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'Cold SSCP': a simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses

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ABSTRACT

A rapid (< 2.5 hrs) method for single-strand conformation polymorphism (SSCP) analysis of PCR products that allows the use of ethidium bromide staining is described. PCR products ranging in size from 117 to 256 bp were evaluated for point mutations and polymorphisms by 'cold SSCP' in commercially available pre-cast polyacrylamide mini-gels. Several electrophoretic parameters (running temperature, buffers, denaturants, DNA concentration, and gel polyacrylamide concentration) were found to influence the degree of strand separation and appeared to be PCR fragment specific. Use of the 'cold' SSCP technique and the mini-gel format allowed us to readily optimize the electrophoretic conditions for each PCR fragment. This greatly increased our ability to detect polymorphisms compared to conventional, radioisotope-labeled 'hot' SSCP, typically run under two standard temperature conditions. Excellent results have been obtained in resolving mutant PCR fragments from human *p53* exons 5 through 8, human HLA-*DQA*, human K-*ras* exons 1 and 2, and rat K-*ras* exon 3. Polymorphisms could be detected when mutant DNA comprised as little as 3% of the total gene copies in a PCR mixture. Compared to standard 'hot' SSCP, this novel non-isotopic method has additional advantages of dramatically increased speed, precise temperature control, reproducibility, and easily and inexpensively obtainable reagents and equipment. This new method also lacks the safety and hazardous waste management concerns associated with radioactive methods.

INTRODUCTION

PCR amplification of DNA segments of suitable length followed by screening for single-strand conformation polymorphisms (SSCP) facilitates rapid identification of genetic polymorphisms, whether resulting from germ-line heterozygosity or somatic cell mutations, at virtually any site within a given moderately long (up to 400 bp) segment of DNA. The original SSCP protocol uses radiolabeled PCR primers or nucleotides to generate a radioactive PCR product, which is then highly diluted, denatured by heat, and electrophoresed in a large-formatted (40×20 cm) nondenaturing gel (1). SSCP bands are then visualized by autoradiography. Although sensitive, this method is both time-consuming and cumbersome. The gel must be run at low voltage (usually overnight) to avoid heat-induced conformational changes. Precise control of the gel temperature has been shown to be important to obtain consistent results (2), but this is often difficult to achieve with large-formatted gels. Autoradiography (usually with multiple exposures) requires additional time to produce a clear image of the resulting SSCP pattern. Furthermore, it is difficult to excise SSCP bands from these gels for sequence characterization since they cannot be visualized directly.

There are now several reports describing non-isotopic protocols for SSCP analysis (3–15). Some require the purchase of expensive additional equipment, or use of silver staining techniques. We describe here a rapid, inexpensive, non-radioactive method for SSCP analysis of standard PCR products. Commercially available pre-cast mini-gels (8.0×8.0×0.1 cm) are used, which are both convenient and inexpensive. Gel temperature, the most critical parameter influencing SSCP band resolution and reproducibility, is precisely controlled through the use of a thermostatically controlled circulator which accurately maintains a predetermined buffer temperature within the gel unit. Individual SSCP bands can be visualized directly by ethidium bromide staining, and can be easily cut from the gel for subsequent reamplification or sequencing. The total time required to perform the electrophoresis, staining, and photography is approximately 1.5–2.5 hrs. The method is compared with radioactive SSCP, and the effects of buffer tank temperature control, composition and volume of loading solutions, denaturants, starting and running voltages, and tank buffer concentration are discussed.

MATERIALS AND METHODS

Preparation of PCR products

PCR products with a size range of 117–256 bp were studied in our laboratory using this method. Human *p53* and K-*ras* products were generated from archival formalin-fixed, paraffin-

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embedded specimens of human gastric adenocarcinomas. Human class II HLA-*DQA* samples were amplified from DNA extracted from human cell lines. Rat K-*ras* exon 3 samples were generated from DNA extracted from methyl (methoxymethyl)nitrosamine-induced primary renal mesenchymal tumors. No special purification of PCR products was performed prior to SSCP. However, to obtain unambiguous results, it was important that a single, clear band of PCR product (double-stranded DNA) was found on gel electrophoresis after PCR.

