Prevention of depurination during elution facilitates the reamplification of DNA from differential display gels

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ABSTRACT

DNA fragments that show a pattern of differential expression on differential display gels must be eluted from the gel matrix and reamplified to enable further analysis. Elution is usually achieved by heating excised gel slices in a small volume of either water or TE. Here we show that this elution step can adversely affect the ability of the eluted DNA to act as a template for PCR reamplification, probably via the process of depurination. Simply switching to an elution solvent designed to minimise depurination (PCR buffer) facilitates the elution of intact DNA fragments. This improvement is likely to be most beneficial when eluting higher molecular weight fragments (e.g. those >500 bp), in situations where the amount of DNA in an excised gel slice is limited or when contaminating differential display products co-migrate with the differentially expressed product.

INTRODUCTION

Differential display is an RNA fingerprinting technique for identifying genes that are differentially expressed between two or more cell populations (1). The technique involves the reverse transcription of mRNA with an ‘anchored’ primer that targets the beginning of the poly(A) tail, followed by PCR amplification under low stringency conditions with the anchored primer in combination with an ‘arbitrary’ primer, to generate a diverse range of 3’-end products. These products are separated on a high resolution polyacrylamide gel and visualised by autoradiography (1,2).

The identity of putatively differentially expressed gene products can be determined by excising bands from the gel and then cloning and sequencing the DNA they contain. Most differential display studies have eluted DNA from gel slices by allowing the DNA to diffuse out of the rehydrated gel matrix (Table 1). This diffusion-mediated elution approach is both simpler than electroelution, the method adopted by Liang and Pardee in their original description of the differential display technique (1), and allows many samples to be processed simultaneously and rapidly. Another alternative is to elute the DNA during the PCR reamplification reaction itself, simply by adding the gel slice directly to a PCR reaction mix (see for example 2–5). However, this approach is limited in that only a single reamplification reaction can be performed, whereas several PCR reactions may be needed if secondary screening, cloning and DNA sequencing of candidates are envisaged.

It has already been noted that if urea is co-eluted from gel slices it can inhibit subsequent PCR reactions (6,7). Here, we describe an equally serious source of reamplification failure and provide a simple solution to the problem.

MATERIALS AND METHODS

Optimised elution technique

Bands were excised from dried differential display gels with a sterile blade and then rehydrated in 100 µl of 2× PCR buffer (20 mM Tris–HCl, pH 9.0 at 25°C, 100 mM KCl, 0.2% Triton X-100; Promega) for 10 min at room temperature. After this time the remaining buffer was removed, taking care not to damage the gel slice, and replaced with a fresh 100 µl aliquot of buffer. Samples were incubated at 94°C for 90 min to elute the DNA. After a brief centrifugation, the DNA-containing eluant (~95 µl) was removed to a fresh tube, diluted 10-fold with 2× PCR buffer and stored at −20°C until use. Typically, 10 µl of the diluted template was used in each 20 µl PCR reamplification reaction.

Diffusion-mediated elution kinetics

Preliminary experiments were carried out to determine the optimal elution temperature and elution duration. Several large volume (50 µl) differential display reactions were performed under standard conditions (8) and 5 µl aliquots of this sample run on a non-denaturing polyacrylamide gel. Replicate high intensity bands (~500 bp) were excised from the dried differential display gel and each gel slice rehydrated in 100 µl of H2O for 10 min at room temperature. After this time the residual H2O was removed and replaced with a fresh 100 µl aliquot. The samples were incubated at 20, 37, 65, 80 or 94°C for 60 min or at 94°C for 0, 15, 30, 45, 60, 90, 120 or 180 min to elute the DNA. Ninety microlitres of eluant was mixed with 10 ml of scintillation fluid (CytoScint; ICN) and scintillation counted. Elution ‘efficiency’ was determined as the amount of radiolabelled DNA extracted from the polyacrylamide gel matrix, as a percentage of the elution level achieved after 60 (Fig. 1A) or 180 min (Fig. 1B) at 94°C. Note that it had previously been demonstrated that all of the eluted radiolabel was ethanol-precipitable and as such did not simply comprise free purine bases (specifically [33P]dATP) released through the process of depurination.

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Reamplification efficacy

The ability of eluted DNA to act as a template for PCR reamplification was assessed as a function of elution time. The ~500 bp fragment that had been eluted in water at 94°C for varying periods of time (see above) was reamplified under conditions similar to those used in the original differential display reaction (8), except for an increased concentration of 50 µM each dNTP. The reaction was subjected to hot start PCR: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The reamplification products were run on an agarose gel, stained with ethidium bromide and the amount of product generated quantified by scanning densitometry, expressed as a percentage of the maximum yield obtained. To enable comparison with elution in water, the efficiency of reamplification of the ~500 bp gel slice was also assessed after elution in PCR buffer at 94°C for various times.

