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Direct Cloning Protocol for Rapid Transformant Selection

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ABSTRACT

This study highlights a reporter-less direct-cloning protocol based on PCR and digestion/ligation reactions. Few manipulations were done allowing the user to reduce the time required for screening the right colony of any transformed cells. The obtained result showed high efficiency compared to the other commercially-available high throughput techniques, especially Gateway.

Key words: Yeast transformation, lambda exonuclease, direct cloning, reporter-less yeast system

INTRODUCTION

There is no doubt that the recent cloning systems considered as a revolution in the molecular science. Such a technological development comes with two main benefits. First, it allows efficient accomplishment of complex cloning protocols in a short period of time for scientists who are already aware of molecular techniques. Second, even non-molecular scientists with low experience in molecular technology can conduct genetic modifications without any hurdles (Marsischky and LaBaer, 2004). The history of the current commercially-available cloning systems was initiated by the homologous recombination like gap-repair cloning in the yeast *Saccharomyces cerevisiae* (Martzen *et al*., 1999; Uetz *et al*., 2000; Datsenko and Wanner, 2000; Zhang *et al*., 2000). After that discovery, several disadvantages were realized which led to further advancement of a new site-specific recombination-based technology (Ito *et al*., 2001; Zhu *et al*., 2001). The site specific recombination technology has many advantages including construction of large ORF clones beside general flexibility (Liu *et al*., 1998, 2000, 2003; Reboul *et al*., 2001; Walhout *et al*., 2000).

However, still there are unobserved aspects to those types of technologies, such as flexibility of the vector-backbone manipulation and the total costs per reaction.

Currently, it is known that the main problem of the reporter-less direct cloning protocol of yeast is screening the transformed colonies for the right insertion. Thus, in the current study TLA polymerase, two restriction enzymes and lmbda exonuclease have been used in order to shortage the yeast classical cloning method by overcomes the bacterial cloning step and directly obtaining the right colony with the target insertion.

MATERIALS AND METHODS

Construction of [CaMV35S+SV40 T antigen] cassette: Overlapping PCR with the CaMV 35S promoter and the SV40 T antigen were used to construct the DNA cassette CaMV35S+T-antigen (Fig. 1a). Primers on the cassette's flanks were containing restriction sites *SacI* and *ApaI* for double

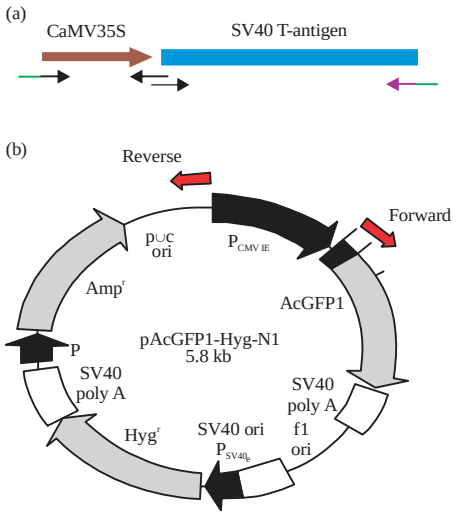

Fig. 1(a-b): (a) Construction of the insert-cassette with primer locations flanked by *SacI* and *ApaI* restriction sites and (b) Primer designed to linearize the plasmid vector pAcGFP-Hyg-N1 vector excluding the CMV promoter

Table 1: Primers used in the current protocol

Primers No.	Primers name	Sequences (5'-3')	Purpose
1	SV40-TAD-F	CAGCGAGCTCTTGGAGGCCTAGGCTTTTG	Primers 1 and 2 are the forward and
2	SV40-TAD-R	GGGCCCGCACGGGCCCGTTAACAACAACAATTGCATTCAT	reverse of the SV40 T-antigen total gene
3	pAC-Recons-R	GAGCTCGGCGGTAATACGGTTATCCA	Primers 3 and 4 are used to open and
4	pAC-Recons-F	GGGCCCGCTCAAGCTTCGAATTCTGC	linearize the pAcGFP-Hyg-N1 vector excluding the CMV promoter
5	SV-TA-pri+ F1	AGAACACGGGTTGGAGGCCTAGGCTTTTG	Primers 5, 6 and 7 designed to amplify
6	CaM35S-R1	AGGCCTCCAACCCGTGTTCTCTCCAAATGA	the CaMV35S promoter from
7	CaMV35S-F	GAGCTCCTGCAGGAGATTAGCCTTTTCA	pRI 201-AN-GUS vector and connect it to the T-antigen via overlapping-PCR

