Chapter 2

In-Fusion® Cloning with Vaccinia Virus DNA Polymerase

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Abstract

Vaccinia virus DNA polymerase (VVpol) encodes a 3′-to-5′ proofreading exonuclease that can degrade the ends of duplex DNA and expose single-stranded DNA tails. The reaction plays a critical role in promoting virus recombination in vivo because single-strand annealing reactions can then fuse molecules sharing complementary tails into recombinant precursors called joint molecules. We have shown that this reaction can also occur in vitro, providing a simple method for the directional cloning of PCR products into any vector of interest. A commercial form of this recombineering technology called In-Fusion® that facilitates high-throughput directional cloning of PCR products has been commercialized by Clontech. To effect the in vitro cloning reaction, PCR products are prepared using primers that add 16–18 bp of sequence to each end of the PCR amplicon that are homologous to the two ends of a linearized vector. The linearized vector and PCR products are coincubated with VVpol, which exposes the complementary ends and promotes joint molecule formation. Vaccinia virus single-stranded DNA binding protein can be added to enhance this reaction, although it is not an essential component. The resulting joint molecules are used to transform *E. coli*, which convert these noncovalently joined molecules into stable recombinants. We illustrate how this technology works by using, as an example, the cloning of the vaccinia N2L gene into the vector pETBlue-2.

Key words: Vaccinia virus, Recombineering, In-Fusion® cloning, DNA polymerase, PCR cloning

1. Introduction

By an odd coincidence, vaccinia virus-encoded enzymes have been utilized in two distinctly different methods for rapid cloning of PCR products, both of which have been commercialized. The first method was invented by Stuart Shuman and is sold as Topo® cloning kits by Invitrogen (1, 2). The Shuman method exploits the reversible association between vaccinia virus topoisomerase I and its

DNA cleavage target, and permits rapid cloning of PCR amplicons into vectors that are supplied bearing molecules of topoisomerase covalently attached to the vector ends. The advantages of this elegant method have been reviewed and compared with other high-throughput cloning technologies (3).

We have described an alternate method for ligase-independent cloning of PCR products, which instead uses vaccinia virus DNA polymerase (VVpol). The principles of the method are shown in Fig. 1. These studies have shown that VVpol can fuse linear DNA molecules into concatemers, in vitro, if the ends of these DNAs share ten or more nucleotides of sequence identity (4–8). The joining reaction depends upon the 3′-to-5′ proofreading exonuclease
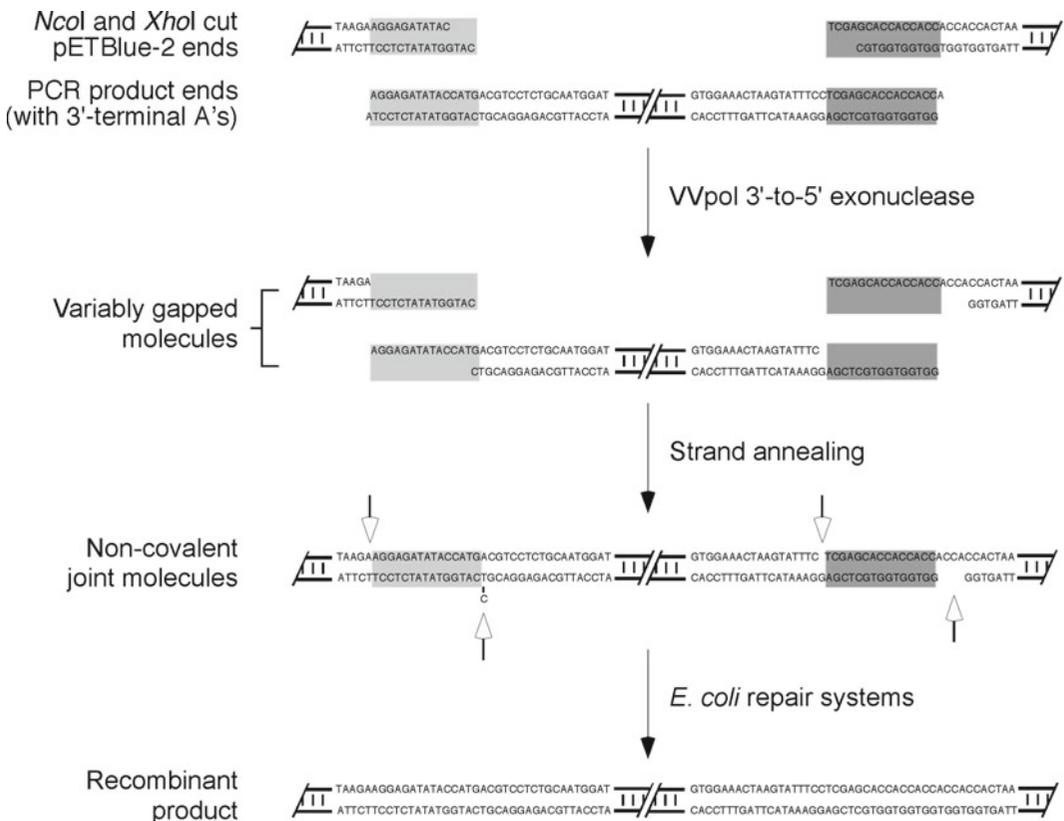

