Chapter 4

Flexi Vector Cloning

Paul G. Blommel, Peter A. Martin, Kory D. Seder, Russell L. Wrobel, and Brian G. Fox

Summary

A protocol for ligation-dependent cloning using the Flexi Vector method in a 96-well format is described. The complete protocol includes PCR amplification of the desired gene to append Flexi Vector cloning sequences, restriction digestion of the PCR products, ligation of the digested PCR products into a similarly digested acceptor vector, transformation and growth of host cells, analysis of the transformed clones, and storage of a sequence-verified clone. The protocol also includes transfer of the sequence-verified clones into another Flexi Vector plasmid backbone. Smaller numbers of cloning reactions can be undertaken by appropriate scaling of the indicated reaction volumes.

Key words: Flexi vector; Gene cloning; High throughput; Expression vectors; Proteomics; Genomics

4.1. Introduction

The availability of sequenced genomes has stimulated investigations into the best high-throughput methods to obtain the encoded proteins and enzymes. Structural genomics *(1–3)*, functional proteomics *(4)*, drug discovery *(5–7)*, agricultural research *(8)*, environmental studies *(9)*, and many other topics of current research in protein biochemistry and enzymology benefit from these efforts.

An essential prerequisite is the establishment of reliable and reproducible protocols for high-throughput cloning. Current methods include recombinational *(10–12)*, ligation-independent *(13–16)*, and ligation-dependent cloning *(17)*. Because of the complexity of protein expression and folding in heterologous hosts, methods to efficiently transfer cloned and sequence-verified genes to many different expression contexts are desirable.

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Flexi Vector cloning is a ligation-dependent method facilitated by selection for the replacement of a toxic gene insert in an acceptor vector *(17)*. Genome-scale restriction mapping has shown that the combination of SgfI and PmeI restriction sites used by the Flexi Vector method allows cloning of 98.9% of all human genes, 98.9% of mouse, 98.8% of rat, 98.5% of *C. elegans*, 97.8% of zebra fish, 97.6% of *Arabidopsis*, and 97.0% of yeast genes, suggesting broad overall utility for use with eukaryotes. This protocol covers Flexi Vector cloning of genes from cDNA directly into an expression vector and the subsequent high-fidelity transfer of the sequence-verified coding region into alternate expression vectors.

4.2. Materials

4.2.1. Flexi Vector Plasmids

Flexi Vector plasmids for bacterial, cell-free, and mammalian cell expression are available from Promega Corporation (Madison, WI). The University of Wisconsin Center for Eukaryotic Structural Genomics (CESG) will be depositing Flexi Vector plasmids and genes cloned by the Flexi Vector method into the Materials Repository of the Protein Structure Initiative at the Harvard Institute of Proteomics (http://plasmid.hms.harvard.edu). Among these are the *Escherichia coli* expression vectors pVP56K and pVP68K, and the wheat germ cell-free expression vector pEU-His-FV.

Figure 4.1 shows a map of the plasmid pVP56K, which is used to create a His8-MPB-target fusion protein. The target protein can be liberated from the N-terminal portion of the fusion by treatment with tobacco etch virus (TEV) protease *(12, 18)*. The Bar-CAT cassette, bounded by SgfI and PmeI restriction sites, consists of the lethal barnase gene to select against the parental plasmid during cloning and the chloramphenicol acetyltransferase gene to select for the presence of the cassette during construction and propagation of the vector. Plasmids containing the lethal barnase gene must be propagated in a barnase-resistant strain (e.g., *Escherichia coli* BR610, which is available through Technical Services, Promega Corporation).

4.2.2. Target Genes

Target cDNA originally cloned by the Mammalian Gene Collection (http://mgc.nci.nih.gov/) can be purchased from Open Biosystems (http://www.openbiosystems.com/), Invitrogen (https://www.invitrogen.com/), or American Type Culture Collection (ATCC, http://www.atcc.org/catalog/molecular/index.cfm). Other sources of eukaryotic cDNAs that the CESG has used are the Kazusa DNA Research Institute (http://www.kazusa.or.jp/eng/index.html), and the Arabidopsis Biological Resource Center (ABRC, http://www.biosci.ohio-state.edu/pcmb/Facilities/

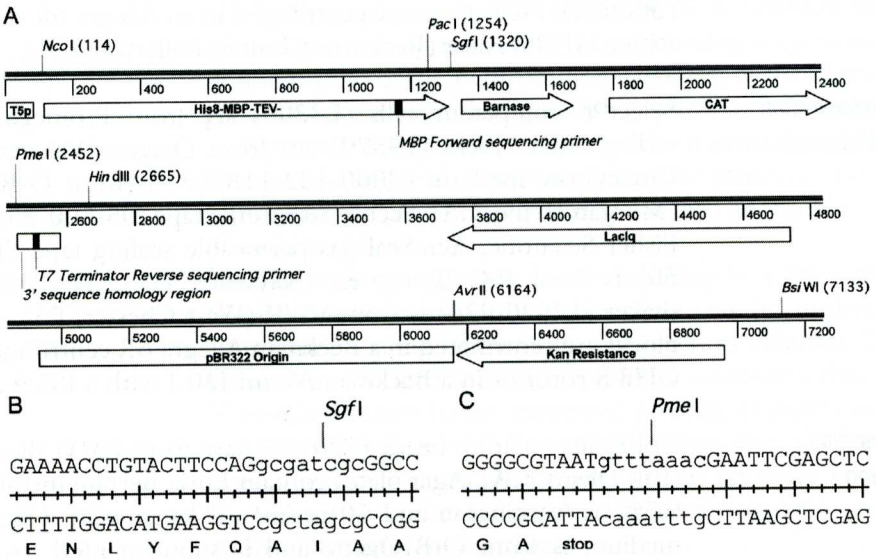

Fig. 4.1. *Escherichia coli* expression vector pvp56k. (a) Linear map showing key features of the vector. (b) Sequence in the region near to the sgfi site. The nucleotide and encoded protein sequence of a portion of the linker between His8-MBP and the target is shown. The TEV protease site is ENLYFQA, where proteolysis occurs between the Q and A residues. After expression of the fusion protein, an N-terminal AIA-target is released by treatment with TEV protease. The identity of the next residues in the target is determined by the PCR primer design. (c) Sequence in the region near to the pmeI site, including the stop codon of the target gene.

