Use of DHPLC for Rapid Screening of Recombinant Clones

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Denaturing HPLC (DHPLC) is a relatively novel technique that has been mostly applied to molecular detection of pathological gene alterations (1,2). The method relies on the identification of heteroduplex molecules, due to mismatches between wild-type and mutant alleles, that are visualized in the form of altered chromatograms. Other nonconventional applications of DHPLC have also been reported, including quantification of mitochondrial heteroplasmy (3) and somatic mosaicism (4), gene mapping (5), and bacterial species identification (6). More recently, DHPLC reliability in mutation detection was validated by two different studies, based on PCR-mediated mutagenesis (7,8). In these two reports, several mutant bacterial clones were cloned, sequenced, and then confirmed by DHPLC for the presence of genomic alterations in MET and p53 genes, respectively.

Here we describe a different application of DHPLC that speeds up the screening step of recombinant clones after site-directed mutagenesis (SDM), by avoiding needless sequencing of non-mutant clones. SDM is a versatile molecular tool for the introduction of specific mutations into target DNA to study gene expression and functional significance of genomic alterations. Several applications of the SDM strategy showed a variable mutagenesis efficiency of 50%–90% (9). Selection of mutant recombinant clones by colony hybridization or sequencing analysis represents the most tedious, time-consuming, and expensive step of the entire procedure. To overcome this cumbersome process, several expedients have been conceived, such as the insertion of silent restriction enzyme cleavage site in mutagenic primers (10). However, even this improvement still requires plasmid DNA isolation/or PCR amplification from several colonies and subsequent screening by restriction enzyme digestion.

Here we report a DHPLC-based method for the rapid discrimination of recombinant bacterial clones versus non-mutant parental clones, after performing two different SDM protocols: one based on "megaprimer PCR" (10) and one based on a commercial kit (QuickChange® Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA). The first protocol was carried out to create a single point mutation (39C→T) in exon 2 of the β-globin gene (HBB), using two PCR amplifications and three primers, as described (10). The amplified products were then cloned into pGEM® vector (Promega, Madison, WI, USA), according to manufacturer's instructions. Conversely, the QuickChange-based protocol was used to create four different missense changes in exons 2 (124A→G), 7 (836A→G), 12 (1403 C→T), and 13 (1508 G→C) of the PTPN11 gene. From both experiments, several bacterial colonies were isolated and grown into 100 µL LB for 30 min at 37°C. Two microliters of growing colonies were then lysed by heating at 98°C for 10 min and used as template for PCR amplification. The reaction was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µL containing 250 µM dNTPs, 0.5 µM each specific HBB and PTPN11 primers, 1.25 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), in 1× reaction buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$). The PCR mixture was held at 94°C for 11 min and then cycled 25 times at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, followed by 7 min at 72°C in the final cycle.

Since each bacterial clone contains only a single allele, to originate DHPLC-detectable heteroduplex DNA molecules, each single colony PCR product was mixed with a homozygous wild-type (HBB or PTPN11) PCR product. These PCR mixtures were denatured at 95°C for 5 min and allowed to cool at 65°C for 30 min. Samples were then analyzed in WAVE DHPLC and DNASep Column (both from Transgenomic™, Crewe, UK) as described previously (11). The gradient was formed by mixing buffer A (0.1

