Chapter 6

The Polymerase Incomplete Primer Extension (PIPE) Method Applied to High-Throughput Cloning and Site-Directed Mutagenesis

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Summary

Significant innovations in molecular biology methods have vastly improved the speed and efficiency of traditional restriction site and ligase-based cloning strategies. "Enzyme-free" methods eliminate the need to incorporate constrained sequences or modify Polymerase Chain Reaction (PCR)-generated DNA fragment ends. The Polymerase Incomplete Primer Extension (PIPE) method further condenses cloning and mutagenesis to a very simple two-step protocol with complete design flexibility not possible using related strategies. With this protocol, all major cloning operations are achieved by transforming competent cells with PCR products immediately following amplification. Normal PCRs generate mixtures of incomplete extension products. Using simple primer design rules and PCR, short, overlapping sequences are introduced at the ends of these incomplete extension mixtures which allow complementary strands to anneal and produce hybrid vector/insert combinations. These hybrids are directly transformed into recipient cells without any post-PCR enzymatic manipulations. We have found this method to be very easy and fast as compared to other available methods while retaining high efficiencies. Using this approach, we have cloned thousands of genes in parallel using a minimum of effort. The method is robust and amenable to automation as only a few, simple processing steps are needed.

Key words: Cloning; Ligase independent; Enzyme free; Site-directed mutagenesis; PIPE; Incomplete primer extension

6.1. Introduction

Contemporary cloning strategies outline mainly iterative protocols based on ligase-independent methods *(1–13)*. Most of these methods require specific sequences for successful cloning. Recombinatorial cloning is only one example where specific sequences must be incorporated and can encode extra, unwanted residues into expressed proteins *(9)*. "Enzyme-free" cloning alleviates sequence

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requirements through a series of PCR steps and product treatments *(10)*. Likewise, the PIPE method also eliminates sequence constraints, and it also reduces cloning and site mutagenesis to a single PCR step and transformation. These combined innovations make the PIPE method very fast, cost effective, and highly efficient. The following protocol includes all the wet lab steps, from making competent cells to submitting samples for sequencing, necessary for successful cloning and mutagenesis. Although the various examples presented here are shown with the expression vector pSpeedET (in-house), the PIPE method can be used with other vectors.

6.2. Materials

6.2.1. Preparation of Competent Cells

1. Milli-Q water (*see* **Note 1**).
2. LB Broth: 25 g of Difco™ LB Broth (Miller) per 1 L of deionized (DI) water and autoclaved for 30 min at 121°C. The media is autoclaved in 2,000 ml Kimax® Baffled Culture Flasks (ThermoFisher Scientific, Waltham, MA).
3. Sterile, disposable Corning Erlenmeyer polycarbonate flasks (500 ml) (available through ThermoFisher Scientific).
4. $MgCl_2$ Solution: 100 mM $MgCl_2$ in Milli-Q water, then sterile filtered. Stored at 4°C.
5. $CaCl_2$ + Glycerol Solution: 100 mM $CaCl_2$ and 15% glycerol in Milli-Q water, then sterile filtered. Stored at 4°C.
6. Microcentrifuge tubes (2 ml).

6.2.2. Preparation of Selective LB Agar Plates

1. Sterile square BioAssay trays with 48-well dividers (Genetix, Boston, MA).
2. LB Agar: 40 g of Difco™ LB Agar (Miller) per 1 L of DI water and autoclaved for 30 min at 121°C.
3. Antibiotics (working concentration): Ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), kanamycin (30 μg/ml).

6.2.3. PCRs for Amplifying Cloning Vectors and Inserts or Generating Site-Directed Mutants

1. Template DNA (20–100 pg per PCR amplification).
   i. For cloning vector amplifications (*V-PIPE*), use a recipient expression plasmid such as pSpeedET (*see* **Fig. 6.1a**).
   ii. For insert amplifications (*I-PIPE*), use genomic DNA, cDNA, PCR product, or miniprepped DNA from a previously generated clone (*see* **Fig. 6.1b**).
   iii. For mutagenic amplifications (*M-PIPE*), use the miniprepped DNA from a previously generated clone (*see* **Fig. 6.1c**).