Fig. 1. Cloning DNA with vaccinia virus DNA polymerase. The target vector is digested with restriction enzyme(s) and the PCR products are prepared using primers that encode homology to the vector ends (*dark boxes*, the recommended minimal length of the homology is 16–18 bp). These products are then mixed together and incubated with VVpol. The 3′-to-5′ exonuclease activity degrades the ends of the DNAs, exposing areas of complementary sequence. These ends can then anneal (in a cloning reaction that is stimulated by vaccinia virus I3 single-strand DNA binding protein) and create noncovalently linked joint molecules that are sufficiently stable to survive transfection of *E. coli*. *E. coli* DNA repair systems convert the joint molecules into stable recombinants. Note that the joint molecules will contain a mix of nicks, gaps, and extra nucleotides (*white arrows*) due to variation in the extent of exonuclease attack. We illustrate the technique using a specific combination of *Nco*I and *Xho*I cut vector (pETBlue-2) and PCR amplicon (the N2L gene), but the method can be used with any combination of homologous ends.

function of the polymerase and is enhanced by adding vaccinia virus single-strand DNA binding protein (the I3 protein) (4, 5, 9). The proofreading exonuclease can attack a variety of duplex ends and the joining reaction depends upon it exposing 5′-overhangs of single-stranded DNA. Such single-stranded DNAs can anneal spontaneously if they encode complementary sequences and will form joint molecules that can be readily converted into covalently joined recombinants after transfection of *E. coli*. One of the properties of VVpol that may help promote these reactions is that it has a reduced capacity to attack joint molecules, once formed, and this may help stabilize such products against continued exonucleolytic degradation (10). These in vitro systems exhibit many of the same properties that characterize in vivo vaccinia virus recombination reactions (i.e., they have the same exonuclease and homology requirements, and the products show evidence of attack by a 3′-to-5′ exonuclease), suggesting that vaccinia virus also uses the DNA polymerase as a recombinase in infected cells (4, 7, 8).

This approach has the advantage that it requires no modification of existing vectors beyond linearizing them, which can be accomplished either by digestion with restriction enzymes(s) or by PCR amplification of the entire vector. This second approach thus permits the cloning of fragments at any location in a vector, even if there are no convenient cloning sites. Significantly, the VVpol 3′ to 5′ exonuclease activity is able to process DNA irrespective of the type of duplex end (5′-overhang, blunt, or 3′-overhang). Thus, the method permits directional cloning of PCR products irrespective of the form of the linear ends of the vector. The method does require extending the PCR primers to add sequences that duplicate the 16–18 bp of sequences flanking the vector cut site, but this costs very little nowadays and is often done anyway to produce clones encoding precisely modified junction sequences (e.g., peptide epitope tags and altered reading frames). The benefit, however, is that the desired sequence is cloned without the addition of any extra bases, since the primer extensions are simply homologous to the ends of the target vector itself. Moreover, there appears to be no significant restriction on the sequence of homology used, permitting cloning at any desired location. In this chapter, we outline this approach and illustrate the method by showing how it was used to clone the vaccinia N2L gene into the protein expression vector pETBlue-2.