abrc/abrchome.htm). Flexi Vector cloning can also be applied to cDNA libraries or genomic DNA prepared from natural organisms or tissues. Genes already cloned by the Flexi Vector method are available from Origene (Rockville, MD) and the Kazusa DNA Institute.

4.2.3. Flexi Vector Reagents

The SgfI/PmeI 10X Enzyme Blend and Buffer (Product No. R1852), high concentration T4 DNA ligase (M1794), Magnesil PCR cleanup kits (A923A), Magnebot II magnetic bead separation block (V8351), and DNA molecular weight markers (PR-67531) are from Promega. (*see* **Note 1**).

4.2.4. PCR Reagents

ORF specific primers (25-nmol synthesis with standard desalting) can be obtained from IDT (Coralville, IA). The dNTP mix (10 mM of each nucleotide, U1515) is from Promega. An MJ DNA Engine, DYAD, Peltier Thermal Cycler (MJ Research, Waltham, MA) can be used. HotMasterTaq DNA Polymerase (0032 002.676) is from Eppendorf (Hamburg, Germany). PCR plates (T-3069-B) are from ISC Bioexpress (Kaysville, UT). Adhesive covers for PCR plates (4306311) are from Applied Biosystems (Foster City, CA). The protocol was originally developed using YieldAce HotStart DNA polymerase (600336) from Stratagene (La Jolla, CA) and can be substituted by Pfu Ultra II Hotstart DNA polymerase

(600672). PCR plates are centrifuged in an Allegra 6R centrifuge with a GH3.8 rotor (Beckman Coulter, Fullerton, CA).

4.2.5. Bacterial Cell Culture Materials

Select96 competent cells (L3300) are from Promega. Deep-well growth blocks (19579) are from Qiagen (Valencia, CA). CircleGrow medium (3000-132-118268) is from Q-BIOgene (Morgan Irvine, CA). Secure Seal sterile tape (05-500-33) is from Fisher Scientific. AeraSeal gas permeable sealing tape (T-2421-50) is from ISC Bioexpress (Kaysville, UT). The microplate shaker (12620-926) is from VWR (West Chester, PA). Growth blocks are centrifuged in a Beckman Allegra 6R centrifuge with a GH3.8 rotor or in a Beckman Avanti J30-I with a JS5.9 rotor.

4.2.6. Plasmid Preparation

ColiRollers plating beads (71013-3) are from EMD Biosciences (San Diego, CA). Agar plates contain Luria Bertani medium plus 0.5% (w/v) glucose and 50 μg/mL of kanamycin. CircleGrow medium is from Q-BIOgene and is supplemented to contain 50 μg/mL of kanamycin. QiaVac96 vacuum plasmid preparation materials are from Qiagen.

4.2.7. DNA Analysis and Sequence Verification

Optical spectroscopy is used to assess DNA concentration and purity *(19)*. In this work, measurements are made with a SpectroMax Plus Model 01269 spectrophotometer (Molecular Devices, Sunnyvale, CA). Samples are measured in UV Star plastic 384-well plates (T-3118-1) from ISC Bioexpress.

PCR is used for qualitative insert size mapping and for DNA sequencing. The vector-directed forward and reverse primers are 5′-GATGTCCGCTTTCTGGTATGC-3′ (MBP Forward sequencing primer, *black* rectangle starting at 1,155 bp, **Fig. 4.1a**) and 5′-GCTAGTTATTGCTCAGCGG-3′, (T7 Terminator sequencing primer, *black* rectangle starting at 2,501 bp, **Fig. 4.1a**), respectively. A 2.5X PCR Mastermix (FP-22-004-10) from Fisher and the 2% E-gel 96 system (G7008-02) from Invitrogen (Carlsbad, CA) are used for insert size determination. Big Dye Version 3.1 sequencing reagents are from Applied Biosystems. DNA sequencing can be performed at the University of Wisconsin Biotechnology Center.

4.3. Methods

Standard molecular cloning techniques are used *(20)*. A comparison of Flexi Vector and Gateway cloning methods has been published *(17)*. Promega also provides detailed instructions for Flexi Vector cloning *(21)*.

The complete protocol consists of PCR amplification of the desired gene to append Flexi Vector cloning sequences, restriction digestion of the PCR products, ligation of the digested PCR products into a similarly digested acceptor vector, transformation and growth of host cells, analysis of the transformed clones, and storage of a sequence-verified clone. The protocol also includes transfer of the sequence-verified clone into another Flexi Vector plasmid backbone.

The following protocol is for cloning in a 96-well format. Smaller numbers of cloning reactions can be undertaken by appropriate scaling of the indicated reaction volumes. This protocol describes production of plasmid constructs that yield an N-terminal fusion to the expressed protein, as illustrated in **Fig. 4.1**. A section is provided on modifications that yield alternative N-terminal constructs, and thus serve to illustrate how expression vector and primer design can be used to provide useful variations of expression constructs.