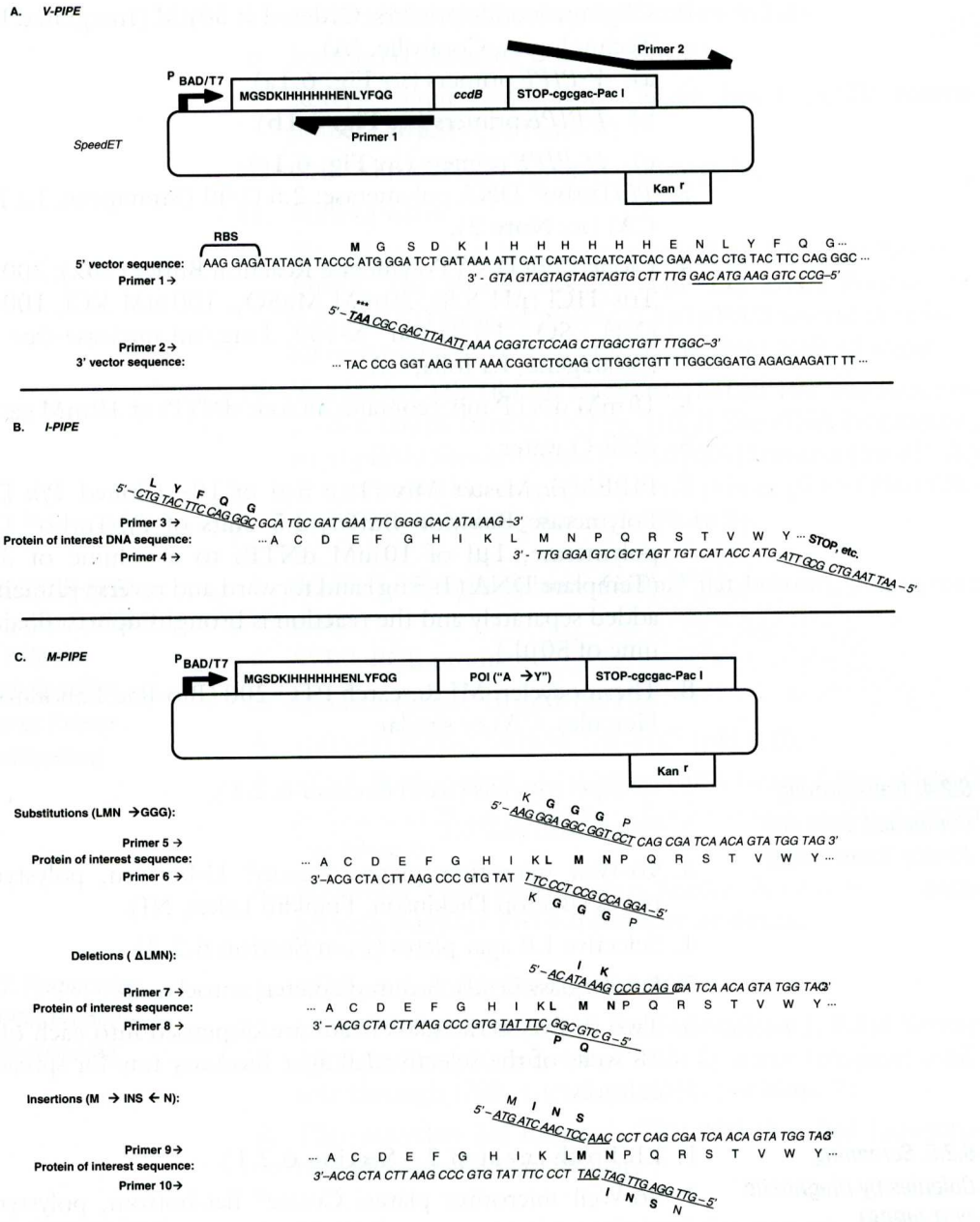

Fig. 6.1. Oligonucleotide design for the three common PCR amplifications used in the PIPE method. The 15 base complementary overlaps are shown as the *underlined* portion of each primer sequence. (**a**) V-PIPE (vector PCR). Primers 1 and 2 are examples of primers that could be used to PCR amplify a vector, such as speedet, in a way suitable for annealing to inserts amplified in (**b**). (**b**) I-PIPE (insert PCR). Primers 3 and 4 are examples of primers that could be used to PCR amplify inserts from various templates in a way suitable for annealing to the vector amplified in (**a**). PIPE cloning works by intermolecular annealing across the two annealing sites of the (**a**) and (**b**) PCRs. (**c**) M-PIPE (mutagenic PCR). Primers 5 and 6 represent primers which could be used to create substitution mutants. Primers 7 and 8 represent primers which could be used to create deletion mutants. Primers 9 and 10 represent primers which could be used to create insertion mutants. PIPE mutagenesis works by intramolecular annealing across the single site of a (**c**) PCR.

2. Oligonucleotide primers: Ordered at 50 µM (Integrated DNA Technologies, Coralville, IA).
   a) *V-PIPE* primers (*see* **Fig. 6.1a**).
   b) *I-PIPE* primers (*see* **Fig. 6.1b**).
   c) *M-PIPE* primers (*see* **Fig. 6.1c**).
3. *Pfu*Turbo® DNA polymerase: 2.5 U/µl (Stratagene, La Jolla, CA) (*see* **Note 2**).
4. Cloned *Pfu* DNA Polymerase Reaction Buffer (10×): 200 mM Tris–HCl (pH 8.8), 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton® X-100, 1 mg/ml nuclease-free BSA (Stratagene, La Jolla, CA).
5. 10 mM dNTP mix (contains all four dNTPs at 10 mM each).
6. Milli-Q water.
7. PIPE *Pfu* Master Mix (1×): 5 µl of 10× Cloned *Pfu* DNA Polymerase Reaction Buffer, 2.5 units of *Pfu*Turbo® DNA polymerase, 1 µl of 10 mM dNTPs to a volume of 35 µl. (Template DNA (1–5 ng) and forward and reverse primers are added separately and the reaction is brought up to a final volume of 50 µl.).
