Chapter 8

Circular Polymerase Extension Cloning

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Abstract

High-throughput genomics, proteomics, and the emerging field of synthetic biology demand ever more convenient, economical, and efficient technologies to assemble and clone genes, gene libraries, and synthetic pathways. Here, we describe an extremely simple, efficient, and cost-effective cloning method, circular polymerase extension cloning (CPEC), for complex, combinatorial, or multi-fragment assembly as well as routine cloning. This method uses a single polymerase to assemble and clone multiple inserts with any vector in a one-step reaction in vitro. No restriction digestion, ligation, or single-stranded homologous recombination is required.

Key words Molecular cloning, Genetic assembly, Library cloning, DNA polymerase

1 Introduction

Molecular cloning is a foundational technology for molecular biology and biotechnology. Pioneered by the restriction digestion and ligation-based methods [1–3], new cloning technologies have continuously been invented and evolved to suit various requirements and applications. Circular polymerase extension cloning (CPEC) is a simple, efficient, and economical circular DNA assembly and cloning method developed to meet the ever-increasing demand from high-throughput genomics, proteomics, and synthetic biology. Compared with existing cloning strategies, either sequence-dependent or -independent, CPEC offers significant benefits by combining simplicity, efficiency, versatility, and cost-effectiveness in one method [4]. In addition to routine single-gene cloning, CPEC is ideal for a wide variety of other applications, including complex gene library cloning, high-throughput expression cloning, and multi-way assembly of genetic pathways [5].

CPEC is a single-tube, one-step reaction that normally takes 5–10 min to complete for everyday laboratory cloning. The method is directional, sequence-independent, and ligase-free.

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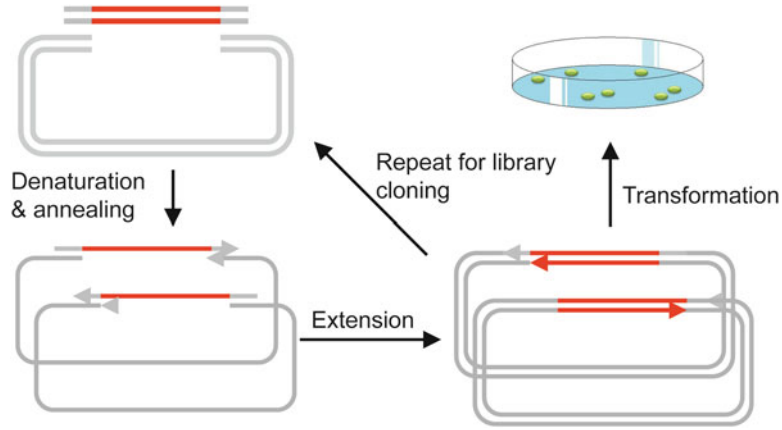

Fig. 1 A schematic diagram of the proposed CPEC mechanism for cloning a single insert. The vector and the insert share overlapping regions at the ends. After denaturation and annealing **(Step 1)**, the hybridized insert and vector extend using each other as a template until they complete a full circle and reach their own 5′-ends **(Step 2)**. The final completely assembled plasmid has two nicks, one on each strand, at the positions marked by an *arrow head*. They can be used for transformation **(Step 3)** with or without further purification. For library cloning, the cycle may be repeated in order to increase the yield of complete plasmids

It uses the polymerase extension mechanism [6] to join overlapping DNA fragments into a double-stranded circular form, such as a plasmid. In a typical CPEC reaction, linear double-stranded insert(s) and vector are first heat-denatured; the resulting single strands then anneal with their overlapping ends and extend using each other as a template to form double-stranded circular plasmids. In CPEC, all overlapping regions between insert(s) and the vector are unique and carefully designed to have very similar and high melting temperatures (*Tm*), which eliminates vector reannealing and concatenation of inserts and makes CPEC very efficient and accurate. The low concentrations of fragments in the reaction favor plasmid circularization and effectively prevent plasmid concatenation. After the CPEC reaction, the perfectly formed double-stranded circular plasmids, with one nick in each strand, can be directly transformed into competent host cells (Fig. 1).