2. Materials

2.1. DNA Substrate Preparation

1. 60 mm dish of BSC-40 cells.

2. Media: minimal-essential media supplemented with 5% FBS and 1% of each of the following: nonessential amino acids, L-glutamine and antibiotic–antimycotic.

3. Cell lysis buffer: 1.2% (w/v) SDS, 50 mM Tris–HCl, pH 8.0, 4 mM EDTA, 4 mM $CaCl_2$, 0.2 mg/mL proteinase K.

4. Buffer saturated phenol.

5. Cold 95% and 70% ethanol.

6. 3 M sodium acetate, pH 5.2.

7. Forward primer: 5′-<u>AGG AGA TAT ACC ATG</u> ACG TCC TCT GCA ATG GAT-3′ (see Notes 1 and 2).

8. Reverse primer: 5′-<u>GGT GGT GGT GCT CGA G</u>GA AAT ACT TAG TTT CCA C-3′ (see Notes 1 and 2).

9. *Taq* DNA polymerase (e.g., Fermentas) (see Note 3).

10. pETBlue-2 plasmid (Novagen).

11. Restriction enzymes.

12. 0.8% Agarose gel in 1× Tris–acetate buffer (TAE).

13. Qiagen gel extraction kit.

14. NanoDrop spectrophotometer.

2.2. Cloning by the Strand Joining Reaction and Plasmid Analysis

1. 10× Reaction buffer: 300 mM Tris–HCl, pH 7.9, 50 mM $MgCl_2$, 700 mM NaCl, 18 mM dithiothreitol, 0.1 mg/mL acetylated BSA.

2. Polymerase dilution buffer: 25 mM potassium phosphate pH 7.4, 5 mM β-mercaptoethanol, 1 mM EDTA, 10% (v/v) glycerol, 0.1 mg/mL acetylated BSA.

3. VVpol, 25 ng/μL in polymerase dilution buffer (see Note 4).

4. I3 single-strand DNA binding protein, 0.5 mg/mL in PBS (see Note 5).

5. 37°C water bath.

6. 55°C heating block.

7. Electro- and chemically competent *E. coli*, strain DH10B (see Note 6).

8. Bio-Rad Gene Pulser and electroporation cuvettes.

9. SOC medium.

10. LB/X-gal/amp plates: LB agar supplemented with 40 μg/mL X-gal, 100 μg/mL ampicillin (see Note 7).

11. LB/amp media: LB supplemented with 100 μg/mL ampicillin.

12. Mini-prep kit (e.g., Fermentas).

13. Ethidium bromide solution: 0.1 μg/mL ethidium bromide in 1× TAE.

14. Kodak Gel Logic 200L photodocumentation system.

15. Reaction stopping solution: 2.4 µL 0.5 M EDTA, 0.6 µL 20 mg/mL proteinase K, 0.2 µL 10% (w/v) SDS. Add 3.2 µL per 20 µL reaction.

3. Methods

3.1. DNA Substrate Preparation

Any DNA can be used, but as an example we used a 553-bp amplicon encoding the N2L gene. This DNA was prepared by PCR using purified vaccinia virus genomic DNA (strain Western Reserve) as a template, *Taq* DNA polymerase and standard PCR reaction and cycling conditions.

