slides. Three replications were performed with over 200 comets analyzed per slide type. The results, obtained by computerized analysis, showed that the damaged DNA was significantly more quantifiable on the custom slides (p = 0.0154). Cells receiving the UV treatment registered an average of 1.42 tail-moment units (see Reference 1 for an explanation of tail moment) higher on custom slides (3.82 vs. 2.40). Because only tail moments greater than 2 are regarded as significantly damaged, and typical undamaged controls are approximately 0.5, this kind of increase could discriminate mildly damaged cells from undamaged ones. Other experiments, using more considerably damaged DNA, exhibited as high as 5.21 U difference in favor of the custom configuration. We attribute this enhanced sensitivity to the decreased background fluorescence, which provides greater contrast and allows for increased gain settings during analysis.

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The SSCP Phenomenon: Addition of HEPES Buffer Dramatically Affects Electrophoretic Mobility

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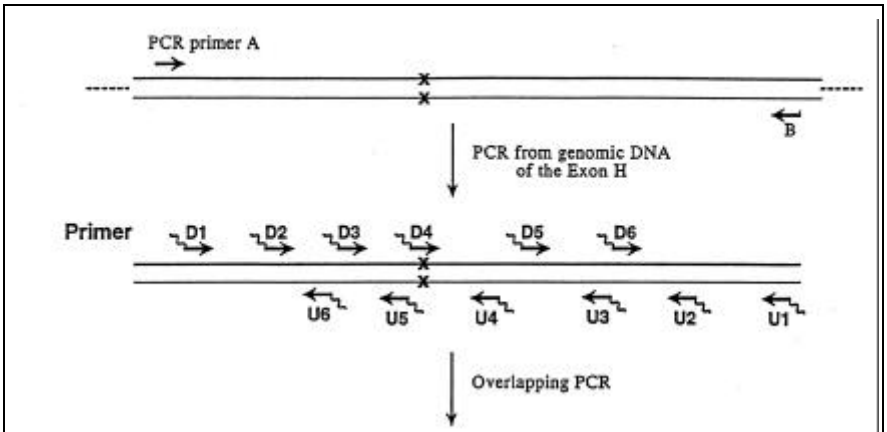
To our knowledge, analysis of single-strand conformation polymorphism (SSCP) (5) is the most widely used means of mutation scanning. SSCPs reveal single-base sequence changes because altered electrophoretic migration of one or both single strands on a nondenaturing gel can be detected. SSCP does not detect all sequence changes with one set of electrophoresis conditions, and sensitivity of detection is a complex function of parameters such as sequence and size (1). The sensitivity of mutation detection by SSCP can be increased by using two electrophoresis conditions, such as electrophoresing at 20° and 8°C. This approach has some disadvantages including: *(i)* smearing of segments at 8°C (especially if the GC content is ≥55%); and *(ii)* inconvenience of loading samples and performing experiments in a cold room.

We show that the addition of 20 mM HEPES to standard 50 mM TBE buffer (HTBE) substantially alters SSCP mobilities, allowing segments to be analyzed under two convenient and essentially independent room temperature conditions.

To compare HTBE and TBE electrophoresis buffers, we analyzed a 1-kb region from exon H of the human factor IX gene [from bp 30 646 to 31 645 using the numbering system of Yoshitake et al. (7)]. Twenty-six overlapping



All the expected twenty-six fragments and their sizes

	D1 *1*	D2 *116*	D3 *216*	D4 *313*	D5 *465*	D6 *591*
U1 *840*	D1U1 852	D2U1 737	D3U1 637	D4U1 539	D5U1 387	D6U1 262
U2 *709*	D1U2 721	D2U2 606	D3U2 506	D4U2 408	D5U2 256	D6U2 131
U3 *591*	D1U3 603	D2U3 488	D3U3 388	D4U3 280	D5U3 138	
U4 *438*	D1U4 450	D2U4 335	D3U4 235	D4U4 137		
U5 *313*	D1U5 325	D2U5 210	D3U5 110			
U6 *209*	D1U6 224	D2U6 109				

