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Rapid Screening of Recombinant Plasmids by Direct Colony Quantitative Real-Time PCR

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Abstract

Quantitative real-time PCR (qPCR) was applied to rapid screening of positive plasmid clones. Insert-specific primer pairs were used in qPCR colony screening, and false positive colonies could easily be distinguished from true positive ones by comparing their Ct values. In addition, qPCR is particularly suitable when amplicon is small (<150 bp). This method is sensitive, simple and fast, obviates the need for gel electrophoresis, and is a cost-effective alternative to the traditional PCR approach.

Keywords

qPCR, Colony PCR, Plasmid Screening

1. Introduction

Plasmid DNA recombination is the core technique in molecular biology, which is essential for many experimental purposes such as DNA amplification, DNA library construction, protein expression, ShRNA gene knockdown, and in the recent CRISPR/Cas 9 mediated mammalian genome editing [1]. Generally, recombinant plasmids are constructed as follows: circular plasmid DNA is cleaved with one or more restriction enzymes and ligated *in vitro* to foreign DNA bearing compatible termini. The ligation mixture is subsequently transformed into an appropriate strain of *E. coli*. The resulting transformed *E. coli* grown on appropriate selective medium contains recombinant plasmids or self-ligated vector colonies, which are subsequently screened for colonies containing the desired DNA insert with the right orientation [2].

Traditionally, plasmid cloning is characterized by picking bacterial colonies, growing

bacteria in selective medium overnight, preparing plasmid DNA, and digesting with restriction enzymes. This remains the definitive and often time tested method [2]. However, it can be very tedious and labor intensive when a large number of colonies have to be screened in some difficult cloning procedures [2]. Over the years, many methods have been devised to distinguish bacteria transformed by recombinant plasmids from those carrying empty wild-type plasmids. The most durable and general one of these methods uses a nondestructive histochemical procedure to detect β-galactosidase activity in transformed bacteria. This procedure is commonly used as a test to distinguish colorless colonies of bacterial cells that carry recombinant plasmids from blue colored ones that carry the wild type plasmids. However, false positive colonies remain a major concern for this technique. Alternatively, *in situ* hybridization methods may be used to identify with certainty bacterial colonies that have been transformed with recombinant plasmids which carry specific sequences of foreign DNA. Other generally useful methods including colony PCR are available to analyze the size of recombinant plasmids and to screen transformed colonies [3]. Today, colony PCR is commonly used to screen bacterial transformants. In this study, we report a sensitive, simple and fast plasmid screening method by using colony quantitative real-time PCR (qPCR).

2. Materials and Methods

2.1. Materials

1) 2 x SYBR Green PCR master mix (life technologies).
2) 1.5uM stock solution of each oligonucleotide primer.
3) PCR-grade water.
4) Real-time thermal cycler (BIO-RAD).

2.2. Methods

1) Following bacterial transformations, check the plates and pick the suitable colonies (1 - 2 mm in diameter) with a sterile 200 ul pipette tip and suspend it in 50 ul sterile water by pipetting up and down a couple of times.

2) Quantitative real-time PCR reaction. 10ul real-time PCR reaction contains the following: 5 ul 2 x SYBR Green PCR master mix, 1 ul forward and reverse primer mixture, 2 ul water and 2 ul bacterial mixture from step 1. Empty vector alone is used as negative control. The cycling conditions were: an initial denaturation step at 95˚C for 10 min followed by 40 cycles of 95˚C (15 s), 60˚C (1 min) in a real-time thermal cycler.

3) 20 ul traditional PCR reaction contains the following: 10 ul 2 x Phusion Flash PCR master mix (Thermo Scientific), 2 ul forward and reverse primer mixture, 4 ul water and 4 ul bacterial mixture from step 1. Empty vector alone is used as negative control. The PCR conditions were programmed as follows: an initial denaturation step at 98˚C for 10 seconds followed by 35 cycles of 98˚C (1 second), 55˚C (5 seconds), 72˚C (30 seconds). After 35 cycles, additional extension at 72˚C for 1 minute was added, then PCR products were separated on agarose gel.