Complex library cloning and multi-way pathway assembly require high cloning efficiency and accuracy. Although other relevant cloning methods only allow the overlapping fragments to anneal or recombine once, CPEC allows multiple annealing–extension cycles that not only increase the chance of hybridization but also permanently join the fragments through polymerase extension, thereby maximizing the cloning efficiency. Whereas the other relevant cloning methods perform the critical annealing/incubation step under ambient temperature, which tends to cause

nonspecific hybridization and leads to compromised cloning efficiency and accuracy, CPEC designs the overlapping ends to have very similar *Tm* (±2 °C) and performs the annealing step at high, stringent temperatures (typically in the range of 55–65 °C) to ensure highest accuracy in multi-way assembly and complex library cloning. Unlike PCR, CPEC does not amplify sequences and therefore does not propagate errors with an increased number of thermal cycles.

The combinatorial library cloning strategy using CPEC is illustrated in Fig. 2. In this example, two libraries are cloned simultaneously into a single vector for expression or functional screens to identify the best combinatorial sequences. It is anticipated that such screens will be performed more and more frequently in synthetic biology applications to construct and identify the optimal macromolecular complexes or gene networks. So far, CPEC is the only in vitro method that works well in our hands for combinatorial library cloning [5]. The successful development of this cloning strategy will highly accelerate the process of protein expression screening using large quantity of library gene variants and hence the development of synthetic biology applications as such screens will be performed more and more frequently to construct and identify the optimal macromolecular complexes or gene networks.

2 Materials

2.1 Reagents

1. Linearized cloning vector: Commercial or custom-designed vectors can be used.
2. Cloning insert or insert library: Cloning insert can be prepared by PCR or restriction digestion from a particular plasmid or DNA template. Insert library is often assembled using oligo libraries synthesized with a DNA synthesizer in house or from commercial providers such as IDT.
3. dNTP mix (dATP, dCTP, dGTP, and dTTP) (e.g., Bioline, cat. no. BIO-39043).
4. Oligonucleotide primers: Custom DNA primer synthesis is available from commercial suppliers such as IDT. Prepare stock solutions of primers (e.g., 100 μM) using sterile DNase/RNase-free water. Prepare aliquots of 10× working solution (e.g., 10 μM) and store at −20 °C to prevent contamination of stock and repeat freeze–thaw cycles.
5. Phusion High-Fidelity DNA polymerase with 5× Phusion HF buffer (Finnzymes, cat. no. F-530).
6. Nuclease-free water (Sigma-Aldrich, cat. no. W4502).
7. Taq DNA polymerase with ThermoPol buffer (New England Biolabs, cat. no. M0267).