Figure 1. Schematic of overlapping PCR and SSCP fingerprinting. A 1000-bp genomic DNA region of exon H is first amplified with primers A and B. The first nucleotide of the amplified segment corresponds to No. 30 738 in the numbering system of Yoshitake et al. (7). An overlapping PCR is performed with six downstream and six upstream primers to produce the twenty-six double-stranded overlapping DNA segments. Each primer contains a short sequence-specific region and a noncomplementary 5′ tail that prevents short segments from megapriming to produce longer segments in subsequent cycles of PCR (6). The locations of the 5′ end of the six downstream primers and the six upstream primers are indicated; e.g., downstream primer D_2 begins at nucleotide 116 from the PCR product AB, while the 5′ end of the upstream primer U_1 is at nucleotide 840. The 26 amplified segments are shown along with their sizes.

segments were generated in one amplification reaction by overlapping PCR (Figure 1). The lengths and positions of the overlapping segments along the 1-kb region are shown in Figure 1. Overlapping PCR products from DNA samples of 40 different hemizygous patients with hemophilia B who had single-base changes within the region were analyzed. Overlapping PCR of 40 DNA samples provided a total of 1026 mutation-containing single-stranded segments per gel for analysis.

Fifty microliters of stop/loading buffer (7 M urea, 50% formamide, 2 mM EDTA) were added to each tube. Electrophoresis through 0.5× MDE™ gels (FMC BioProducts, Rockland, ME, USA) (45 cm × 37.5 cm × 0.35 mm) was performed with TBE buffer (50 mM Tris-borate, 1 mM EDTA, pH 8.3) or with HTBE buffer (20 mM HEPES in TBE, pH 8.0) at 12 W. The temperature of the plate was maintained at 20°C (3). The water-cooled Poker Face™ apparatus (Hoefer Pharmacia Biotech, San Francisco, CA, USA) was used at room temperature, and a fan was used with the SequiGen® GT apparatus (BioRad, Hercules, CA, USA). In other experiments, a fan was used for cooling at room temperature. After a preliminary electrophoresis for 60 min, 1.5-µL aliquots were loaded and electrophoresed for 6 h. The gels were dried and autoradiographed (Figure 2).

The altered pattern of segments can be attributed to either an amplification component or an SSCP component. Under the conditions of multiplex amplification, mutations located in or nearby a primer (samples 7, 14, 15, 25, 26, 28, 29 and 39) result in the absence of amplification from that primer (informative amplification component). However, it is alterations in mobilities due to the SSCP component that are of interest for this analysis. The presence of segments with altered mobilities was determined by visual analysis of the autoradiograms in comparison with normal controls. Unequivocal mobility changes were scored (typically, a migration change of 1/2 bandwidth or more, except near the bottom of the gel where the bands are broader, and a 1/4 bandwidth change can be detected).

The autoradiographs in Figure 2 (A, B) show the effect of adding HEPES to standard TBE buffer. In TBE buffer, 50.8% of all the 1026 single segments with a mutation had an abnormal mobility, while 53.3% of all the segments had such when HTBE buffer was used. When the SSCP efficiency was analyzed for each condition by the type of mutation (e.g., transitions vs. transversions), no simple relationship was discerned. This was similar to earlier results (2–4).

For a given mutation, the efficiencies of the SSCP component varied markedly between the two buffers and often in a complementary fashion (Figure 3). For example, the SSCP component efficiency in sample 17 was 54.8% of the 37 mutation-containing single-stranded segments for TBE and 0% for HTBE buffer, while the SSCP component efficiency for sample 31 was 0%

of the 25 segments in TBE buffer and 60% in HTBE. The efficiencies were not well-correlated (r = 0.46, and the 95% confidence interval overlaps with zero). The data suggest that mobilities are altered in an independent or nearly independent manner.

In a second analysis, we take each sense and antisense pair of segments as the unit for calculation. The focus is on the 312 segment pairs that contain mutations and are less than 550 bp (Table 1). With MDE as the gel matrix, the sensitivity of SSCP decreased only moderately with the size of the segment. In total, the average sensitivity of SSCP with TBE buffer was 75%; i.e., when one or two strands of a double-stranded segment were shifted. With HTBE buffer, the average sensitivity was similar (67%). Table 1 shows that the two buffers were essentially independent of one another; 90% of the 312 double-stranded segments with mutations showed an altered mobility of one

Table 1. SSCP Sensitivity with Size in Two Buffers

Segment Size[a]	No. of Sense/ Antisense Segment Pairs[b]	SSCP Sensitivity of Double-Stranded Segment[c] with TBE Buffer	with HTBE Buffer	with Both Buffers
≤250 bp	56	80%	80%	95%
251–350 bp	71	86%	76%	96%
351–450 bp	90	64%	58%	84%
451–539 bp	95	73%	61%	87%
Total	312	75%	67%	90%

[a]The number of double-stranded mutant segments is divided into four groups according to size. The segments larger than 539 bp are not included.