3.1.1. Isolation of Vaccinia Virus Genomic DNA

1. Infect a 60-mm dish of BSC-40 cells with vaccinia virus (strain Western Reserve) at an M.O.I. of five.

2. Twenty-four hour after infection, remove the media and add 1 mL of cell lysis buffer.

3. Incubate at 37°C for 3–4 h.

4. Transfer the mixture to a 1.5-mL tube and add 0.5 mL of buffer saturated phenol. Mix well and then centrifuge at room-temperature for 10 min at $18,000 \times g$.

5. Transfer 0.3 mL of the aqueous layer to a new tube and add 0.05 mL of 3 M sodium acetate and 1.25 mL 95% ethanol.

6. Allow the DNA to precipitate for 15 min at –80°C before centrifuging at $18,000 \times g$ for 15 min.

7. Remove the supernatant and wash pellet with 70% ethanol.

8. Allow pellet to air-dry for 20–30 min at room temperature before resuspending in water.

9. Check OD and dilute DNA to 25 ng/µL.

3.1.2. PCR Amplification of VACV Gene and Plasmid DNA Preparation

1. To PCR amplify the N2L gene two 50 µL PCR reactions were assembled as recommended by the supplier of *Taq* polymerase, using 25 ng of vaccinia virus DNA as a template. The cycling parameters were as follows: 2 min initial denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s. This was followed by a final elongation step of 72°C for 7 min.

2. Digest pETBlue-2 plasmid DNA with *Nco*I and *Xho*I.

3. Gel-purify both the PCR product and plasmid digestion on a 0.8% agarose gel (see Note 8).

4. Bands of the correct size were excised and purified using a Qiagen gel extraction kit.

5. A NanoDrop spectrophotometer was used to determine the yield and purity of the DNA.

3.2. Cloning Reaction and Plasmid Confirmation

In parallel to the "home-made" reactions outlined below, we also assembled a cloning reaction using In-Fusion® enzyme and incubated the mix as directed by the supplier (Clontech). The commercial reaction is simpler and more efficient, as discussed in Subheading 3.4.

1. Cloning reactions were prepared as outlined in Table 1 using a 3:1 molar ratio of insert to vector.

2. The cloning reactions were started by adding 25 ng of VVpol last and then incubating for 20 min at 37°C.

3. The cloning reactions were stopped by heating to 55°C for 15 min.

4. After heat inactivating each strand-joining cloning reaction, 1 μL of each product from cloning reactions 1–5 was used to transform electrocompetent DH10B *E. coli* cells using a Bio-Rad Gene Pulser (see Subheading 3.3 for what to do with remaining material in each cloning reaction tube).

5. We added 0.25 mL of SOC medium to each electroporation cuvette, and the bacteria were incubated for 1 h at 37°C before being plated in duplicate on LB/X-gal/amp plates (see Note 9).

6. The plates were scored next day for the yield of blue or white colonies (see Table 2 for an example of the results and discussion).

7. Five white colonies and one blue colony were selected from the transformants produced in cloning reaction 5, Table 1, and inoculated into 3 mL of LB/amp media and cultured overnight at 37°C.

Table 1
Cloning reaction composition

| Component | Cloning reaction number | | | | |
	1	2	3	4	5
10× Reaction buffer	2 μL	2 μL	2 μL	2 μL	2 μL
Vector (50 ng/μL)	2 μL	2 μL	2 μL	2 μL	2 μL
Insert (25 ng/μL)	–	–	2 μL	2 μL	2 μL
VVpol (25 ng/μL)	–	1 μL	–	1 μL	1 μL
I3 (0.5 mg/mL)	–	–	–	–	1 μL
Water	16 μL	15 μL	14 μL	13 μL	12 μL
Total	20 μL	20 μL	20 μL	20 μL	20 μL

Table 2
Cloning efficiency

Cloning reaction # (from Table 1)	1	2	3	4	5
Cloning reaction composition					
Vector	✓	✓	✓	✓	✓
PCR amplicon		✓	✓	✓	✓
VVpol				✓	✓
I3					✓
Cloning reaction yield					
Blue colonies	25	14	9	19	2
White colonies	9	22	17	35	304
Percent white	26%	61%	65%	65%	99%

The addition of VVpol and I3 greatly increased the number of transformants as well as the proportion of white clones. DNA isolated from In-Fusion® cloning reactions were transfo✓rmed in parallel (see Subheading 3.4)

8. The recombinant plasmids were purified using a mini-prep kit.

9. The plasmid concentrations determined by spectrophotometry, and 0.5 μg of each DNA cut with restriction enzymes. In the example here, the plasmid DNA was digested with either *Bam*HI or *Nco*I or *Xho*I.