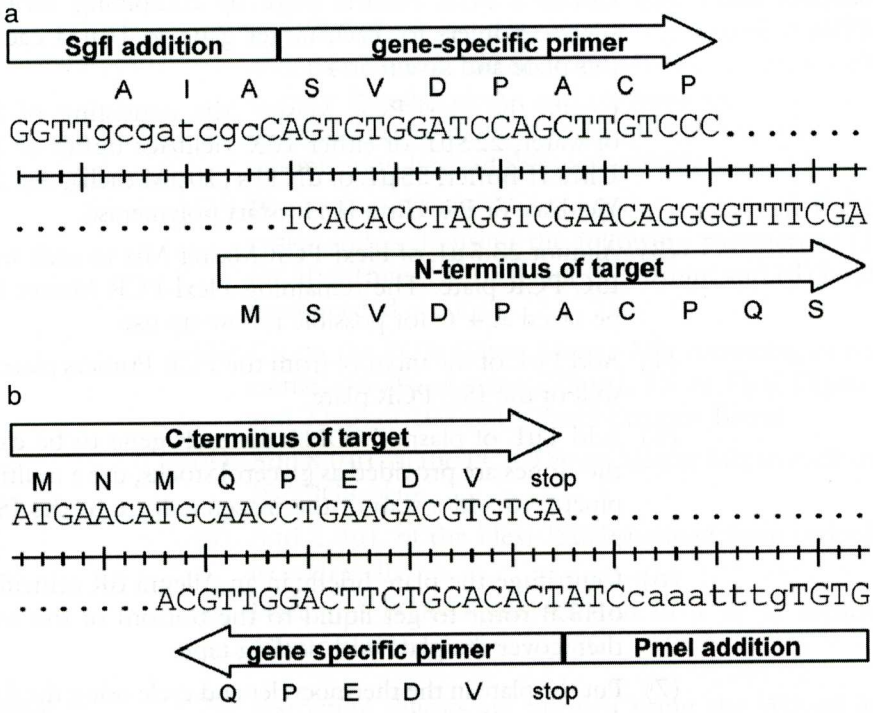

Fig. 4.2. An example of 5′ coding and 3′ reverse complementary strand primers created for Flexi Vector cloning. (a) The 5′ primer consists of an exact match of the desired gene-specific sequence and an additional sequence encoding an sgfi site (34 nucleotides). (b) The 3′ complement reverse primer consists of an exact match of the gene-specific sequence including the stop codon, a primer-encoded stop codon and an additional sequence adding a pmel site (33 nucleotides).

4.3.1. Attachment of Flexi Vector Cloning Sequences

4.3.1.1. PCR Primers

In the Flexi Vector cloning approach described in this section, target genes are amplified using a single-step PCR. **Figure 4.2** shows an example of primers designed to clone a structural genomics target, human stem cell Nanog protein (NM_024865), into pVP56K. In general, the forward and reverse primers are 28–36 nucleotides in length. The gene-specific portion includes 14–23 nucleotides that exactly match the target gene beginning at the second codon. Whenever possible, the gene-specific primers end with a C or G nucleotide to enhance DNA polymerase initiation. The invariant sequence 5′-GGTTgcgatcgcC-3′(including an SgfI site, *lower* case) is added to the 5′ end of the forward primer. The reverse primer consists of the invariant sequence 5′-GTGTgtttaaacCTA (including a PmeI site, *lower* case) followed by the reverse complement of the 3′ gene-specific 14–23 nt including the stop codon. The additional nucleotides are added to the 5′end of these sequences to promote restriction nuclease digestion of the PCR products. For this example, the synthesis of primers containing a total of 68 nucleotides is required.

4.3.1.2. PCR Amplification

The following steps are used to PCR amplify the desired gene and append the sequences required for cloning (*see* **Note 2**).

(1) Create a PCR Primers plate by combining forward and reverse primers for each target gene to 10 μM each. Label this plate and save it at 4°C.

(2) Create the Flexi-PCR Master Mix consisting of 2.23 mL of water, 225 μL of either 10X YieldAce buffer or 10X Pfu Ultra II Buffer, 55 μL of dNTPs (10 μM each), and 23 μL of YieldAce or Pfu Ultra II Hotstart polymerase.

(3) Aliquot 23.5 μL of Flexi-PCR Master Mix to each well of an ISC PCR plate. The remaining Flexi-PCR Master Mix can be saved at 4°C for possible follow-up use.

(4) Add 1 μL of the mixture from the PCR Primers plate to each well of the ISC PCR plate.

(5) Add 1 μL of plasmid cDNA for each gene to be cloned. If the clones are provided as glycerol stocks, use a multichannel pipette to add a stab of the frozen culture to the ISC PCR plate.

(6) Centrifuge the plate briefly in an Allegra 6R centrifuge and 6H3.B rotor to get liquid to the bottom of the wells and then cover the plate with sealing tape.

(7) Put the plate in the thermocycler and cycle using the following parameters for reactions using YieldAce polymerase: (1) 95°C for 5.00 min; (2) 94°C for 30 s; (3) 50°C for 30 s; (4) 72°C, 1.00 min/kb; (5) repeat **steps 2–4** for 4 more times; (6) 94°C for 30 s; (7) 55°C for 30 s; (8) 72°C for 1.00 min/kb plus 10 s per cycle; (9) repeat **steps 6–8** for 24 more times; (10) 72°C for 30.00 min; and (11) 4°C and hold. For amplification of

~1 kb genes, this PCR takes ~3 h to complete. Use the following conditions for PfuUltraII polymerase: (1) 95°C for 3.00 min; (2) 95°C for 20 s; (3) 50°C for 20 s; (4) 72°C, 15 s/kb; (5) repeat steps 2–4 for 4 more times; (6) 95°C for 20 s; (7) 55°C for 20 s; (8) 72°C for 15 s/kb; (9) repeat steps 6–8 for 24 more times; (10) 72°C for 3 min; and (11) 4°C and hold.

