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Fast and easy method for construction of plasmid vectors using modified Quick-change mutagenesis

Jin Woo Bok* and Nancy P. Keller

Department of Medical Microbiology and Immunology and Department of Bacteriology, University of Wisconsin, Madison, WI 53705

Abstract

Plasmid vector construction is an essential step for molecular microbiology yet often a time-consuming process. Manipulation of the fungal genome to express genes to activate secondary metabolite production often requires creation of plasmid constructs in a reiterative fashion. Here we introduce a modified Quick-change site directed mutagenesis method that allows for rapid and accurate construction of fungal transformation vectors.

Keywords

plasmid vector; restriction enzyme; polymerase chain reaction; Quick-change site directed mutagenesis

1. Introduction

Plasmids are self replicable extrachromosomal circular DNAs containing genes of resistance to microbes, degradation of complex organic compounds and production of toxins and enzymes (1). Plasmids were quickly recognized as a convenient means to package foreign DNA for molecular cloning strategies allowing for mass production of certain genes and/or encoding proteins in a desired host (2–4). PCR technology has resulted in improved techniques in plasmid creation (5–8), however there are limits in terms of speed due to a multitude of protocol steps, desired DNA sequence changes and/or restriction enzyme digestibility (9, 10). In vitro site-directed mutagenesis has been broadly used to selectively change DNA sequences for vector modification in lieu of PCR methods (11–15) but these approaches such as use of single-stranded DNA (ssDNA) as the template (16–18) can be time consuming and/or technically difficult.

An advance in plasmid creation has been the newly developed Quick-change site-directed mutagenesis method by Agilent Technologies which is specifically optimized for site-specific mutation in virtually any double-stranded plasmid (19). Here we provide a modified Quick-change site directed mutagenesis, which is applicable for the insertion or replacement of up to several kb DNA into a plasmid vector by a single PCR amplification (20, 21). This method does not need single-stranded DNA template, subcloning, unique restriction enzyme sites, multiple transformations or in vitro vector methylation pre-treatments. We also present a fungal genomic DNA extraction method, a modified high-yield gel extraction method, and a simple plasmid mini-preparation method which when combined with our Quick-change modifications lead to fast and reproducible vector constructions for studies in fungal molecular biology.

*corresponding author, jwbok@facstaff.wisc.edu.

2. Materials

2.1. *Aspergillus nidulans* genomic DNA isolation

1. 20 X salts solution: NaNO₃ 120 g, KCl 10.4 g, MgSO₄·7H₂O 10.4 g, KH₂PO₄ 30.4 g, and then add ddH₂O to 1 liter, autoclaved and stored at room temperature.
2. Trace elements: ZnSO₄·7H₂O 2.2 g, H₃BO₃ 1.1 g, MnCl₂·4H₂O 0.5 g, FeSO₄·7H₂O 0.5 g, CoCl₂·5H₂O 0.16 g, CuSO₄·5H₂O 0.16 g, (NH₄)₆Mo₇O₂₄·4H₂O 0.11 g, Na₄EDTA 5.0 g, added in order to 80 mL of H₂O, dissolving each completely before adding the next. Heat the solution to boiling, cool to 60° C, adjust the pH to 6.5–6.8 with KOH pellets. Cool to room temperature and adjust volume to 100 mL with ddH₂O.
3. *Aspergillus* glucose minimal medium (GMM): 20× salt solution (50 mL/L), trace elements (1 mL/L), D-glucose (10 g/l), agar (for solid media) (15 g/L) in 1 liter ddH₂O, adjust pH to 6.5 and then autoclave 15 min, 121°C on liquid cycle.
4. *Aspergillus* wild type strain (or strain of choice).
5. Phenol:CHCl₃:isoamyl alcohol=25:24:1 (Ambion, Austin, TX, cat. no. 9732), abbreviated as PCI.
6. RNase (5 Prime, Gaithersburg, MD, cat. no. 2500130).

