BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Heteroduplex Cleavage Analysis Using S1 Nuclease

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A variety of methods have been developed to screen for genetic mutations. Most rely on the comparative analysis of polymerase chain reaction (PCR) products. Single-strand conformational polymorphism (4), the most widely used technique, relies on sequence-dependent conformational electrophoretic mobility differences between wild-type (WT) and mutant strands; whereas, heteroduplex analysis (5,8), the second most popular technique, is based on differential mobility between homoduplexes and heteroduplexes in gel electrophoresis. Both techniques require exacting electrophoretic conditions. Another approach to mutation screening relies on cleavage of unpaired nucleotides in self-folded DNA or heteroduplexes between WT and mutant forms as in RNase A cleavage of, for example, RNA/DNA heteroduplexes (1,3,9). None of the techniques is fully applicable to all situations. Each method has its own advantages and disadvantages; however, all require multiple steps subsequent to PCR and/or specialized electrophoretic conditions, size restrictions or do not provide information on the relative location of the mutation.

Here, we describe a version of heteroduplex cleavage analysis of PCR products based on the ability of S1 nuclease to cleave base pair mismatches in DNA/DNA heteroduplexes (6,7). The procedure requires minimal sample, no purification steps, a short 30-min incubation and can be visualized on simple agarose minigels in <20 min. As used in our laboratory, small aliquots of PCR product spanning the suspected site of mutation and reference (WT) PCR products are mixed, heated and cooled to effect annealing. An equal volume of S1 nuclease in digestion buffer is added to each tube. Following incubation, the samples are electrophoresed in agarose minigels to visualize digestion products.

PCR amplification products were produced by incubating DNA template using standard PCR conditions and reagents [5 U *Taq* DNA Polymerase (Life Technologies, Gaithersburg, MD, USA) in the manufacturer's PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) with 1.5 mM $MgCl_2$, 0.2 mM each dNTP mixture and 0.5 mM of each primer]. In the basic S1 nuclease digestion protocol, samples to be digested consisting either of WT (control reference) or mutant PCR amplification products, or a 1:1 mixture of reference and mutant products were incubated under mineral oil at 95°C for 5 min (denaturation) followed by a gradual cooling to 65°C (re-annealing stage) over 5 min. Four-microliter samples were deoiled by pipetting onto Parafilm®, dragging the drop over the film with a clean pipet tip and placing the droplet into 0.5-mL microcentrifuge tubes placed on ice and containing 2.7 µL H_2O, 0.8 µL 10× S1 buffer (80 mM NaCl, 0.5 M Na acetate, pH 4.5, 10 mM $ZnSO_4$, 5% glycerol) and 0.5 µL of a 1:20 dilution (25 U) of S1 nuclease (Life Technologies) in the dilution buffer supplied by the manufacturer. Reactions were incubated 30 min at 37°C and immediately placed on ice. Samples were mixed with running buffer and electrophoresed in 1.5% agarose and stained with ethidium bromide. Gels were viewed by UV transillumination and photographed.

Of the five reference/mutant amplification product combinations tested (Table 1), all showed a single band following amplification and after melting and re-annealing and subsequent incubation in the digestion buffer in the absence of the enzyme (data not shown). In the presence of S1 nuclease, the heteroduplex combination with a contiguous three-base mismatch (77/81) showed both the homodimer band (higher molecular weight [mol wt] and two lower mol wt bands) corresponding to the cleavage products of the heterodimer (Figure 1, lane C). Of the two heteroduplex combinations in which there was only a single base mismatch, one (KG2/KG4) digested using the standard protocol (Figure 1, lane B), and the other (77/93) did not. The pair differing by a single deletion (213-1/213-2) also did not digest.

In an attempt to increase the sensitivity of the reaction to single-base mismatches, we varied the digestion conditions and digestion buffer and tested these conditions on the various duplex combinations. The following observations were made: *(i)* Digestibility was not affected by additional glycerol or the addition of betaine or dimethyl sulfoxide (DMSO). *(ii)* Sensitivity was increased by lowering the NaCl concentration of the S1 buffer. *(iii)* Maximal digestion was achieved when the NaCl concentration in the reaction mixture was in the 8.0 to 0.0 mM range (data not shown). *(iv)* The addition of diox-