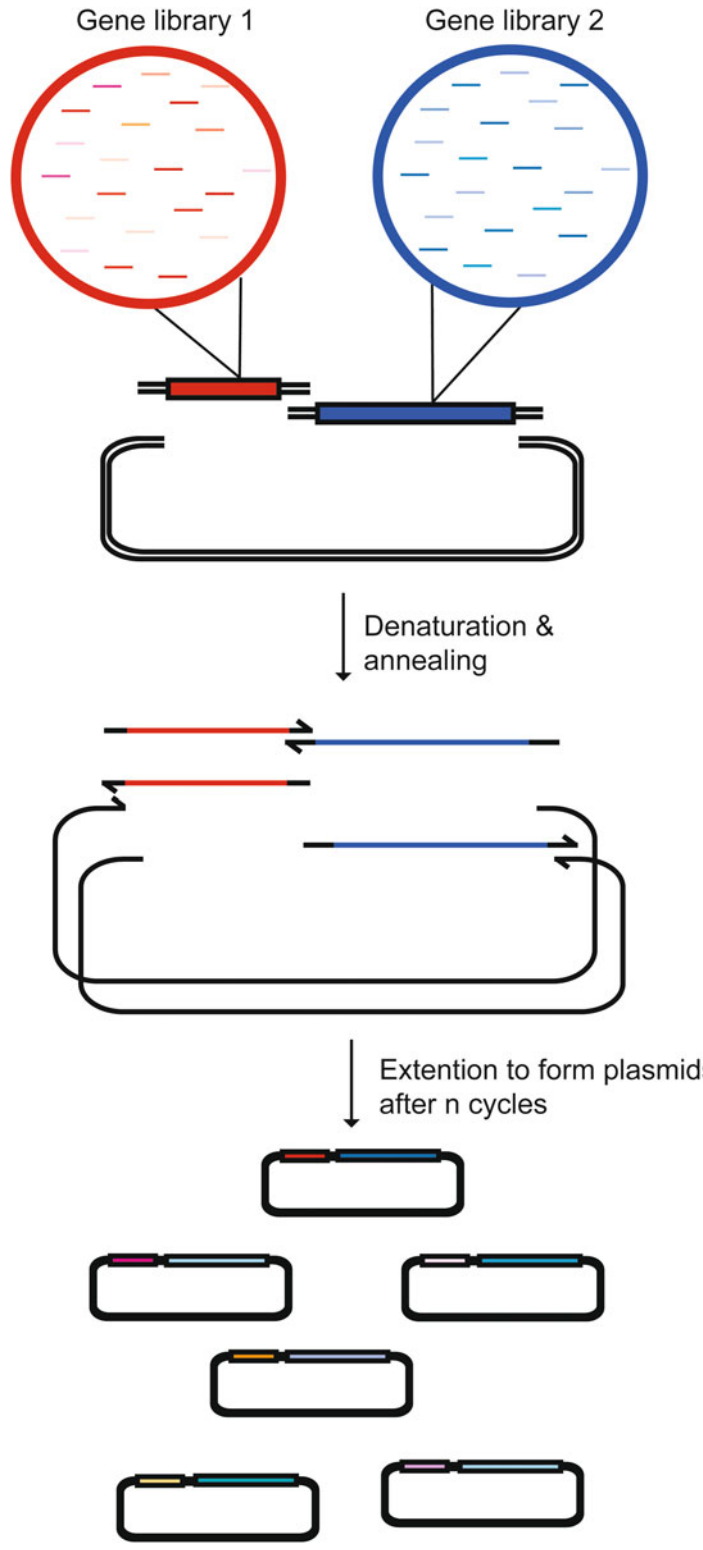

Fig. 2 Schematic diagram of CPEC of combinatorial gene libraries. Two gene libraries are cloned in frame into a vector. The vector and the inserts share overlapping regions at the ends. In each CPEC cycle, after denaturation and annealing, the hybridized inserts and vector extend using each other as a template until they complete a full circle. The assembled plasmid library can be directly used for transformation into competent cells

8. 1× Tris Acetate-EDTA buffer: Mix 900 mL DNase/RNase-free water with 100 mL 10× TAE (Sigma-Aldrich, cat. no. T9650).
9. Ethidium bromide solution (Sigma-Aldrich, cat. no. E1510).
10. 1–2 % (wt/vol) agarose gel with ethidium bromide: Weigh 1.5–3 g agarose (Denville Scientific Inc., cat. no. CA3510-8) and mix with 150 mL 1× Tris Acetate-EDTA buffer in a flask. Heat in a microwave for 1 min and shake the flask. Repeat two more times or until agarose is completely dissolved. Add ethidium bromide to the gel solution to a final concentration of 0.5 μg/mL. Let the solution cool to about 60 °C and then pour it into a casting tray containing a comb and allow it to solidify at room temperature.
11. 6× gel loading dye, blue (New England Biolabs, cat. no. B7021S).
12. 1 kb DNA ladder (New England Biolabs, cat. no. N3232S; Bio-Rad, cat. no. 170–8204) (*see* **Note 1**).
13. 100 bp DNA ladder (New England Biolabs, cat. no. N3231S) (*see* **Note 1**).
14. LB agar (Sigma, cat. no. L3027-1KG).
15. GC5 competent cells (Genesee Scientific, cat. no. 42–653).
16. S.O.C. medium (Cellgro, cat. no. 46-003-CR).
17. Qiaprep Spin MiniprepKit (Qiagen, cat. no. 27106).
18. E.Z.N.A. gel extraction kit (Omega, cat. no. D2501).
19. ExoSAP-IT for PCR clean-up (Affymetrix, cat. no. 78200).

