

Improvements in gel composition and electrophoretic conditions for broad-range mutation analysis by denaturing gradient gel electrophoresis

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ABSTRACT

Denaturing gradient gel electrophoresis (DGGE) is believed to be the most powerful pre-screening method for mutation detection currently available, being used mostly on an exon-by-exon basis. Broad-range DGGE for the analysis of multiple fragments or an entire gene is rarely applied. We and others have already shown that one or two DGGE conditions are usually sufficient to analyse an entire gene. Conditions, however, have never been profoundly tested and compared with alternative methods suggested in the literature. Trying to do so in this study, we found significant differences between the various gel systems. The optimal conditions we found for broad-range DGGE include 9% polyacrylamide for the gel, a denaturing gradient with a difference of 30–50% between the lowest and the highest concentration of denaturant, and electrophoresis in 0.5× TAE buffer at a voltage >100 V and <200 V.

INTRODUCTION

Denaturing gradient gel electrophoresis (DGGE), developed by Fischer and Lerman in 1983 (1), is a highly sensitive technique based upon differential melting of double-stranded DNA molecules in a gradient with an increasing concentration of denaturant (urea and formamide). The melting behaviour is highly sequence dependent. It allows for the resolution of DNA fragments differing by as little as a single nucleotide. As double-stranded DNA is electrophoresed through the denaturing gradient, it will melt, i.e. change its conformation in such a way that the mobility of the molecule is dramatically reduced. To prevent complete strand dissociation and to facilitate the detection of mutations in the higher melting domains, a GC-rich fragment (GC-clamp) is introduced during fragment amplification. The GC-clamp increases the percentage of single base changes detectable by DGGE to theoretically 100% (2,3). Nevertheless, the success of DGGE is highly dependent on the melting profile of the DNA molecule and the choice of the primers and gel systems used. Computer algorithms have been designed to analyse the melting behaviour of the DNA fragment, thus facilitating optimal primer selection (4). Primer design, including the length, position and nucleotide sequence of the GC-clamp, contribute greatly to the success of the DGGE assay. We have recently investigated these aspects (5).

In addition to the primer design, there are other practical aspects that influence the efficiency of mutation detection. A literature study revealed that no standard exists with regard to gel composition and electrophoretic conditions for DGGE analysis. Assays described include the following gel compositions: denaturing gradient gels with concentrations of polyacrylamide ranging from 6 to 12.5% polyacrylamide, porosity gradients, addition of glycerol or glycerol gradients, and denaturing gradients ranging from as little as 5% to as much as a 70% urea/formamide (UF) gradient. Marked differences in electrophoretic conditions occur, ranging from as little as 40 V to a maximum of 200 V, with electrophoresis times ranging from 2 to 23 h. Buffer conditions also varied, though not dramatically, with most groups using 1× TAE and some 0.5× TAE or 0.5× TBE.

Several groups, including our own, have proposed specific DGGE approaches to reduce the number of experimental conditions. Guldberg and Guttler (1994) proposed a so-called 'broad-range' DGGE for single-step mutation scanning of entire genes (6). We and others have used two-dimensional DNA electrophoresis, which involves size separation in combination with denaturing gradient electrophoresis (7–10). These 'single-step' DGGE approaches have been facilitated by the application of multiplex PCR or combination (pooling) of the amplicons before electrophoresis (11–13). No studies have, however, compared the performance of the different gel systems. Therefore, in this study we have tested what we considered the most relevant variables with respect to DGGE methodology.

MATERIALS AND METHODS

Mutations analysed

Genomic DNA was amplified from either blood or from paraffin-embedded tumour material using standard procedures (13). Known mutations were analysed from seven different amplicons from five different genes, selected on the basis of their different melting temperatures (different positions in the

