ABC cloning: An efficient, simple, and rapid restriction/ligase-free method

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A B S T R A C T

DNA cloning remains the primary step before the further investigation of gene function. Restriction enzyme-based cloning methods are still widely used and numerous restriction-free cloning techniques are available as alternatives. Here we describe a PCR-based cloning method named ABC cloning. This method uses PCR to combine three overlapping DNA fragments into a recombinant vector that can be immediately transformed into competent cells. This technique uses only a thermostable DNA polymerase and is more rapid and efficient than previously described methods.

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Specifications table

Subject Area	● *Biochemistry, Genetics and Molecular Biology*
More specific subject area:	*DNA cloning*
Method name:	*ABC cloning*
Name and reference of original method	*not applicable*
Resource availability	*not applicable*

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Method details

DNA cloning involves the insertion of a DNA fragment into a suitable plasmid to generate a recombinant vector. For decades, 'cutting and pasting' using restriction enzymes and DNA ligase was the method of choice in molecular biology laboratories. To overcome the inconvenience of restriction enzyme-based cloning methods, several attempts have focused on recombination-based cloning which consists of preparing a PCR product flanked by regions of homology to the target vector. A second PCR is then performed using the previous PCR product as a megaprimer. This step is followed by DpnI digestion to cleave methylated DNA that can be the source of false positive clones after transformation [1]. Another cloning strategy [2] also relies on the use of megaprimers corresponding to the gene of interest to replace a negative selection marker, the coupled cell division B gene (ccdB). CcdB is a toxin that interferes with bacterial DNA gyrase, causing un-repairable chromosomal damage. However, the use of this cloning method is limited to plasmids harboring the ccdB gene.

Several restriction free cloning methods have been previously described. TOPO (TI) cloning exploits the terminal transferase activity of Taq polymerase to add a single adenosine (A) overhang to the 3′ end of the PCR product. Topoisomerase I is added to generate a final recombinant vector through hybridization between the 3′ overhang of the PCR product and the 5′ T overhang of the TOPO backbone [3]. However, the use a non-proofreading polymerase can be a potential source of mutations, and additional hemi-phosphorylation of both T-vector and insert are required for directional cloning. Gateway cloning uses the bacteriophage lambda site-specific recombination system to shuttle a cloned gene of interest into a compatible destination vector [4]. This method is limited to specific plasmids and requires the use of an additional enzyme, clonase, for recombination.

In an attempt to improve the efficiency of restriction-free cloning methods that use megaprimers, recombination-assisted megaprimer (RAM) cloning was developed [5]. This method differs from the restriction-free method by adding primers that allow exponential amplification of the recombinant plasmid. However, similar to the restriction-free method [1], RAM also includes a DpnI digestion step prior to transformation. Another restriction-ligation-free method [6] consists of co-transforming E. coli with an insert and a linearized vector that share sequence homology. This method takes advantage of the E. coli recombination system to generate the desired recombinant vector inside the cell, though with less efficiency than other methods. A similar cloning strategy in yeast was developed to advantage of the yeast homologous recombination system to assemble multiple DNA fragments into a suitable cloning vector [7]. This is an efficient and cost effective but requires vector modification by including a Yeast cloning cassette (YCC) that contains the yeast 2 μm origin of replication (2 μm ori) and the ura3 gene prior to the DNA assembly. Sequence and Ligase Independent Cloning (SLIC) includes an additional step by using the exonuclease activity of the T4 DNA polymerase to create complementary overhangs [8]. A method similar to SLIC, named Gibson assembly, uses the 5′ exonuclease activity of the T5 exonuclease [9]. Seamless Ligation Cloning Extract (SLiCE) is similar to SLIC and Gibson assembly, with the exception that SliCE uses bacterial extracts to allow in vitro insert and vector assembly via homologous recombination [10].

Both SLIC and Gibson assembly use at least one additional enzyme (T4 polymerase for SLIC and T5 exonuclease plus ligase for Gibson assembly) to achieve the cloning. The efficiency of these methods is dependent upon the DNA sequences of both insert and plasmid since they require single stranded DNA at the ends of the insert and vector. Repeats or hairpins create DNA structures that can lead to aberrant incorporation of the insert into the vector.