2.2. Plasmid preparation

1. LB medium: tryptone 10g, yeast extract 5g, NaCl 10g in 1 liter ddH₂O, adjust pH to 6.5 and then autoclave 15 min, 121°C on liquid cycle.
2. Resuspension buffer: 50mM Tris-HCl, pH8.0, 10mM EDTA, 20 µg RNase A.
3. Lysis buffer: 200mM NaOH, 1% SDS.
4. Neutralization buffer: 3M potassium acetate, pH5.5
5. Isopropanol
6. 70% ethanol
7. 10mM Tris-HCl, pH 8

2.3. Gel extraction

1. Qiagen gel extraction kit (Valencia, CA, cat. no. 28706)

2.4. Quick change

1. dNTP mixture (10mM of each dATP, dCTP, dTTP and dGTP, Promega, Fitchburg, WI, cat. no. U151B)
2. PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, Santa Clara, California, cat. no. 6000672-51)
3. pME3188 YFP split vector (22)
4. *Aspergillus nidulans* genomic DNA
10. G50 spin column (GE Healthcare, Buckinghamshire, UK, cat. no. 27-5340-03).

3. Methods

The key step in creating plasmid vectors for fungal transformations is a reliable mutagenesis method. In addition, it is important to have good quality of template DNA for PCR steps and good recovery of PCR products from agarose gel for vector construction. The basic procedure utilizes a supercoiled double-stranded plasmid DNA vector and a PCR product containing the desired DNA fragment with flanking sequences (see Fig. 1). The flanking sequences of PCR product recovered by modified gel extraction, each complementary to opposite strands of the vector, are extended during temperature cycling by PfuUltra II Fusion HS DNA polymerase (23). Extension of the PCR template generates nicked circular DNA molecules. Following temperature cycling, the reaction mixture is treated with *Dpn* I endonuclease (target sequence: 5'-Gm6ATC-3') used to digest the methylated parental DNA template and to select for target gene containing nonmethylated synthesized DNA (24, 25). The nicked plasmid vector DNA incorporating the desired mutations is then transformed into competent cells for nick repair. The mutated plasmid vector will be extracted from transformed bacteria by a modified simple mini-plasmid preparation. The mutated region is confirmed by PCR amplification followed by sequencing of the amplicon.

3.1. A. *nidulans* genomic DNA isolation

1. Add 20 ml GMM with appropriate supplements and yeast extract at 5g/L into a petri dish.
2. Inoculate with several loopful of *Aspergillus* conidia from solid media.
3. Incubate at 37 °C for 16–24 hrs. (*see* Note 1).
4. Pour off media and scrape hyphal material together. Put material in lid of petri dish and remove as much liquid as possible.
5. Take cell material and place between paper towels and squeeze out remaining liquid. Put cell material in eppendorf tube and freeze in –80° C for at least 30 min or in liquid nitrogen for several min.
6. Lyophilize overnight (*see* Note 2).
7. Break up lyophilized hyphae into a fine powder using toothpicks.
8. Add 700µl of LETS buffer containing 20 mM EDTA (pH8), 0.5% SDS, 10mM Tris-HCl (pH8) and 0.1M LiCl. Mix by using toothpick as well as inverting the tubes several times and leave the samples on bench for 5min.
9. Add 700µl of PCI. Mix by shaking 10–15 times. Let samples on bench for 5 min.
10. Spin 10 minutes at 4° C at maximum speed of microcentrifuge.
(Optional: Transfer supernatant (SN) to a new tube, and add equal volume of PCI and repeat Step. 9–10)
11. Transfer SN to a new tube and add 1ml 95% ethanol. Mix well and spin 5 minutes to pellet DNA.
12. Decant SN and add 70% ethanol to wash pellet.
13. Spin 1 minutes at maximum speed to pellet DNA.
14. Discard SN and dry pellet on bench for 5min. (*see* Note 3).
15. Resuspend in 50–70µl 10mM Tris-HCl (pH8) and add 2µl RNase (10mg/ml stock). (*see* Note 4).