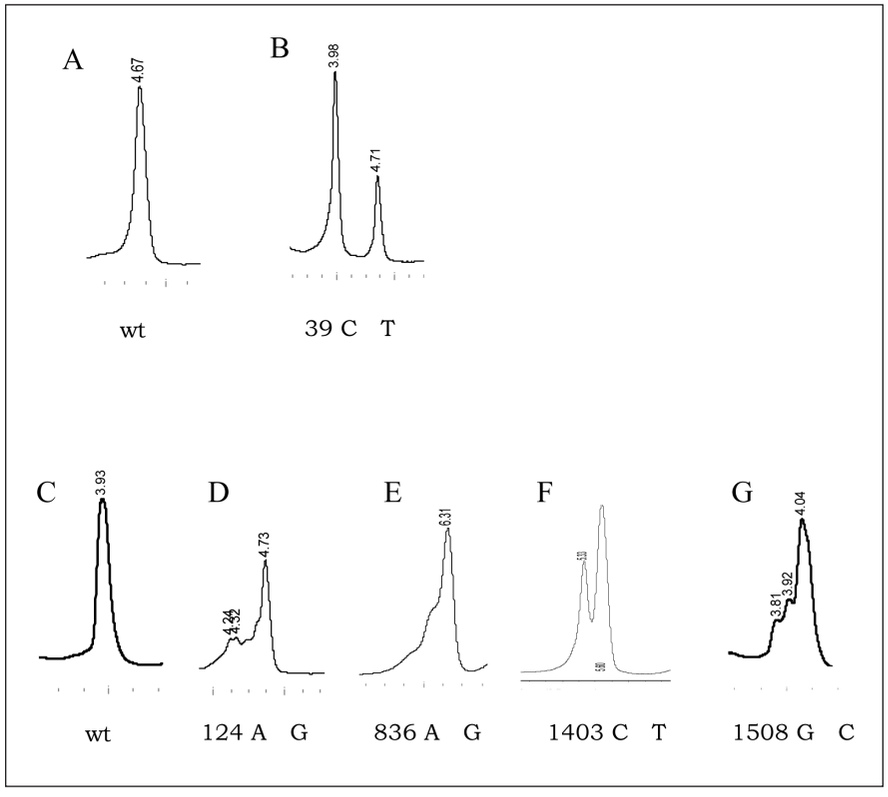

Figure 1. DHPLC elution profiles of wild-type and mutant clones. (A) Parental HBB clone; (B) recombinant HBB mutant clone after "megaprimer PCR"-based SDM; (C) parental PTPN11 clone; (D–G) four different recombinant PTPN11 mutant clones after using the QuickChange Site-Directed Mutagenesis Kit.

mM TEAA) and buffer B (0.1 M TEAA, 25% acetonitrile), and analysis was carried out at a flow rate of 0.9 mL/min and buffer B gradient increase of 2% per min for 4 min. Start and end concentration of buffer B were 61°C–71°C for HBB exon 2; 51°C–59°C for PTPN11 exon 2; 55°C–63°C for PTPN11 exon 7; 58°C–66°C for PTPN11 exons 12 and 13. Oven temperatures for optimal heteroduplex separation, determined by the WAVE Maker software 4.1.40, were 60.2°C for HBB exon 2; 59.7°C, 57.3°C, and 59.4°C for PTPN11 exons 2, 7, and 12 and 13, respectively. The visualization of an altered elution profile, compared with a wild-type homozygous elution profile used as reference, suggested the presence of a mismatch between a mutant allele and a parental allele, thus discriminating the recombinant clones versus the parental non-mutant clones (Figure 1). Sequencing reactions carried out for each abnormal elution profile confirmed this hypothesis.

In detail, we screened 15–20 bacterial clones for each mutagenesis experiment, identifying by DHPLC from 6–10 mutant clones, each of them carrying the introduced mutation and no other sequence variations. All mutant bacterial clones that presented the same point mutation gave rise to identical elution profiles (data not shown). Altogether these results confirmed the high rate of reliability and reproducibility of DHPLC (1,7). No preventive or additional processing of PCR products, such as agarose gel running, enzymatic digestion, or carryover primer removal, was needed in our experimental procedure. Provided that amplification reactions from bacterial clones were successful, our protocol did not require gel-quantification of PCR products before DHPLC analysis, as previously reported (7,8). The presence of at least 10% of mutant DNA in post-mixture samples was sufficient for DHPLC to detect sequence alterations (data not shown). Nevertheless, this additional step could indeed increase the chances of having detectable mutant sequences when colony PCR conditions have not been optimized. Finally, the use of a semi-automated DHPLC protocol allowed us to save both time and costs of analysis, compared to conventional recombinant colonies screening methods. Apart from common experimental steps (including SDM, cloning, and PCR amplification), the total time and cost of each DHPLC run were 2.5 min/sample and less than $1/sample, respectively. In contrast, traditional methods used to distinguish mutant versus parental colonies (i.e., enzymatic digestion or direct sequencing) are much more time consuming (at least 60 min are needed for enzymatic digestion and 4 h for sequencing), tedious, and expensive (variable costs depending on the restriction enzyme and $10–$15/sample for sequencing). Obviously, all these costs are subjected to considerable increases when SDM efficiency is not very high and requires the simultaneous screening of a large number of bacterial clones.

In conclusion, the protocol presented here proved the efficacy of DHPLC in speeding up the screening step of recombinant clones after two different SDM-based strategies, by avoiding additional sequencing of non-mutant clones. Similar procedures have been proven to be useful also for "random mutagenesis-PCR" (7), "overlap-extension PCR" (8), and other applications needing the isolation of single alleles in bacterial clones. One example being represented by mutational screening analysis of patients with hereditary disorders for the characterization of complex genomic rearrangements (i.e., small deletions, insertions, and duplications) or the determination of the presence in *cis* or *trans* of two different mutations/polymorphisms (personal unpublished observations).

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