8. Thermocycler: MJ Research PTC-200 (Bio-Rad Laboratories, Hercules, CA) or similar.

6.2.4. Transforming Competent Cells and Plating Transformed Cells

1. Competent cells (from **Section 6.2.1**).
2. Water bath (42°C).
3. 96-Well microtiter plates: Falcon® U-bottom, polystyrene plates (Becton Dickinson, Franklin Lakes, NJ).
4. Selective LB agar plates (from **Section 6.2.2**).
5. Sterile glass beads, 5-mm diameter, autoclaved.
6. Two to five sterile glass beads are dispensed into each of the 48 wells of the selective LB agar BioAssay tray for spreading cultures.

6.2.5. Screening Colonies by Diagnostic PCR (dPCR)

1. LB Broth (*see* **step 2 – Section 6.2.1**).
2. 96-Well microtiter plates: Costar® flat-bottom, polystyrene plates.
3. 96-Well PCR plates: Costar® Thermowell®, polypropylene plates.
4. Thermocycler: MJ Research PTC-200 (Bio-Rad Laboratories, Hercules, CA) or similar.
5. *Taq* Reaction Buffer (10×): 100 mM Tris–HCl (pH 8.8), 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin.
6. *Taq* DNA Polymerase (*see* **Note 3**).

7. dNTPs (10 mM) (*see* **step 5 – Section 6.2.3**).
8. Forward sequencing primer (50 µM).
9. Reverse sequencing primer *or* an insert-specific reverse primer (50 µM) (*see* **Note 4**).
10. Cells cultured from isolated colony picks.
11. Milli-Q water.
12. *Taq* Master Mix (for 1 reaction): 5 µl 10× *Taq* Reaction Buffer, 1 µl 10 mM dNTPs, 1 µl *Taq* DNA Polymerase, 0.5 µl pBAD forward primer, 0.5 µl pBAD reverse or universal insert-specific reverse primer and 39 µl Milli-Q water.
13. *Taq* Master Mix (for 96 reactions): 510 µl 10× *Taq* Reaction Buffer, 102 µl 10 mM dNTPs, 102 µl *Taq* DNA Polymerase, 51 µl pBAD forward primer, 51 µl pBAD reverse (for dPCR) or a universal insert-specific reverse primer (for SYBR-PCR) and 3,978 µl Milli-Q water (*see* **Note 5**).

