BIOCHEMISTRY
including biophysical chemistry & molecular biology

ARTICLE

pubs.acs.org/biochemistry

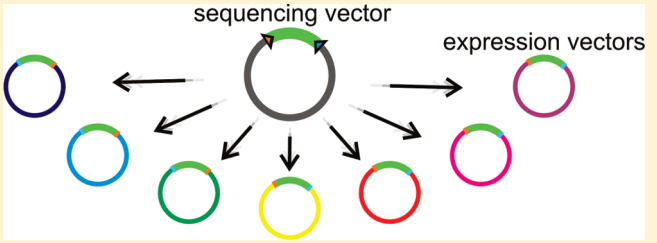
A Versatile and Efficient High-Throughput Cloning Tool for Structural Biology

Eric R. Geertsma* and Raimund Dutzler*

Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Supporting Information

ABSTRACT: Methods for the cloning of large numbers of open reading frames into expression vectors are of critical importance for challenging structural biology projects. Here we describe a system termed fragment exchange (FX) cloning that facilitates the high-throughput generation of expression constructs. The method is based on a class IIS restriction enzyme and negative selection markers. FX cloning combines attractive features of established recombination- and ligation-independent cloning methods: It allows the straightforward transfer of an open reading frame into a variety of expression vectors and is highly efficient and very economic in its use. In addition, FX cloning avoids the common but undesirable feature of significantly extending target open reading frames with cloning related sequences, as it leaves a minimal seam of only a single extra amino acid to either side of the protein. The method has proven to be very robust and suitable for all common pro- and eukaryotic expression systems. It considerably speeds up the generation of expression constructs compared to traditional methods and thus facilitates a broader expression screening.



Progress in structural genomics initiatives and in challenging structural biology projects, such as membrane protein structure determination, is tied to the possibility to screen for the overexpression and biochemical stability of many different family members to identify ones with superior biochemical properties.[1−3] In later stages it might be necessary to modify a particular protein by removing unstructured termini and loop regions to improve its crystallization behavior.[4,5] Both strategies require robust methods to generate large numbers of constructs for different expression systems. Ideally, such a high-throughput cloning method is reliable, inexpensive, and straightforward in its use. It should be independent of the sequence of the target gene, and the primers should be easy to design. A single PCR amplification should suffice to clone into several different vectors since expression levels are often sensitive to the location of fusion products such as reporter genes and purification tags and also show a strong dependence on the expression host and the promoter system. Finally, the addition of cloning related sequences to an open reading frame (ORF) should be kept at its minimum since such sequences frequently affect the expression and crystallization behavior of the protein.

Traditional restriction and ligation-based cloning strategies are in this respect unsatisfactory. These methods are of limited efficiency and labor intensive in their use as they require the purification of intermediate cleavage products. Moreover, a uniform cloning strategy for a large set of different ORFs is prevented by the frequent occurrence of restriction sites in the sequences of the target genes.

Alternative approaches that circumvent the limitations of restriction-based cloning are either recombination-based or based on ligation-independent cloning methods (reviewed in ref 6). Recombination-based methods, such as Gateway,[7] Creator,[8] and MAGIC,[9] make use of site-specific recombination to insert an open reading frame (ORF) into an expression vector. Ligation-independent cloning methods, such as LIC[10] and In-Fusion,[11] use the controlled digestion of DNA to generate long complementary single-stranded overhangs on the vector and insert, which allows cloning by base complementation without the need of a ligation step. Of these high-throughput cloning strategies, only Gateway and Creator allow subcloning of an ORF into several expression vectors. Seamless cloning is possible only for MAGIC and In-Fusion, though these methods require separate PCR products for each combination of tags or fusion proteins and are thus not suitable for high-throughput approaches. Apart from MAGIC and In-Fusion, all methods lead to the extension with cloning-related sequences ranging from 12 to 15 (LIC) up to 21−25 base pairs (Gateway) on either side of the ORF.[6] Amino acid stretches resulting from such additional nucleotides are generally unstructured and are likely to interfere with crystallization. Despite the lack of subcloning options and significant extension of the ORF, many structural genomics initiatives employ LIC due to its flexibility and low cost.[12,13]

In this communication we present a superior alternative to existing high-throughput cloning strategies and demonstrate its applicability. The method is based on the use of type IIS restriction endonucleases (REs), which cleave DNA at a fixed

Received: February 4, 2011
Revised: March 16, 2011
Published: March 17, 2011

distance outside their asymmetric recognition site.[14] The actual sequence of the resulting overhang can be any of the 16, 64, or 256 combinations possible for cohesive ends of 2, 3, or 4 nucleotides, respectively. The unique properties of class IIS REs has led to several applications (reviewed in ref 14) including cloning procedures.[15−18] We have applied critical improvements to these methods, resulting in an efficient high-throughput tool that allows initial cloning and subcloning while limiting the flanking extensions of the ORF to three base pairs.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors Compatible with FX Cloning. Removal of the SapI site near the pBR322 origin of replication (ORI) in pET26[19] was done by Quikchange mutagenesis. To construct FX cloning compatible derivatives of pBAD vectors,[20] the two endogenous SapI sites around the pBR322 ORI and the P_{BAD} promoter in pBADnLIC[21] were removed by PCR, yielding pBADnLICΔSapI. To construct derivatives of pRE vectors,[21] the SfiI fragment containing the sole SapI site located near the pBR322 ORI was replaced with a corresponding SapI-free SfiI fragment obtained via PCR using pBADnLICΔSapI as template.

