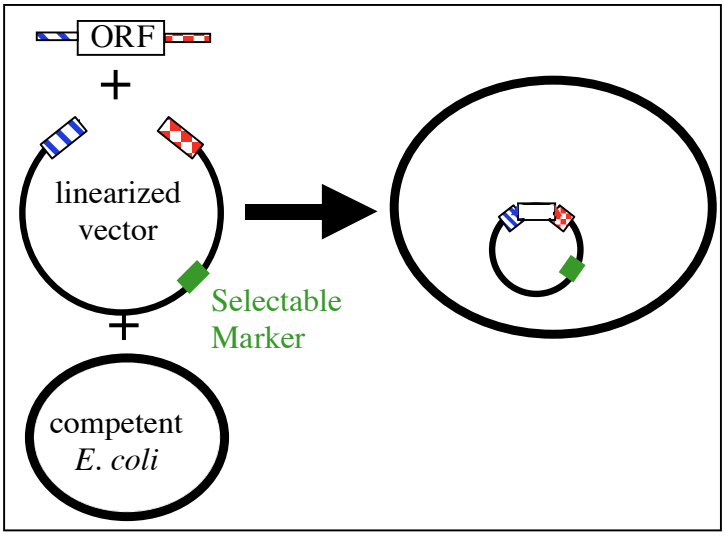
Recombination Cloning in Bacteria

The following approach to high throughput subcloning using recombination in bacteria was developed in the Finley lab at Wayne State University by Jodi Parrish and Tom Limjindaporn (1).

Introduction

Co-transformation of a *recA*$^+$ *E. coli* strain with a linearized plasmid and a linear fragment of DNA having ends homologous to the plasmid, can result in insertion of the DNA fragment into the plasmid by recombination. This approach has been used to subclone open reading frames (ORFs) and other DNA fragments into plasmid vectors (1,2,3). We have optimized this as an approach to rapidly subclone PCR products into a variety of vectors, including yeast vectors and *E. coli* expression vectors. We have shown that PCR products with as little as 21 bp of homology to the vector at either end can be subcloned by recombination with good efficiency and fidelity. In one project, for example, we have subcloned 1,184 different ORFs. The cloning efficiency observed was 75% (*E. coli* transformant colonies (%) containing recombinant plasmids with inserts). Cloning fidelity was around 93% (based on DNA sequence of the region around the 5' insertion site for 1126 clones). Included below are protocols adapted for high throughput subcloning and also for cloning individual and small sets of ORFs into expression vectors.



Critical Parameters

1. The vector should be linearized by digesting with one or more unique restriction enzymes to completion. Uncut vector in the prep will lead to a high background of *E. coli* transformants having no inserts in the vector.
2. We generally digest the vector with two different restriction enzymes that have nearly adjacent recognition sites in the cloning region. This produces non-compatible sticky ends, which reduces the background of vector recircularization without insert. If digesting with a single enzyme, treatment with alkaline phosphatase is recommended. After linearization purification of the vector is recommended.
3. ORFs tagged with the vector homology regions can be generated by PCR using primers containing an additional 21 bases of the vector sequence 5' to the ORF-specific sequence. We refer to the vector homology regions as Recombination Tags (5RT and 3RT). The 21 bases should correspond to the vector sequence adjacent to the restriction site, but not including the sticky ends. We have found that longer tags are usually not necessary. However, recombination tags longer than 21 bases increases the transformation efficiency. Longer tags can be added in the PCR primer or by performing a secondary PCR.
4. The PCR products can be used directly in the transformation without further purification. However, using ethanol precipitated PCR products increases the transformation efficiency (1,2). Other methods of purification may also result in increased efficiency (2).

5. The total amount of vector, and the ratio of vector to insert are important and may vary with the vector and insert used. For a typical 10 kb yeast vector or a 6 kb *E. coli* pET-derived vector, and inserts from 100 to 3000 bp, we have used 50 ng vector and 50 ng of PCR product.
6. We have tried a number of different *E. coli* strains and found that KC8 works best under some conditions. KC8 outperformed BUN10 (*recB21 recC22 sbcA23*), V324 (*recD1009*), MG1655, and DH5α(1).
7. Other methods for transformation of *E. coli* may be used (2,3). The chemical method outlined below is particularly effective for high throughput cloning because it enables small volume transformations in 96-well plates.