6.2.6. SYBR-PCR Assay (for Optional Use with the Insert-Specific Reverse Primer Amplification)

1. 96-Well microtiter plates: Costar® flat-bottom, polystyrene plates.
2. SYBR-PCR products.
3. Quench Buffer: 10 mM EDTA.
4. Dilution Buffer: 50 mM Tris–HCl (pH 8.0).
5. SYBR Buffer: 40 µl of 10,000× SYBR Green I Dye (Sigma) is diluted in 20 ml of Dilution Buffer to make a 20× solution (*see* **Note 6**).
6. Microtiter Plate Fluorescence Reader: Spectramax Gemini XS with SoftMax Pro 4.6 software or similar.

6.2.7. Preparation of dPCR Products for Sequencing

1. PCR product (*see* **Section 6.2.5**).
2. Exo/SAP Solution *(14)*: 0.5 µl Exonuclease I, 0.5 µl Shrimp Alkaline Phosphatase, 4.0 µl Milli-Q water (enzymes available through USB, Cleveland, OH) (*see* **Note 7**).
3. Thermocycler: MJ Research PTC-200 (Bio-Rad Laboratories, Hercules, CA) or similar.

6.2.8. Glycerol Stock Archival of Putative Clones

1. 96-Well microtiter plates: Costar® flat-bottom, polystyrene plates.
2. Glycerol (80% (v/v)): 1 kg of 100% glycerol ($\rho = 1.25\ g/cm^3$) is diluted to 1 L with Milli-Q water, then autoclave.
3. Cultures (*see* **step 5 – Section 6.3.5**).
4. Aluminum foil lids: Biomek® Seal and Sample (#538619, Beckman Coulter, Fullerton, CA) or similar.
5. Freezer (–80°C).

6.3. Methods

The PIPE method is based on the observation that, contrary to popular assumption, normal PCR amplifications result in mixtures of products which are not fully double stranded *(15)*. The 5′ ends of such products are left variably unpaired by incomplete 5′ → 3′ primer extension caused by sequence-specific stalling and changes in the reaction equilibrium (less dNTPs available, more template copies to synthesize) in the final cycles of PCR. These unpaired 5′ ends on the PCR products are the same 5′ ends on the synthetic amplification primers. Therefore, a simple oligonucleotide design rule can control the sequences of these ends in a way that promotes easy cloning and mutagenesis.

The first 15 bases on the 5′ ends of the primers are designed to be directionally complementary such that the resultant PCR fragment(s) can anneal as desired and become viable plasmids upon transformation. In basic PIPE cloning, the vector is linearized by *V-PIPE* PCR amplification and contains two distinct 5′ ends (**Fig. 6.1a**). The inserts are *I-PIPE* PCR amplified with primers which contain 5′sequence complementary to the two distinct ends of the amplified vector (**Fig. 6.1b**). In this manner, annealing occurs directionally and creates a viable plasmid. In basic PIPE mutagenesis (*M-PIPE*), the entire plasmid is amplified (**Fig. 6.1c**). The two primers used are designed to create the mutation (a substitution, deletion, or insertion) and to be complementary to each other so that the linearized PCR product can self-anneal to recreate a viable, mutant plasmid. The following protocol describes PIPE cloning and mutagenesis in a 96-well plate.

6.3.1. Preparation of Competent Cells

1. Start a 10-ml overnight culture of cells in LB Broth at 37°C for a 1-L batch.
2. On the following morning, seed 1 L of LB Broth with the 10-ml overnight culture.
3. Incubate the culture at 37°C while shaking at 250 rpm until the optical density measured at 600 nm (OD_{600}) reaches 0.4–0.6. (The remainder of this preparation is done on ice or in the cold room.).
4. Split the 1-L culture into 2 × 500 ml sterile, disposable Corning Erlenmeyer polycarbonate flasks and pellet the cells by chilled centrifugation at 2,500 × *g* for 20 min (*see* **Note 8**).
5. Decant and discard the LB Broth.
6. Add $MgCl_2$ Solution at one-tenth the original volume (100 ml) to the pellet.