2.2 Equipment

1. Thermal Cycler.
2. Centrifuge.
3. Electrophoresis apparatus.
4. UV transilluminator.
5. FluoChem multi-Imaging system (Alpha Innotech).
6. NanoDrop spectrophotometer (ND-1000, Thermo Scientific).
7. 37 °C shaker.
8. 37 °C cabinet incubator.
9. Water bath.
10. Pipettes.
11. Pipette tips.
12. Microcentrifuge tubes (1.7 mL; Axygen Scientific, cat. no. MCT-175-C).
13. PCR tubes (Denville Scientific, cat. no. 18064).
14. Petri dish (15 × 150 mm; BD, cat. no. 351058).
15. Culture tubes (17 × 100 mm; VWR, cat. no. 60818–703).

16. Cell spreaders.
17. Microplate reader and software (GENios-Basic, Tecan Group).
18. Microwave.

3 Methods

3.1 Preparation of Insert or Insert Library(s)

1. Design PCR primers for the insert or insert library so that they hybridize with the ends of linear vector. The hybridizing parts can either be already incorporated in the insert DNA or be added in this PCR step as overhangs in the primers. The homolog overhangs can range from 0 to 40 bp in order to make the annealing temperature of the hybridizing sequence between vector and insert to be over 55 °C.
2. Set up the PCR on ice as described below. Note that the amount of library DNA that is needed as template is generally larger than that required for standard PCR (*see* **Note 2**).

Initial concentration	Volume per 50 µL reaction	Final amount in 50 µL reaction
Phusion HF buffer (5×)	10 µL	1×
dNTP mix (40 mM)	1 µL	0.8 mM
Phusion High-Fidelity DNA polymerase (2 U/µL)	0.5 µL	1 U
Forward primer (10 µM)	2.5 µL	0.5 µM
Reverse primer (10 µM)	2.5 µL	0.5 µM
DNA template (variable)	Variable	Variable (*see* **Note 2**)
Nuclease-free water	Up to 50 µL	–

3. Run the PCR under the following conditions. For the Phusion enzyme, the annealing temperature should be 3 °C higher than the lower *Tm* of the two primers; *Tm* should be calculated using the part of primer that hybridizes with the insert and using the nearest-neighbor method [7].

Cycle number	Denature	Anneal	Extend
1	98 °C, 30 s		
2–31	98 °C, 10 s	(*Tm*+3)°C, 30 s	72 °C, 15 s/kb
32			72 °C, 5 min

4. Combine 50 µL of the finished PCR with 10 µL of 6× DNA loading dye and run the product on a 1–1.5 % agarose gel. The time required for electrophoresis is dependent on the length of the expected product.

5. Visualize the resolved product with a UV transilluminator or a gel imaging system. A prominent and unique band at the desired position of the length of the insert should be seen.
6. Cut the PCR product band from the gel with a clean sharp razor and purify the insert DNA using a commercial kit (e.g., E.Z.N.A. gel extraction kit). Measure the concentration of the purified insert DNA with the NanoDrop spectrophotometer.

3.2 Preparation of Linear Vector

1. Design PCR primers for the vector so that they hybridize with the ends of the insert DNA. The hybridizing parts can either be already incorporated in the vector or be added in this PCR step as overhangs in the primers. The homolog overhangs can range from 0 to 40 bp in order to make the annealing temperature of the hybridizing sequence between vector and insert to be over 55 °C.
2. Set up the PCR on ice as tabulated below:

Initial concentration	Volume per 50 μL reaction	Final amount in 50 μL reaction
Phusion PCR buffer (5×)	10 μL	1×
dNTP mix (40 mM)	1 μL	0.8 mM
Phusion High-Fidelity DNA polymerase (2 U/μL)	0.5 μL	1 U
Forward primer (10 μM)	2.5 μL	0.5 μM
Reverse primer (10 μM)	2.5 μL	0.5 μM
Vector DNA template (variable)	Variable	1 pg–10 ng
Nuclease-free water	Up to 50 μL	

3. Run the PCR with the following conditions. Again, for the Phusion enzyme, the annealing temperature should be 3 °C higher than the lower *Tm* of the two primers; *Tm* should be calculated using the part of primer that hybridizes with the insert and using the nearest-neighbor method [7].