A mixture consisting of 5 μl of PCR product (roughly 20−100 ng of DNA), 0.4 μl of 1 M methylmercury hydroxide (Johnson Matthey Electronics, Inc., War Hill, MA), 1.0 μl of 15% w/v Ficoll (M.W. 400,000) loading buffer containing 0.25% bromphenol blue and 0.25% xylene cyanol, and 13.6 μl of 1×TBE buffer (90 mM Tris, 92 mM boric acid, 2.5 mM EDTA) was prepared to yield a total volume of 20 μl (a 1:4 dilution of the PCR product). This mixture was heated to 85°C for 4 minutes, and then plunged into ice prior to loading the entire 20 μl onto the gel.

Gel conditions

A 20% or a 4−20% gradient polyacrylamide TBE gel (8.0×8.0 cm × 1 mm; 39:1 acrylamide to bis-acrylamide cross-linking), was used with the matching gel electrophoresis unit (Novex, San Diego, CA). The buffer chamber was filled with 1×TBE buffer for 4−20% gradient gels or 1.5×TBE for 20% gels. A thermostatically controlled refrigerated circulator (Multitemp 2209, LKB Products, Bromma, Sweden) was used to maintain a constant (±0.5°C) preset temperature (5−42°C). The circulator was equipped with a Y-tube on the fluid output to allow it to supply the inner and outer buffer chambers of the gel apparatus simultaneously. These tubes had an internal diameter of 4.8 mm, and each supplied 1350−2000 ml/min. This rapid rate of flow allowed the circulator easily to maintain a constant temperature within the gel unit. (We have also found that inexpensive miniature peristaltic pumps can be used to recirculate buffer chilled by passage through long coils of tubing immersed in appropriate temperature water in an ice bucket or water bath.)

Twenty μl of the mixture prepared above was loaded onto the gel. The gel was run at 300 volts (38 volt/centimeters)(FB570 power supply, Fisher Biotech, Pittsburgh, PA) until the bromphenol blue marker reached the bottom of the gel. The running time was approximately 40 minutes to 2 hours. Temperature, gel type and fragment size affected the chosen running time. The temperature of the buffer chamber nearest the gel was monitored with a thermistor during the gel run.

Gel staining

Gels were stained with a 0.5 μg/ml solution of ethidium bromide in 1×TBE buffer for 20 minutes, and then destained in distilled water for 5 minutes. Ethidium bromide-stained bands were visualized using a 340 nm UV viewing box and photographed.

RESULTS AND DISCUSSION

Results of SSCP analyses of *p53* exon 7 PCR products (117 bp) from human gastric carcinomas are shown in Figure 1. Conventional radioactive SSCPs run on large formatted gels at ambient temperature (21°C)(Fig. 1A) or in the cold room (6 to 8°C)(Fig. 1B) are shown along with 3 unlabeled, 'cold' SSCPs run under various conditions (Fig. 1C−1E). SSCP bands can be clearly discerned in a number of tumor samples, in contrast

