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Rapid construction of recombinant plasmids by QuickStep-Cloning

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i. Summary

QuickStep-Cloning is a novel molecular cloning technique that builds upon the concepts of asymmetric PCR and megaprimer-based amplification of whole-plasmid. It was designed specifically to address the major drawbacks of previously reported cloning methods. The fully optimized protocol allows for a seamless integration of a long DNA fragment into any position within a plasmid of choice, in a time-efficient and cost-effective manner, without the need of a tedious DNA gel purification, a restriction digestion and an enzymatic ligation. QuickStep-Cloning can be completed in less than 6 hours, significantly faster than most of the existing cloning methods, while retaining high efficiency.

ii. Keywords

Recombinant DNA, Megaprimer PCR, Ligation-independent cloning, Plasmid construction, Synthetic biology, Protein engineering, Metabolic engineering

1. Introduction

DNA cloning is, undoubtedly, one of the most fundamental molecular biology techniques. It is routinely employed in various disciplines related to genetic manipulation and is central to synthetic biology experiments. Conventionally, molecular cloning has been achieved by conducting a restriction enzyme digestion, followed by an enzymatic ligation. This approach, despite receiving unceasing support from the scientific community, is a resource-intensive procedure that depends on the availability of unique restriction sites and potentially results in addition of undesired amino acid(s). To address these problems, many sequence-independent cloning methods have been proposed in recent years (1). Among these techniques, megaprimer-based cloning methods, such as restriction-free (RF) cloning (2) and MEGAWHOP cloning (3), are most popular for their simplicity and robustness. However, most megaprimer-based cloning methods are based upon linear amplification of whole plasmid and the use of self-annealing megaprimers, which compromise their overall efficiency and significantly hinder their widespread adoption.

QuickStep-Cloning utilizes asymmetric PCR to overcome the problem of self-annealing megaprimers and enables exponential amplification of recombinant plasmid (4). QuickStep-Cloning consists of five simple steps (Fig. 1). The method begins with two parallel asymmetric PCRs. In each of these PCRs, DNA fragment of interest is amplified by two primers of unbalanced concentrations (1:50 ratio is used), resulting in predominantly single-stranded DNAs. Primers are designed in such a way that these single-stranded products carry a 3'-terminal region that corresponds to the integration site on the recipient plasmid. The two PCR mixtures are then purified separately. When mixed in an equimolar ratio, for the next PCR stage, the single-stranded products of the two asymmetric PCRs form megaprimer pairs, with 3'-overhangs that are complementary to the recipient plasmid. This allows megaprimers to anneal to the recipient plasmids, even when the two megaprimer strands self-anneal. Such

a primer design also facilitates exponential amplification of whole plasmid (4), which results in the production of nicked-circular plasmids, with DNA fragment of interest integrated at the desired position. After a brief DpnI digestion to remove methylated/hemimethylated parental plasmid, PCR mixture is used directly for bacterial transformation and protein expression. For a standard experiment involving cloning of a 1-kb DNA fragment into a 7-kb recipient plasmid, the aforementioned procedure can be completed in less than 6 hours, without the need of lengthy enzymatic reactions or DNA gel purification (4). Overall, QuickStep-Cloning is a robust method of constructing recombinant plasmids in a sequence-independent manner, with a reported cloning efficiency of over 90% (4).

2. Materials

2.1. Bacterial strains

1. Competent E. coli cells (see **Note 1**).

2.2. Nucleic acid

1. Donor plasmid (or other source of DNA insert; see **Note 2**).
2. Recipient plasmid.
3. PCR primers (Eurofins Genomics, Ebersberg, Germany) – see section 3.1. for detailed information on primer design.

2.3. PCR components

1. Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, USA; see **Note 3**).
2. Q5 Reaction Buffer (New England Biolabs, Ipswich, USA).
3. Deoxynucleotide (dNTP) Solution Mix (New England Biolabs, Ipswich, USA).
4. Ultrapure water.

5. 0.2 ml PCR tubes.
6. Thermal cycler (Mastercycler personal, Eppendorf, Hamburg, Germany).