digestion (Table 1). All PCR reactions were carried out using the high fidelity TLA polymerase (Bioneer Inc., Daejeon, Korea). The PCR program was denaturation at 94°C for 10 sec, at 60°C for 20 sec, extension at 72°C for 2 min, for a total of 15 cycles.

Linearization of the pACGFP1-Hyg-N1 vector excluding the CMV promoter: Linearization and modification of the pAcGFP1-Hyg-N1 plasmid vector (Clontech) was done using TLA polymerase. Two primers were designed to start from the MCS region excluding the CMV promoter (Fig. 1b). The primers were containing the restriction sites *SacI* and *ApaI* for double digestion reaction (Table 1). The PCR carried out as follows denaturation at 95°C for 30 sec, annealing/extension at 68°C for 4 min, for a total of 15 cycles.

Digestion/ligation reaction for the linearized vector and the DNA cassette: Two endo-nuclease digestion reactions were carried out using the two enzymes *SacI* and *ApaI* according to NEB double-digest finder. The first digestion reaction was performed in two tubes by separately digesting each fragment alone. The second digestion was performed by gathering a ratio 1:3 from the linear vector: cassette insertion into one tube. After purification, fragments were ligated using T4-DNA ligase (Bioneer Inc., Daejeon, Korea).

Table 2: Stains and plasmids used in the current protocol

Strains/plasmids	Relevant characteristics	References or sources
Strains		
Escherichia coli K12	Wild type	Deokgok-Je reservoir, Josan-ri, Soramyeon, Yeosu, Korea
Saccharomyces cerevisiae	Wild type	Deokgok-Je reservoir, Josan-ri, Soramyeon, Yeosu, Korea
Escherichia coli-CaMV	*E. coli* harboring plasmid pAcGFP1-Hyg-N1-CaMV35S cassette	This study
Escherichia coli-Original	*E. coli* harboring plasmid pAcGFP1-Hyg-N1	This study
Saccharomyces cerevisiae-CaMV	*Saccharomyces cerevisiae* harboring plasmid pAcGFP1-Hyg-N1-CaMV35S cassette	This study
Plasmids		
pAcGFP1-Hyg-N1	CMV promoter, AcGFP, Hyg^r, *ori*SV40	Clontech, CA, USA
pRI 201-AN-GUS	CaMV 35S promoter, GUS, NOS promoter,	Takaka Bio. Inc., Shiga, Japan
pAcGFP1-Hyg-N1-cassette2	CaMV35S promoter, Complete T-antigen, AcGFP, SV40pro-Hyg^r, *ori*SV40	This study

Ligation confirmation by PCR: About 4 μL from the non-purified ligation product was used in PCR with two flank primers to confirm the ligation process (Table 1).

Purifying the circular vectors from the ligation mixture: After ligation and without purifying the ligation product, digestion reaction was carried out in a 25 μL reaction volume using lambda exonuclease for 1.5 h to start digesting the 5'end of the positive strand of the empty linear vector.

***Escherichia coli* transformation and colony PCR:** Hit competent cells, been used with 10 μL of the non-purified product of the exonuclease digestion reaction (Table 2). Transformation was done through heat-shock. Transformation followed by refreshing the cells for two hours in liquid medium, then plating on LB medium supported with 50 μg mL^{-1} ampicillin. Colony PCR was done after 24 h to screen for the right insertion. Five colonies were selected randomly for colony PCR as described previously (Osek, 2001).