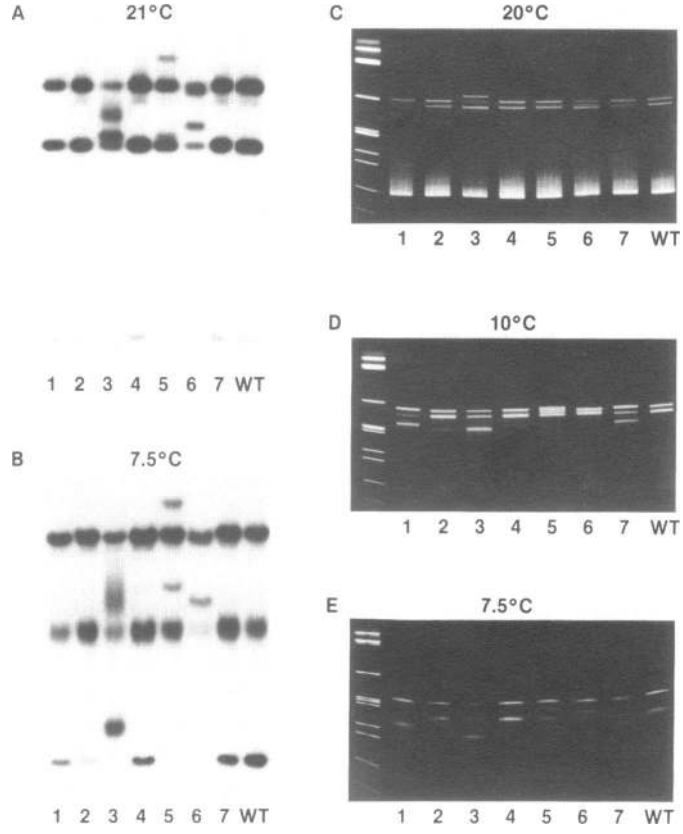

Figure 1. Radioactive SSCP (left side) and non-radioactive SSCP (right side) of *p53* exon 7 PCR fragments. The numbers below the lanes identify various human gastric carcinoma specimens. The upper radioactive SSCP (**A**) was run on a 40×33 cm MDE (MDE, J.T. Baker, Inc., Phillipsburg, NJ) gel with 5% glycerol added for 14 hrs at 6 watts at ambient temperature. The autoradiograph was exposed for 17 hrs at ambient temperature. The lower radioactive SSCP (**B**) was run on the same type of gel (glycerol was omitted) for 15.5 hrs at 5.5 watts in a 7.5°C cold room. The autoradiograph was exposed for 17 hrs at ambient temperature. The non-radioactive SSCPs ('cold' SSCPs, right side) were run on commercially prepared pre-cast 8.0×8.0×0.1 cm 20% polyacrylamide TBE gels (Novex, Inc., San Diego, CA) at 300 V for 1.5 to 2 hrs. In this and each of the figures showing these gels, the leftmost lane is a Phi×174 Hae III-digest molecular weight marker. The topmost 'cold' SSCP gel (**C**) was run at 20°C, the middle gel (**D**) at 10°C, and the lower gel (**E**) at 7.5°C. All of the 'cold' SSCP gels were stained with 0.5 μg/ml ethidium bromide in 1×TBE for 20 minutes, then destained in distilled water for 5 minutes. Note that the 10°C buffer temperature produced the best defined SSCP bands for this particular PCR fragment.

to only two bands normally seen in wild-type samples. Direct dideoxy sequencing of tumor PCR products confirmed that single point mutations were present in samples showing polymorphisms (data not shown). Mutations included G→A or C→T single point mutations at codons 237, 243, 248, 249 or 250. A single base pair deletion was also detected at codon 251 in one sample (lane 6).

To compare the sensitivity of the 'cold' SSCP and radioactive SSCP techniques, other *p53* exons from additional human gastric carcinomas were studied in parallel utilizing 'cold' SSCP, radioactive SSCP (1), and direct dideoxy sequencing. In *p53* exons 5, 6, and 8, 100% of mutations detected by sequencing were also detected by our non-radioactive method, while 94% were detected by conventional radioactive SSCP. In exon 7, 100% of mutations detected by sequencing were found using the

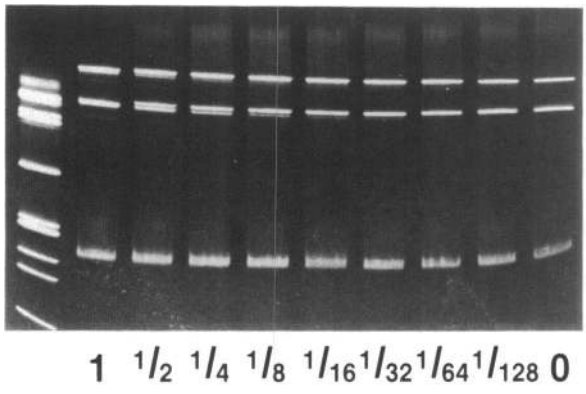

Figure 2. The sensitivity of 'cold' SSCP to detect p53 mutations was evaluated using serial dilutions of a *p53* exon 5 PCR product from a human gastric carcinoma (heterozygous for a single base pair point mutation) diluted with a wild-type *p53* exon 5 PCR product. A 20% TBE gel was run at 300 V for 70 min, with a buffer temperature of 25°C. Numbers at the bottom of each lane show the ratio of tumor-derived PCR product (heterozygous for the point mutation) relative to the total amount of PCR product. The leftmost lane is a molecular weight marker (PhiX174 HaeIII-digest). The gel was stained as in Figure 1.