2.4. DpnI digestion and DNA purification

1. DpnI (New England Biolabs, Ipswich, USA).
2. QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) or equivalent (see **Note 4**).
3. 1.5 ml or 2 ml microcentrifuge tubes.
4. Benchtop centrifuge (MiniSpin plus, Eppendorf, Hamburg, Germany).
5. Optional Spectrophotometer (VersaWave, Expedeon, Cambridge, UK).

2.5 Transformation and clone analysis

1. Ice.
2. 2×TY media: 16 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl.
3. Agar plates supplemented with an appropriate antibiotic, e.g., TYE agar plates: 10 g/l tryptone, 5 g/l yeast extract, 8 g/l NaCl and 15 g/l agar.
4. Sterile toothpicks.
5. 1.5 ml microcentrifuge tubes.
6. 50 ml sterile conical tubes.
7. QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) or equivalent.
8. Heat block (ThermoMixer C, Eppendorf, Hamburg, Germany) or water bath.
9. Shaking incubator (VWR Incubating Mini Shaker, VWR International, West Chester, USA).
10. Incubator (INCU-Line, VWR International, West Chester, USA).

3. Methods

3.1.Primer design

1. Identify DNA fragment to be cloned.
2. Using standard primer design guidelines, design a pair of oligonucleotides for amplification of the DNA fragment of interest (denoted herein as Fwd primer and Rev primer). Make sure that there is no significant difference between the melting temperatures of the two oligonucleotides (see **Note 5**).
3. Choose a DNA insertion point on the recipient plasmid.
4. Identify two megaprimer annealing sites (about 25 bp long) on both sides of the DNA insertion point and denote them as A and B (Fig. 2).
5. Check the melting temperatures (T_m) of both regions and adjust their length to obtain comparable melting temperatures (see **Note 5**).
6. Design primers IntA-Fwd and IntB-Rev according to Fig. 2. Remember to provide primer sequences in 5'→3' direction while placing the primer order.

3.2. Asymmetric PCR

1. Create 100 µM stock solutions of Fwd, Rev, IntA-Fwd and IntB-Rev primers by resuspending the lyophilized oligonucleotides in ultrapure water (see **Note 6**). By diluting the 100 µM stock solution with ultrapure water, prepare 10 µM working solutions of Fwd and Rev primers and 0.2 µM working solutions of IntA-Fwd and IntB-Rev primers.
2. Prepare asymmetric PCR mixtures I and II (see Table 1 and Table 2) in two separate 0.2 ml PCR tubes. Both mixtures should be prepared concurrently.
3. Transfer the two PCR tubes to a thermal cycler (see **Note 8**) and initiate the thermocycling program given in Table 3.

4. Purify the two PCR products using QIAquick PCR Purification Kit (see **Note 4**). Elute with 30 µl of ultrapure water.
5. Quantify the DNA concentrations of the two purified products using a VersaWave spectrophotometer (see **Note 9**). If no product is detected, consult **Note 10**.
6. Product of asymmetric PCR can be stored overnight at 4°C (if longer storage is required, place in -20°C freezer).

3.3. Megaprimer PCR

1. Prepare megaprimer PCR mixture (Table 4) in a 0.2 ml PCR tube.
2. Transfer the PCR tube to a thermal cycler (see **Note 8**) and initiate the thermocycling program given in Table 5 (see **Note 13**).
3. After the completion of PCR, add 2 µl of 20 U/µl DpnI directly into the 50 µl PCR product and incubate at 37°C for 15 min (see **Note 14**).
4. For short-term storage, DpnI-digested PCR product can be kept at 4°C. If longer storage is required, place in -20°C freezer.

3.4. Transformation

1. Add 5 µl of the DpnI-treated reaction mixture (see **Note 15**) to 50-100 µl competent E. coli cells. Mix gently.
2. Incubate on ice for 30-60 min. Perform heat shock by placing the tube at 42°C for 1 min. After the heat shock, transfer the tube immediately to ice for an additional 3 min incubation.
3. Add 1 mL of 2×TY media (pre-warmed to 37°C) and incubate with shaking for 1 h at 37°C.