The two SapI sites required for cloning and flanking the sequences coding for tags, fusion proteins, or start and stop codons were introduced by a PCR of the vector backbone. For this, the forward primer was extended on its 5′ end by the sequence 5′ *atatatGCTCTTCtGCAnnn*. The *nnn* codon represents the first triplet of the desired expression element that will follow the C-terminal Ala residue. The reverse primer was extended on its 5′ end by the sequence 5′ *atatatGCTCTTCaCTnnn*. Here, the *nnn* codon represents the reverse complement of the triplet of the desired N-terminal expression element preceding the Ser residue. Lowercase letters in the extensions can be replaced by any nucleotide to prevent stable secondary structure elements. The fragment containing the *ccdB* and *cat* markers was amplified from pREBACK[21] using forward primer 5′ *tatataAGTTGAAGAGCGACCTGCAGACTGGCTGTGTATAA* and reverse primer 5′ *atatatTGCAGAAGAGCTGAACTAGTGGATCCCCAAAAAAG*. To create the FX-cloning compatible expression vector, the DpnI-treated PCR product of the vector (∼100 ng) was mixed with the gel-purified PCR product of the *ccdB* and *cat* markers to a final molar ratio of 1:5 (vector:insert). After the addition of 1.5 μL of 10x buffer (200 mM Tris-acetate, 500 mM potassium acetate, 100 mM magnesium acetate, 10 mM dithiothreitol, pH 7.9), the volume was adjusted to 14 μL, and the digestion was started by the addition of 1 μL of SapI (2U). The mixture was digested for 1 h at 37 °C, and the enzyme was heat inactivated by a 20 min incubation at 65 °C. After cooling to RT, ligation of the fragments was started by the addition of 1.9 μL of 10 mM ATP-mix (10 mM Na_2-ATP, 10 mM $MgSO_4$, 50 mM KPi, pH 7.0 adjusted to pH 6.5−7.0 with NaOH) and 1.9 μL of T4 ligase. Fragments were ligated for 1 h at 25 °C followed by heat-inactivation of the enzyme by a 20 min incubation at 65 °C. After cooling to RT, 5 μL of the mixture was transformed to chemically competent cells of a *ccdB*-resistant *E. coli* strain such as DB3.1.[7] Aliquots were plated on LB-agar supplemented with the appropriate antibiotics, and colonies were obtained after overnight incubation at 37 °C. Relevant regions were verified by sequencing and subsequently recloned into the SapI-free parent vector to avoid potential errors introduced by the PCR of the vector backbone. Plasmids described in this study are detailed in Table S3.

Construction of pINITIAL. A fragment containing the *sacB* counterselection marker flanked by SfiI sites was amplified by PCR using pEA_sacB as template. A fragment containing the pBR322 ORI and kanamycin resistance gene, flanked by SfiI sites, was amplified by PCR using a pET28-derivative as template. A fragment containing the *ccdB* and cat markers, flanked by SapI sites, was amplified from pREBACK using primers containing SfiI sites. All fragments were mixed, digested with SfiI, and subsequently ligated and transformed to *E. coli* DB3.1 cells. The orientation of the three fragments was fixed by the choice for three different types of overhangs resulting from SfiI digestion. In the resulting plasmid, the SapI site near the pBR322 origin of replication (ORI) was removed by Quikchange mutagenesis, leading to pINITIAL.

FX Cloning of PCR Products. Open reading frames coding for SLC12 transporters devoid of their start and stop codons were amplified by PCR using a forward primer extended on its 5′ end by 5′ *atatatGCTCTTCtAGTnnn* and a reverse primer extended on its 5′ end by 5′ *tatataGCTCTTCaTGCnnn*. The *nnn* triplet indicates the position of the codon flanking the start and stop codon in the forward and reverse primer, respectively. Lowercase letters in the extensions can be replaced by any nucleotide to prevent stable secondary structure elements. PCR products were gel-purified.