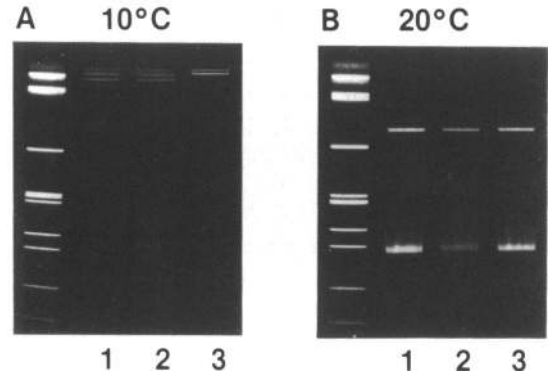

Figure 3. The effect of differing buffer temperatures on SSCP band profiles is shown for rat K-*ras* exon 3 PCR fragments. Lanesä 1 and 2 contain heterozygous mutant samples, while lane 3 contains a wild-type sample. The right hand gel (20% polyacrylamide TBE gel, Novex, Inc., San Diego, CA) was run at 20°C, while the left hand gel was run at 10°C. Gels were stained as described in Figure 1. Molecular weight markers (PhiX174 HaeIII-digest) are in the leftmost lane of each gel. Note the large difference in mobility and definition of the SSCP bands.

non-radioactive SSCP, while only 57% were found using conventional radioactive SSCP. In this exon, temperature control was particularly important to allow resolution of SSCP bands. The large-formatted gel used for radioactive SSCP could be run at ambient temperature or in a 6 to 8°C cold room, but precise intermediate temperatures were not obtainable. It is probable that maintaining the radioactive SSCP gel at a precise intermediate temperature using commercially available but expensive equipment would have increased its sensitivity.

Both methods occasionally showed positive results (especially in *p53* exon 5) which could not be confirmed by direct sequencing of the original PCR mixture. However, weak bands were easily reamplified from the 'cold' SSCP gel, and mutations could then be readily detected by direct sequencing. This suggested that the sensitivity of 'cold' SSCP was somewhat greater than that of direct sequencing in detecting a small subpopulation of mutant sequences against a background of wild-type sequence. This was confirmed in experiments in which a mutant PCR product was serially diluted with wild-type product prior to SSCP analysis. Mutant SSCP bands could be clearly discerned when as little as 3% of the gene copies contained the mutation (Fig. 2).

Alterations in several gel conditions profoundly affected the SSCP results. The effects of buffer chamber temperature, DNA concentration, loading volume, denaturants, loading buffers, gel unit buffer concentration and type, gel type, and starting and running voltages were systematically studied.

Temperature. Precise control of the temperature within the gel unit proved to be especially important for consistent detection of SSCP bands. Panel C in Figure 1 shows the result of running the gel at too high a temperature (20°C) versus running at a more effective temperature of 10°C (Fig. 1D). Continuous measurement of the buffer temperature during the gel run showed that recirculation of buffer maintained at a constant temperature using a thermostatically controlled circulator or peristaltic pumps produced superior temperature control compared to simply maintaining the gel unit in ice or in a cold room. Using the circulator or the pumps, temperature could be controlled within plus or minus 0.5°C. The ideal temperature for SSCP varied among individual PCR products and required empirical study to obtain optimal results for each product. Figure 1 shows the effect of temperature on the production of SSCP bands in exon 7 of the *p53* gene. Note that the best results for this PCR product were obtained at an intermediate temperature (10°C) (Fig. 1D); temperatures lower (7.5°C)(Fig. 1E) and higher (20°C) (Fig. 1C) resulted in blurring or loss of SSCP bands. Figure 3 shows the dramatic effect of temperature on SSCP band mobility in PCR fragments generated from exon 3 of the rat K-*ras* gene. SSCP bands cannot be visualized for the mutant samples (lanes 1 and 2) at 20°C (Fig. 3B), but are quite clear at 10°C (Fig. 3A). Wild-type samples should produce two distinct bands when run at the proper temperature (Fig. 3A, lane 3). If only a single band is noted or there are more than two bands, the temperature needs to be adjusted.

Dilution of PCR products and loading volumes. Dilutions of fixed amounts of DNA from 5:6 to 1:6 were studied and are shown in Figure 4A. In each case, 5 μl of a mutant PCR product plus 0.4 μl of 1 M methylmercury hydroxide and 1.0 μl of 15% Ficoll was diluted in an appropriate volume of 1×TBE to produce the desired final volume. Dilutions of less than 3:6 resulted in significant amounts of double stranded DNA and blurred bands in the SSCP gel, probably due to the rapid kinetics of complete and partial reannealing that occurs at high DNA concentrations. The 1:4 and 1:6 dilutions of the PCR product (20 and 30 μl total volume lanes) produced the sharpest bands on the SSCP gel, but this will depend on the DNA concentration in the PCR product.