7. Resuspend the pellet by gentle swirling the $MgCl_2$ Solution over the pellet. Pipette up and down to break up dislodged pellet, if necessary (*see* **Note 8**).
8. Incubate the resuspended cells on ice for 30 min.
9. Pellet the cells by chilled centrifugation at 2,500 × *g* for 20 min.
10. Decant and discard the supernatant.
11. Add $CaCl_2$ + Glycerol Solution at one-fiftieth the original volume (20 ml) to the cell pellet.
12. Resuspend the pellet by gentle swirling the $CaCl_2$ + Glycerol Solution over the pellet. Pipette up and down to break up dislodged pellet, if necessary.
13. Divide the competent cells into 2 ml aliquots using 2-ml microcentrifuge tubes.
14. Flash freeze the aliquots in liquid nitrogen.
15. Store aliquots at –80°C.

6.3.2. Preparation of Selective LB Agar Plates

1. LB Agar plates: 200 ml of liquefied LB Agar is supplemented with an appropriate antibiotic at 55°C and then poured into the BioAssay tray with the 48-well divider removed from the tray (*see* **Note 9**).
2. After the LB agar has been poured, the 48-well divider is placed back into the tray and partially submerged into the LB agar creating 48 separate squares.
3. The LB agar is allowed to solidify at room temperature.
4. The plates are then dried overnight by propping the lids slightly open to allow sufficient gas exchange while minimizing potential contamination.

6.3.3. PCRs for Amplifying Cloning Vectors and Inserts or Generating Site-Directed Mutants

1. Dilute the 50-μM oligonucleotide primer stocks to 10 μM with Milli-Q water.
2. Set up the PIPE PCRs.
   a) (*V-PIPE*) Tranfer 5 μl of both 10 μM forward and reverse primers (*see* **step 2a – Section 6.2.3**) into PCR tubes and then add 5 μl of template (*see* **step 1i – Section 6.2.3**) and 35 μl of PIPE *Pfu* Master Mix (*see* **Notes 10** and **11**).
   b) (*I-PIPE*) Tranfer 5 μl of both 10 μM forward and reverse primers (*see* **step 2b – Section 6.2.3**) into a PCR plate and then add 5 μl of template (*see* **step 1ii – Section 6.2.3**) and 35 μl of PIPE *Pfu* Master Mix (*see* **Note 10**).
   c) (*M-PIPE*) Tranfer 5 μl of both 10 μM forward and reverse primers (*see* **step 2c – Section 6.2.3**) into a PCR plate and then add 5 μl of template (*see* **step 1iii – Section**

6.2.3) and 35 µl of PIPE *Pfu* Master Mix (*see* **Notes 12** and **13**).

3. Thermocycler conditions for PIPE amplifications.
   a) (*V-PIPE*) 95°C for 2 min, then 25 cycles of 95°C for 30 s, 55°C for 45 s and 68°C for 14 min, and finally a 4°C hold.
   b) (*I-PIPE*) 95°C for 2 min, then 25 cycles of 95°C for 30 s, 55°C for 45 s and 68°C for 3 min, and finally a 4°C hold (*see* **Note 14**).
   c) (*M-PIPE*) 95°C for 2 min, then 25 cycles of 95°C for 30 s, 55°C for 45 s and 68°C for 14 min, and finally a 4°C hold.
4. Confirm successful amplifications by gel electrophoresis.