PROTOCOLS:

I). Preparation of Competent Cells[1]

1. Streak the appropriate *E.coli* strain (preferably KC8) on an LB plate[2] and incubate overnight at 37ºC.
2. Inoculate a single colony in 5 ml of LB[2] and incubate overnight at 37ºC in a shaking incubator.
3. Transfer the 5-ml culture to 500 ml of LB and incubate for 3 h or until the OD600 is 0.5.
4. Centrifuge the cells in two sterile 250-ml tubes for 15 min at 4ºC at 3500 rpm.
5. Gently resuspend the cells in 25 ml of ice-cold, filter-sterilized TSB.
6. Incubate for 10 min on ice
7. Aliquot the cells (500µl/tube) and quick-freeze in dry ice-ethanol.
8. Store at –80ºC.
9. Determine the transformation efficiency. Use protocol below, but use uncut vector and no insert DNA.

II). High-throughput Cloning via Transformation of PEG-DMSO Competent Cells

1. Array 10 µl (≈50 ng) of each of the tagged PCR products into a 96-well PCR plate.
2. Add 25 µl of 2X KCM and 10 µl of sddH2O to each well and mix by pipetting up and down 3 times.
3. Add 5 µl (≈50 ng) of linearized vector DNA and mix by pipetting up and down 3 times.
4. Thaw the *E. coli* chemo-competent cells on ice.
5. Add 50 µl of cells to each well of the 96-well PCR plate and mix by pipetting up and down 3 times.
6. Seal the plate with foil and incubate for 30 min in an ice-ethanol bath.
7. Incubate for 10 min at room temperature.
8. Add 80 µl of LB, mix by pipetting up and down 3 times. Incubate for 2 h at 37ºC.

9. Plate all (195 µl) of the cells onto dried 6-well culture LB plates[3] containing 3.2 ml LB plus antibiotic in each well.
10. Incubate overnight at 37°C[4].

III). Cloning individual or small sets of ORFs via Transformation of PEG-DMSO Competent Cells (low throughput)

1. Thaw the chemocompetent cells on ice.
2. To 25 µl of 2x KCM add 5µl (50 ng) of linearized vector DNA, and 5 µl (50 ng) of tagged PCR product (insert). Raise volume to 50µl with sddH2O (add 15 µl sddH2O).
3. Add 50 µl of cells, mix gently, and incubate for 20 min on ice.
4. Incubate for 10 min at room temp.
5. Add 500 µl of LB, mix, and incubate for 1-1.5 h at 37°C.
6. Pellet the cells in microcentrifuge. Pour off most of the supernatant except for about 200 µl. Use this to resuspend the pellet.
7. Plate all of the cells on one LB plate containing the appropriate antibiotic.
8. Incubate overnight at 37°C[4]

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[1]This transformation protocol uses the Chemo-competent Bacteria Preparation & Transformation Protocol described in: Walhout et al, 2000, Methods in Enzymology, 328:575-592.

[2]If using *E. coli* KC8, then include kanamycin (20 µg/ml).

[3]We use Costar 6-well cell culture cluster plates from Corning Incorporated (catalog number 3506).

[4]We have observed for two-hybrid vectors cut with two enzymes (*Bam*H1 & *Eco*R1), and with PCR inserts containing 21 bp homology tags, anywhere from 4–80 colonies. Typically around 80% of these contain vector + insert (determined using PCR). Note that a control transformation using the linearized vector but no insert ideally should yield no greater than 1 – 10 colonies.

Reagents

TSB

1. LB-HCL (pH6.1)
   *** get better result in pH 6.1
2. 10% (w/v) PEG (mol wt = 3,400)
3. 5% (v/v) DMSO
4. 10% (v/v) glycerol
5. 10mM $MgCl_2$
6. 10mM $MgSO_4$

5xKCM

1. 0.5 M KCL
2. 150 mM $CaCl_2$
3. 250 mM Mg Cl_2

Literature Cited

1. Parrish, J. R., T. Limjindaporn, R. Finley, Jr. High-throughput cloning of *Campylobacter jejuni* ORFs by *in vivo* recombination in *Escherichia coli*. J. Proteome Research. 3:582-586 (2004).

2. Oliner, J. D., K. W. Kinzler, and B. Vogelstein. *In vivo* cloning of PCR products in *E. coli*. Nucleic Acids Research. 21:5192-5197 (1993).
3. Bubeck, P., M. Winkler, and W. Bautsch. Rapid cloning by homologous recombination *in vivo*. Nucleic Acids Research. 21:3601-3602 (1993).

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