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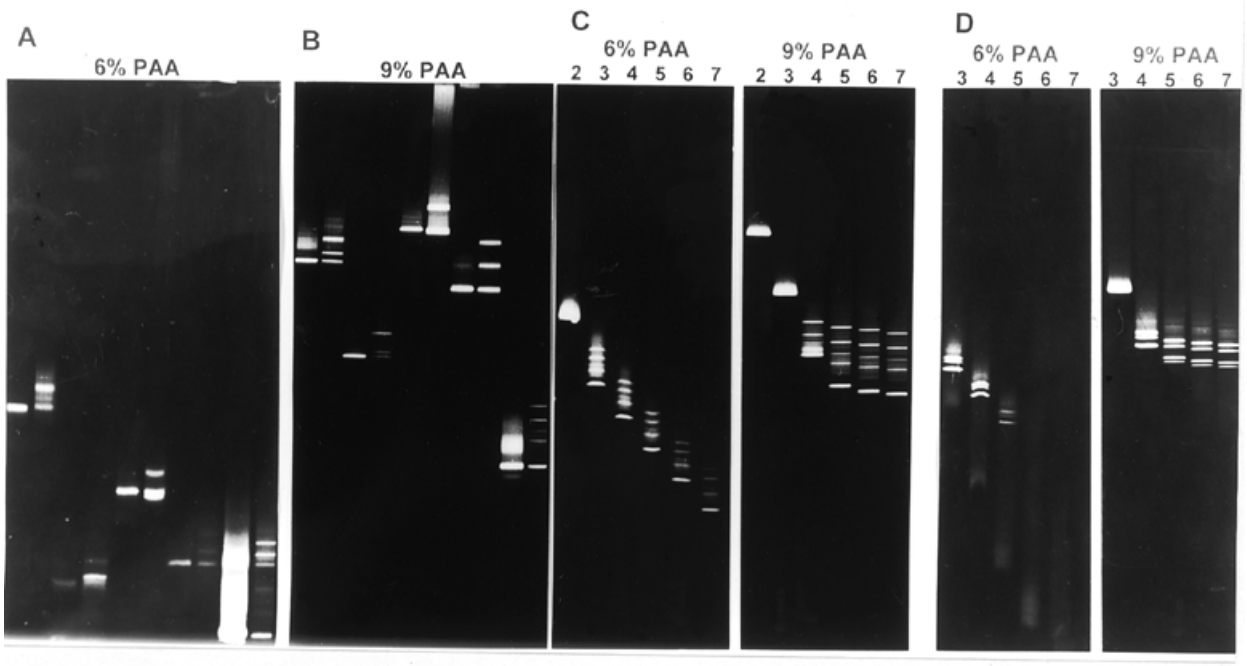

Figure 1. Porosity comparisons of the '*combination I*' mutations (M1–M5, from left to right and adjacent to a normal control), in DGGE gels with a 25–65% UF gradient, after electrophoresis at 150 V for 7 h at 59°C (0.5× TAE). (**A**) A 6% polyacrylamide gel solution was used and (**B**) a 9% polyacrylamide gel solution was used. Time travel gels are shown, with 35–75% UF gradients, electrophoresed at 150 V for a total of 7 h at 59°C (0.5× TAE). Amplicons were loaded at hourly intervals and show different electrophoretic mobilities in gels with porosities of 6 and 9% polyacrylamide, respectively. (**C**) The double mutation M5 (*TP53* amplicon 4.1). (**D**) Mutation M8 (*TP53* amplicon 7).

gel) and the nature of the mutation (e.g. insertion, deletion, transition, transversion). The mutations to be analysed were divided into two combinations and ordered according to their positions in the gel (left to right). '*Combination I*' (M1–M5) included M1, *KRAS* exon 1 (203 bp) G-A transition (Gly13-Asp); M2, *EDN3* exon 4 (204 bp) 1 bp insertion (598insA); M3, *DMD* exon 6 (161 bp amplicon 6A) 1 bp deletion (585delA); M4, *RB1* exon 8 (190 bp amplicon 8A) C-G transversion (IVS7-9); M5, *TP53* exon 4 (224 bp amplicon 4.1) double mutation G-A transition (Pro36Pro) and a G-C transversion (Arg72-Pro). All mutations in '*combination II*' (M6–M10) were *TP53* mutations. M6, exon 6 (209 bp) G-T transversion (Pro222Pro), and four exon 7 (229 bp) mutations; M7, A-G transition (Asn235-Asp), M8, T-C transition (IVS6-15), M9, C-T transition (Thr253Thr), M10, G-A transition (Ser261-Asn). DNA used for the analysis of all *TP53* mutations and the *KRAS* mutation was extracted from paraffin-embedded tumour material.