Denaturants. The use of methylmercury hydroxide as a denaturant is important to the success of this method. Only with such a strong denaturant could a sufficiently large amount of DNA be loaded to readily allow the visualization of weakly-staining single-stranded bands with ethidium bromide. Methylmercury hydroxide prevented excessive reannealing of the strands prior to entry into the gel (16). Volumes of 1M methylmercury hydroxide ranging from 0.2 to 2.0 μl/20 μl of total volume were compared (Fig. 3B). Volumes of 1.0 μl or greater resulted in progressive blurring of the SSCP bands for unknown reasons.

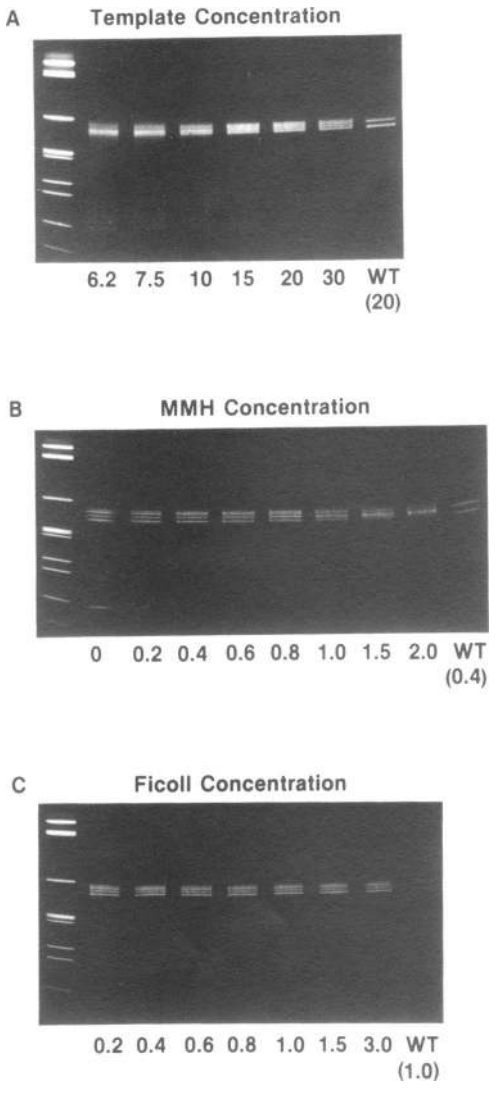

Figure 4. Effects of DNA concentration (**A**), methylmercury hydroxide (MMH) concentration (**B**), and Ficoll concentration (**C**). In 4A, all lanes contained 5 μl of PCR product, diluted in loading solution to produce the series of total volumes shown by the numbers below the lanes (higher numbers reflect greater dilution of PCR product). In 4B and 4C, the numbers show the concentration of methylmercury hydroxide or Ficoll, respectively, expressed as μl per total loading volume of 20 μl. The gels were 20% TBE gels run for 2 hrs at 300 V at 10°C. The same PCR fragment (No. 5 from Fig. 1) was used to facilitate these comparisons. A wild type (WT) sample is also shown. Molecular weight markers (PhiX174 HaeIII-digest) are in the leftmost lane of each gel. Gels were stained as described in Figure 1.