16. Inactivate DNase and digest RNA at 65° C for 30 min.

3. 2. Plasmid preparation (see Note 5)

1. Inoculate 3ml LB medium containing proper antibiotic with bacterial clone, incubate overnight at 37° C and 200rpm, and harvest bacteria by spinning at maximum speed for 30sec.
2. Remove supernatant as much as possible (see Note 6) and resuspend the pellet completely in 100µl resuspension buffer.
3. Add 100 µl lysis buffer and mix gently 3–4 times by inversion.
4. Add 125 µl neutralization buffer and mix gently for 3min.
5. Spin down to remove bacterial debris by centrifugation at maximum speed for 2min and transfer supernatant in 1.5ml new centrifuge tube.
6. Add 200µl isopropanol and mix well to pellet plasmid DNA. Leave the sample for a couple of min on bench.
7. Collect DNA pellet by spin down at maximum speed for 2 min and decant supernatant.
8. Add 500µl 70% ethanol to wash pellet.
9. Collect the DNA pellet by spinning at maximum speed for 30 sec and remove SN (see Note 7).
10. Air dry the pellet for 3min on bench and add 50 µl 10mM Tris-HCl buffer, pH8.0. (see Note 8).

3.3. Gel extraction

1. Excise the DNA band from the agarose gel with a clean, sharp scalpel and put into a 1.5 ml microfuge tube (see Note 9).
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel for DNA fragments. (see Note 10).
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing every 2–3 min during the incubation. (see Note 11)
4. Check that the color of the mixture is yellow.

If the color of the mixture is orange or purple, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color should turn to yellow. The adsorption of DNA to QIAquick membrane is only efficient at pH 7.5. Buffer QG now contains a pH indicator which is yellow at pH7.5, and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix
Do not centrifuge the sample at this stage (see Note 12).
6. To bind DNA. Apply the sample to the QIAquick column with a 2ml collection tube and centrifuge for 1min (see Note 13).
7. Discard the flow-through and place QIAquick column back in the same collection tube.
8. Add 0.5ml of buffer QG to QIAquick column and spin for 1 min.

This step will remove all traces of agarose.

9. To wash, add 750 μ l of Buffer PE to QIAquick column and centrifuge for 1 min.
10. Discard the flow-through and centrifuge QIAquick column for additional 1min at maximum speed.
11. Place QIAquick column into a clean 1.5ml microfuge tube (*see* Note 14).
12. To elute DNA, add 50 μ l of 10 mM Tris-Cl, pH 8.5 or H₂O to the center of the QIAquick membrane and centrifuge the column for 1min at maximum speed. Alternately, for increased DNA concentration, add 30 μ l of elution buffer to the column, let the column stand for 1min, and then centrifuge for 1min. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH is within this range, and store DNA at -20° C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) but the EDTA may inhibit subsequent enzymatic reactions.
13. Eluted samples are loaded on gel to check quality (Fig. 3) (*see* Note 15).

3.4. Quick change

3.4.1. Primer design—IDT provide good quality primers, of course, you can order primers through your vender. Insertion or replacement of DNA fragments in plasmid vector needs two primers for PCR amplification. Keep primer length between 45 and 60 bp. You should not try to exceed 60 bp because above that size IDT forces you to order 100 nmol scale with the corresponding price increase. Ideally, you'd want 25–30 bp on either side of the mismatch, approximately equal melting temperature (TM) for both “arms” of the primer (*see* Note 16). The overall primer TM (**not counting the mismatched pairs**) should be in the range of 60–75° C.

3.4.2. PCR condition—Contained PCR tubes are

- (i) 25–50 ng template (*see* Note 17 and 18)
- (ii) 250ng PCR product (*see* Note 19)
- (iii) 0.75 μ l dNTP mix
- (iv) 2.5 μ l 10 $\times$ PfuUltra II Fusion (“Pfu Fusion” for brevity) buffer (*see* Note 20)
- (v) 1.0 μ l DMSO (optional) (*see* Note 21)
- (vi) 0.5 μ l of Pfu polymerase
- (vii) Add sterile water to 25 μ l and one drop mineral oil (*see* Note 22).