4) The rest of bacterial mixture of positive colonies can be transferred to 5 ml LB

media containing appropriate antibiotics to grow overnight for plasmid DNA mini-preparations.

5) Mini-preparation plasmids were digested by restriction enzymes and sequenced.

3. Results and Discussion

Following qPCR, the Ct curves of positive colonies started going up between 10 and 20 cycles (Figure 1) compared to negative control and wild type plasmids. The positive colonies were validated by subsequent mini-preparation, restriction enzyme digestion and DNA sequencing (data no shown).

Occurrence of false positives as high as 90% is a major disadvantage associated with traditional colony PCR [3] [4] when only insert-specific primer pairs are used for screening. The false positives have been attributed to unligated insert from ligation mixture, picked up with the colony from the plate, worked as a template, and amplified

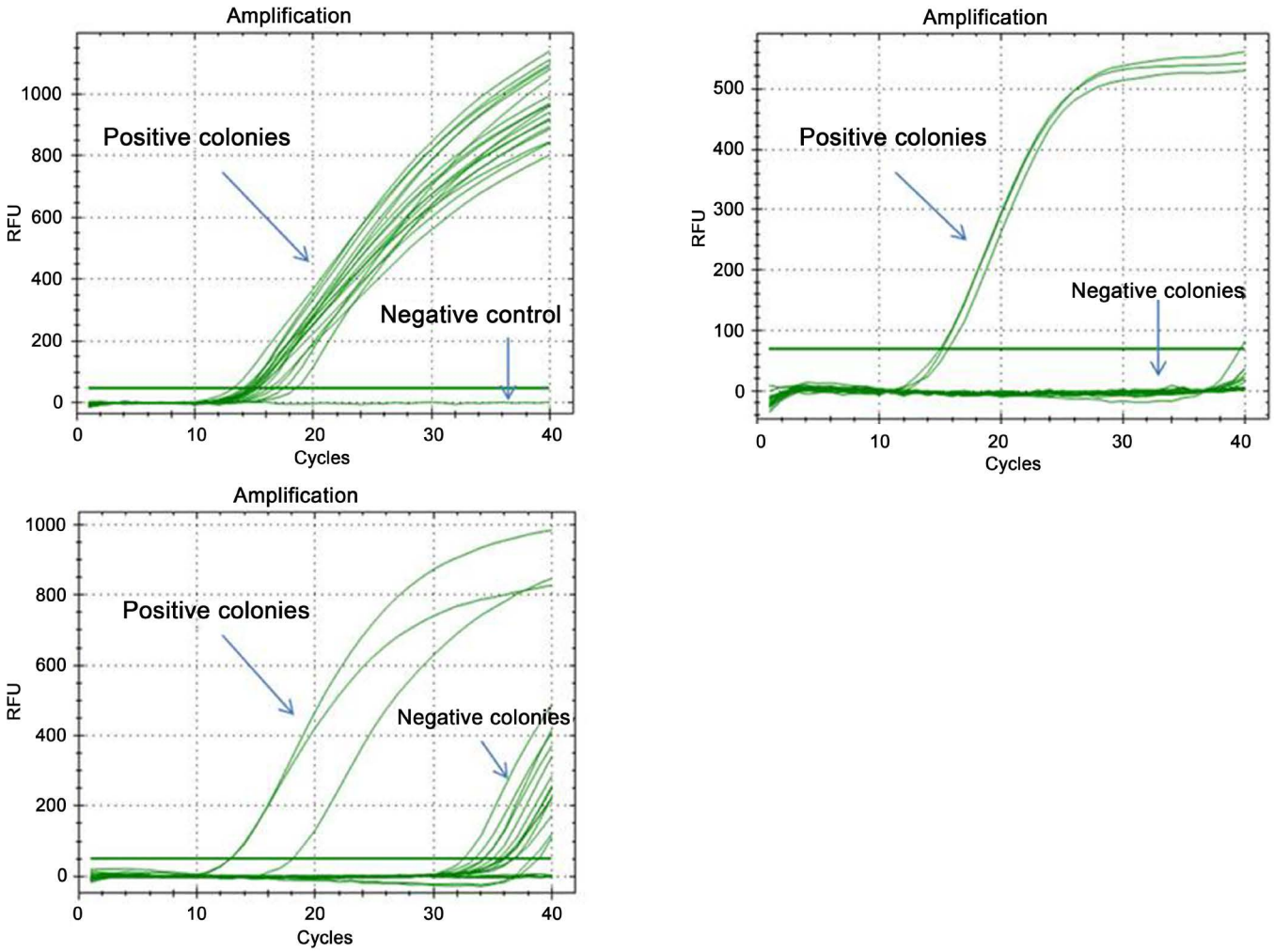

Figure 1. Three different gene coding sequences were cloned into pCMV-3FLAG vector (stratagene) at EcoRI and XhoI sites, and transformed into Dh5α competence bacteria. Vector-based T3 forward and gene specific reverse primer pairs were used for quantitative real-time PCR colony screening.

in the PCR reaction [4]. Although the use of vector-specific and insert primer pair eliminates false positives, this requirement limits the use of colony PCR to plasmids where vector-specific primers are available [3] [4]. Another technique to avoid false positives is to use a simple single-tube technique involving pre-PCR nuclease incubation [4]. A recent work by Skarratt *et al.* [5] shows that false positives can easily be distinguished from true positives by comparing Ct values derived from qPCR amplifications curves. In agreement with this observation, we found that when we only used insert-specific primer pairs for transformant screening, amplification cycle after 27 cycles always indicated a negative clone, while clones amplified within 20 cycles was confirmed to be positive by restriction digestion after mini-preparation (**Figure 2(b)**, **Figure 2(d)**). Also, no false positive clones were detected by qPCR. However, traditional colony PCR results showed that all colonies contained amplified bands, even though in some negative colonies the PCR bands looked weaker compared to positive ones (**Figure 2(c)**). Our data further confirm that colony qPCR eliminated false positive results, and insert-