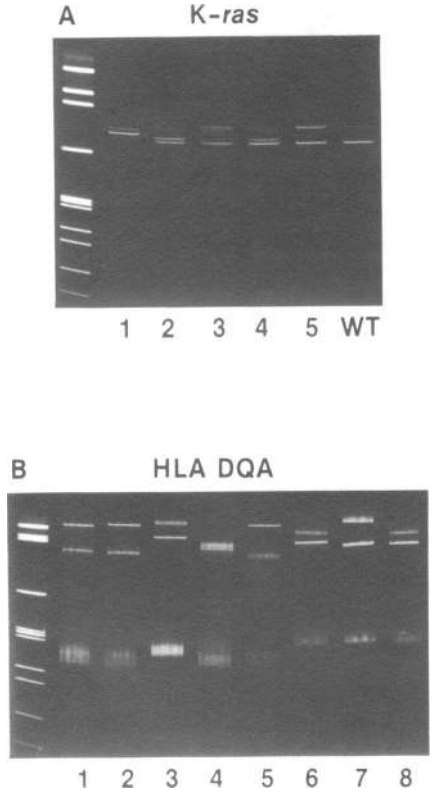

Figure 5. 'Cold' SSCP of K-*ras* exon 1 samples from human gastric carcinoma (upper) and various HLA-*DQA* polymorphisms (lower). The numbers below the lanes denote various sample numbers or wild type (WT). The gel at the top is an 8.0×8.0×0.1 cm 4−20% polyacrylamide gradient TBE gel (Novex, Inc, San Diego, CA) which was run at 300 V for 1 hr with 1×TBE buffer at 21°C. The lower gel is a 20% polyacrylamide TBE gel which was run at 300 V for 2 hours with 1.5×TBE buffer at 8.5°C. Molecular weight markers (PhiX174 HaeIII-digest) are in the leftmost lane of each gel. Both gels were stained as described in Figure 1.

The optimal amount was 0.4 μl/20 μl (0.02 M). Caution should be used with other loading buffer formulations to prevent neutralizing the alkalinity of the methylmercury hydroxide. Sodium hydroxide (3,5) may be a suitable denaturant in some cases, but has not yielded as consistent results in our hands as methylmercury hydroxide. Urea (5−750 mM) was also examined as a potential denaturant, however it proved much less effective than methylmercury hydroxide. Gel lanes often showed additional artifactual bands comprised of reannealed double-stranded DNA or metastable 'ghost' bands of incompletely denatured single strands.

Loading buffers. Buffers composed of 15% Ficoll with no formamide, or 95% formamide with no Ficoll, were studied. Reasonably sharp bands were produced with both, but the SSCP bands 'smile' when formamide only was used. The amount of Ficoll generally used was 1.0 μl per 20 μl of total volume. When the optimum amount of methylmercury hydroxide was used, varying the added Ficoll volume from 0 to 3 μl caused little observable difference in band sharpness (Fig. 4C).

Buffer type and concentration. The type and concentration of buffer in the buffer chamber of the gel unit was another important parameter, affecting the clarity of the SSCP bands. Concentrations of TBE buffer ranging from 0.25 to 2×standard concentrations were tested for effects on various PCR products. In most cases 1×TBE yielded the sharpest SSCP bands when using 4−20% gradient gels. With 20% gels, 1.5×TBE usually produced superior clarity. TAE (40 mM Tris-acetate, 1 mM EDTA) and TGE (25 mM Tris, 250 mM glycine, 1 mM EDTA) buffers were also used at 1×concentration but tended to produce bands of lesser clarity compared to TBE (data not shown).

Gel types. Twenty-percent polyacrylamide TBE gels showed the sharpest SSCP bands most consistently, but 4−20% gradient gels were found to be more convenient because band mobilities were almost 50% faster and gel run times were consequently shorter.

For some PCR products, both types of gel produced equivalent resolution. The commercially prepared gels we used contained 2−4% glycerol, which may have been beneficial in resolving SSCP bands.

Starting and running voltage. Various combinations of starting (first 3−5 minutes) and running voltages were tested. Running voltages of 40 to 400 V were examined. In some experiments, 20% TBE gels were run at voltages of less than 80 volts in the cold room overnight (without a buffer circulator or pumps). This resulted in a sensitivity approximately equal to that of conventional radioactive SSCP run on a large-formatted gel in the cold room. These gel running conditions could be used for PCR fragments with an optimal temperature below 8°C. The use of the circulator or pumps allowed the use of higher voltages (more rapid runs) without heating the gel. Running voltages of 300 or 400 V produced SSCP gels with the best sharpness and mutation sensitivity when the gel was maintained at the proper temperature. Runs at lower voltages (160−200 V) or at varying voltages (300 to 400 V) with a constant temperature resulted in no improvement in band resolution. A starting voltage of 400 V for the first 3−4 minutes of the run followed by a running voltage of 200 V for the remainder of the separation did not enhance resolution compared to electrophoresis at a constant 300−400 V.

The protocol described in the Methods section incorporates the results of these experiments and represents the best combination of parameters we could devise. When further optimizing conditions for SSCP analysis of a new PCR product, determination of the optimal running temperature and gel concentration should be the principal parameters considered for empirical testing. TBE buffer concentrations (we suggest a range of 0.5× to 2×) and the use of certain additives, such as glycerol, may have benefit. The speed and convenience of this protocol allows for rapid optimization of resolving conditions. In contrast, the time-consuming and tedious nature of the large-formatted radioactive technique generally precludes such routine 'fine-tuning' of conditions.