For initial cloning of PCR products, 50 ng of the pINITIAL vector was mixed with the PCR product to a final molar ratio of 1:5 (vector:insert). After addition of 1 μL of 10x buffer (200 mM Tris-acetate, 500 mM potassium acetate, 100 mM magnesium acetate, 10 mM dithiothreitol, pH 7.9), the volume was adjusted to 9 μL, and the digestion was started by the addition of 1 μL of SapI (2U). The mixture was digested for 1 h at 37 °C. The enzyme was subsequently heat-inactivated by a 20 min incubation at 65 °C and cooled to 25 °C. Fragments were ligated by the addition of 1.25 μL of 10 mM ATP-mix and 1.25 μL of T4 ligase (1 U/μL) for 1 h at 25 °C followed by heat-inactivation of the enzyme by a 20 min incubation at 65 °C. A 5 μL aliquot of the mixture was transformed to chemically competent *E. coli* MC1061,[22] a non-*ccdB*-resistant *E. coli* strain. Aliquots were plated on LB-agar supplemented with 50 μg kanamycin/mL, and colonies were obtained after overnight incubation at 37 °C. The pINITIAL-derivative obtained was sequence-verified and used for subsequent subcloning of the ORF to different expression vectors. If initial sequence verification is not required, cloning into pINITIAL can be skipped and PCR products can be cloned immediately in an expression vector using the same procedure.

Subcloning Using FX Cloning. For subcloning, 50 ng of the desired FX-cloning compatible expression vector was mixed with the pINITIAL-derivative containing the inset to a final molar ratio of 1:4 (expression vector:pINITIAL-derivative). The same procedure as described above was followed, except that after transformation to chemically competent cells of a non-ccdB-resistant *E. coli* strain, such as *E. coli* MC1061,[22] aliquots were plated on LB-agar supplemented with 7% (w/v) sucrose and the appropriate antibiotic for the expression vector. The intermediate pREXNH3 and pREXC3H vectors were converted into lactococcal expression vectors using the VBEx procedure.[21]

Overexpression and Characterization of SLC12 Transporters. *E. coli* MC1061[22] and BL21(DE3)[19] containing derivatives of P_{BAD}-controlled (pBXNH3 and pBXC3H) or P_{T7}-controlled

(p7XNH3 and p7XC3H) expression vectors, respectively, were cultivated on Terrific Broth containing 0.75% (w/v) glycerol and supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin when appropriate. Cultures of 700 μL were inoculated with a 1% (v/v) inoculum of an overnight culture and grown in 96-well plates sealed with gas-permeable seals at 37 °C with vigorous shaking (300 rpm). After 1.5 h growth, the temperature was slowly adjusted to 25 °C over the course of 1 h. Cells were subsequently induced at OD_{600} = 0.4−0.6 with 1.10^{-2} % (w/v) L-arabinose or 0.5 mM IPTG for P_{BAD}- or P_{T7}-controlled expression vectors, respectively. Cultivation was continued for ∼16 h. *L. lactis* NZ9000[23] containing derivatives of P_{nisA}-controlled (pNZXNH3 and pNZXC3H) expression vectors were cultivated on M17[24] supplemented with 0.5% (w/v) glucose and 5 μg/mL chloramphenicol. Cultures of 4 mL were inoculated with a 1% (v/v) inoculum of an overnight culture and grown semianaerobically in a sealed 24-well plate at 30 °C with mild shaking (50 rpm). Cells were subsequently induced at OD_{600} = 0.4−0.6 with 4×10^{-4} volume of spent medium of *L. lactis* NZ9700,[25] corresponding to a final nisin A concentration of 4 ng/mL, and cultivation was continued for ∼16 h. Cell disruption for *E. coli* and *L. lactis*, solubilization of the membrane fragments with 1% (w/v) dodecyl-β-D-maltoside, and subsequent differential sedimentation and detection by immunoblotting were performed as described.[2,26]

RESULTS

Description of the Cloning Procedure. Uniform cloning strategies for a large set of ORFs based on restriction endonucleases (REs) are generally prohibited as the length of the recognition sites are insufficient to prevent their occurrence in a fraction of the target sequences. This complication is even more severe due to the need of two different restriction enzymes to secure the orientation of the inset in a plasmid vector. However, these difficulties mainly apply to the commonly used type IIP REs, which recognize palindromic sequences and cleave the DNA within their recognition site, thus producing an invariable overhang.[27] Although type IIS REs have recognition sites of similar lengths as type IIP REs, their recognition sequence is nonpalindromic and cleavage occurs at a defined number of base pairs outside their recognition site[14] (Figure 1A). The resulting overhang can thus be of any sequence, and directional cloning can be accomplished with the use of a single type IIS RE. Unlike internal IIP recognition sites that will always produce a compatible overhang and consequently lead to a gene truncation, an internal type IIS recognition site is only problematic in rare cases where the produced overhang is complementary to that of the vector.