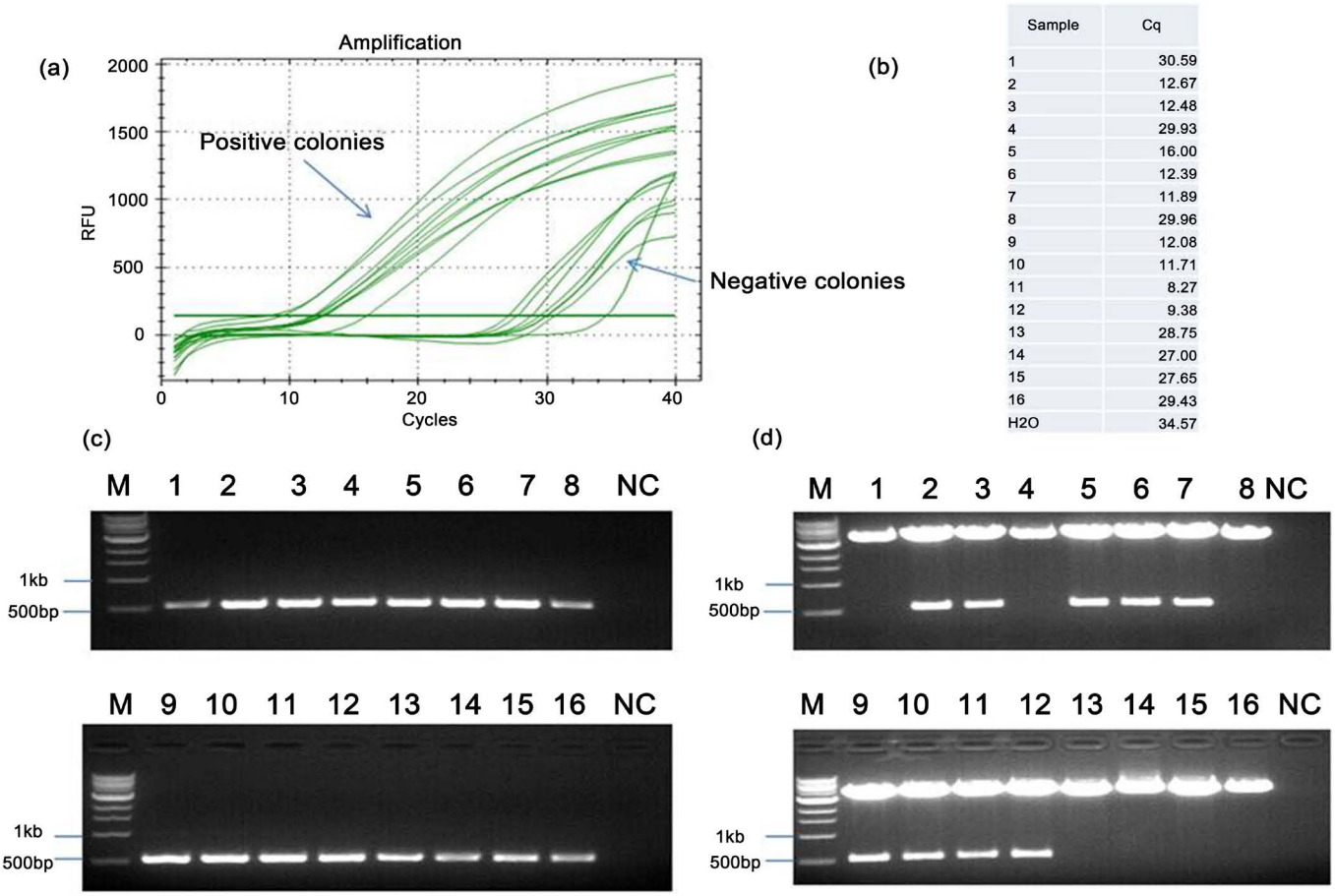

Figure 2. Human genomic micro RNA 136 stem loop and flanking sequences (520 bp) were cloned into PCDH lentiviral vector (SBI) by PCR-based strategies at NheI and NotI sites, and transformed into Dh5*α* competence bacteria. (a) 16 colonies were screened by qPCR with cloning PCR primers. (b) Ct value of each colony. (c) 4 ul bacterial mixture was amplified in 20 ul traditional PCR reaction for 35 cycles and separated on 1.2% agarose gel. (d) Mini-preparation plasmid DNA from the colonies were digested with NheI and NotI at 37˚C for 1 hour then separated on 1.2% agarose gel.

specific primers could be exploited for screening bacterial transformants in this system.

The optimal amplicon length in a SYBR Green qPCR is 75 - 150 bp [6]. It rarely meets this requirement when insert-specific primer pairs are used for screening, unless new screening primers are designed and synthesized. In this study, we used PCR cloning primers for screening; if amplicon was bigger than 1 kb, we extended annealing and extension time to 2 minutes. We tried the latter method in a screening when the amplicon was about 1.5 kb. We did observe decreased qPCR reaction efficiency when amplicon was bigger than 1 kb, since both Ct values increased for positive and negative colonies when amplicons became larger. However, this did not compromise the sensitivity of the technique, as there existed significant Ct differences between positive colonies and negative ones (data not shown).

CRISPR/Cas9 system is a novel powerful tool for mammalian genome editing by simply specifying a 20-nt targeting sequence within its guide RNA [1]. Currently we use this system extensively in our laboratory to establish gene knockout cell lines. Construction of an expression plasmid for single-guide RNA (sgRNA) is simple and rapid, involving a single cloning step with a pair of partially complementary oligonucleotides. The oligo pairs encoding the 20-nt guide sequences are annealed and ligated into a CRISPR/Cas9 vector [7]. Since this kind of insert is very small, it is usually not feasible to screen positive recombinants by traditional restriction enzyme digestions. Colony quantitative real-time PCR is especially convenient for this kind of plasmid screening. We used vector-based U6 forward sequencing primer (recommended by the vendor) and reverse oligo insert as screening primers, as shown in **Figure 3**, two of the five