Here we describe what we believe to be a simpler method, ABC cloning. This method relies upon overlap PCR using a high fidelity thermostable DNA polymerase. Overlap PCR is a commonly used DNA amplification technique to combine two or more overlapping DNA fragments into a single DNA segment [11]. The ABC cloning method is inspired from overlap PCR and consists of using two overlapping primers to generate a single large circular DNA fragment from three smaller DNA fragments that overlap with each other (Fig. 1A). The 'A' fragment corresponds to the portion of the vector that includes the promoter downstream from which the gene of interest will be cloned. The 'B' fragment corresponds to the gene of interest. The 'C' fragment corresponds to the remainder of the plasmid. We chose to amplify the vector in two parts (A) and (C) rather than one piece (AC) for PCR
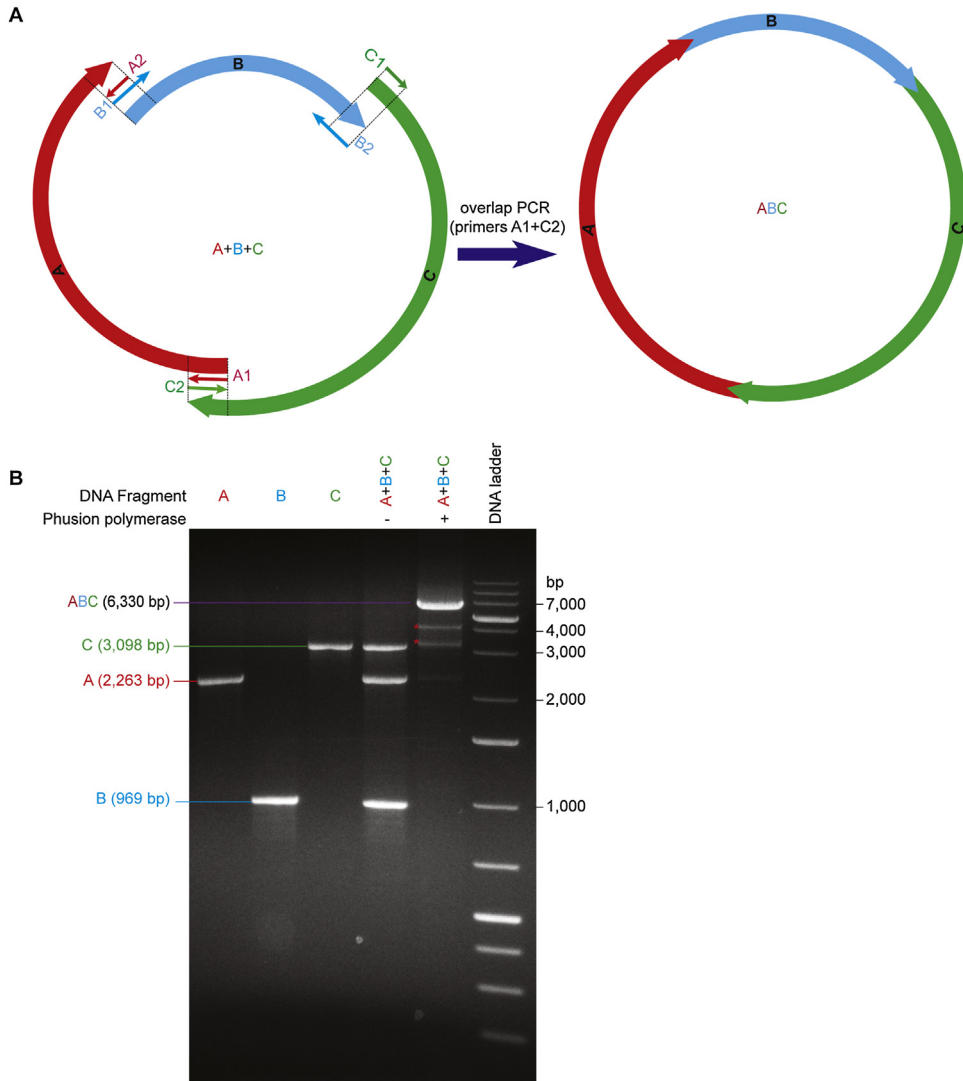
Fig. 1. ABC Cloning. A. Schematic of the ABC cloning method applied to pET28. The three overlapping fragments are used as templates in an overlap PCR reaction using primers A1 and C2 to generate the desired recombinant plasmid. The B fragment corresponds to the gene to be expressed, the A and C fragment correspond to the destination vector. The complementary region of the overlapping primers is bordered by dashed lines. B. Agarose gel illustrating the ABC cloning of the Human *crkl* gene (Fragment B) into pET28a represented by fragments A and C. The three overlapping DNA fragments A, B, and C, are used as templates for overlap PCR to produce the final PCR product. Intermediate fragments are marked with red asterisks.

fidelity and efficiency, as well as to avoid contaminating the PCR product with the template plasmid during gel extraction.

Three pairs of primers are used to achieve the cloning, Primers B1 and B2 are 50 nucleotides in length and are designed in such a way that the upstream 30 nucleotides are homologous to the vector and the remaining 20 nucleotides are specific to the gene of interest. Primers A2 and C1 are 30 nucleotides in length and their sequences are complementary to the first 30 nucleotides of B1 and B2. C2 and A1 are 30 nucleotides in length and are complementary to each other (Table 1). The three sets

Table 1
ABC cloning primers. Complementary regions of overlapping primers are labeled with the same color.