(8) Analyze the completed Flexi-PCR reactions on a 2% E-gel 96 by loading 15 µL of the gel running buffer plus 5 µL of the reaction samples. Load 5 µL of PCR molecular weight markers with 15 µL of the gel running buffer.

(9) Retry the PCR for any genes that fail to amplify.

(10) Create a master PCR plate of all successfully amplified genes, label the plate, and begin the restriction digestion step or store the plate at −20°C until needed.

4.3.2. Restriction Digestion of PCR Products

The following steps are used to digest the acceptor vector and PCR products with SgfI and PmeI prior to the ligation (*see* **Note 3**).

4.3.2.1. Digestion Reaction

(1) Create the Acceptor Vector Digest Master Mix consisting of 158.3 µL of sterile, deionized water, 44.0 µL of 5X Flexi-Digest Buffer, 2.20 µL of 10X SgfI/PmeI Enzyme Blend, and 13.5 µL of Acceptor Vector (e.g., purified pVP56K at a concentration of 150 ng/µL). Mix the solution well, as the enzyme blend is dense and tends to settle. Substitution of individual preparations of SgfI and PmeI will require extensive optimization beyond the scope of this protocol.

(2) Place the Acceptor Vector digest reaction in the thermocycler and cycle using the following parameters: (1) 37°C for 40.00 min; (2) 65°C for 20.00 min; and (3) hold at 4°C until needed.

(3) Create the PCR-Digest Master Mix consisting of 638 µL of sterile, deionized water, 220 µL 5X of Flexi-Digest Buffer, and 22 µL of 10X SgfI/PmeI Enzyme Blend.

(4) Add 8.0 µL of the PCR-Digest Master Mix to each well of an ISC PCR plate.

(5) Add 2.0 µL of the Flexi-PCR obtained from procedure 3.1 to each well.

(6) Place the PCR-Digest reaction in a 37°C incubator for 40 min and then move to 4°C.

4.3.2.2. Cleanup

The restriction digests are purified using the Wizard Magnesil PCR Cleanup system and a Magnebot II plate. An important part of the cleanup is to thoroughly dry the sample after the final wash step to evaporate all residual ethanol.

(1) Add 10 µL of well-mixed Magnesil Yellow to each well of the PCR digest plate.

(2) Mix the 20 µL of solution 4 times, incubate for 45 s, and then mix four more times. If any bubbles are present, briefly centrifuge the plate.

(3) Place the PCR digest plate on the Magnebot II magnetic stand, wait 30 s for the magnetic beads to adhere to the right side of the plate, and remove and discard the liquid. The PCR product is now bound to the Magnesil Yellow beads.

(4) Remove the plate from the Magnebot II, and add 20 µL of Magnesil Wash Solution to each well. Mix each well 4 times, wait 60 s, and then mix 4 more times.

(5) Place the plate on the Magnebot II, wait 30 s for the magnetic beads to adhere to the right side of the plate, and remove and discard the liquid.

(6) Wash the beads two more times using 30 µL of 80% ethanol as described in **steps 4** and **5**.

(7) Place the PCR digest plate on a 42°C heating block for 10 min or until all of resin has dried.

(8) To elute the DNA, add 10 µL of water, mix well, and wait 60 s.

(9) Place the plate on the Magnebot II, remove the Magnesil particles, and save the supernatant for use in the ligation reaction.

4.3.3. Ligation of PCR Products into an Acceptor Vector

Ligation reactions are performed in a 96-well PCR plate using the restriction-digested and purified PCR products and Acceptor vector prepared in **step 3.2**. The following steps are used.

(1) Create the Ligation Master Mix containing 225 µL of sterile, deionized water, 110 µL of 10X T4 Ligase Buffer, and 50 µL of T4 DNA Ligase HC.

(2) Add 5.0 µL of cleaned-up PCR product digest, 2.0 µL of Acceptor vector digest, and 3.5 µL of Ligation Master Mix to each well of a new PCR plate.

(3) Store the clearly labeled plate of leftover cleaned-up PCR product at −20°C.

(4) Incubate the reaction in a thermocycler at 25°C for 3 h. Proceed to the transformation step (**Section 4.3.4**) or the reaction can be left overnight at 4°C.

4.3.4. Transformation and Growth of Host Cells

The material from the ligation reaction is used to transform Select96 competent cells by the following steps.

(1) Thaw four strips of Select96 cells on ice. Distribute 15 µL into each well of a prechilled PCR plate. Begin warming SOC medium in a 37°C incubator.

(2) Add 1 µL of the ligation reaction to the Select96 cells, cover the PCR plate and incubate at on ice for 20 min.

(3) Heat shock at 42°C for 30 s on a heat block.

(4) Chill on ice for 1 min.

(5) Add 90 µL of prewarmed SOC medium.

(6) Cover the plate and incubate at 37°C for 1 h with no shaking.

(7) Label Luria Bertani agar plates containing 0.5% (w/v) glucose and 50 µg/mL of kanamycin with the corresponding plate position numbers (A1-H12 for a 96-well plate).

(8) Add 5–10 sterile ColiRoller glass beads to each plate.

(9) Apply the entire volume of the transformation reaction onto the glass beads.

(10) Shake the plates horizontally for 15 s and then dump the glass beads off the plate into a beaker of 80% ethanol. The beads can be washed with 1% (v/v) nitric acid, rinsed extensively with deionized water, and then sterilized and dried for reuse.

(11) Incubate the plates overnight at 37°C.

4.3.5. Analysis of Plasmid DNA

A colony PCR amplification step provides a qualitative check for the presence of the target gene before more labor-intensive plasmid preparation, quantification by optical spectroscopy, and DNA sequence verification. (*see* **Note 4**).