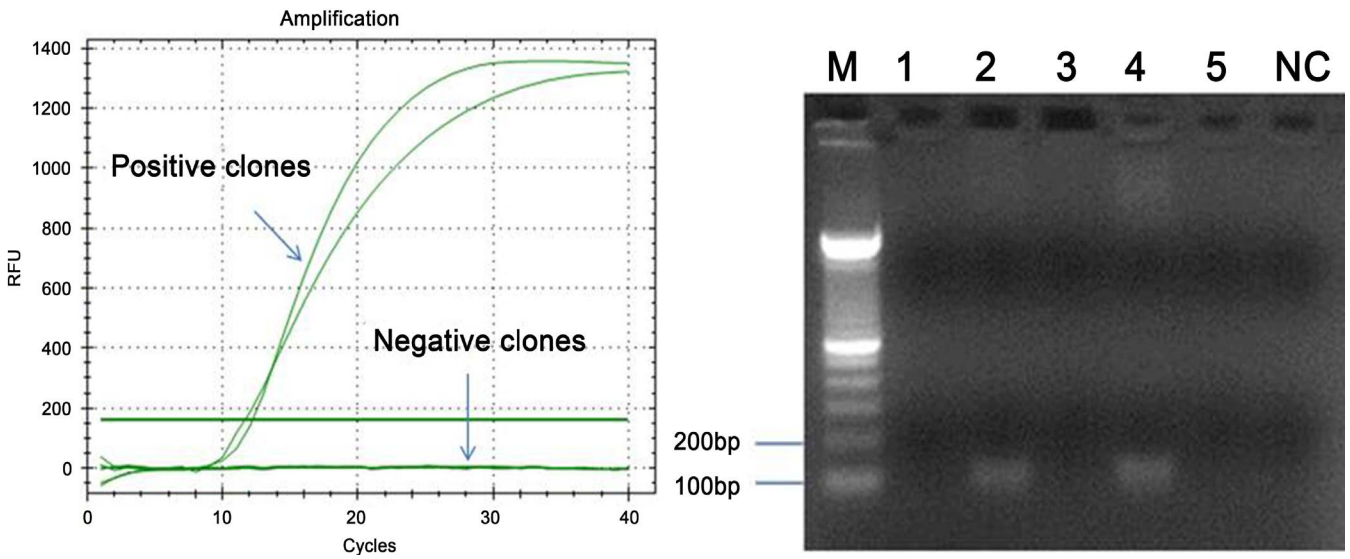

Figure 3. (a) Oligos of sgRNA against human GATA2 were ordered from Fisher (Forward: 5' CACCGAGGGGTTGCCCTGCGAGTCG 3' and Reverse: 5' AAACCGA CTC GCA GGG CAA CCC CTC3'). The oligos were annealed and ligated into the pX458 vector digested with BbsI, then transformed into Dh5α competence bacteria. Five colonies were picked and screened by qPCR with U6 forward primer (5' GAGGGCCTATTTCCCATGATTCC 3') and above-mentioned reverse primer. B, 4 ul bacterial mixture was amplified in 20 ul traditional PCR reaction for 35 cycles with the same primer pairs in qPCR and separated on 2% agarose gel.

tested colonies were positive in qPCR and traditional PCR, suggesting that these two colonies contained the desired recombinant. This result was further confirmed by DNA sequencing. However, traditional PCR showed very weak band even after 35 cycle amplification, because the amplified product was small (110 bp) and factored by input DNA. We believe that quantitative real-time PCR is extremely suitable for small-size insert plasmid screening especially when amplicon is small (<150 bp), while small amplicon detection is relatively difficult for traditional PCR.

To test the viability of inoculated bacteria in sterile water (method step 1) with time, we stored the bacterial mixture at room temperature for 2 or 8 hours, then grew them in 5 ml LB containing appropriate antibiotics overnight, and the plasmid mini-preparation didn't show any difference between them, suggesting that storage time was not a big concern for the viability of bacteria in sterile water.

qPCR colony screening is fast and simple, with results visualize "real-time" by using a computer. Generally we know the results within one hour of plate-run on the machine rather than 2 - 4 hours for traditional PCR combined with gel electrophoresis. It also allows screening a large number of colonies in one experiment.

In conclusion, insert-specific primer pairs can be used in qPCR colony screening. This method obviates traditional PCR detection techniques circumventing the duration and is economical for analysis on a regular basis.

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