Cycle number	Denature	Anneal	Extend
1	98 °C, 30 s		
2–31	98 °C, 10 s	(*Tm*+3)°C, 30 s	72 °C, 15 s/kb
32			72 °C, 5 min

4. Combine 50 μL of the finished reaction with 10 μL of 6× DNA loading dye and run the product on a 1–1.5 % percent agarose gel. The time for electrophoresis is dependent on the length of the product.

5. Visualize the resolved product with a UV transilluminator or a gel imaging system. A prominent and unique band at the desired position of the length of the linear vector should be seen.
6. Cut the PCR product band from the gel with a clean sharp razor and purify DNA using a commercial kit (e.g., E.Z.N.A. gel extraction kit). Measure the concentration of the purified vector DNA with the NanoDrop spectrophotometer.

3.3 CPEC

3.3.1 CPEC of a Single Insert

1. Set up the cloning reaction on ice as below. The amount of insert required will be dependent on its size and should be calculated to maintain an insert:vector molar ratio between 1:1 and 2:1.

Initial concentration	Volume per 20 μL reaction	Final amount per 20 μL reaction (*see* Note 3)
Phusion HF buffer (5×)	4 μL	1×
dNTP mix (40 mM)	0.4 μL	0.8 mM
Phusion High-Fidelity DNA polymerase (2 U/μL)	0.2 μL	0.4 U
Vector DNA (variable)	Variable	50–100 ng
Insert DNA (variable)	Variable	–
Nuclease-free water	Up to 20 μL	

2. Run the CPEC reaction using the following conditions (*see* **Note 4**) (Fig. 3):

Cycle number	Denature	Anneal	Extend
1	98 °C, 30 s		
2	98 °C, 10 s	(*Tm*+3)°C, 30 s (*see* **Note 5**)	72 °C, 15 s/kb (*see* **Note 6**)
3			72 °C, 5 min

3.3.2 CPEC of a Single Library

1. Set up the cloning reaction on ice as below. The amount of insert required will be dependent on its size and should be calculated to maintain an insert:vector molar ratio between 1:1 and 2:1.

Initial concentration	Volume per 20 μL reaction	Final amount per 20 μL reaction (*see* Note 3)
Phusion HF buffer (5×)	4 μL	1×
dNTP mix (40 mM)	0.4 μL	0.8 mM
Phusion High-Fidelity DNA polymerase (2 U/μL)	0.2 μL	0.4 U
Vector DNA (variable)	Variable	50–100 ng
Insert library DNA (variable)	Variable	–
Nuclease-free water	Up to 20 μL	

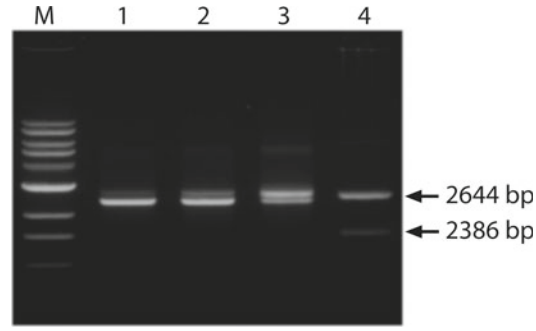

Fig. 3 CPEC of a 258 bp gene. The image shows gel electrophoresis analysis of the CPEC reaction product after 1, 2, and 5 cycles (lanes 1–3) together with a full-length plasmid (lane 4) and 1 kb DNA ladder (lane M, New England Biolabs). The assembled full-length plasmid is 2,644 bp shown as the *upper band* of the two prominent bands close to each other. The *lower band* of the two is the empty vector of 2,386 bp that has not been incorporated within reaction cycles