The nucleotide sequence of mutation-specific SSCP bands detected by the 'cold' SSCP technique can be readily determined by selectively isolating visible mutant bands from the stained gel and subjecting these to direct dideoxy sequencing. If necessary, these bands can easily first be reamplified by PCR. In this manner, mutant sequence can be clearly identified even if the mutant DNA comprises only a minor fraction of the total PCR product. Isolating such bands by aligning autoradiograms and dried gels is clearly more problematic.

Silver staining has previously been used to visualize SSCP bands (4,6,7,10−12). In our hands, ethidium bromide staining of SSCP bands, first described by Yap and McGee (3,5), was equally sensitive in a direct comparison (data not shown). Other investigators (13,14) have successfully used ethidium bromide staining for SSCP analysis after generation of a single stranded product by asymmetric PCR.

The photographic technique is important in obtaining maximum sensitivity from our method. Generally, we used a long exposure (1 sec) and shielded the camera from stray UV light by masking the portions of the viewing box which were outside the boundaries of the gel itself. This improved contrast and sensitivity in the resulting photograph. In general, we find that the ethidium bromide procedure requires many fewer manipulations than silver staining, is much more rapid (25 min versus 1−2 hrs.), is considerably less expensive, and does not require the acquisition of additional new reagents or equipment.

Known mutant K-*ras* exon 1 and 2 samples from human gastric carcinomas, mutant rat K-*ras* exon 3 tumor samples, and a series of polymorphic HLA-*DQA* samples were also analyzed using 'cold' SSCP. 'Cold' SSCP readily detected *ras* mutations and differentiated the HLA types. These results are shown in figures 3 and 5, illustrating the power and versatility of this new method as a screening tool for the detection of mutations or allelic polymorphisms.

We can infer from our extensive observations of the major parameters that affect strand mobility that the critical events of strand separation occur at the gel interface. As the DNA leaves the denaturing environment, it must rapidly begin to undergo intramolecular folding based on the thermal stability of stretches of complementary nucleotides. As long as the thermal and ionic conditions remain constant, this conformation is maintained and all molecules of identical sequence migrate as a sharp band. If the temperature is allowed to fluctuate during migration, the shape, and hence the relative migration rate, will be altered. This alteration will not occur simultaneously for all molecules, resulting in diffuse bands. Therefore, a constant temperature is essential for band sharpness and reproducibility of strand separations.

In summary, we have described a novel modification of the SSCP procedure which is simple, rapid and non-radioactive. We have shown the critical importance of accurate temperature control and suggested a simple method to achieve this. The impact of other aspects of the protocol (denaturants, gel types, loading solutions, etc.) have been systematically studied and summarized to assist other researchers to apply this new technique. The reagents and components are easily and inexpensively obtained from commercial sources, and the results compare favorably with those obtained with conventional radioactive large-formatted gel SSCP. Autoradiographic, silver staining or colorimetric detection techniques are readily adaptable to this protocol.

We have not yet completely evaluated the effects of acrylamide cross-linking, or the use of alternative gel materials, gel-thickness, or intermediate gel concentrations. With self-contained, highly efficient, internally-cooled electrophoresis apparatus, we cannot theoretically foresee any upper limit to the amount of voltage which could be applied to the gel, and hence even shorter run times should be possible. Therefore, in conjunction with certain extremely rapid PCR protocols, the technique of rapid 'cold' SSCP is now entering the realm of potential 'real-time' usefulness in clinical diagnosis, pre-surgical decision-making, post-surgical therapy determinations, and prenatal screening for genetic therapy of inherited and congenital diseases.

We did not evaluate the upper size limits of PCR fragments which can be efficiently screened for polymorphisms by 'cold' SSCP. Fragments of 256 bp size worked well in our protocol. Hayashi (17) has suggested that the ability of SSCP to detect mutations declines as PCR fragments approach 400 bp in size. However, we have noted that when using denatured molecular weight marker DNA that sharp resolution of single strands up to 1.35 Kb in length is possible. Many useful human polymorphisms, both natural and disease-state, are multi-nucleotide based (18−21). Although these differences may not be large enough to detect as altered migration of long double-stranded DNA bands, these polymorphisms might potentially be detected on SSCP under these conditions. This may be one potentially powerful new use of 'cold' SSCP as a rapid screening tool.

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