Yeast transformation colony PCR: In this step the purification of the Exonuclease digestion product took place using PCR purification kit (Bioneer Inc., Daejeon, Korea). Followed by electroporation of *Saccharomyces cerevisiae*, according to Delorme (1989) and Gunn *et al*. (1995). Transformation followed by cell recovery for 2 h in YPD liquid-medium, then plating on YPD plate supported with 50 μg mL^{-1} hygromycin. Colony PCR was done after 48 h to screen for the right insertion. Five colonies were selected randomly for colony PCR as described previously (Mirhendi *et al*., 2007; Luo and Mitchell, 2002) (Table 2).

RESULTS AND DISCUSSION

In regards to cassette and vector construction, increasing the template concentration resulted in some smear with some non-specific bands. However, the right bands were in much higher concentrations compared to the others (Fig. 2a). After double digestion, the ligation was confirmed using flank primers to obtain whole fragment insertion with 30 bp from the two vector's flank-regions (Fig. 2b).

Colony PCR showed the right insertion in all colonies of *E. coli* culture and yeast culture as well (Fig. 2c-d). According to the screening, there was no colonies appeared without the right insertion, except for the microsatellite colonies which are usually appears smaller and beside the long-term growing recombinant colonies.

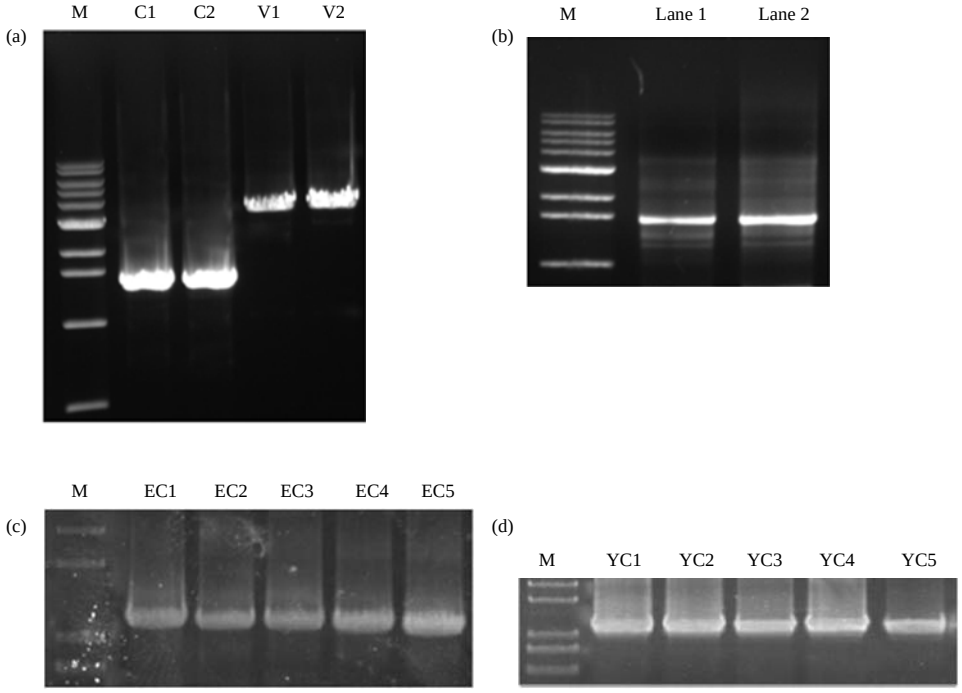

Fig. 2(a-d): Gel agarose showing, (a) Amplified fragments "vector and cassette" with *SacI* and *ApaI* restriction sites. M: 1kb DNA ladder. Lane C1 and C2: SV40-T antigen's cassettes flanked by *SacI* and *ApaI* restriction sites. Lane: V1 and V2, are linear vector flanked by *SacI* and *ApaI* restriction sites, (b) Amplified fragment of T-antigen for ligation confirmation of the insertion within linear vector. M: 1 kb DNA ladder Bioneer. Lane 1: Confirmed product using the separately digested vector and cassette. Lane 2: Confirmed product using the gathered digested vector and cassette, (c) Colony PCR products for the five *E. coli* colonies that appeared on the LB plate without lambda Exonuclease treatment and (d) Colony PCR products for the five yeast colonies that appeared on the YPD plate with lambda exonuclease treatment

The purpose of the current study is to highlight the equal efficiency of both the classical cloning- and high-throughput techniques (Esposito *et al*., 2009; Walhout *et al*., 2000).