[b]Segment D_4U_4 (Figure 1) is absent with HTBE buffer, presumably because the segment is dispersed (smeared). Segment D_6U_2 (Figure 1) was associated with three single-stranded segments, presumably because of partial non-template addition by *Tth* DNA polymerase. For several other double-stranded PCR products, only one single-stranded segment was seen, presumably because of dispersal of the segment or co-migration. The above-mentioned segments were short, and only a few mutations occurred in those segments.

[c]The SSCP sensitivity means that a double-stranded segment is scored if one or both single-stranded segments are shifted.

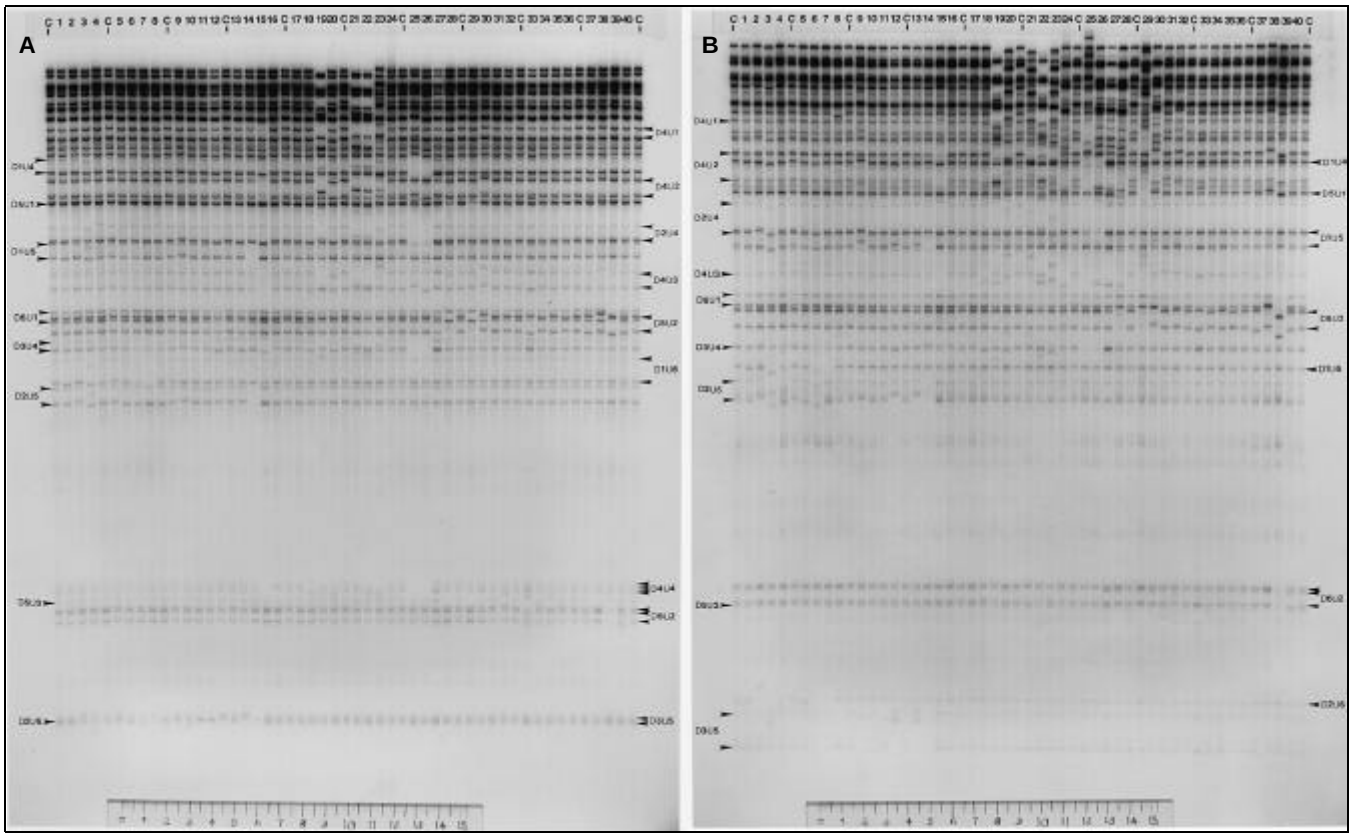


Figure 2. Autoradiograph of SSCP fingerprinting. (A) With TBE running buffer. (B) With HTBE running buffer. Segments were identified and numbered by the segment size and by absent segments in the informative amplification component. Lane C: wild-type DNA; lanes 1–40: 40 samples from patients with hemophilia B (see text). The mobilities of the specific segments were determined in pilot experiments with one or a few of the segments (data not shown).

