Benchmarks

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Large-Scale Colony Screening and Insert Orientation Determination Using PCR

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Though many diagnostic PCR methods have been described, few techniques have been developed to rapidly and efficiently screen transformed bacterial colonies for plasmid presence (1). Unfortunately, PCR screening of a large number of colonies is time consuming and requires substantial amounts of polymerase and primers. In this report, we describe a two-step large-scale bacterial colony screening method that we have labeled "cracking PCR". Cracking PCR includes (i) size screening of cracked bacterial colonies (i.e., selecting lysed clones displaying a band similar to the expected recombinant size) in an agarose gel and (ii) PCR screening of gel-determined potential positives directly from the cracking buffer/colony mixture. This method offers several advantages. First,

the initial use of conventional cracking methods permits many colonies to be screened quickly in agarose, rather than the several hours required for PCR amplification. Second, since only the apparent positive clones will be used as template in PCR, fewer reactions will be necessary, thus reducing time and cost requirements. Finally, cracking PCR serves to reconfirm the presence of positive recombinants. The initial cracking step assists to verify that a band present in the PCR amplification reaction is a true band, rather than an artifact resulting from contamination from another colony or an anomaly of the reaction.

Figure 1 illustrates a flowchart of the cracking PCR procedure. Bacterial colonies are lysed using a protocol based on a previously described method (2). This method has been significantly modified. Briefly, 1 mm² of each colony is picked from a masterplate with a sterile toothpick and twisted in 50 µL cracking buffer (in 200 mL: 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1% SDS, 27.4 g sucrose, 20 mg bromophenol blue, and 20 mg xylene cyanol) in 0.5-mL microcentrifuge

Figure 1. Flowchart of the cracking PCR method.

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Figure 2. PCR products from a bacterial transformation (*TOP10F* strain) after performing the cracking PCR method. Lane 1 is a 1-kb λ ladder (Life Technologies). Products in lanes 2–5 were amplified using one forward primer internal to the insert (GB5-3) and one reverse primer internal to the vector (3'AOX1; Invitrogen, Carlsbad, CA, USA). Lane 2 is transformed pGAPZ α A (Invitrogen). Lanes 3 and 4 contain a 1928-bp fragment amplified from two different clones of transformed pGAPZ α A•GB5-3 [pGAPZ α A containing a glucocerebrosidase (EC 3.2.1.45.) insert] with the insert in the correct orientation. Lane 5 contains a 1932-bp fragment amplified from transformed pGAPZ α A•GB5-3 with the insert in the incorrect orientation. Products in lanes 6–8 were all amplified using two reverse primers: one internal to the vector (3'AOX1), the other internal to the insert (GB2). Lane 6 is transformed pGAPZ α A•GB5-3 with the insert in correctly. Lane 7 is pGAPZ α A•GB5-3 with the insert in incorrectly. Lane 8 is the negative control. Lane 9 is the positive control of transformed pGAPZ α A•GB5-3 confirmed by sequence analysis, containing a 1928-bp segment amplified with primers GB5-3 and 3'AOX1.

tubes. The tubes are vortex mixed briefly, incubated at room temperature for 20 min, and centrifuged at 13000 $\times$ g for 15 min. Then, 45 μ L of the mixture are loaded into a 0.7% agarose gel with ethidium bromide and electrophoresed at 70 V for 45 min. After the gel is photographed, it is analyzed for evidence of possible positive recombinants (i.e., a band of similar size to the expected recombinant size). From all the potential positive clones, 0.5 μ L of the buffer/colony mixture is used as template for PCR amplification in 0.2-mL thin-walled microcentrifuge tubes (Gordon Technologies, Mississauga, ON, Canada) containing 5 μ L 10 $\times$ PCR Buffer (Life Technologies, Rockville, MD, USA), 8 μ L 25 mM MgCl₂, 5 μ L 2.5 mM dNTPs, 4 μ L 5 μ M each of the specific primers (combination of insert forward primer GB5-3: 5'-ACTCGAATTCTTCATCTAAG GACCCTGAGG-3' and vector reverse primer 3'AOX1: 5'-GCAAATGGCAT-TCTGACATCCTC-3' to test for correct orientation, or combination of insert reverse primer GB2: 5'-GCGGG AATTCTTTAATGCCAGGCTGAG CC-3' and 3'AOX1 to test for incorrect orientation), and 2 U *Taq* DNA Poly-

merase (Life Technologies) in a total volume of 50 μ L. The PCR program used in Figure 2 was an initial denaturation at 94°C for 2.5 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1.5 min, and 72°C for 1.5 min, with a final extension at 72°C for 7 min. The products were electrophoresed in a 1.0% agarose gel.

It is important to note that inclusion of a primer to screen for the insert in the incorrect orientation is not necessary in this cracking PCR method, unless one wants to confirm a clone as a control for later expressions. The use of a primer internal to the vector coupled with an opposite primer internal to the insert will amplify only those clones with inserts in the correct orientation. For those clones in which no band appeared in the subsequent electrophoresis, it can be assumed that either the insert was in the incorrect orientation or that no insert was actually present.

In conclusion, this cracking PCR method permits rapid large-scale screening of bacterial colonies and the determination of insert orientation without vector purification. This technique can easily be applied to screen diverse bacterial colonies for different types of vec-

tors containing various inserts, as long as one internal vector primer and one internal insert primer with compatible annealing temperatures are available.

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