4. Plate transformed cells on TYE agar plates supplemented with an appropriate antibiotic. Incubate overnight at 37°C.

3.5.Colony analysis

1. Inspect the plate for the presence of colonies (if no colonies are observed, consult **Note 16**).
2. Inoculate 3 colonies in separate 50 ml conical tubes containing 5 ml 2×TY media supplemented with an appropriate antibiotic. Incubate overnight at 37°C with shaking.
3. Isolate the plasmids using QIAprep Spin Miniprep Kit.
4. Send the three plasmid samples for DNA sequencing to verify the presence of an insert at the desired position.

4. Notes

1. To prepare competent cells, traditional $CaCl_2$-based method was used (5). It was found that, in QuickStep-Cloning, direct transformation of E. coli expression strain C41 (DE3) results in a higher number of transformants in comparison to transformation of DH5α cells. Competent cells prepared via more complicated protocols or purchased directly from a manufacturer (e.g., New England Biolabs) are fully compatible with the QuickStep-Cloning method and, more often than not, will result in a higher number of transformants. The use of commercially available ultracompetent cells is encouraged when attempting more challenging cloning experiments (e.g., in the case of cloning very long DNA fragments).
2. Sources of DNA insert other than a plasmid, e.g., a linear DNA fragment, can be used. However, it should be noted that the recommended DNA concentration, given in Table 1 and Table 2, has been optimized for use with a 4-kb plasmid carrying a 1-kb DNA

fragment of interest. When DNA fragments of significantly different molecular mass are to be used in the reaction, DNA concentration should recalculated accordingly (knowing that the concentration given corresponds to 0.1 fmol molecules of cloned DNA fragment present in a 50 µl PCR mixture). When cloning very long DNA fragments (or other challenging amplicons), consult **Note 16**.

3. The protocol given has been carefully optimized for use with Q5 High-Fidelity DNA Polymerase from New England Biolabs and, consequently, its use is strongly recommended. Other high-fidelity polymerases are, in principle, compatible with the concept of QuickStep-Cloning, however, their use necessitates a careful adjustment of PCR conditions.
4. Other kits and alternative PCR purification methods can be used.
5. New England Biolabs provides a T_m calculator on its website, the use of which is strongly recommended.
6. When reconstituting the primers and preparing the oligonucleotide working solutions, TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) can be used instead of ultrapure water.
7. The annealing temperature should be calculated based on the sequence of Fwd and Rev primers (remember that only parts of IntA-Fwd and IntB-Rev primers anneal to the amplified DNA). Use of the New England Biolabs Tm Calculator is strongly recommended for this purpose. Time of the elongation step should be determined based on the length of the cloned DNA fragment (and not the length of a donor plasmid).
8. The two reaction mixtures should be transferred to a thermal cycler (preheated to 98°C) immediately after their preparation.
9. Measuring DNA concentration at this point is strongly recommended as it helps to detect any potential problems with asymmetric PCRs and make sure that the amount of both

megaprimers is sufficient for the second PCR stage. However, if the DNA quantification cannot be performed or is highly undesirable (e.g., in high-throughput applications), this step can be omitted.

10. Make sure that the primers have been correctly designed. The yield of asymmetric PCRs can be potentially improved by using a lower annealing temperature and a higher concentration of the donor plasmid (see **Note 14**).
11. The megaprimer concentrations, given in Table 4, have been optimized for use with a 1-kb insert. For longer inserts, use higher amounts of the megaprimer. If the two concentrations have not been measured or the yield of asymmetric PCR is insufficient to achieve the concentration given, use as high concentration as possible (i.e., if 1 µl of recipient plasmid is used, use 18.75 µl of purified asymmetric PCR product I and 18.75 µl of purified asymmetric PCR product II). The recipient plasmid concentration has been optimized for use with a 7-kb plasmid. If a recipient plasmid of significantly different molecular mass is used, recalculate the concentration accordingly.
12. The annealing temperature should be calculated based on the sequence of the two megaprimer annealing sites (denoted as A and B in Fig. 2). Use of the New England Biolabs Tm Calculator is strongly recommended for this purpose. The time of elongation step should be determined based on the total length of the recipient plasmid.
13. Higher yields of megaprimer PCR can be achieved by separating the two megaprimers for the first five PCR cycles. Two reaction mixtures (25 µl each; containing 1× Q5 Reaction Buffer, 200 nM of each dNTP, 200 ng of either purified asymmetric PCR product I or II, 10 ng of recipient plasmid and 0.5 U Q5 High-Fidelity DNA Polymerase) are subjected to the thermocycling conditions of megaprimer PCR described in Table 5. After 5 cycles, they are mixed together and subjected to the remaining 20 cycles of the PCR program.