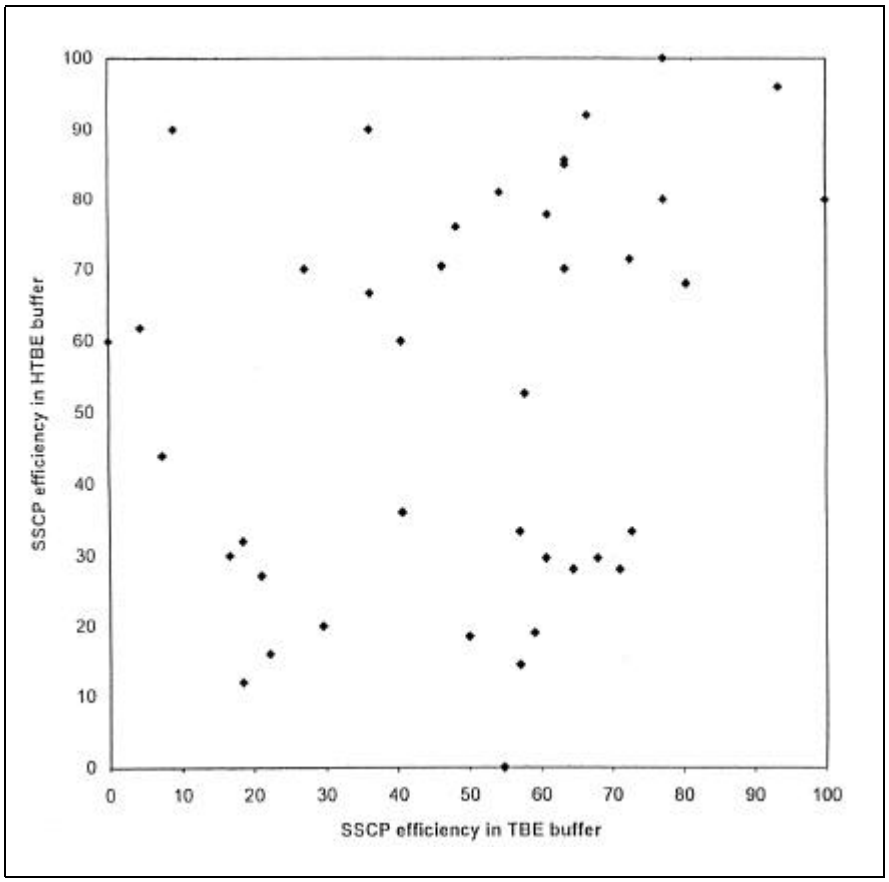


Figure 3. Map plot of SSCP component efficiency in the two electrophoresis buffers. The *x* axis shows SSCP component efficiency (%) in TBE buffers for a specific mutation. The *y* axis shows corresponding SSCP component efficiency (%) for the same mutation in HTBE buffer. The SSCP efficiencies were calculated in the following manner. For each of the 40 mutations, the number of single-stranded segments with a mutation was determined. This varied from 12 to 32 (average = 29), depending on the position of the mutation. When all the single-stranded segments with a mutation showed an altered mobility, the efficiency was 100%. At the other extreme, when none of the segments with mutation showed an altered mobility, the efficiency was zero. For each of the 40 mutations, the SSCP efficiency with TBE and HTBE electrophoresis buffers was plotted.

or both strands in at least one of the two buffers, whereas 92% would be predicted if the average sensitivity were independent of the buffer used.

In conclusion, HTBE buffer offers a convenient condition to augment the sensitivity of SSCP. By performing the analysis described here (Figure 1 and Table 1), it is possible to screen for other conditions that show mobility changes independent of the conditions described as TBE and HTBE buffers. The independence of the mobilities in the two buffer systems are hard to explain if SSCP mobilities are primarily determined by secondary structure. The data provide further indirect evidence that SSCP mobilities are primarily the result of sugar/base and sugar/sugar interactions.

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