Primer	Sequence (5'-3')
A1 (pET28a)	AGATGTAGGTGTTCCACAGGGTAGCCAGCA
A2 (pET28a)	GCGACCCATTTGCTGTCCACCAGTCATGCT
C1 (pET28a)	CACCACCACCACCACCACTGAGATCCGGCT
C2 (pET28a)	GGATGCTGCTGGCTACCCTGTGGAACACCT
cdc34 (Human) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGGCTCGGCCGCTAGTGCC
cdc34 (Human) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGGGACTCCTCCGTGCCAGAGT
cdc37 (Human) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGGTGGACTACAGCGTGTG
cdc37 (Human) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCACACTGACATCCTTCTCAT
crkl (Human) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGTCCTCCGCCAGGTTCGA
crkl (Human) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGTCACTCGTTTTCATCTGGGT
crp (*E. coli*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCGGATCCATGGTGCTTGGCAA
crp (*E. coli*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTAACGAGTGCCGT
gapdh (*E. coli*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGACTATCAAAGTAGGTAT
gapdh (*E. coli*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGTTTAGAGATGTGAGCGATCA
gapdh (Human) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGGGGAAGGTGAAGGTCGG
gapdh (Human) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCCTTGGAGGCCATGTGGG
hcpA (*A. hydrophila*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGCCAACTCCATGTTATAT
hcpA (*A. hydrophila*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGGCCTCGATCGGAGCAC
nleA (*E. coli*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGAACATTCAACCGAACAT
nleA (*E. coli*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGACCCTTGTTTCTTGGA
nleB (*C rodentium*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGTTATCTCCATTAAATGT
nleB (*C. rodentium*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCCCATGAACTGTTGGTAT
nleB1 (*E. coli*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGTTATCTTCATTAAATGT
nleB1 (*E. coli*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCCATGAACTGCTGGTATACA
nleF (*E. coli*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGTTTGGTATGTTACCAGC
nleF (*E. coli*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGCCAATTTGCAAAGATC
nmpC (*E. coli*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGAATATTTATCGCGCAGT
nmpC (*E. coli*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGAACTGGTAAACCAGAC
ogt (Human) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGGCGTCTTCCGTGGGCAA
ogt (Human) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCTGCTGACTCAGTGACTT
siah1 (Human) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGAGCCGTCAGACTGCTAC
siah1 (Human) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGACACATGGAAATAGTTACAT
siah2 (Human) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGAGCCGCCCGTCCTCCAC
siah2 (Human) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGACAACATGTAGAAATAGTAA
sseK1 (*S. enterica*) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGTTTTCTCGAGTCAGAGG
sseK1 (*S. enterica*) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGTCTCCAGGAGCTGATAGTCA
traf2 (Human) B1	AGCATGACTGGTGGACAGCAAATGGGTCGCATGGCTGCAGCTAGCGTGAC
traf2 (Human) B2	AGCCGGATCTCAGTGGTGGTGGTGGTGGTGGAGCCCTGTCAGGTCCACAA

of oligonucleotides are designed in a way to obtain three mutually overlapping fragments (Fig. 1). The 3′ arm of A recognized by primer A2 shares homology with the 5′ arm of B. The 3′ arm of B is homologous to the 5′ arm of C that hybridizes with primer C1. The 3′ arm of C recognized by primer C2 is homologous to the 5′ arm of A that hybridizes with primer A1. The three fragments are used as PCR templates with two overlapping primers that generate a circular vector that can be immediately used to transform competent cells. The success of the ABC cloning method can be easily visualized on agarose gels.

The PCR primers and gene templates used in this study are listed in Tables 1 and 2, respectively. Primers A1-A2, B1-B2, and C1-C2 were used to amplify the fragments A, B, and C in a 50 μl reaction containing 0.5 μM of each primer, 200 μM dNTPs, 3% DMSO, 1x Phusion HF buffer, 100 ng of template DNA and 1 unit of Phusion DNA polymerase (New England Biolabs). Thermo-cycling conditions were as follows: 30 s at 98 °C for initial denaturation, 30 cycles of (15 s at 98 °C, 15 s at 56 °C, and 30 s/kbp at 72 °C), followed by a final extension of 5 min at 72 °C. PCR products were loaded on 1% agarose gels and extracted using a Gel DNA extraction Kit (IBI scientific). Overlap PCR was then performed in a 25 μl reaction volume containing 0.5 μM of primer C2, 0.5 μM of primer A1, 200 μM dNTPs, 3% DMSO, 1x Phusion HF buffer, 30 ng of fragment A (≈ 0.8 nM), 30 ng of fragment B (≈ 1.8 nM), 50 ng of fragment C (≈ 1 nM), and 0.5 unit of Phusion DNA polymerase. Thermo-cycling conditions were as follows: 30 s at 98 °C for initial denaturation, 16 cycles of (15 s at 98 °C, 15 s at 58 °C, and 1 min/kbp at 72 °C), followed by a final extension of 10 min at 72 °C. Five μl of the PCR product was electrophoresed through a 1% agarose gel to visualize the results.