Heat the reaction to 95° C for 5 min to denature. Perform 36 cycles of PCR [95° C for 1 min. (denaturation), 58° C for 1 min (annealing) and 68° C for 1.5 min/ kbp template (elongation)]. Followed by final elongation at 65° C for 10 min and hold at 4° C (*see* Note 23–26).

3.4.3. Dpn I treatment and transformation (*see* Note 27)—After removing mineral oil from PCR sample by rolling on parafilm, pass through the sample by G-50 column to eliminate salts from previous reaction. Load 3 μ l of sample in agarose gel to see success of the reaction (Fig. 2). Set up *Dpn*I digestion for 3 hrs as follows: (*see* Note 28)

Sample	13 μ l

NEB Buffer #4	3 μ l
<i>DpnI</i> (NEB)	1.5 μ l
Sterile distilled water	13 μ l

- Use 15 μ l for transformation with chemical competent cell or use 3–5 μ l for transformation with electroporation competent cell after passing G50 column (*see* Note 29), and then immediately add 0.5 ml SOC, let cells recover for 30 min at 37°, plate 200 and 20 μ l (*see* Note 30).

4. Conclusions

Traditional plasmid vector construction has been commonly used in many fields yet it is still a time consuming process and is of limited use due to awkward placement of restriction sites and cumbersome mutagenesis methods. Quick-change site directed mutagenesis is the simplest and most broadly applicable tool to change DNA sequences in plasmid vectors without using restriction enzymes. Modification of the Quick-change kit as described here provides several advantages in plasmid construction by decreasing the cost of materials while increasing the time in plasmid construction.

5. Notes

1. The wild type *veA* allele results in decreased DNA extraction quality. To get better DNA, culture the fungus in light but harvest prior to sporulation.
2. Pierce the lid of the eppendorf tubes.
3. After discarding SN, quick spin and take out residual SN with pipette before drying on bench.
4. Tris-HCl (pH8) makes DNA more stable than sterile water.
5. You can use left-over buffers in commercial plasmid prep kits such as Qiagen mini prep kit or Biorad min prep kit or you can make your own buffers. All of steps can be done at room temperature using a microcentrifuge.
6. Decant culture SN, quick spin to remove residual SN by pipetting out.
7. Instead of drying pellet in the tube by turning it upside down, quick spin to remove residual SN by pipetting after decantation of culture SN.
8. To inactivate possible DNase contamination, incubate your sample at 65° C for 10 min and preserve your sample at –20° C until you need it).
9. Make 0.8% agarose gel for separation. This provides you better results because lower % agarose is easy to melt completely.
10. If gel weight is under 150 mg, just add 3 volumes of QG. Even if gel weight is over 150 mg, do not exceed a total volume more than 600 μ l. For example, if gel weight is 200 mg, add 400 μ l QG.
11. You can incubate at 65° C for short time (2–3min) to dissolve the gel completely.
12. Mix well immediately to prevent resolidification of agarose, which can cause clogging of column. Do not centrifuge because centrifugation can result in precipitation of your DNA.
13. Reapply the flow-through to the column and centrifuge at 3380 $\times$ g for 15 sec. Repeat this step one more time to maximize recovery of DNA (total 3 times for flow-through).