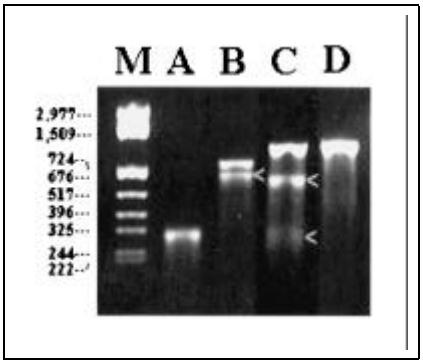

Figure 1. S1 digestion of heteroduplexes. Heteroduplexes were produced and digested with S1 nuclease in S1 buffer and electrophoresed as indicated in the text. Arrow heads indicate digestion products. Lane A, 213-1/213-2 mixture showing no digestion. Lane B, KG2/KG4 mixture showing digestion, the upper band is the undigested homoduplex, and the lower band is the large digestion product (the smaller fragment is not visible). Lane C, 77/81 mixture showing digestion; the upper band is the undigested homoduplex, and the middle and lower bands are the digestion products. Lane D, 77/93 mixture showing no digestion. 77/90A (not shown) was identical to 77/93. Lane M = size markers.

Table 1. Reference/Mutant Amplification Product Combinations

Amplification Product Combination (Form[a])	Size (bp)	Target Sequence[b]	Digestion without Dioxane	Digestion with Dioxane
213-2 (wt)	280/	...GGCATT**T**TGGAAC...	no	no
213-1 (mut)	279	...GGCATT-TGGAAC...		
KG2 (wt)	820	...CTGTAA**C**TCCTGG...	yes	yes
KG4 (mut)		...CTGTAA**T**TCCTGG...		
77 (wt)	936	...CCGGTG**ACG**GTGTCG...	yes	yes
81 (mut)		...CCGGTG**TGC**GTGTCG...		
77 (wt)	936	...CACCAAG**G**GCCCAT...	no	yes
93 (mut)		...CACCAAG**T**GCCCAT...		
77 (wt)	936	...CACCAAG**G**GCCCAT...	no	no
90A (mut)		...CACCAAG**A**GCCCAT...		

[a]wt = wild-type; mut = mutant.

[b]Divergent sequences are underlined and bold.

ane had a dramatic effect by converting the refractory heteroduplex, 77/93, into a digestible reaction (Figure 2). *(v)* Dioxane concentrations up to 40% yielded increasing digestion; however, toward the higher end of the concentration range, nonspecific digestion and smearing of the remaining bands were observed. At concentrations higher than 40%, only faint smears were evident. *(vi)* Incubation at 37°C for >30 min also lead to smearing, as did overnight storage of the dioxane-enhanced digestion products at 4°C, and incubation under oil resulted in over-digested smears. *(vii)* Reactions not containing dioxane could be run with oil overlays at 37°C. Dioxane did not result in digestion of 77/90A or 213-1/213-2 (data not shown).

The conditions that proved optimal were those that are known to destabilize DNA duplexing, specifically lower salt concentration (the KCl in the PCR buffer contributes 25 mM to the final reaction mixture) and the addition of dioxane (2). Presumably, these conditions promote "breathing" of the duplex adjacent to the mismatch, thus increasing the size of the single-stranded target for S1 digestion. This advantage is somewhat offset by the increased breathing of the ends of the DNA strands leading to random shortening, which, we presume, leads to the smearing of the samples in those reactions with more than 30%–40% dioxane and upon prolonged incubation or storage of dioxane-containing reactions.

There is no obvious explanation as to why some of the amplification products cleaved and some did not. One would expect at least some single-base insertions and deletions as well as point mutations to be susceptible and even more so for larger mutations. Though not applicable in all cases, the simplicity of the technique makes it especially attractive when screening large numbers of amplification products for known mismatches or when attempting to map the location of mutant residues.

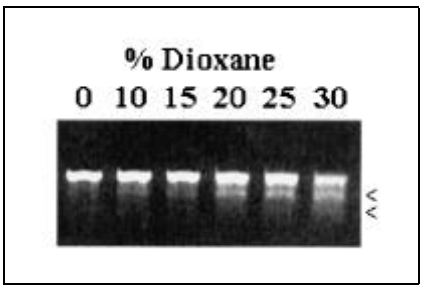

Figure 2. The effect of dioxane on the digestion of the resistant duplex, 77/93. The percentage of dioxane in each reaction mixture is indicated. The locations of the digestion products in the 20%, 25% and 30% dioxane samples are indicated by arrow heads.

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