We reasoned that these attractive properties of type IIS REs could be exploited for a uniform high-throughput cloning procedure. The overall cloning strategy, termed FX cloning (for fragment exchange cloning), is outlined in Figure 1B,C. Initially, the target gene is amplified by PCR using a pair of comparably short primers, each containing a type IIS RE site. The recognition sites are located at the respective 5′ ends and are oriented so that they are physically separated from the ORF upon cleavage (Figure 1D). Though the sequence of the short single-stranded overhangs at both ends of the cleaved PCR product can be freely chosen, they should differ sufficiently from each other to allow directional cloning and prevent self-ligation of the vector. The cleaved ORF can be received by an intermediate sequencing vector (pINITIAL) or cloned immediately into one or more expression vectors. Both classes of vectors contain the same pair of type IIS RE sites flanking the counterselection gene *ccdB*.[28] Importantly in pINITIAL the sites have the same direction as in the PCR product, oriented toward the counterselection marker. In this case the respective recognition sites remain on the plasmid after cleavage. In the expression vectors, in contrast, the direction of the cleavage sites is reversed (Figure 1E). The recognition sites are thus lost after digestion, which ensures that the cloning-related sequences added to the expressed constructs remain small. After cleavage, both classes of vectors contain complementary overhangs which hybridize with the insert and are finally joined by ligation. Subsequent transformation of the ligation mix to a non-*ccdB*-resistant *E. coli* strain allows only cells with daughter plasmids containing the target ORF to survive. Taken together, these steps eliminate the need for purification of cleaved products and thus allow the entire reaction to be performed in one cup.

The generation of different expression constructs from a single ORF usually proceeds in two steps. In a first step the PCR product is cloned into pINITIAL and confirmed by sequencing (Figure 1B). In a second reaction, the intermediate vector pINITIAL, containing the ORF, takes over the role of the PCR product (Figure 1C). The reaction proceeds in the same way, with the respective expression vector serving as acceptor. Plating on sucrose-containing media after transformation to a non-*ccdB*-resistant *E. coli* strain selects for expression vectors containing the insert, as the pINITIAL backbone carries the counterselection marker *sacB* which renders *E. coli* sensitive to sucrose.[29]

Selection of the Type IIS RE and Overhang Sequence. To establish the FX cloning procedure, we selected the most suitable type IIS RE out of the set of 532 available enzymes in the REBASE database (March 2008).[30] Next to being commercially available, we searched for enzymes with long and thus infrequently occurring recognition sequences, which produce a single-stranded overhang of preferably three bases. The latter would result in the addition of only a single amino acid on either terminus of the protein. The criteria were satisfied by only four isoschizomers—BspQI, LguI, PciSI, and SapI—that all recognize the same seven base pair long sequence (Figure 1A). Though we established the procedure using SapI, in principle any of these enzymes can be used.

The distribution of SapI sequences was analyzed in 1121 prokaryotic genomes and 345 plasmids larger than 100 kb present in the NCBI database (September 2010; Figure S1). We found that, in agreement with our expectations, these sites are rare as in over 97% of the cases the average distance between neighboring SapI sites is larger than 2.5 kb. This ensures that the vast majority of ORFs are not targeted by this enzyme.

As terminal single-stranded overhangs we selected the sequences AGT and GCA, coding for the small and uncharged amino acids serine and alanine, respectively. These triplets are incompatible with each other, which prevents self-ligation of the vector and insertion of the target in the undesired direction. Since ORFs with internal SapI sites that produce overhangs complementary to those of the vector will complicate the cloning procedure, we determined the fraction of affected sequences and found that only about 7% of the SapI sites present in prokaryotic ORFs (NCBI database, September 2010) give rise to compatible overhangs (Figure S2). As most ORFs contain either no (86%) or a single recognition site (12% of which 93% result in overhangs not compatible to those in the vector), a total