This method is particularly useful in instances where low product yields are anticipated (e.g., in the case of cloning very long DNA fragments).

14. When using concentrations of donor or recipient plasmid that are significantly higher than the ones given in the protocol, it is recommended to increase DpnI digestion to 1 hr to make sure all parental plasmids are properly digested.
15. As the transformation efficiency for intact cloning vectors is significantly higher than that for nicked plasmids, subjecting the product of megaprimer PCR to enzymatic phosphorylation and ligation can potentially increase the overall number of transformants.
16. The QuickStep-Cloning protocol has been optimized with the purpose of obtaining a significant number of transformants in the shortest time possible (at the same time, making sure that a high percentage of colonies contain a recombinant plasmid of interest). As a result, there are several simple strategies of modifying the protocol to increase the total number of transformants (in return for a more time-consuming procedure) that can be easily incorporated whenever the standard protocol does not provide desirable results (see **Note 13** and **Note 15**). Moreover, the yield of megaprimer PCR, and consequently the final number of transformants, can potentially be improved by using a lower annealing temperature, a higher concentration of the megaprimer and/or a higher concentration of the recipient plasmid (see **Note 14**). Use of ultracompetent cells might also prove helpful (see **Note 1**).

5. References

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Figure Captions

Fig. 1. Overview of the QuickStep-Cloning method.

Fig. 2. Outline of primer design for the QuickStep-Cloning.

Tables

Table 1. Composition of asymmetric PCR mixture I (see **Note 2**).

Component	Stock solution	Volume (µl)	Final concentration
Q5 Reaction Buffer	5x	10	1x
dNTP mix	10 mM each	1	0.2 mM each
Fwd primer	10 µM	2.5	0.5 µM
IntB-Rev primer	0.2 µM	2.5	0.01 µM
Donor plasmid	variable	variable	4 pg/µl
Q5 Polymerase	2 U/µl	0.5	0.02 U/µl
Ultrapure water		to 50 µl	

Table 2. Composition of asymmetric PCR mixture II (see **Note 2**).

Component	Stock solution	Volume (µl)	Final concentration
Q5 Reaction Buffer	5x	10	1x
dNTP mix	10 mM each	1	0.2 mM each
IntA-Fwd primer	0.2 µM	2.5	0.01 µM
Rev primer	10 µM	2.5	0.5 µM
Donor plasmid	variable	variable	4 pg/µl
Q5 Polymerase	2 U/µl	0.5	0.02 U/µl
Ultrapure water		to 50 µl	

Table 3. Asymmetric PCR thermocycling conditions (see **Note 7**)

Step	Temperature	Time	Cycles
Denaturation	98°C	30 s	1
Denaturation	98°C	7 s	30
Annealing	50-72°C	20 s	
Elongation	72°C	30 s/kb	
Cooling	4-8°C	-	-

Table 4. Composition of megaprimer PCR mixture (see **Note 11**).

Component	Stock solution	Volume (µl)	Final concentration
Q5 Reaction Buffer	5x	10	1x
dNTP mix	10 mM each	1	0.2 mM each
Purified product of asymmetric PCR I	variable	variable	4 ng/µl
Purified product of asymmetric PCR II	variable	variable	4 ng/µl
Recipient plasmid	variable	variable	0.4 ng/µl
Q5 Polymerase	2 U/µl	0.5	0.02 U/µl
Ultrapure water		to 50 µl	

Table 5. Megaprimer PCR thermocycling conditions (see **Note 12**).