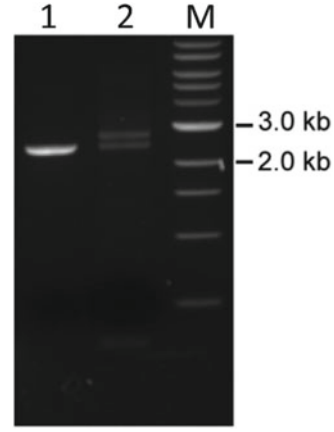

Fig. 4 Agarose gel examination of the CPEC product for a 258 bp gene library after 5 thermal cycles (lane 2) together with linear vector (lane 1) and 1 kb DNA ladder (lane M, New England Biolabs), respectively

2. Run the CPEC reaction using the following conditions (*see* **Note 7**) (Fig. 4):

Cycle number	Denature	Anneal	Extend
1	98 °C, 30 s		
2–6	98 °C, 10 s	(*Tm*+3)°C, 30 s (*see* **Note 5**)	72 °C, 15 s/kb (*see* **Note 6**)
7			72 °C, 5 min

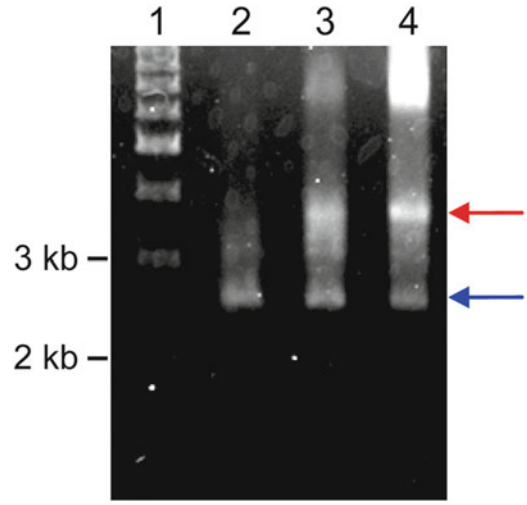

Fig. 5 CPEC of combinatorial gene libraries. Lanes 2, 3, and 4 show the CPEC reaction products after 5, 10, and 20 thermal cycles. The *upper arrow* indicates the full-length cloning product of a 306 bp gene library and a 741 bp gene library as well as the vector (2.5 kb); the *lower arrow* indicates the remaining empty vector. Lane 1 is the 1-kb ladder from Bio-Rad

3.3.3 CPEC of Combinatorial Gene Libraries

1. Set up the cloning reaction on ice as below. The amount of insert required will be dependent on its size and should be calculated to maintain an insert:vector molar ratio between 1:1 and 2:1 for each insert library.

Initial concentration	Volume per 20 μL reaction	Final amount per 20 μL reaction (*see* Note 3)
Phusion HF buffer (5×)	4 μL	1×
dNTP mix (40 mM)	0.4 μL	0.8 mM
Phusion High-Fidelity DNA polymerase (2 U/μL)	0.2 μL	0.4 U
Vector DNA (variable)	Variable	50–100 ng
Insert library DNA (variable)	Variable	–
Nuclease-free water	Up to 20 μL	

2. Run the CPEC reaction using the following conditions (*see* **Note 8**) (Fig. 5):

Cycle number	Denature	Slow ramp anneal	Anneal	Extend
1	98 °C, 30 s			
2–31	98 °C, 10 s	70 to (*Tm*+3)°C (0.1 °C/s)	(*Tm*+3)°C, 2 min (*see* **Note 5**)	72 °C, 15 s/kb (*see* **Note 6**)
32				72 °C, 5 min