10. The resulting cloning reaction products were size fractionated by electrophoresis using a 0.8% agarose gel, stained with ethidium bromide, and imaged using a Kodak Gel Logic 200L photo documentation system (see Fig. 2 and Note 10).

3.3. Detection of End-Joining by Agarose Gel Electrophoresis

To detect the production of joint molecules, one can use the remainder of the cloning reactions which were not used for bacterial transformation, to determine the success of the end-joining step. To do this (detailed below) the DNA is incubated with SDS and proteinase K to remove the VVpol and I3 proteins. The material is fractionated by electrophoresis on a 1.2% agarose gel and visualized with ethidium bromide. The exonucleolytic activity of VVpol is readily detected in many reactions as a blurring of the bands and a shift to smaller sizes (e.g., see Fig. 3, cloning reaction 2). When both insert and vector are present, the formation of slower migrating bands, indicative of the presence of joint molecules, is also seen. The greatest yield of joint molecules is seen in reactions containing VVpol plus I3 protein (see Fig. 3, cloning reaction 5). Several different products can be seen in these cloning reactions, which appear to comprise a mixture of concatemers and circles.
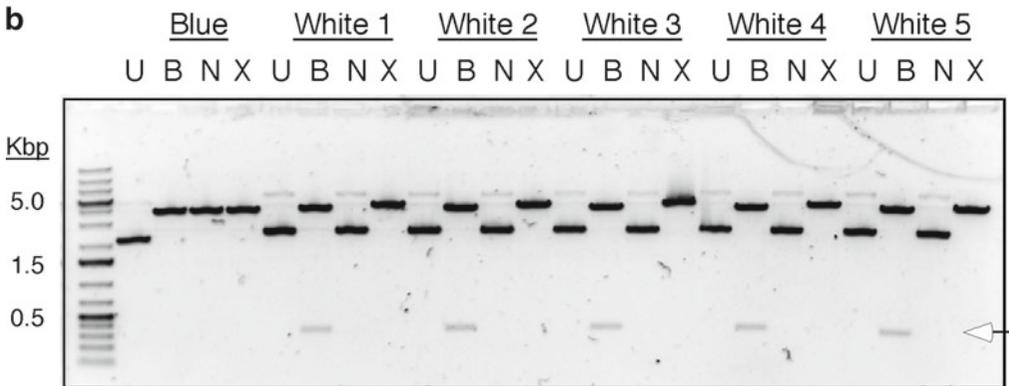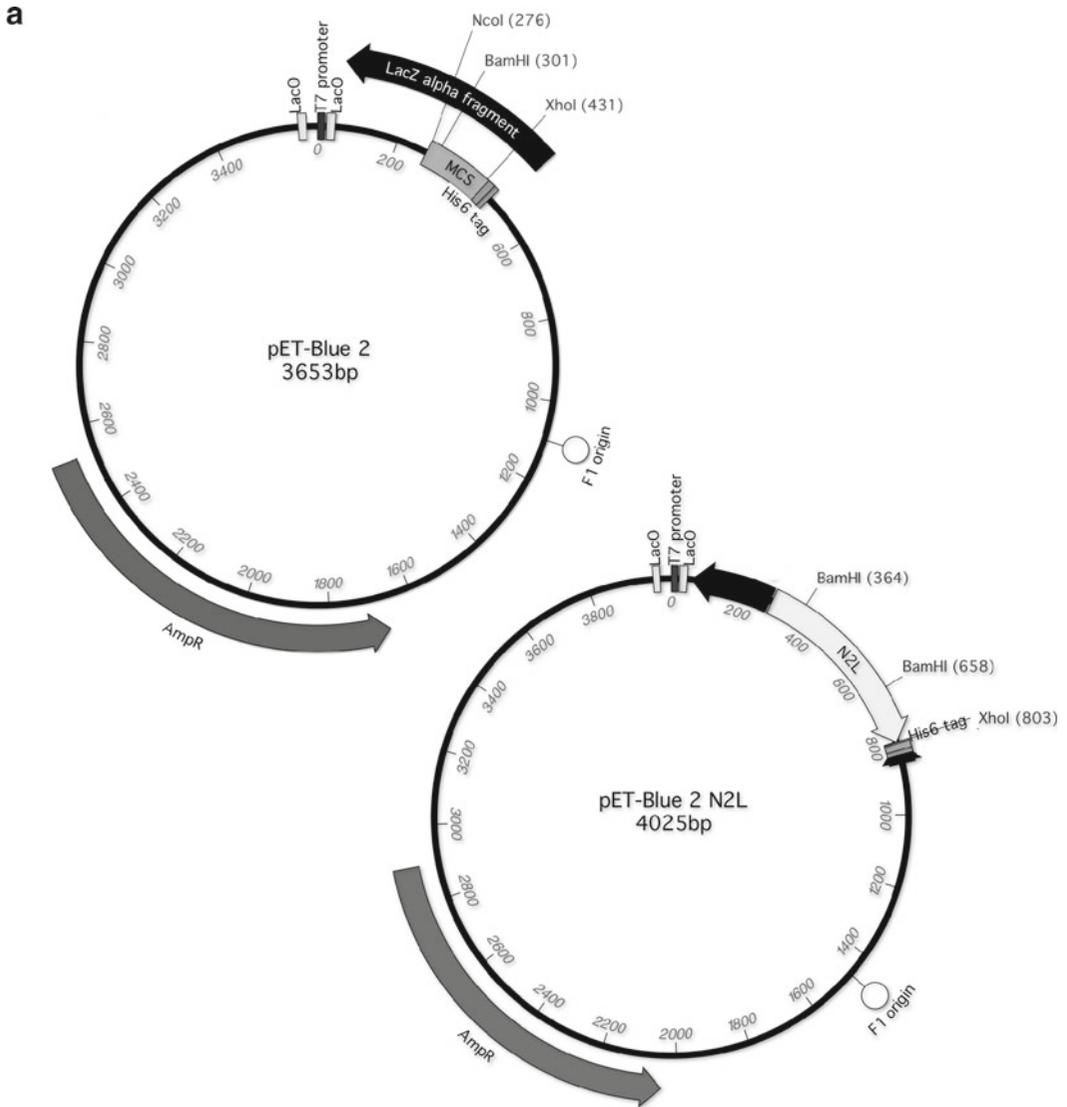
Fig. 2. Restriction analysis recombinant plasmids. (**a**) Plasmid maps showing the original vector (pETBlue-2) and predicted recombinant (pETBlue-2 N2L). (**b**) Restriction analysis of recombinant clones. Plasmid DNA was extracted from six colonies (5 white and 1 blue) and digested with *Bam*HI (B), *Nco*I (N), or *Xho*I (X). The products were separated on a 0.8% agarose gel along with uncut (U) DNA and stained with ethidium bromide. All five clones from the white colonies encoded N2L inserts (*arrow*). The blue colony appeared to be the original vector.
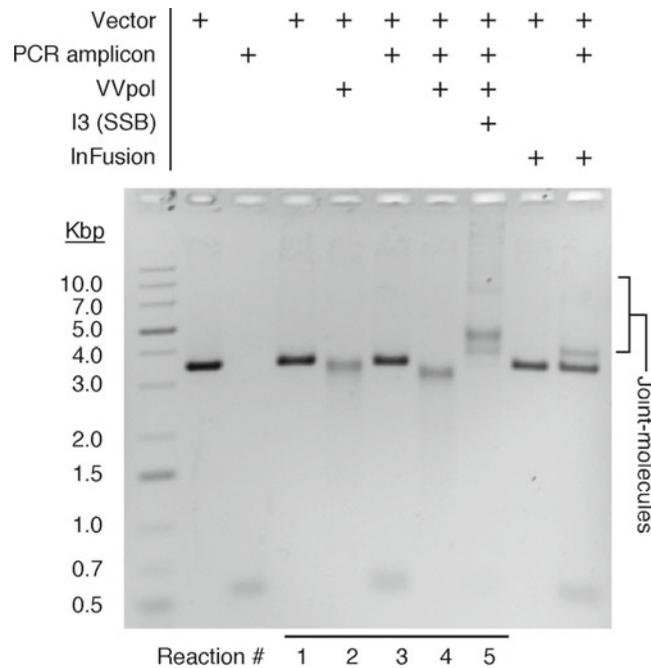
Fig. 3. Agarose gel analysis of cloning reaction products. The cloning reaction products were deproteinized and then separated on a 1.2% agarose gel. Under these conditions, the exonuclease activity causes a reduction in apparent mass and blurring of the bands. The greatest yield of joint molecules is seen in cloning reactions containing both VVpol and I3 single-strand DNA binding protein (cloning reaction #5). Although the commercial In-Fusion® cloning reactions produce lesser numbers of joint molecules (*last lane*), they actually produce a far greater yield of recombinants.