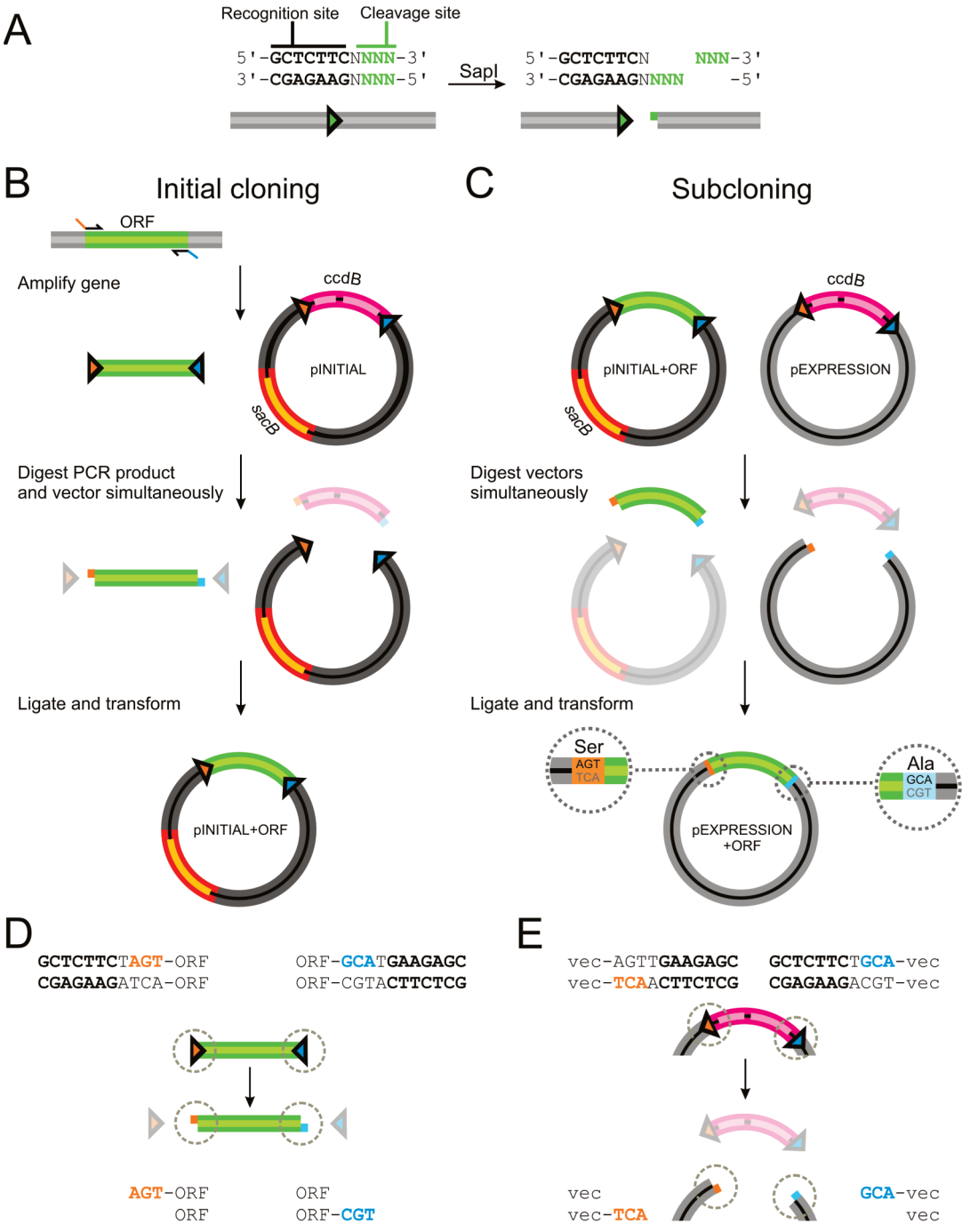


Figure 1. Schematic overview of the FX-cloning method. (A) SapI restriction site. The recognition site is shown in bold letters; nucleotides of the three base-pair single-stranded overhangs are colored in green. N describes any of the four nucleotides. A schematic view of the cleavage is shown. Arrows indicate the direction of the restriction site. (B) Cloning of a PCR product into pINITIAL. The amplified open reading frame (ORF) is shown in green. The direction of the IIS restriction sites is indicated by arrows, which are colored corresponding to their respective generated overhangs after cleavage. The genes coding for the counterselection markers ccdB and sacB on pINITIAL are colored in magenta and orange, respectively. (C) Subcloning of an ORF into an expression vector (pEXPRESSION). Color coding is as in (B). The three nucleotides added to either terminus of the ORF are shown as insets (circle). (D) Orientation of the SapI cleavage sites in the PCR product and pINITIAL and (E) in expression vectors. The single-stranded overhangs generated upon cleavage are shown in orange and magenta.

of 97% of the analyzed ORFs can be cloned in a straightforward manner (Table S1). The addition of the small three base-pair sequences connecting the target ORF with the expression elements of the vector allows the use of a single PCR product to generate fusions at both termini of the translated protein. The use of different primer sets for N- and C-terminal fusions to prevent translation of the long cloning-related sequences, a common practice for most high-throughput cloning strategies, can thus be bypassed. The preparation of any expression vector for FX cloning involves only minor adaptations. Internal SapI