DNA amplification and denaturing gradient gel electrophoresis

A standard 30-cycle amplification was performed followed by a heteroduplexing step, involving denaturation at 96°C for at least 5 min and re-annealing for at least 15 min. Primers and conditions used can partly be found in reference 13 and are further available upon request. PCR products were loaded on a 20 × 27 cm, 0.75 mm-thick polyacrylamide gel (acrylamide:bisacrylamide, 37.5:1) containing a linear denaturing gradient (100% UF = 7 M urea/40% deionised formamide). The percentage of polyacrylamide varied between the experiments. A 9% polyacrylamide stacking gel is poured to create solid slots for efficient loading of the PCR products, preventing difficulties caused by the (high) urea concentration. Electrophoresis was performed in TAE buffer (1× TAE = 40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0), at 59°C. For all experiments presented in this study, fresh buffer was used and only a single experimental condition was changed per test. Time-travel parallel DGGE was performed according to an established protocol (14). Gels were stained with ethidium bromide and photographed under a UV transilluminator.

RESULTS AND DISCUSSION

Percentage polyacrylamide and polyacrylamide gradients

The percentage polyacrylamide used in the DGGE gel will effect its porosity and therefore mutation detection. From the literature, most laboratories use either a 6 or 6.5% polyacrylamide gel for DGGE analysis. Using '*combination 1*' of mutations, we compared the performance of 6, 9 and 12% polyacrylamide DGGE gels, in gels containing a 25–65% denaturing gradient. As was expected under identical electrophoretic conditions, amplicons move faster through the 6% polyacrylamide gel (Fig. 1A) than through the 9% gel (Fig. 1B), while migration in 12% gels proved to be too slow to obtain optimal band separation

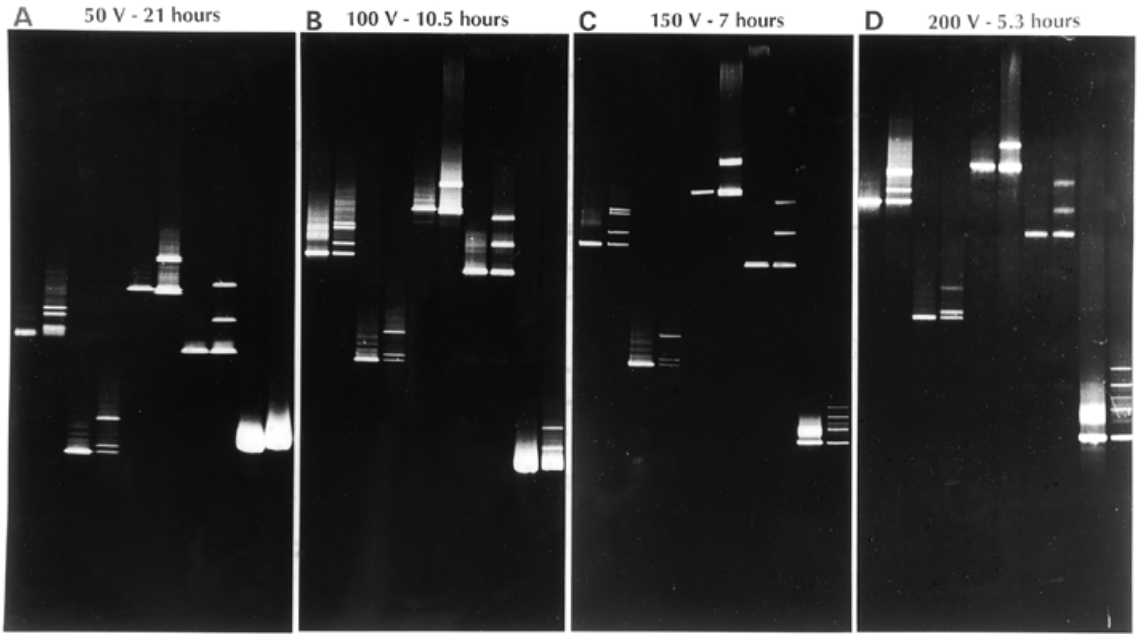

Figure 2. Comparison of voltage applied to the '*combination I*' mutations (M1–M5, from left to right and adjacent to a normal control) electrophoresed at 1050 V h, at 59°C (0.5× TAE), in a 9% polacrylamide gel containing a 25–65% UF gradient and 5% glycerol. Electrophoresis was performed at (**A**) 50 V for 21 h, (**B**) 100 V for 10.5 h, (**C**) 150 V for 7 h and (**D**) 200 V for 5.3 h.