The In-Fusion® cloning reaction mix produces primarily a single new joint molecule in a manner dependent upon the presence of both vector and PCR amplicons (see Fig. 3).

1. To measure the efficiency of strand joining, 3.2 μL of the reaction stopping solution was added to the remainder of each of cloning reactions 1–5.

2. Tubes were incubated for 30 min at 37°C.

3. The cloning reaction products were then separated by electrophoresis for 4 h at 40 V/cm in a 1.2% agarose gel.

4. The DNA was stained with ethidium bromide solution for 30 min and imaged using a Kodak Gel Logic 200L photodocumentation system (see Fig. 3 for an example of results).

3.4. Conclusions

We have shown that VVpol can be used in conjunction with vaccinia virus single-strand DNA binding protein to catalyze a ligase-independent method for cloning PCR products. This approach is adaptable to any vector, allows for the rapid directional cloning of DNAs, and readily facilitates the engineering of flanking regulatory

and other sequence elements. While we illustrated the joining of two pieces of DNA, this approach has also been used to clone multiple pieces of DNA together at once (11, 12).

Clontech has optimized this method to generate a more efficient reaction. The commercial method produces many more (>50-fold) transformants than do our original methods. For example, the reactions we assembled including vector, insert, VVPol, and I3 yielded 304 white and 2 blue transformants from 1/20th of the DNA (see Table 2, reaction 5), whereas the vector plus insert In-Fusion® cloning reaction yielded 410 white and 3 blue colonies from 1/800th of the DNA. The commercial method yielded just a single white colony when supplied with only vector DNA. Both methods produce about the same proportion of white clones (>99%) when cloning reactions contain both vector and insert DNA.

This method produces very few aberrant clones, as illustrated by the observation that all five of the plasmids isolated from white colonies encoded an insert. Clontech's internal quality control data indicates that >90% of constructs produced in their proprietary approach contain inserts, even in the absence of blue–white or other selection methods. Furthermore, when Clontech analyzed one side of the junction region in a much larger library, only 43 out of 3,650 clones (1.1%) were found to contain mutations in the region of homologous sequence common to the vector and PCR product. Assuming the error rate is the same for both junctions, the likely percentage of clones that have at least one mutation in either end is just 2.3%. This highlights the accuracy of this process. We have noted some very rare examples of codons being lost within repetitive sequences (e.g., CAT repeats associated with His_6 tags), presumably by a process resembling the Streisinger frameshift error mechanism (13). None of these error frequencies are high enough to routinely inconvenience the investigator, but illustrate why one should still always sequence any new clone as a matter of good lab practice.