6.3.4. Transforming Competent Cells and Plating Transformed Cells

1. Thaw 2 ml aliquot of competent cells (*see* **step 15 – Section 6.3.1**) on ice for 10–15 min.
2. Chill a 96-well microtiter plate on ice for 10–15 min.
3. Transfer 2 µl of each of the PCRs into wells of a prechilled microtiter plate.
   - When cloning, first mix 2 µl from the V-PIPE and 2 µl I-PIPE reactions together (*see* **Note 15**).
   - For mutagenesis, 2 µl from the M-PIPE reaction can be used directly (*see* **Note 16**).
4. Dispense 20 µl of competent cells into each well. Pipette up and down ONCE to ensure DNA has mixed with the cells.
5. Incubate the DNA-cell mixture on ice for 15 min.
6. Heat shock the cells by floating the microtiter plate in a 42°C water bath for 45 s (*see* **Note 17**).
7. Immediately return the microtiter plate to ice.
8. Dispense 100 µl of LB Broth (no antibiotic) into each well.
9. Recover the transformed cells by incubating at 37°C while shaking at 250 rpm for 1 h.
10. Dispense 100 or 40 µl of the recovered cells into the respective wells of the selective LB agar trays with glass beads.
11. Shake (by hand) the trays enough to move the glass beads and evenly distribute the cells across the entire well.
12. Invert the tray to drop the glass beads off of the LB agar and onto the lid.
13. Remove the glass beads from the lid.
14. Incubate the inverted trays overnight (12–16 h) in a stationary 37°C incubator to grow the bacterial colonies (*see* **Note 18**).

6.3.5. Screening Colonies by Diagnostic PCR (dPCR) or SYBR-PCR

1. Dispense 200 µl of LB Broth with appropriate antibiotic into the wells of flat-bottom 96-well plate.
2. Using aseptic technique, pick and transfer 1–4 isolated colonies per transformation (*see* **step 14 – Section 6.3.4**) into unique wells of the microtiter plate.
3. Incubate the plate at 37°C while shaking at 250 rpm for at least 3 h (up to overnight).
4. Transfer 3-µl samples from each culture into a 96-well PCR plate.
5. Put the remainder of the cultures (~197 µl/well) back into the shaking incubator to continue growth for future glycerol stock archival.
6. Add 47 µl of *Taq* Master Mix to each well containing cells in the PCR plate (*see* **Note 19**).
7. Place the PCR plate into a thermocycler.
8. Amplify the DNA fragments using the following cycling conditions: 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 3 min, then finally a 4°C incubation.

6.3.6. SYBR Assay (for Optional Use with the Universal Insert-Specific Reverse Primer Amplification)

1. Dispense 50 µl of Quench Buffer in a flat-bottom 96-well plate.
2. Transfer 5 µl of each SYBR-PCR product (*see* **step 8 – Section 6.3.5**) into the wells with Quench Buffer (*see* **Note 20**).
3. Dispense 150 µl of SYBR Buffer into each well.
4. Prepare an unamplified sample for a negative control.
5. Measure fluorescence of each sample using a microtiter plate fluorescence reader. Excitation: 485 nm. Emission: 525 nm. Auto-cutoff enabled.
6. SYBR results are determined on a relative scale with the positive wells having at least fourfold higher fluorescence than the negatives or the control.

6.3.7. Preparation of dPCR Products for Sequencing

1. Dispense 5 µl of Exo/SAP Solution[15] directly into the dPCR positive wells (determined by gel analysis, SYBR assay, or simply assumed to be positive).
2. Incubate the Exo/SAP reaction at 37°C for 30 min, then 75°C for 15 min.
3. Submit these samples directly for sequencing.

6.3.8. Glycerol Stock Archival of Putative Clones

1. Add 50 µl of 80% (v/v) glycerol (20% final) to 150 µl of each culture (*see* **step 5 – Section 6.3.5**) in a 96-well microtiter plate.
2. Mix the glycerol into the culture by pipetting up and down.