dx.doi.org/10.1021/bi200178z |*Biochemistry* 2011, 50, 3272–3278

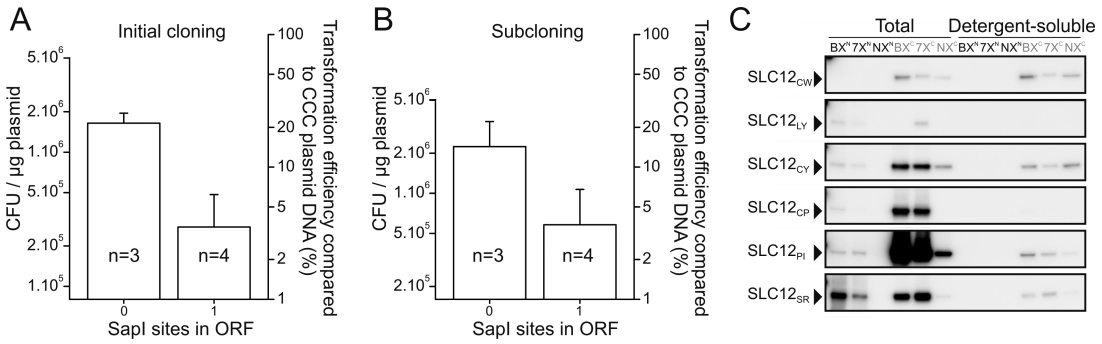


Figure 2. FX-cloning efficiency. (A) Efficiency of the initial cloning step of a PCR product into pINITIAL expressed in colony forming units (CFU) per μg vector (left axis) and as a fraction (%) of the transformation efficiency of covalently closed circular (CCC) plasmid DNA (pINITIAL containing an insert; right axis). Values and standard deviations are shown for inserts containing either no (left) or one SapI site (right). (B) Efficiency of the subcloning step of ORFs from pINITIAL to the expression vector pBXC3H expressed in CFU per μg pBXC3H (left axis) or as a fraction of the transformation efficiency of covalently closed circular (CCC) plasmid DNA (pBXC3H containing an insert; right axis). (C) Immunoblots indicating relative expression levels for SLC12 homologues from *Crocosphaera watsonii* (CW), *Lyngbya* sp. (LY), *Cyanothece* sp. (CY), *Candidatus Protochlamydia amoebophila* (CP), *Psychromonas ingrahamii* (PI), and *Salinibacter ruber* (SR). Membrane proteins were expressed in *E. coli* using the P_{BAD} (BX) and P_{T7} (7X) promoters or in *L. lactis* using the P_{NisA} (NX) promoter. Superscripts N and C indicate an N- or C-terminal location of the His-tag, respectively. For each homologue, equivalent amounts of crude cell lysate were used to assess the total expression levels. Total: samples containing whole cell lysates. Detergent-soluble: fractions solubilized with dodecylmaltoside. Proteins were detected using an anti-His-tag antibody.

sites have to be removed and a pair of restriction sites has to be introduced by PCR, followed by an FX-cloning step in which the *ccdB* marker is inserted. To date, we have prepared pINITIAL and several common expression vectors for that purpose.

FX Cloning of SLC12 Transporters for Expression Screening. To illustrate the versatility of this approach, we have cloned several genes of ~2.3 kb each coding for prokaryotic homologues of the SLC12 membrane transporter family[31,32] into six different expression vectors. For both the initial cloning step of the PCR product into pINITIAL (Figure 2A) and subsequent subcloning steps into different expression vectors (Figure 2B) we have obtained high transformation efficiencies only 1−2 orders of magnitude less than transformation of covalently closed circular plasmid DNA. These values are similar to those reported for efficient high-throughput cloning systems such as LIC[10] and Gateway.[7] Importantly, for both steps the presence of an internal SapI site only lead to a minor, ~5-fold decrease of efficiency. In all cases the amount of DNA used (50 ng plasmid) was an order of magnitude lower compared to classical restriction-type cloning methods. All 56 analyzed colonies contained the desired plasmid (Table S2). Subsequent characterization of expression conditions was performed only few days after the initial PCR reactions. The broad screening allowed the selection of several combinations of expression-host, vector, and target proteins amenable to overexpression and detergent solubilization within 1 week of work (Figure 2C and Figure S3). In comparison, an earlier attempt to carry out this screening by traditional restriction and ligation-based methods has taken several months.

DISCUSSION

The FX-cloning procedure described here presents an efficient and inexpensive high-throughput cloning strategy that is applicable to all established prokaryotic and eukaryotic expression systems. Although parts of the method are conceptually similar to procedures described before,[15,17,18] it includes important improvements that are critical for its performance as a reliable, uniform, and robust method. The choice of the appropriate restriction enzyme with a comparably long recognition site, and a three-base-pair overhang ensures a low frequency of internal cleavage sites in target genes and limits the addition of cloning related sequences to either end of the expressed protein to one amino acid each. The inclusion of appropriate counterselection markers keeps the background of untransformed vectors low and allows unambiguous selection of positive clones in each step. FX cloning requires minimal handling and takes place in one cup within a 3 h period. It does depend neither on comparably expensive proprietary enzymes such as used in Gateway[7] nor on critical enzyme treatments such as required for LIC.[10] In contrast to LIC, subcloning between different vector systems is possible and does not require an additional amplification reaction. The robustness of the method makes it suitable even to researchers new to molecular cloning. During the past two years FX cloning was used in our and selected other laboratories to clone several hundreds of genes into expression vectors for prokaryotic and eukaryotic expression systems and has proven to work reproducibly and with exceptionally high efficiency. This has allowed us to investigate more expression constructs in a considerably shorter time frame than previously when using traditional cloning methods. We thus find FX cloning to be superior to all other available strategies for the generation of large numbers of expression constructs for challenging structural biology projects.