4.3.5.1. Colony PCR Screening of Transformants

Two colonies are selected from the transformation plate and used for colony PCR screening. The same colonies are also used to prepare an inoculum for subsequent plasmid isolation. The preparation of the colony replicates and the colony PCR screening are accomplished as follows.

(1) Prepare 50 mL of CircleGrow medium containing 50 µg/mL of kanamycin.

(2) Pour the medium into a sterile multichannel reagent reservoir.

(3) Use a multichannel pipette to aliquot 200 µL of the CircleGrow medium into each well of two deep-well growth blocks. Label the blocks "Screening Block 1" and "Screening Block 2."

(4) Pipette 10 µL of water into each well of two PCR plates. Label the PCR plates "PCR Screen Plate 1" and "PCR Screen Plate 2."

(5) Pick a colony off the transformation plate with a pipette tip and dab the tip into the water in a well of the PCR plate labeled "PCR Screen Plate 1" and then eject the same pipette tip into the deep-well growth block labeled "Screening Block 1." Repeat the procedure with a second colony into "PCR Screen Plate 2" and "Screening Block 2."

(6) Cover Screening Blocks 1 and 2 with AeraSeal breathable sealing tape.

(7) Place the Screening Blocks 1 and 2 on an orbital shaker at 37°C and aerate vigorously (800 rpm on the VWR microplate shaker) for ~16 h.

(8) Create the Colony PCR Screen Master Mix containing 880 µL of sterile, deionized water, 2.20 mL of 2.5X Eppendorf Hot Master Mix, and 110 µL of MBP Forward sequencing primer (10 µM).

(9) Add 14.5 µL of Colony PCR Master Mix to each well of PCR Screen Plate 1 and PCR Screen Plate 2.

(10) Add 0.5 µL of the gene-specific reverse primers (10 µM) used in **step 3.1** (e.g., the primer designed as implied in **Fig. 4.2b**) to each well of PCR Screen Plate 1 and PCR Screen Plate 2.

(11) Centrifuge the PCR plate to eliminate air bubbles.

(12) Put the two PCR Screen plates in a thermocycler and use the following parameters: (1) 95°C for 5.00 min; (2) 94°C for 30 s; (3) 50°C for 30 s; (4) 72°C for 1.00 min/kb; (5) repeat **steps 2–4** for 19 more times; (6) 72°C for 10.00 min; (7) 4°C and hold. For amplification of ~1 kb genes, this PCR takes ~90 min to complete.

(13) Analyze the completed colony PCRs on a 2% E-gel 96 by loading 15 µL of the gel running buffer plus 5 µL of the reaction sample. Load 5 µL of PCR molecular weight markers plus 15 µL of the gel running buffer.

4.3.5.2 Growth of Individual Isolates

The following steps are used to obtain a 1-mL culture of individual isolates from the transformation plate that are found by colony PCR to have an insert of appropriate size for the gene cloned.

(1) Prepare 200 mL of CircleGrow medium containing 50 µg/mL of kanamycin.

(2) Aliquot 1 mL of CircleGrow medium per well of a Qiagen flat-bottom growth block.

(3) Inoculate the growth block with 5 µL of a culture in the Screening Block 1 or 2 that was identified to have an insert of appropriate size by the colony PCR analysis. Fill any empty wells of the growth block with culture medium or water to help assure the vacuum miniprep procedure used later.

(4) Cover the growth block with AeraSeal Plate Sealers.

(5) Shake the growth block overnight at 37°C orbital plate shaker set at the maximum value (800 rpm on the VWR microplate shaker) for ~16 h.

(6) Store the growth block at 4°C for later use.

4.3.5.3. QiaPrep 96 Turbo Plasmid DNA Purification

This section uses reagents from the QiaPrep 96 kit from Qiagen. Several steps described later are modified from the manufacturer's protocol. These modifications are essential to prepare plasmid DNA of sufficient quantity and purity for subsequent use in Flexi Vector reactions.

(1) Add RNase A to buffer P1 (Qiagen). P1 buffer is stored at 4°C.

(2) Check to make sure that the buffer P2 (Qiagen) has not precipitated during storage. If it has, warm it to 37°C until all precipitate has been redissolved.

(3) The growth block obtained in **Section 4.3.5** is centrifuged either at 2,100 × *g* for 30 min in an Allegra 6R centrifuge with a GH3.8 rotor or at 5,000 × *g* for 15 min in an Avanti J30-I with a JS5.9 rotor.

(4) Discard the supernatant.

(5) *Resuspension*: Add 250 µL of buffer P1 to each well of the growth block. Seal the block with tape and vortex thoroughly to resuspend the cells. Ensure that no cell clumps remain.

(6) *Alkaline lysis*: Add 250 µL of buffer P2 to each sample. Use a clean, dry paper towel to dry the top of the growth block. Seal the growth block tightly with aluminum tape seal and gently invert the Blocks 4–6 times to mix. Incubate the growth block at room temperature for no more than 5 min.

(7) *Neutralization*: Add 350 µL of buffer N3 (Qiagen) to each sample. Dry the top of the growth block and tightly seal the block with a new sheet of aluminum tape. Gently invert the Blocks 4–6 times. To avoid localized precipitation, mix the samples gently but thoroughly immediately after addition of buffer N3. The solution will become cloudy.

(8) Place a Turbofilter plate (white) on the top of a QiaPrep Plate (blue) seated together on top of an empty growth block. Apply 850 µL of neutralized lysate from previous step to the top most Turbofilter plate. Centrifuge at 3,000 × *g* for 5 min to filter the lysate and bind the plasmid to the membrane of QiaPrep Plate. Discard the supernatant captured in the growth block.