Chemically competent *E. coli* DH5α [12] was prepared using calcium chloride [13] and transformed with 5 μl of the overlap PCR product. Cells were incubated on ice for 20 min, heat shocked for 1 min at 42 °C, and then incubated on ice for 2 min. LB broth (1 ml) was added to the cells and incubated for 1 h at 37 °C without shaking. The culture was then centrifuged for 1 min at 10,000 g and plated on LB agar supplemented with appropriate antibiotics. Positive clones were screened using colony PCR in a 20 μl reaction volume containing 0.5 μM of vector-specific primers 200 μM dNTPs, and 0.5 unit of Taq DNA polymerase (New England Biolabs). Plasmids were extracted from positive clones and the B fragment was subjected to DNA sequencing using T7 promoter and T7 terminator primers.

To quantify the efficiency of the ABC cloning method, 17 genes with sizes ranging from 0.5 to 3.1 kbp were cloned into the pET28a vector (Novagen). Primers B1 and B2 are 50 nucleotides in length and were designed in such a way that the upstream 30 nucleotides are homologous to the vector and the remaining 20 nucleotides are specific to the gene of interest. Primers A2 and C1 are 30 nucleotides in length and their sequences are identical to the first 30 nucleotides of B1 and B2. C2 and A1 are 30 nucleotides in length and are complementary to each other (Table 1). Fragment A (2263 bp), fragments

Table 2
Genes used to test the ABC cloning method.

Gene	Size (bp)	Positive clones/8
cdc34 (Human)	708	8
cdc37 (Human)	1,134	8
crkl (Human)	909	8
crp (E. coli)	630	8
gapdh (E. coli EDL933)	999	8
gapdh (Human)	1,005	8
hcpA (Aeromonas hydrophila)	516	8
nleA (E. coli EDL933)	1,239	8
nleB (Citrobacter rodentium)	984	8
nleB1 (E. coli EDL933)	978	7
nleF (E. coli EDL933)	567	8
nmpC (E. coli ETEC)	1,065	8
ogt (Human)	3,108	8
siah1 (Human)	846	8
siah2 (Human)	939	7
sseKl (Salmonella enterica)	1,023	7
traf2 (Human)	1,503	8

B (variable size) and C (3098 bp) were prepared during a first PCR using the primer pairs A1-A2, B1-B2, and C1-C2, respectively. Gel-purified fragments (30–50 ng each) were used as templates for overlap PCR using primers C2 and A1 to generate the full length recombinant vector. We show an example of cloning Human *crkl* gene into pET28a (Fig. 1B). The three fragments A (2263 bp), B (*crkl* open reading frame; 969 bp), and C (3098 bp) were amplified by PCR and purified using a DNA gel extraction kit. The three fragments were successfully assembled into the desired vector with the expected molecular weight corresponding to the sum of the three fragments (6.6 kbp). Since no parental vector was used as template in the overlap PCR, the PCR product was immediately used to transform competent *E. coli* DH5α and no DpnI digestion step was needed. Transformation results are shown in Table 2. Eight colonies were subjected to PCR screening and positive clones were validated by sequencing. A high percentage of positive clones were obtained in each case (88–100 %). Taken together, out of 136 colonies tested, 133 (97%) were positive. No point mutations were detected in any case. This method can be performed in a relatively short time, consisting of 2 PCR reactions (2 h each), with intermediate fragment purification, followed by bacterial transformation (1.5 h). Thus, the ABC cloning method seems to be highly efficient regardless of the size and sequence of the cloned gene.

Intermediate fragments, AB, BC, and AC (Fig. 1B) can also be generated during the PCR reaction. Their abundance is much lower than the final ABC product, which is amplified exponentially, because they follow a linear and megaprimer-based amplification and also because they have tendency to be combined into the full length recombinant vector during overlap PCR. Single fragments and binary intermediates cannot circularize and are not transformed efficiently.

The advantage of the ABC cloning strategy as compared to other methods is that the cloning is conducted with a single DNA polymerase, without the need for subsequent enzymatic steps. The simplicity, low-cost, and rapid nature of the ABC cloning method make it generalizable to many aspects of molecular biology research.

Acknowledgements

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