for broad-range DGGE analysis (data not shown). Some mutations were not or only poorly detectable in the 6% polyacrylamide gel, as opposed to the 9% polyacrylamide gel. Six different mutations were analysed using time travel gels containing 6 or 9% polyacrylamide. Two of these mutation-containing fragments are depicted in Figure 1. In all cases tested, amplicons in the 6% polyacrylamide gel, although melted, continued to move through the gel and, in most cases (for example Fig. 1C), completely disappeared after several hours of electrophoresis. The 9% polyacrylamide gels, however, provided a porosity able to keep the DNA fragments at their optimal melting temperature, even after 7 h of electrophoresis. In addition, greater band resolution was obtained in the 9% polyacrylamide gel than in the 6% gel. These findings probably explain why laboratories using 6% polyacrylamide gels may require many different experimental conditions, as mutation detection for some amplicons is only possible at specific times of electrophoresis.

Recently, a double-gradient (DG) DGGE has also been described (15). This technique is based on the combination of two linear gradients, a primary denaturing gradient (urea and formamide) and a collinear secondary porosity gradient (polyacrylamide). This secondary gradient was suggested to suppress band broadening during electrophoresis and thus improve the resolution of the DGGE banding patterns. We compared DG-DGGE gels with porosity gradients of 6–12%, 6–9% but also 9–6% and 12–6% polyacrylamide to a standard 9% polyacrylamide gel (data not shown). Our results clearly showed that mutations with different melting profiles cannot be appropriately detected using a single DG-DGGE condition.

Electrophoretic conditions

Optimal DGGE banding patterns and the application of broad-range DGGE can be dramatically influenced by electrophoretic factors, including voltage, buffer type and concentration and buffer temperature, all affecting the overall gel temperature. Most DGGE assays are performed at temperatures ~58–60°C. We tested the influence of voltage and buffer concentrations on mutation detection capability of broad-range DGGE. As the voltage increases, so the temperature of the gel increases and the amplicons reach optimal melting temperature earlier in the gel (Fig. 2, '*combination I*' of mutations). The buffer concentration used will also affect the gel temperature. Gels electrophoresed in 0.5× TAE buffer show an increased overall gel temperature, with amplicons reaching optimal gel temperature earlier in the gel, when compared to gels electrophoresed in 1× TAE buffer (Fig. 3, '*combination I*' of mutations). This can be explained by a decrease in ion concentration resulting in an increase in resistance. Comparison of both voltage (50, 100, 150 and 200 V) using 1050 V h (Fig. 2) and buffer concentrations (Fig. 3) in gels run at a constant gel temperature of 59°C, showed insufficient band separation at 50 V (Fig. 2A), 100 V (Fig. 2B), and when using 1× TAE (Fig. 3B) for the double mutation M5. This may be explained by an overall decreased gel temperature, either due to lower voltages (50 and 100 V) or higher ion concentration (1× TAE), thus preventing the amplicon from reaching its optimal melting temperature. Time travel gels for this mutation show (Figs 3C and D) that even after 11 h of electrophoresis in 1× TAE, mutation detection was not possible. *TP53* amplicon 4.1 (224 bp, 57% GC-content) double mutation requires a sufficiently high gel temperature