(9) Place a QiaPrep plate (blue) on the top of the vacuum manifold. The white plastic reservoir should be placed beneath the plate to collect the flow-through waste.

(10) Wash the QiaPrep plate with 0.5 mL/well of Buffer PB.

(11) Wash the QiaPrep Plate with 0.7 mL/well of Buffer PE.

(12) Prepare to centrifuge the QiaPrep plate by placing the QiaPrep plate on top of a used Qiagen 96-well elution plate. Prepare an accurate counterbalance with another elution plate and the used Turbofilter plate.

(13) Spin the balanced plates at 5,000 × *g* for 5 min on the Avanti J30-I centrifuge with a J30-I rotor. The membrane will be dry after this step. Discard the flow-through liquid.

(14) *Elution*: To elute the plasmid DNA, ensure that the QiaPrep plate is in place over a clean elution plate and then add 100 µL of buffer TE (Qiagen) to the center of the filter well. Let the plate stand for 1 min.

(15) Elute the dissolved plasmid DNA by spinning at 6,000 × *g* for 5 min in the Avanti J30-I centrifuge with a JS5.9 rotor.

4.3.5.4. Determination of DNA Concentration and Purity

The concentration and purity of plasmid preparations used for Flexi Vector cloning must be determined. This can be accomplished using UV-visible spectroscopy *(19)*. The minimum useful concentration of plasmid DNA for subsequent work is 25 ng/µL, and the ratio of A_{260}/A_{280} must be between 1.8 and 2.0. Ratios outside this range indicate contamination that will interfere with subsequent sections.

(1) Add 95 µL of water to each well that will be used in a UV Star 384-well plate.

(2) Insert the plate into the spectrophotometer and obtain a reference setting using water as the blank.

(3) Using the multichannel pipette, add 5 µL of the purified plasmid sample to wells (dilution 1:20). Mix carefully to avoid creating air bubbles.

(4) Insert the plate into the spectrophotometer and read the absorbance values at 260 and 280 nm.

(5) Calculate the plasmid concentration (ng/µL) by the following formula: (A_{260} of the sample – A_{260} of the blank) × 1,000. Alternatively, the plasmid concentration can be calculated by the following approach. Measure the A_{260} value of a 1:20 dilution of a 100 ng/µL plasmid DNA standard. Multiply A_{260} measured for the unknown sample by (100/A_{260} of the diluted standard). This calculation also gives a concentration estimate in ng/µL. The plasmid concentration must be greater than or equal to 25 ng/µL. Typical plasmid concentrations from this procedure are ~100 ng/µL in a typical volume of ~100 µL.

(6) Calculate the ratio of A_{260}/A_{280}. This value should be between 1.8 and 2.0. Values that deviate from this range have a suspect concentration estimate and also likely contain contaminants that will interfere with subsequent Flexi Vector transfer reactions.

4.3.5.5. Sequence Analysis of Plasmid DNA

Previous work with genes from eukaryotes has revealed the necessity for DNA sequence verification before extensive downstream studies of protein expression are undertaken *(12)*.

(1) Prepare the Forward Sequencing Master Mix containing 660 µL of sterile, deionized water, 165 µL of 2.5X Buffer 3.1, 110 µL Big Dye v3.1, and 27.5 µL of MBP Forward sequencing primer (10 µM).

(2) Prepare the Reverse Sequencing Master Mix containing 660 µL of sterile, deionized water, 165 µL of 2.5X Buffer 3.1, 110 µL of Big Dye v3.1, and 27.5 µL of T7 Terminator sequencing primer (10 µM).

(3) Aliquot 8.75 µL of each master mix into two separate PCR plates.

(4) Aliquot 1.25 µL of purified plasmid DNA into both the forward and the reverse reactions.

(5) Spin down both plates in the Allegra 6R centrifuge with 6H3.B rotor.

(6) Put the PCR plates into a thermocycler and cycle using the following parameters: (1) 95°C for 3.00 min; (2) 94°C for 10 s; (3) 58°C for 4.00 min; (4) repeat **steps 2–4** for 50 more times; (5) 72°C for 10.00 min; (6) hold at 4°C. This PCR takes ~5 h to complete.

(7) When the PCR is complete, the materials are suitable for submission to a DNA sequencing facility for automated sequence analysis.

4.3.6. Creation of Glycerol Stocks

Glycerol stocks of sequence-verified clones are prepared in the following manner.

(1) Add 20 µL of 80% sterile glycerol to a new PCR plate.

(2) Add 80 µL of culture containing the sequence-verified clones obtained in **Section 4.3.5.2** to create one plate.

(3) Mix the culture and the glycerol stock well with the pipette.

(4) Add an appropriate barcode or other labeling to the PCR plate.

(5) Cover the PCR plate with foil tape and store the plate at −80°C.

4.3.7. Flexi Vector Transfer Reaction

Cloned genes are moved between different Flexi Vectors by restriction digestion and ligation. The donor and acceptor plasmids must encode resistance to different antibiotics in order to permit positive selection of an acceptor plasmid that has accepted an insert and negative selection of the unchanged donor plasmid. The lethal barnase gene will provide selection against the acceptor plasmid that has not been digested. For 96-well operation, the following steps accomplish the transfer reaction. For smaller number of reactions, the volumes should be scaled to avoid waste of reagents. It is essential to have the highest quality plasmid DNA preparations for these transfer reactions. Plasmid

contaminated with *E. coli* genomic DNA will yield false positive colonies, and plasmid contaminated with residuals from the plasmid preparation will have lower efficiency of gene transfer (*see* **Note 4**).