3. Seal the plate using the aluminum foil lid.
4. Store the plate in a −80°C freezer.

6.4. Notes

1. Milli-Q water is purified to a resistivity of 18.2 MΩ cm and contains total organics at less than five parts per billion using the Milli-Q Synthesis System (Millipore, Billerica, MA).
2. Thermostable DNA polymerases from *Pyrococcus furiosus* (*Pfu*), *Thermococcus kodakaraensis* (KOD), and *Thermus aquaticus* (*Taq*) as well as Phusion™ DNA Polymerase have all been used successfully. However, the majority of our experience and, therefore, success has come from using *Pfu*Turbo DNA Polymerase. We also observe spurious mutations using *Taq* DNA Polymerase which have not been observed using the proofreading enzymes.
3. *Taq* DNA Polymerase works very well for amplifying DNA directly from cell cultures. The ability to PCR from these cells grown from isolated colonies eliminates the need to miniprep DNA for sequencing. Amplifying DNA from cell cultures using *Pfu*Turbo DNA Polymerase has NOT worked well for us.
4. The insert-specific reverse primer is designed such that successful PCR amplification is dependent on the insert annealing to the vector to form a viable colony. Conditional PCR can be used in series with a fluorescence assay (SYBR) to determine insert-containing plasmids without running a DNA gel.
5. This master mix is actually made at 102× to account for volume losses in the multiple transfer steps.
6. Although SYBR dyes are reported to be far less carcinogenic than ethidium bromide, they still bind tightly to DNA. Appropriate care should be observed in handling and disposal.
7. Alternate Exo/SAP protocols may use 4 µl of buffer instead of 4 µl of water, but our sequencing results have not suffered.
8. Use centrifugation vessels which maximize the surface area across which the cells are pelleted. This makes it much easier to resuspend the cells without excessive stress to the cells.
9. Subjecting antibiotic(s) to the LB agar at temperatures greater than 55°C for extended periods of time can decrease relative effectiveness on susceptible cells.
10. Our vector pSpeedET contains the *ccdB* gene which is toxic to our expression strain (HK100). This creates a negative

selection against vector template DNA. Strains carrying the *ccdA* antidote on the F′ episome are not susceptible to this negative selection. In the V-PIPE PCR, the entire plasmid is amplified except for the *ccdB* cassette.

11. For large cloning projects, amplifying the vector separately from the inserts is logistically best. It is possible, however, to amplify both the vector and the insert (from the same or different templates) in the same reaction (IV-PIPE). This is done by adding both templates and all four primers to the same 50 µl PCR. Each primer is added at half the original concentration so that the total primer concentration in the reaction remains fixed at 2 µM.
12. Mutagenesis is not limited to a single substitution, deletion, or insertion per reaction. We have made up to five substitutions using a single primer pair when the sites are all in close proximity. We have made four codon substitutions using four primer pairs in the same reaction when the sites were disbursed. We have made N- and C-terminal insertions and deletions separately and simultaneously. In all cases, the total primer concentration in the reactions is 2 µM.
13. In most cases, the M-PIPE templates encode the same antibiotic resistance as the desired mutant plasmids will have. We have found that ~1 ng (20 pg/reaction) is typically low enough to eliminate background transformants. In the cases where PCR amplification is only successful using higher amounts of template, *DpnI* may be used to digest the template DNA to reduce background.
14. Use the thermocycling conditions for V-PIPE PCR when trying to amplify the vector and insert in the same reaction.
15. The PCR products are used directly out of the thermocycler. There are no post-PCR treatments to the PCR products unless the background is determined to be too high (*DpnI* treatment, **Note 13**). If the vector and the insert were amplified together (IV-PIPE), then 2 µl from that reaction is sufficient.
16. The PCR products are used directly out of the thermocycler. There are no post-PCR treatments to the PCR products unless the background is determined to be too high (*DpnI* treatment).
17. Efficient heat shock requires direct water to well contact. Be careful to avoid introducing air pockets between the plate and water.
18. If satellite colonies are present, incubate the trays overnight at 30°C instead of 37°C.
19. For SYBR-PCR, the universal insert-specific reverse primer is designed to anneal to any amplified DNA insert. PCR amplification is conditional on the insert fragment annealing to the

vector in the correct orientation. Therefore, PCR is successful on insert-containing plasmids but not with background transformants such as vector only or PCR template contamination.

20. This assay is not helpful when using two vector-specific primers since amplification will occur with or without the insert. Although the assay is very quick in identifying insert-containing plasmids (compared to agarose gel), the sizes of the PCR products cannot be determined. In our experience, the SYBR assay and PCR products of the correct size have at least an 80% correlation.

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