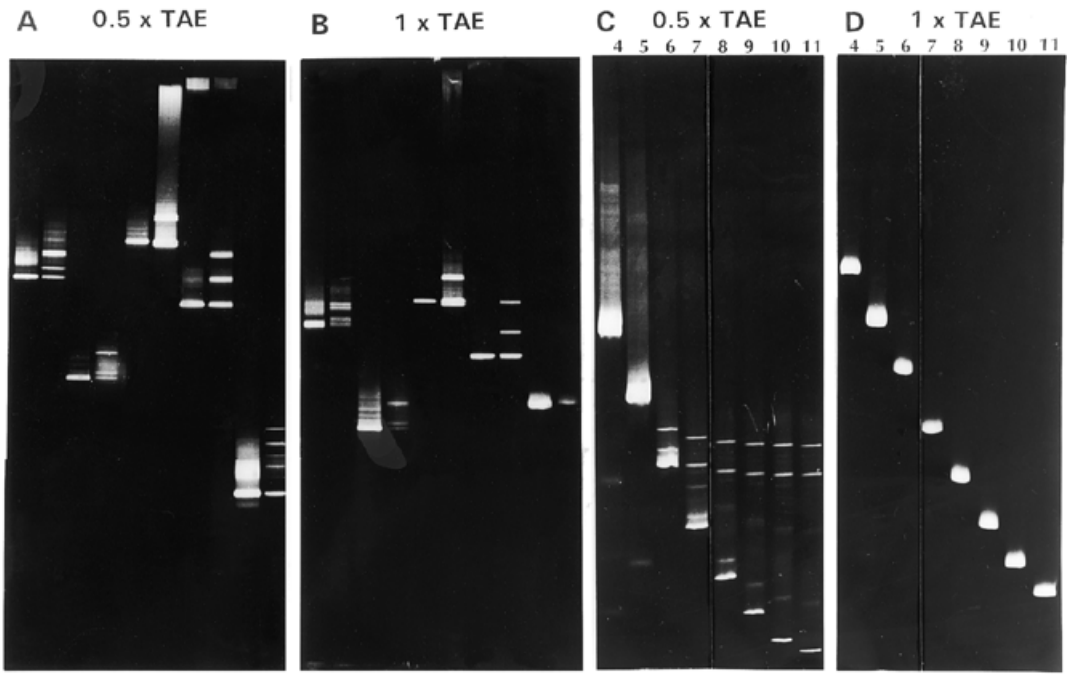

Figure 3. Comparison of buffer concentration applied to the '*combination I*' mutations (M1–M5, from left to right and adjacent to a normal control) in 9% polyacrylamide gels containing a 25–65% UF gradient, electrophoresed at 150 V for 7 h, at 59°C in (**A**) 0.5× TAE buffer solution and (**B**) 1× TAE buffer solution. Time travels of the M5 double mutation (amplicon 4.1 *TP53*), for 4–11 h of electrophoresis under the same conditions and gel compositions as previously mentioned, in (**C**) 0.5× TAE buffer solution and (**D**) 1× TAE buffer solution. All gels were adapted according to the buffer concentrations.

for optimal melting behaviour. Although at 200 V band separation was good, the bands became fuzzier at this higher voltage (Fig. 2D). Increasing the buffer temperature to ~65°C is a possible solution, which has previously been tested (16). However, maintaining such high temperatures accurately is difficult and unfavourable for broad-range DGGE as some amplicons may melt immediately at 65°C. For broad-range DGGE of fragments with both high and low GC-contents, we therefore recommend electrophoretic conditions of between 100 and 200 V and the use of 0.5× TAE.

Additional conditions

Several other parameters that might influence mutation detection using broad-range DGGE, were also tested (results not shown). The range of denaturing gradient was tested for its effect on mutation detection. Urea and formamide gradients used vary greatly per laboratory and in many cases also per amplicon. In one report, 15 different denaturing gradients were used to analyse 24 amplicons of the insulin receptor gene (17). Ideally, one would like to combine all amplicons into a single DGGE gradient. We tested a series of DGGE gradients with differences in denaturant concentrations from 10 to 60%. Band resolution deteriorated when small gradients were applied, while band separation became more difficult with larger gradients. Although the mutations could be detected in all gradients tested, a gradient with a difference of 30–50% between the lowest and highest concentration of denaturant gave optimal resolution appropriate for broad-range DGGE analysis. Other parameters tested include the use of glycerol or glycerol gradients, and the substitution of polyacrylamide with Mutation Detection Enhancer (MDE) solution, which has been shown to improve mutation detection in SSCP analysis. We found that the addition of glycerol or glycerol gradients did not substantially improve mutation detection, while the use of an MDE gel solution proved to be inappropriate substitution.

Conclusions

When establishing a broad-range DGGE assay for mutation detection, a number of criteria need to be taken into consideration. These include optimal DGGE primer design (5) and gel composition and electrophoretic conditions. For the latter two, we found that the use of 9% polyacrylamide gels, a denaturing gradient with a difference of 30–50% between the lowest and highest concentration of denaturant, and electrophoresis in 0.5× TAE buffer at a voltage >100 V and <200 V enables optimal mutation detection.

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