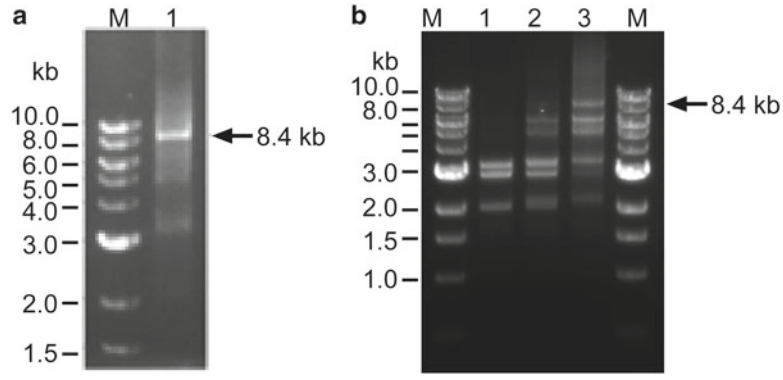

Fig. 6 (**a**) Gel electrophoresis analysis of the final assembly product after a 20-cycle CPEC for four fragments of 3,280, 2,959, 2,040, and 171 bp, respectively. The final full-length plasmid is 8,360 bp as shown in lane 1. (**b**) Gel electrophoresis analysis of the multi-way CPEC reaction. 20 μL is taken out of the reaction after 2, 5, and 10 cycles and separated on a 0.8 % agarose gel (lanes 1, 2, and 3). The starting lengths of the four fragments are 3,280, 2,959, 2,040, and 171 bp, respectively. Discrete bands representing extension products joining neighboring pieces to form longer and longer intermediates are clearly visible. The 171-bp band is not visible from the gel image. Lane M in both figures is 1 kb DNA ladder (New England Biolabs)

3.3.4 CPEC Assembly of Multicomponents

1. Set up the cloning reaction on ice as below. The amount of insert required will be dependent on its size and should be calculated to maintain an insert:vector molar ratio between 1:1 and 2:1 for each insert library.

Initial concentration	Volume per 20 μL reaction	Final amount per 20 μL reaction (*see* Note 3)
Phusion HF buffer (5×)	4 μL	1×
dNTP mix (40 mM)	0.4 μL	0.8 mM
Phusion High-Fidelity DNA polymerase (2 U/μL)	0.2 μL	0.4 U
Vector DNA (variable)	Variable	50–100 ng
Insert library DNA (variable)	Variable	–
Nuclease-free water	Up to 20 μL	

2. Run the CPEC reaction using the following conditions (*see* **Note 9**) (Fig. 6):

Cycle number	Denature	Anneal	Extend
1	98 °C, 30 s		
2–21	98 °C, 10 s	(*Tm* + 3) °C, 2 min (*see* **Note 5**)	72 °C, 15 s/kb (*see* **Note 6**)
22			72 °C, 5 min

3. Combine 10 μL of the finished cloning reaction with 2 μL of 6× DNA loading dye and run the product on a 1–1.5 % agarose gel to assess whether the CPEC reaction is successful. The time for electrophoresis is dependent on the length of the expected product. The rest of the cloning product can be stored at −20 °C for up to 1 year.
4. Visualize the resolved products with a UV transilluminator or a gel imaging system (*see* **Note 10**).

3.4 Transformation of the Cloning Product

1. Thaw the required number of tubes containing high-efficiency competent cells of your choice on ice. We recommend following the manufacturer's recommended procedures for transformation; **Steps 2–9** are based on the supplier's protocol for transformation of GC5 competent cells.
2. Add 1–5 μL of the cloning reaction to 50 μL of GC5 competent *Escherichia coli* cells in a microcentrifuge tube and gently tap the tube to ensure an even distribution of DNA in the solution.
3. Incubate the tubes on ice for 30 min.
4. Heat shock the cells for 45 s in a 42 °C water bath and then leave the tubes on ice for 2 min (*see* **Note 7**).
5. Add 450 μL of room temperature S.O.C. medium to each transformation reaction.
6. Shake the tubes in a shaker incubator at 225 rpm at 37 °C for 1 h.
7. Spread 50–100 μL of transformation reaction evenly on each 15-cm LB-agar plate containing appropriate antibiotics (depending on the vector used) and incubate at 37 °C overnight (16–18 h).
8. The next day, pick a desired number (varies according to the application) of isolated colonies from each plate and culture each colony in 3–10 mL of LB broth with antibiotics for 16–18 h (overnight).
9. Take 700 μL of the overnight bacterial culture and mix it well with 300 μL of 50 % (vol/vol) sterile glycerol in a cryovial to prepare 15 % (vol/vol) glycerol stocks and store at −80 °C.