ASSOCIATED CONTENT

Supporting Information. Average distance between SapI sites in 1121 prokaryotic genomes and 345 large plasmids (Figure S1); frequencies of overhangs and their complement resulting from SapI digestion (Figure S2); fraction of prokaryotic open reading frames with no, one, or multiple SapI sites (Table S1); FX-cloning efficiencies (Table S2); plasmids compatible with FX cloning described in the study (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail e.geertsma@bioc.uzh.ch, tel +41 44 635 5546, fax +41 44 635 6834 (E.R.G.); e-mail dutzler@bioc.uzh.ch; tel +41 44 635 6550; fax +41 44 635 6834 (R.D.).

Funding Sources

The research leading to these results has received funding from a grant from the Swiss National Science Foundation (SNF) and from the NCCR Structural Biology. E.R.G. has received support from long-term fellowships from the Federation of European Biochemical Societies and the Human Frontier Science Program.

■ ACKNOWLEDGMENT

We acknowledge Iwan Zimmermann and Thomas Heitkamp for participation in the vector construction and Stefan Warmuth and Yvonne Neldner for the kind gift of plasmids.

■ ABBREVIATIONS

ORF, open reading frame; RE, restriction endonuclease; ORI, origin of replication; CFU, colony forming units; CCC plasmid DNA, covalently closed circular plasmid DNA.

■ REFERENCES

(1) Lewinson, O., Lee, A. T., and Rees, D. C. (2008) The funnel approach to the precrystallization production of membrane proteins. *J. Mol. Biol. 377*, 62–73.

(2) Geertsma, E. R., Groeneveld, M., Slotboom, D. J., and Poolman, B. (2008) Quality control of overexpressed membrane proteins. *Proc. Natl. Acad. Sci. U.S.A. 105*, 5722–5727.

(3) Kawate, T., and Gouaux, E. (2006) Fluorescence-detection size-exclusion chromatography for precrystallization screening of integral membrane proteins. *Structure 14*, 673–681.

(4) Lesley, S. A. (2009) Parallel methods for expression and purification. *Methods Enzymol. 463*, 767–785.

(5) Gileadi, O., Burgess-Brown, N. A., Colebrook, S. M., Berridge, G., Savitsky, P., Smee, C. E., Loppnau, P., Johansson, C., Salah, E., and Pantic, N. H. (2008) High throughput production of recombinant human proteins for crystallography. *Methods Mol. Biol. 426*, 221–246.

(6) Hartley, J. L. (2006) Cloning technologies for protein expression and purification. *Curr. Opin. Biotechnol. 17*, 359–366.

(7) Hartley, J. L., Temple, G. F., and Brasch, M. A. (2000) DNA cloning using in vitro site-specific recombination. *Genome Res. 10*, 1788–1795.

(8) Colwill, K., Wells, C. D., Elder, K., Goudreault, M., Hersi, K., Kulkarni, S., Hardy, W. R., Pawson, T., and Morin, G. B. (2006) Modification of the Creator recombination system for proteomics applications--improved expression by addition of splice sites. *BMC Biotechnol. 6*, 13.

(9) Li, M. Z., and Elledge, S. J. (2005) MAGIC, an in vivo genetic method for the rapid construction of recombinant DNA molecules. *Nature Genet. 37*, 311–319.

(10) Aslanidis, C., and de Jong, P. J. (1991) Coincidence cloning of Alu PCR products. *Proc. Natl. Acad. Sci. U.S.A. 88*, 6765–6769.

(11) Berrow, N. S., Alderton, D., and Owens, R. J. (2009) The precise engineering of expression vectors using high-throughput In-Fusion PCR cloning. *Methods Mol. Biol. 498*, 75–90.

(12) Graslund, S., Nordlund, P., Weigelt, J., Hallberg, B. M., Bray, J., Gileadi, O., Knapp, S., Oppermann, U., Arrowsmith, C., Hui, R., Ming, J., dhe-Paganon, S., Park, H. W., Savchenko, A., Yee, A., Edwards, A., Vincentelli, R., Cambillau, C., Kim, R., Kim, S. H., Rao, Z., Shi, Y., Terwilliger, T. C., Kim, C. Y., Hung, L. W., Waldo, G. S., Peleg, Y., Albeck, S., Unger, T., Dym, O., Prilusky, J., Sussman, J. L., Stevens, R. C., Lesley, S. A., Wilson, I. A., Joachimiak, A., Collart, F., Dementieva, I., Donnelly, M. I., Eschenfeldt, W. H., Kim, Y., Stols, L., Wu, R., Zhou, M., Burley, S. K., Emtage, J. S., Sauder, J. M., Thompson, D., Bain, K., Luz, J., Gheyi, T., Zhang, F., Atwell, S., Almo, S. C., Bonanno, J. B., Fiser, A., Swaminathan, S., Studier, F. W., Chance, M. R., Sali, A., Acton, T. B., Xiao, R., Zhao, L., Ma, L. C., Hunt, J. F., Tong, L., Cunningham, K., Inouye, M., Anderson, S., Janjua, H., Shastry, R., Ho, C. K., Wang, D., Wang, H., Jiang, M., Montelione, G. T., Stuart, D. I., Owens, R. J., Daenke, S., Schutz, A., Heinemann, U., Yokoyama, S., Bussow, K., and Gunsalus, K. C. (2008) Protein production and purification. *Nature Methods 5*, 135–146.