(1) Create the Flexi Transfer Master Mix from 305 µL of deionized, sterile water, 110 µL 5X Flexi-Digest Buffer, 5.5 µL of 10X SgfI/PmeI Enzyme Blend, and 22.0 µL of purified Acceptor Vector (nominal DNA concentration of 150 ng/µL).

(2) Add 4.0 µL of Flexi Transfer Master Mix to each well of a 96-well ISC PCR plate stored on ice.

(3) Add 1.0 µL of donor vector (nominal DNA concentration of 30 ng/µL) to each well of the 96-well plate, cover the plate with an adhesive cover, and centrifuge the plate for 1 min in the Allegra 6R centrifuge and 6H3.8 rotor.

(4) Incubate the plate for 40 min at 37°C in the thermocycler.

(5) Incubate the plate for 20 min at 65°C in the thermocycler to inactivate the restriction enzymes.

(6) Create the Ligation Transfer Master Mix from 440 µL deionized, sterile water, 110.0 µL of 10X Ligase Buffer, and 55 µL of T4 DNA Ligase HC.

(7) Add 5.5 µL of Ligation Transfer Master Mix to each well of the plate containing the heat-inactivated acceptor vector digests. Mix the contents of the plate thoroughly, cover the plate with an adhesive cover, and centrifuge the plate for 1 min in an Allegra 6R centrifuge and 6H3.8 rotor.

(8) Incubate the plate for 1 h at 25°C in the thermocycler to complete the ligation reaction.

(9) The ligation reaction can be stored at –20°C until needed for transformation as described in **Section 4.3.4**.

(10) Sequence verification is not routinely necessary after Flexi Vector transfers *(17)*.

4.3.8. Alternative Constructs for Flexi Vector

The following sections provide information on approaches to develop custom plasmids with Flexi Vector capabilities and to provide primer design examples for the production of other types of expression constructs and fusion proteins.

4.3.8.1. Creation of an Antibiotic Resistance Cassette

The vectors recently developed at CESG reserve the AvrII and Bsi WI restriction sites to define an antibiotic resistance cassette (**Fig. 4.1**). By use of these restriction sites, the kanamycin resistance gene and promoter can be swapped with either the ampicillin resistance gene and promoter or other antibiotic resistance genes and promoters. For workers interested in creating new Flexi Vector backbones, these sites should be created by PCR mutagenesis before the Flexi Vector barnase cassette is introduced. Other

antibiotic resistance genes and promoters can be introduced into this site after similar PCR mutagenesis, digestion, and ligation. Plasmids containing the lethal barnase gene must be propagated in a barnase-resistant strain (e.g., *Escherichia coli* BR610, which is available through Technical Services, Promega Corporation).

4.3.8.2. Design of 3′ Sequence in Flexi Vector Plasmids

Self-ligation of the vector backbone through the SgfI and PmeI sites can be reduced by including a region of sequence identity adjacent to either the 3′ or 5′ end of the Flexi Vector cloning cassette *(17)*. This region of identity acts to inhibit replication by forming of an extensive DNA palindrome when two vectors with substantial sequence identity ligate to each other *(22)*. Therefore, inclusion of a region of either 3′ or 5′ sequence identity of ~100 bp or longer should be included in the design when different vector backbones are customized for use with the Flexi Vector system. One example is the transfer pairing of CESG plasmids having a kanamycin resistance marker (originally a Qiagen pQE80 backbone) with pEU plasmids having an ampicillin resistance marker (Cell-Free Sciences, Yokohama, Japan).

The pVP56K vector shown in **Fig. 4.1** includes 131 bp of the DNA sequence 3′ from the PmeI site of pF1K (Promega) as the 3′ homology region. This fragment can either be cloned by PCR from pF1K or moved from CESG vectors to other compatible vectors as a separate piece obtained by PmeI and HindIII digestion. The 3′ homology region can also be included with the BarCAT cassette by restriction digestion of CESG vectors with SgfI and HindIII.

4.3.8.3. Two-Step PCR for Fusion Protein Expression

pVP56K encodes the tobacco etch virus protease recognition site in a 5′ position relative to the SgfI site used for Flexi Vector cloning (**Fig. 4.1**). The protein sequence of this site is ENLYFQA, where proteolysis occurs between Q and A. Thus, when TEV protease is used to proteolyse the His8-MBP-target fusion protein produced from pVP56K, an AIA-target protein is liberated. In some circumstances, this modified N-terminal may be undesirable.

Figure 4.3 shows a variation of the vector backbone, pVP68K, and a primer design that allows liberation of S-target after TEV protease processing of the His8-MBP-target fusion protein. Since a significant fraction of natural proteins have a serine as the second residue, CESG primers used for high-throughput cloning encode this residue. However, TEV protease is relatively tolerant of substitution of other residues at the P1 position *(23)*, so a native N terminus (after bacterial N-terminal Met processing) can be engineered through primer design in many cases.

This approach requires a two-step PCR procedure similar to that we have previously used for Gateway cloning *(24)*. In the example shown in **Fig. 4.3b**, the first PCR forward primer contains 14 gene-specific nucleotides (**Fig. 4.3b**). An invariant