3.5 Colony PCR and/or Sequencing

1. Colony PCR is performed to determine whether the picked colonies have insert(s) of the correct size. Colonies picked directly from the plate or from overnight cultures can be used as the template. Set up a PCR on ice as follows:

Components	Final volume per 30 μL reaction	Final amount per 30 μL reaction
ThermoPol reaction buffer (10×)	3 μL	1×
dNTP mix (40 mM)	0.6 μL	0.8 mM
Taq DNA polymerase (5 U/μL)	0.3 μL	1.5 U

(continued)

(continued)

Components	Final volume per 30 μL reaction	Final amount per 30 μL reaction
Bacteria colony or overnight culture	1 μL if using colony culture	–
Vector forward primer (10 μM)	0.6 μL	0.2 μM
Vector reverse primer (10 μM)	0.6 μL	0.2 μM
Nuclease-free water	Up to 30 μL	

2. Run the PCR using the following conditions. The *Tm* will depend on the vector–primer pair.

Cycle number	Denature	Anneal	Extend
1	95 °C, 5 min		
2–26	95 °C, 30 s	*Tm* °C, 30 s	68 °C, 1 min/kb
27			68 °C, 5 min

3. Combine 10 μL of the finished reaction with 2 μL of 6× DNA loading dye and run the product on a 1–1.5 % (wt/vol) agarose gel. The time for electrophoresis is dependent on the length of the expected product.
4. Visualize the resolved product with a UV transilluminator and determine the presence of inserts and their size. Inserts of correct sizes can be further used for sequencing or restriction digestion if needed.
5. Prepare sequencing samples by first purifying the colony PCR with ExoSAP-IT. Set up a reaction on ice as following the manufacturer's recommended procedures:

Components	Final volume per 7 μL reaction
Colony PCR product	5 μL
ExoSAP-IT	2 μL

6. Incubate the mixture at 37 °C for 15 min to degrade remaining primers and nucleotides.
7. Incubate at 80 °C for 15 min to inactivate ExoSAP-IT. Purified colony PCRs are now ready for direct sequencing, either in house or by commercial services.

4 Notes

1. Both 1 kb and 100 bp DNA ladder are diluted to 250 μg/mL with distilled water and 6× gel loading dye (supplied with the DNA ladder). For a 5 mm wide lane, 2 μL of the mixture

should be loaded onto the agarose gel. The amount of the mixture should be scaled up or down, depending on the width of the agarose gel.

2. For plasmid DNA, the amount in a 50-μL reaction is 1 pg–10 ng. For assembled library DNA, the amount in a 50-μL reaction is 1–50 ng depending on the length of the insert. Usually for library DNA, testing a series of template concentrations is recommended.
3. The 20-μL reaction volume has been tested for cloning of at least two complex gene libraries. For cloning simpler inserts, such as a single fragment or one library, lower volumes can be used. The volume can also be scaled up to 50 μL, especially if the researcher wants to analyze partial cloning product on an agarose gel.
4. For single-gene CPEC, Cycle 2 can be repeated 1–9 more times to increase the amount of cloning product. For gene insert of longer than 1 kb, at least 5 cycles are recommended.
5. *Tm* is calculated based on each pair of the hybridizing parts of the insert and vector and use the lowest *Tm* of all *Tm*s.
6. Extension time is calculated based on the full length of the cloning product. For example, if a 1 kb insert is to be cloned into a 4 kb vector, then extension time should be calculated based on 5 kb and hence 75 s for Phusion DNA polymerase.
7. For single-library CPEC, Cycles 2–6 can be extended to up to 20 cycles to increase the amount of cloning product. For gene insert of longer than 1 kb, at least 10 cycles are recommended.
8. Due to the complexity of the combinatorial library cloning, at least 30 cycles should be used.
9. For multicomponent CPEC, slow ramp annealing is not necessary. However, if the cloning product is less than expected, slow ramp annealing as in combinatorial library CPEC should be attempted.
10. This step is to confirm the success of the cloning process. A prominent band should be present on the agarose gel representing the total length of the vector plus inserts. Sometimes a high-molecular-weight smear and/or additional bands representing excessive or unincorporated vector or inserts may also be visible but should not affect the subsequent transformation step.

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