(13) Love, J., Mancia, F., Shapiro, L., Punta, M., Rost, B., Girvin, M., Wang, D. N., Zhou, M., Hunt, J. F., Szyperski, T., Gouaux, E., MacKinnon, R., McDermott, A., Honig, B., Inouye, M., Montelione, G., and Hendrickson, W. A. (2010) The New York Consortium on Membrane Protein Structure (NYCOMPS): a high-throughput platform for structural genomics of integral membrane proteins. *J. Struct. Funct. Genomics 11*, 191–199.

(14) Szybalski, W., Kim, S. C., Hasan, N., and Podhajska, A. J. (1991) Class-IIS restriction enzymes--a review. *Gene 100*, 13–26.

(15) Padgett, K. A., and Sorge, J. A. (1996) Creating seamless junctions independent of restriction sites in PCR cloning. *Gene 168*, 31–35.

(16) Fromme, T., and Klingenspor, M. (2007) Rapid single step subcloning procedure by combined action of type II and type IIs endonucleases with ligase. *J. Biol. Eng. 1*, 7.

(17) Engler, C., Kandzia, R., and Marillonnet, S. (2008) A one pot, one step, precision cloning method with high throughput capability. *PLoS One 3*, e3647.

(18) Kotera, I., and Nagai, T. (2008) A high-throughput and single-tube recombination of crude PCR products using a DNA polymerase inhibitor and type IIS restriction enzyme. *J. Biotechnol. 137*, 1–7.

(19) Studier, F. W., and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol. 189*, 113–130.

(20) Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol. 177*, 4121–4130.

(21) Geertsma, E. R., and Poolman, B. (2007) High-throughput cloning and expression in recalcitrant bacteria. *Nature Methods 4*, 705–707.

(22) Casadaban, M. J., and Cohen, S. N. (1980) Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. *J. Mol. Biol. 138*, 179–207.

(23) de Ruyter, P. G., Kuipers, O. P., and de Vos, W. M. (1996) Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. *Appl. Environ. Microbiol. 62*, 3662–3667.

(24) Terzaghi, B. E., and Sandine, W. E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol. 29*, 807–813.

(25) Kuipers, O. P., Beerthuyzen, M. M., Siezen, R. J., and De Vos, W. M. (1993) Characterization of the nisin gene cluster nisABTCIPR of Lactococcus lactis. Requirement of expression of the nisA and nisI genes for development of immunity. *Eur. J. Biochem. 216*, 281–291.

(26) Geertsma, E. R., and Poolman, B. (2010) Production of membrane proteins in Escherichia coli and Lactococcus lactis. *Methods Mol. Biol. 601*, 17–38.

(27) Roberts, R. J., Belfort, M., Bestor, T., Bhagwat, A. S., Bickle, T. A., Bitinaite, J., Blumenthal, R. M., Degtyarev, S., Dryden, D. T., Dybvig, K., Firman, K., Gromova, E. S., Gumport, R. I., Halford, S. E., Hattman, S., Heitman, J., Hornby, D. P., Janulaitis, A., Jeltsch, A., Josephsen, J., Kiss, A., Klaenhammer, T. R., Kobayashi, I., Kong, H., Kruger, D. H., Lacks, S., Marinus, M. G., Miyahara, M., Morgan, R. D., Murray, N. E., Nagaraja, V., Piekarowicz, A., Pingoud, A., Raleigh, E., Rao, D. N., Reich, N., Repin, V. E., Selker, E. U., Shaw, P. C., Stein, D. C., Stoddard, B. L., Szybalski, W., Trautner, T. A., Van Etten, J. L., Vitor, J. M., Wilson, G. G., and Xu, S. Y. (2003) A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res. 31*, 1805–1812.

(28) Bernard, P., Gabant, P., Bahassi, E. M., and Couturier, M. (1994) Positive-selection vectors using the F plasmid ccdB killer gene. *Gene 148*, 71–74.

(29) Recorbet, G., Robert, C., Givaudan, A., Kudla, B., Normand, P., and Faurie, G. (1993) Conditional suicide system of Escherichia coli

released into soil that uses the Bacillus subtilis sacB gene. *Appl. Environ. Microbiol. 59*, 1361–1366.

(30) Roberts, R. J., Vincze, T., Posfai, J., and Macelis, D. (2010) REBASE--a database for DNA restriction and modification: enzymes, genes and genomes. *Nucleic Acids Res. 38*, D234–236.

(31) Gamba, G. (2005) Molecular physiology and pathophysiology of electroneutral cation-chloride cotransporters. *Physiol. Rev. 85*, 423–493.

(32) Warmuth, S., Zimmermann, I., and Dutzler, R. (2009) X-ray structure of the C-terminal domain of a prokaryotic cation-chloride cotransporter. *Structure 17*, 538–546.