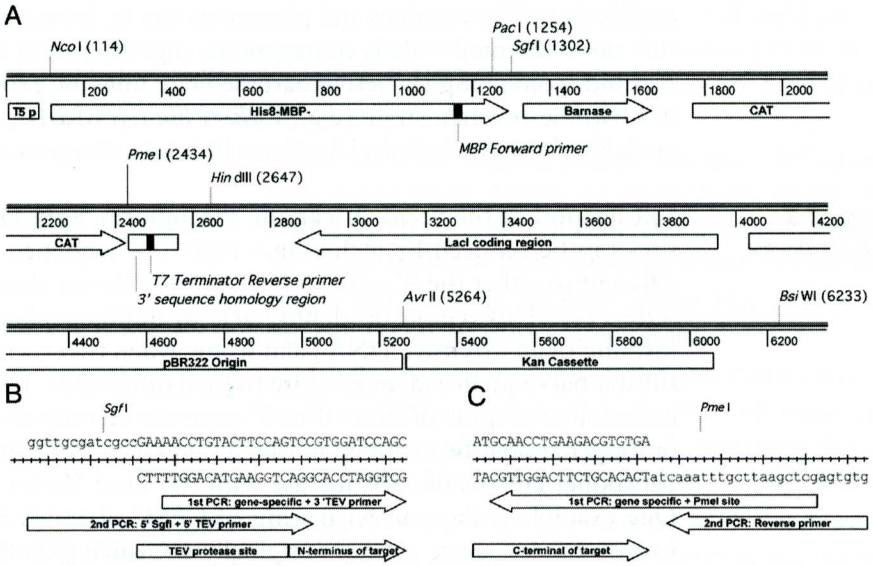

Fig. 4.3. *Escherichia coli* expression vector pvp68k. (a) Linear map showing key elements in the vector. (b) Sequence in the 5′ region near to the sgfi site. The first round forward PCR primer includes a gene-specific sequence and a portion of the TEV protease site (29 nucleotides). The second round forward PCR primer is a universal sequence that completes the TEV protease site and appends a 5′ sgfi sequence (34 nucleotides). The first round 3′ reverse complement primer includes a gene-specific portion, an additional stop codon and the pmeI site and a redundant ecori site (39 nucleotides). The second round reverse complement PCR primer is a universal sequence that duplicates the pmeI and EcoRI sites (24 nucleotides). After expression of the His8-MBP-target fusion protein, an N-terminal S-target can be released by treatment with TEV protease.

sequence is added to the first PCR forward primer to encode a portion (15 nucleotides) of the TEV protease site. The design of the first step 3′ reverse complement primer (**Fig. 4.3c**) is the similar to that described in **Fig. 4.2** and an example is shown in **Fig. 4.3c**. The first step PCR is as described in **Section 4.3.1.2** using 0.2 μM of the appropriately designed forward and reverse primers. For the second PCR, a universal forward primer (39 nucleotides) is used to add the nucleotides required to complete the TEV site and add the SgfI site, and a universal reverse PCR primer is used to duplicate the PmeI site and add additional nucleotides. For the second step PCR, one-fifth of the first-step reaction is added into a new PCR using 0.2 μM of the universal forward primer and reverse primers. The second-step reaction is then completed as described in **Section 4.3.1.2**. For this example, the synthesis of primers containing a total of 126 nucleotides is required.

4.3.8.4. Native N Terminus

The example of **Fig. 4.4** is compatible with use of pFK1 (Promega). Primer design places the native start codon immediately downstream from the SgfI site and ~35 residues downstream from the promoter. This example also places the SgfI site

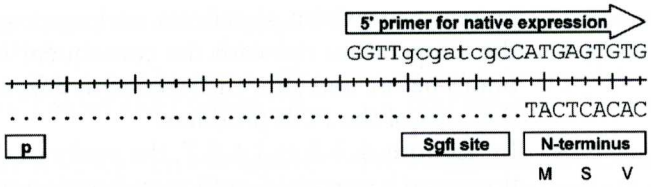

Fig. 4.4. A primer design example for expression of a native protein using Flexi Vector cloning. The promoter region is located upstream of the sgfi site and the start codon encoded by the 5′ primer.

so that the consensus-10-region is retained. For newly engineered plasmids, the nucleotide sequence between the promoter and the desired start codon should not encode alternative start codons in the other translation frames. The design of the 3′ reverse complement primer is the same as described in **Fig. 4.2**. For this example, the synthesis of primers containing a total of 58 nucleotides is required.

4.3.8.5. C-Terminal Fusion Proteins

C-terminal fusion proteins can be produced by Flexi Vector cloning into vectors such as pFC7K (C-terminal HQ tag) or pFC8K (C-terminal HaloTag). This is accomplished by digestion of the C-terminal fusion acceptor vector by SgfI and the alternative blunt end restriction enzyme Eco IRCI and digestion of an existing Flexi Vector clone with SgfI and PmeI. Ligation of the insert will place the C terminus of the target in frame with the fusion protein encoded by the acceptor vector, but will destroy the PmeI site in the ligated product. Vectors created by this approach can no longer be used for transfer to another Flexi Vector, so this approach should not be used as the first step in assembling a family of Flexi Vector expression contexts.

4.4. Notes

1. Use of the Promega blend of SgfI and PmeI is simpler than trying to optimize a mixture of separately purchased SgfI and PmeI.
2. The majority of errors in cloned genes occur in sequence associated with primers. It is advisable to us high-quality primers to minimize the number mutations introduced in these regions, which are critical for successful cloning and gene transfer. It may be necessary to sequence several clones in order to find those without errors.
3. The timing of **step 6** is important as overdigestion can lead to lowered efficiency of cloning. Also, do not heat inactivate the PCR digest because the residual DNA polymerase

present can exhibit significant endonuclease activity during the temperature rise with the consequent removal of nucleotides before and after the restriction enzymes have digested the ends of the PCR products.

4. In **Sections 4.3.5** and **4.3.7**, the purity of the DNA preparations used is essential for efficient digestion and ligation during initial cloning and subsequent transfer reactions. It is advisable to prepare large stocks of the plasmids and verify their purity and function. Care in the preparation of the inserts must be taken to avoid transfer of guanidinium, detergents, or solvents from the plasmid and DNA preparations into the restriction digestion reactions, as these enzymes can be inactivated by these contaminants.

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