The first key-point of the current protocol is eliminating the vector self-ligation problem, which usually appears in the restriction enzyme-cloning technique (Costa *et al*., 1994; Upcroft and Healey, 1987; You *et al*., 2012). Avoiding the later problem was done by linearizing the plasmid vector using non-phosphorylated primers and high-fidelity DNA-polymerase.

Although, the current experiment was done based on the sticky-end ligation, however same experiment could be done on the basis of the blunt-end ligation by simply 5' phosphorylating the ends of the insertion DNA, besides elongating the ligation period.

Exonuclease has been reported to purify circular DNA by degrading double stranded linear-DNA (Balagurumoorthy *et al*., 2008; Demple and Harrison, 1994; Mol *et al*., 1994). In the current protocol, lambda exonuclease was used to purify circular plasmids via degrading all linear DNAs which remained in the sample after ligation including the linear empty vector. The usage of the exonuclease in the current protocol is unnecessary with *E. coli*, in fact the only

Table 3: Comparison between the current protocol and Gateway cloning system

Factors	Presented protocols	Gateway cloning system
Time	3:4 h PCR, 20 min purification, 1.30 h endo-digestion, 20 min purification, 1.30 h ligation, 1.30 h exo-digestion, 12 h transformation Total time to obtain the right colony is 21 h	3:4 h PCR, 20 min purification, 60 min BP clonase reaction, 10 min proteinase K, 12 h transformation then spreads on LB plate, ~4 h cultivating the right colony in LB broth media, 30 min plasmid mini prep, 60 min LR reaction, 10 min proteinase K, 12 h transformation then spreading on LB plate Total timing until getting the right colony, >35 h
Requirements	High fidelity DNA polymerase (TLA, Ex-taq, PFU, etc), primers with restriction sites, Suitable restriction enzymes, T4 DNA ligase and exonuclease III PCR purification kit and competent cells (DH alpha)	DNA polymerase, primers with restriction sites, BP CLONASE Enzyme Mix, donor vector, destination vector, LR CLONASE Enzyme Mix, PCR purification kit and competent cells (DH alpha).
Reactions required	PCR, DNA purification, endonuclease digestion, DNA purification, ligation, exonuclease digestion, transformation.	PCR, DNA purification, BP clonase reaction, LR clonase reaction, DNA purification and transformation
Transferring the insert from one vector to another	This is the only defect in the current method. But it depends on the purpose of the transferring. If the purpose is just inserting the same fragment into another vector, so simply it can be done by storing the purified PCR product	Easy to handle and it also shortage the timing if starts from the ready donor vector step.
Efficiency	99% of the appeared colonies with right insertion, with circular form plasmid	99% of the appeared colonies with right insertion, with circular form plasmid
Others	Not restricted method, all vectors can be used. Easy preparing the required vector/insert. The vector backbone can be submitted to some modifications like replacing the promoter, reporter gene, selection gene, or terminator	Restricted on the donor and destination vectors which are supported by the company. Incase of propagating the donor or the destination vector, additional requirement is needed (*gyr*A462 strain of *E. coli*, Library Efficiency DB3.1 Competent Cells)
Cost per reaction	Total cost per reaction is 3.5 USD. The vector is not included	Total cost per reaction is 20 USD Destination vector is not included

purpose of the circular DNA-purification step is to void any possibility of linear-heterogonous DNA recombination with any kind of recA-mutant cells. Advantages and disadvantages determined as the most important factors required by the user are shown in Table 3.

CONCLUSION

The current protocol shows the classical cloning system as a highly efficient, low-cost cloning technique as the other high-throughput systems. We focused on comparing the provided method with the Gateway cloning product by Invitrogen.

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