Step	Temperature	Time	Cycles
Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	
Annealing	50-72°C	20 s	25
Elongation	72°C	30 s/kb	
Elongation	72°C	2 min	1
Cooling	4-8°C	-	-

Figure 1.

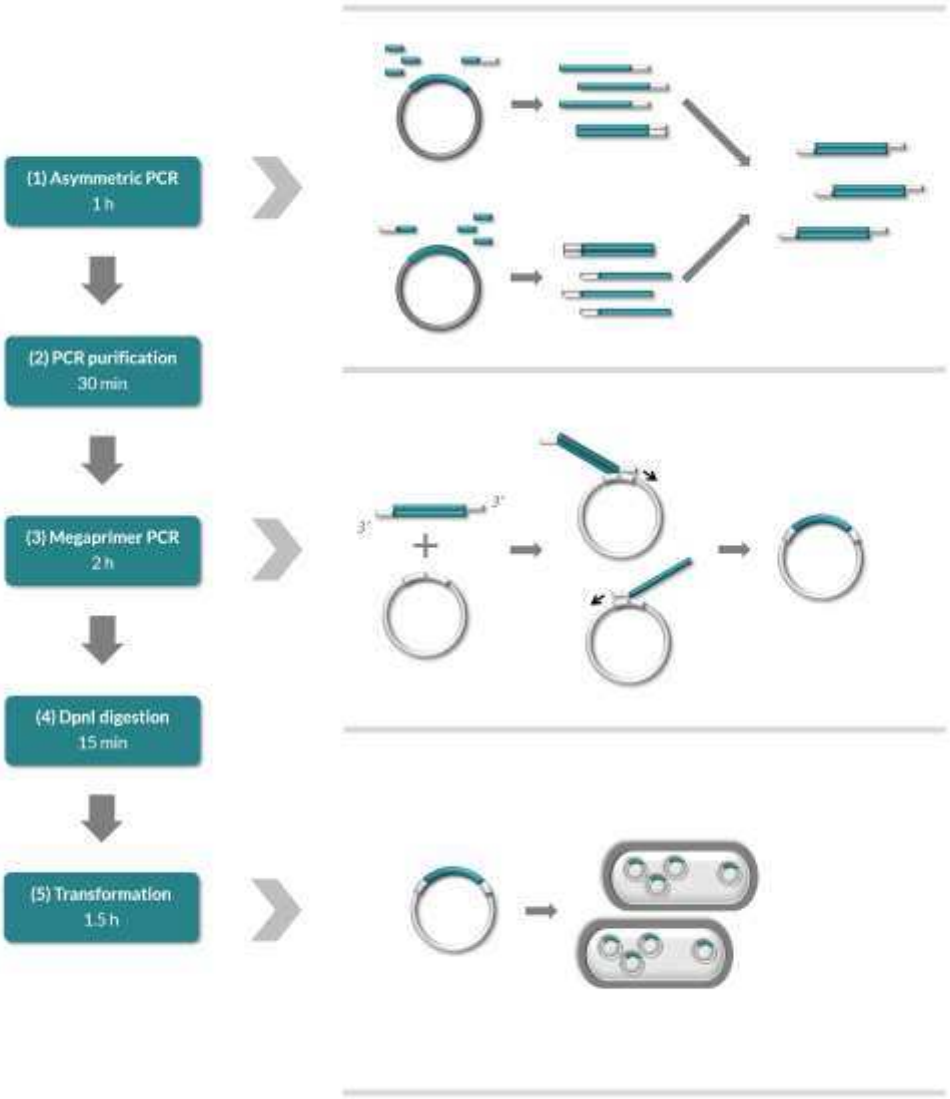
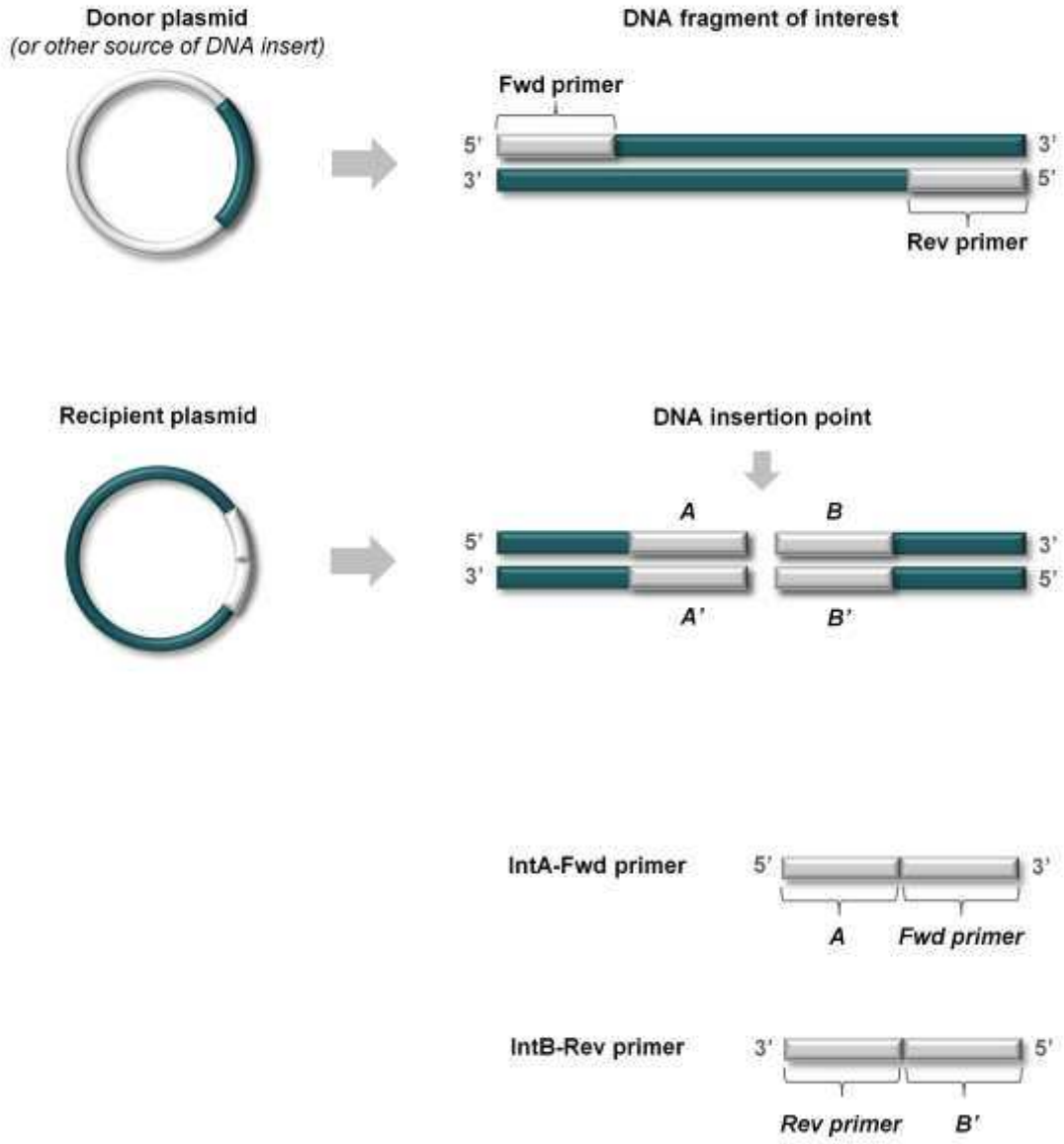

(1) Asymmetric PCR
1 h
(2) PCR purification
30 min
(3) Megaprimer PCR
2 h
(4) DpnI digestion
15 min
(5) Transformation
1.5 h

Figure 2.


Donor plasmid
(or other source of DNA insert)
DNA fragment of interest
Fwd primer
5'
3'
3'
5'
Rev primer
Recipient plasmid
DNA insertion point
A
B
5'
3'
3'
5'
A'
B'
IntA-Fwd primer
5'
3'
A
Fwd primer
IntB-Rev primer
3'
5'
Rev primer
B'