14. To remove residual ethanol, spin 1 min with open lid at $3380 \times g$.
15. To get more recovery of DNA from column, add 65°C elution buffer into the column and immediately open and close the lid several times to make the buffer absorbed well into the column materials.
16. The 3' end is more significant because that's where DNA synthesis starts; try to end the primer with a couple of G/Cs.
17. *Amount of template.* The stated 50 ng is simply lifted from the standard Quick-change protocol, 100 ng/50 μl , and was not tested experimentally. 0.2–2 μl of miniprep sample was used in the 25 μl reaction. The advantages of higher template concentration are more product with less number of cycles and higher chance of priming when annealing is suboptimal, however, the disadvantages of having too much of the template are introducing more impurities into the reaction (some of which might be inhibitory), increased background if the *Dpn* I treatment is incomplete, and increase in the misprimed side reactions. There is an optimal balance between all of these factors which each practitioner must establish themselves.
18. *Quality of template.* Most of time current modified plasmid preparation method worked well. However, steps to increase quality using the Qiagen mini-prep kit include: i) using 1 ml culture instead of 3 ml if your plasmid is high copy number; ii) using a PB solution volume that covers all areas of the column that are in contact with cell lysate; iii) using 750 μl PE for wash twice.
19. Good quality of PCR product is very important with high concentration. To achieve this goal, we suggest to use 30 μl elution buffer of 65°C and heat inactivation at 65°C for 10 min. Usually the volume of 250 ng will be contained in 4–6 μl .
20. The Pfu Fusion polymerase works faster and makes several-fold less errors than Pfu Ultra.
21. DMSO is optional but it is helpful for decreasing melting temperature of DNA duplexes with high GC content and it reduces primers' secondary structure. You can use up to 4% DMSO and make sure to mix DMSO well with other components before adding polymerase.
22. One drop of mineral oil is very important to have reproducible result. The mineral oil keeps 25 μl PCR reaction from evaporating.
23. The larger number of cycles gives better results but we have found 20 – 36 cycles can work.
24. *Annealing temperature.* Pfu Fusion works in a broad range of annealing conditions including 5–10 degrees below the calculated primer T_M based on GC content (number of GC $\times 4$ + number of AT $\times 2$). However, 56 – 58°C should work in the vast majority of cases.
25. *Extension time.* The recommended 1.5 min per kbp at 68° definitely works for Pfu Fusion. Round the number of minutes upwards and stay with this value. We have had success with 1 min/kbp in some cases. Pfu Fusion has some strand displacement activity that may require 2 min per kbp at 65°C for some reactions.
26. *Miscellaneous.* You may increase the primer concentration (~two-fold), increase concentration of dNTPs, add extra MgCl_2 in the reaction all when using high fidelity Pfu Fusion.

27. While plasmid DNA isolated from almost all of the commonly used *E. coli* strains (*dam*⁺) is methylated and is a suitable template for mutagenesis, plasmid DNA isolated from the exceptional *dam*[−]*E. coli* strains, including JM110 and SCS110, is not suitable.
28. This is very critical step to digest parental plasmid DNA. Some protocols says that the digestion can be done over night at 37° C but there is a greater chance for DNA to be degraded and the reaction volume can be reduced by evaporation. This is especially true of small volumes where 37° C incubations in the water bath or incubator are hard to control and may not be reproducible because of evaporation/condensation, etc. Therefore we suggest a shorter incubation time than overnight. Or alternatively, you can leave your samples in a PCR machine if you want to incubate your samples over night.
29. Gel filtration with G50 is very important to remove salts for electroporation. Too much salts or too much cells cause an electric arc and kill cells.
30. Sometimes cell recovery after chemical transformation or electroporation is not consistent. We recommend to try the chemical method first as it is cheaper. If this method does not work, you can try electroporation or you may try using LB instead of SOC, or LB with extra MgSO₄ and glucose. A plasmid vector with kanamycin resistance marker needs to incubate longer (1hr) than ampicillin marker (30min) to help competent cell recovered.

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Figure 1.
Overview of plasmid construction by the modified Quick-change method

Figure 2.

PCR amplified plasmid vectors by modified Quick-change method and their confirmation by PCR.

A. 3 μ l load of PCR product after modified Quick-change; lane 1: 1kb size marker, lane 2: *veA* replaced with AN2012 in pME3188, lane 3: *veA* replaced with AN10356 in pME3188, lane 4: *veA* replaced with AN4252 in pME3188, lane 5: *veA* replaced with AN4500 in pME3188. B. PCR confirmation of each vector obtained by modified plasmid DNA preparation; lanes 2, 3, 4 & 5 are matched to the same gene in Fig. 2 A respectively.

Figure 3.
Gel extraction of PCR products by Qiagen protocol and modified Qiagen protocol; lane1.
1kb size marker, lanes 2 & 4: Qiagen protocol, lanes3 & 5: modified Qiagen protocol.