biochemistry* 2011, 50:3272–3278.
17. Bernard P, Coutourier M: Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *J Mol Biol* 1992, 226:735–745.
18. Opara T, Hiraqa S, Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc Natl Acad Sci U S A* 1983, 80:4784–4788.
19. Castanheira M, Deshpande LM, Mathai D, Bell JM, Jones RN, Mendes RE: Early Dissemination of NDM-1 and OXA-181-Producing Enterobacteriaceae in Indian Hospitals: Report from the SENTRY Antimicrobial Surveillance Program, 2006–2007. *Antimicrob Agents Chemother* 2011, 55:1274–1278.
20. Otoo J, Hernandez JM, Espasa M, Pleters A, Saez D, Bautista V, Perez-Vazquez M, Fernandez-Garcia MD, Delgado-Irribarren A, Sanchez-Romo I, Garcia-Picazo L, Moral MD, Solis S, Aznar E, Trujillo G, Mediavilla C, Fontanals D, Rojo S, Vindel A, Campos J: Emergence of OXA-48-producing Klebsiella pneumoniae and the novel carbapenemases OXA-244 and OXA-245 in Spain. *J Antimicrob Chemother* 2011, 67:317–321.
25. Borra PS, Samuelsen Ø, Spencer J, Walsh TR, Lorentzen MS, Leiros H-KS: Crystal structures of pseudomonas aeruginosa GIM-1: active-site plasticity in metallo-β-Lactamases. Antimicrob Agents Chemother 2013, 57:848–854.

26. French C, Keshavarz-Moore E, Ward J: Development of a simple method for the recovery of recombinant proteins from the Escherichia coli periplasm. Enzym Microb Technol 1996, 19:332–338.

27. Giuliani F, Docquier JD, Riccio ML, Pagani L, Rossolini GM: OXA-46, a new class D beta-lactamase of narrow substrate specificity encoded by a blaVIM-1-containing integron from a Pseudomonas aeruginosa clinical isolate. Antimicrob Agents Chemother 1973–1980, 2005:49.

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