Although few research laboratories would have the technical capacity (or desire) to prepare VVpol in practice, the availability of a commercial version of the method makes it readily accessible to interested researchers, and the utility of the method has been documented in a number of large-scale cloning exercises (14–16).

4. Notes

1. Although one could use many different vectors, in this chapter we choose pETBlue-2 because it permits blue/white screening of inserts and regulated expression of a His_6-tagged protein from an IPTG-inducible T7 RNA polymerase promoter. The two primers that were used to amplify the N2L gene are bipartite in nature. The 3′-ends of these primers encode the N- and

C-terminal ends of the N2 protein and were designed using standard PCR design principles. The 5′-ends of these primers encode sequences that are homologous to the ends of *Nco*I- and *Xho*I-digested pETBlue-2 DNA. The forward primer encodes promoter sequences found upstream of the *Nco*I cut site and the reverse primer encodes the His$_6$-tag found downstream of the *Xho*I site. The underlined nucleotides in the primer denote nucleotides that are identical to the pETBlue-2 sequence. It should be noted that the forward primer was designed to delete the *Nco*I site and place the N2L gene in frame with the vector start codon, while the *Xho*I site was retained in the reverse primer. Vector maps were designed using MacVector 11.0.

2. Clontech provides a program that can be used to design primers for In-Fusion® cloning. It is available at http://bioinfo. clontech.com/infusion/convertPcrPrimersInit.do.

3. While *Taq* DNA polymerase was used here, high-fidelity polymerases can also be used to prepare the PCR amplicons. The method is not affected by the presence or absence of 3′-A residues.

4. Vaccinia DNA polymerase can be purified to homogeneity using methods developed by Traktman and her colleagues (17). The purity was ascertained by SDS-PAGE and the concentration determined using a Bradford assay (Bio-Rad). A working stock of polymerase was prepared by diluting it to a concentration of 25 ng/μL in polymerase dilution buffer. This reagent is part of the In-Fusion® cloning kit.

5. A C-terminal His$_6$-tagged form of recombinant vaccinia single-strand DNA binding protein (I3) can be expressed in *E. coli* strain BL21 and purified using nickel affinity columns as described by Tseng et al. (18). The purity was confirmed by SDS-PAGE, and the concentration determined using a Bradford assay. To produce a working stock, the I3 DNA binding protein was diluted to a concentration of 0.5 mg/mL in PBS.

6. We recommend using *E. coli* with a competency of at least 1×10^8 cfu/μg in order to increase the number of clones obtained.

7. LB plates containing X-gal permits blue–white screening of colonies if beta-galactosidase cassette included in the plasmid.

8. We recommend purifying the PCR products because the dNTPs in the PCR reaction can inhibit the 3′-to-5′ exonuclease and thus inhibit joint molecule formation (9). Although this step is not strictly necessary, gel purification also minimizes the later recovery of vector plasmid or cloning of undesirable PCR products.

9. The In-Fusion® cloning reaction (10 μL) was diluted to 100 μL with 10 mM Tris–HCl, pH 8 and 1 mM EDTA, and 2.5 μL

was used to transform chemically competent *E. coli*, and 1/20th of the mix plated in a similar fashion.

10. The plasmid DNA was digested with either *Bam*HI, *Nco*I or *Xho*I. These enzymes each cut pETBlue-2 once, although the *Bam*HI site is deleted during a *Nco*I and *Xho*I double digest. There are no *Nco*I and *Xho*I sites in the N2L gene and *Bam*HI is found twice (see Fig. 2, panel a). The plasmids recovered from the five white colonies all exhibited the restriction patterns expected if they encoded an N2L gene insert and the presence of this insert, and its proper orientation, was later confirmed by DNA sequencing (data not shown). The DNA extracted from a blue colony had a restriction pattern identical to pETBlue-2, suggesting that it was derived from an uncut plasmid.

5. Acknowledgments and Disclosures

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