To examine the suitability of TE (10 mM Tris–HCl, pH 8.0 at 25°C, 1 mM EDTA) as an elution solvent, replicate ~370 bp
RESULTS AND DISCUSSION
The kinetics of diffusion-mediated elution in water was examined as a function of both temperature and time. Unsurprisingly, smaller DNA fragments were eluted more rapidly than larger fragments (data not shown), with elution becoming more efficient at higher temperatures (Fig. 1A). As shown in Figure 1B, a 90 min incubation at 94°C resulted in the elution of ~90% of the available DNA (there was no further increase in yield beyond 3 h incubation). However, when these conditions (90 min in water at 94°C) were used to elute 62 putatively differentially expressed fragments from differential display gels, only 37 of the samples (60%) could be reamplified. Interestingly, 83% of fragments <500 bp could be reamplified (n = 36 samples), whereas the success rate for fragments >500 bp was only 27% (n = 26). After excluding parameters and conditions relating to the PCR process as causes of reamplification failure, the possibility of DNA degradation during elution was investigated.

Differential display fragments were eluted under conditions designed to limit the extent of DNA degradation; specifically, maintaining the pH of the eluting solvent at 2pH 7.0 at the elution temperature (9,10). This was achieved simply by eluting in standard ‘2× PCR buffer’ (see Materials and Methods) rather than in water. All 25 of the reamplification reactions that had failed when fragments were eluted in water at 94°C for 90 min could be reamplified successfully when eluted in PCR buffer, including fragments >1.7 kb in length. Since only a small proportion (~1%) of the eluant was required as a template for each reamplification reaction, several reamplification reactions could be performed in order to generate sufficient DNA for reverse northern analysis, DNA sequencing and cloning.

To examine the (presumed) depurination effect further, DNA integrity was assessed as a function of elution time. Replicate gel slices containing an ~500 bp fragment were incubated in water at 94°C for up to 3 h and then the ability of the eluted DNA to act as a template for PCR reamplification was determined. Whereas the elution of DNA increased with time at 94°C, reamplification efficiency declined, to the extent that after 2 h at 94°C the eluted DNA was unable to serve as a template for reamplification (Fig. 1B, open circles). In contrast, the same fragment could be reamplified in high yield after elution in PCR buffer, at all of the time points tested (Fig. 1B, triangles).

Depurination is thought to be one of the main factors that limits the amplification of long targets by PCR, since DNA polymerases such as Taq are unable to extend through apurinic sites (11). At any given temperature and pH, depurination occurs at a constant rate. However, it is more frequent both at higher temperatures (especially those above the melting temperature of the template) and at lower pH (12). Barnes (9) has estimated that at 95°C and pH ~6.5, one apurinic site is created every 20–30 kb of single-stranded DNA per minute. Thus, during a 90 min elution at 94°C, most targets >500 bp are likely to lose at least one purine residue (note that the rate of depurination may be even higher in water than in the Tris-buffered solution used in Barnes’ model).

It is interesting that few previous studies have reported difficulty in reamplifying DNA fragments extracted from differential display gels. One key reason for this may be that the depurination effect is more problematic for longer templates than shorter ones. Liang and Pardee (1) state that most of the differential display fragments generated by their method are <500 bp in length and as a consequence researchers using this protocol probably do not suffer greatly from depurination effects. Furthermore, the 40 PCR cycles proposed by Liang and Pardee may generate substantially more product than the 18–25 cycles of ‘second generation’ differential display methods, such as that used here, so that even if depurination does occur during elution, a sufficient number of intact template molecules may remain to permit reamplification. The common use of TE as an elution solvent (Table1), rather than water, also appears to limit the extent of depurination at high temperatures (Fig. 2). However, at 94°C the pH of TE will be ~5.9, assuming a temperature coefficient of ~0.03 pH units per degree for Tris (9) and that the EDTA has minimal proton buffering capacity itself and a negligible effect on the buffering capacity of Tris. Thus depurination probably occurs at a significant rate in TE at 94°C, explaining the superior performance of PCR buffer as an elution solvent.

A final potential reason for the scarcity of reports describing reamplification failure is highlighted in Figure 3. This shows the results of an analysis designed to assess whether the reamplified transcripts produced from water-eluted templates represented full-length or truncated products. Rf values were calculated for each fragment on both the original differential display gel and the agarose gel used to visualise reamplification products. Most of the points on the Rf versus Rf plot (Fig. 3) were found to lie along the line y = x, suggesting that the majority of fragments had yielded full-length reamplification products. However, four of the 37 points (11%) fell a considerable distance below this line of unity, suggesting that they represented truncated reamplification products (presumably the result of mispriming at one or more internal sites on the template). For gel slices containing co-migrating DNA fragments in addition to the differentially expressed product of interest, this truncation effect could be serious. For instance, if the truncated form of a contaminating sequence was formed early on in a PCR reamplification reaction, it could out-compete the bona fide full-length product and consequently (upon later analysis) lead to the erroneous conclusion that the differentially expressed band
was a false positive. All four fragments that generated truncated products when eluted in water subsequently yielded full-length reamplification products when the elution solvent was switched to PCR buffer.

In summary, our results suggest that many cases of differential display reamplification failure are due to the depurinating effects of elution in either water or TE. Fortunately, the problem can be solved simply by eluting gel slices in PCR buffer, which minimises the extent of depurination. The improvement is likely to be most beneficial for higher molecular weight fragments (e.g. those >500 bp), in situations where the amount of DNA in any given gel slice is limited or when contaminating products co-migrate with the differentially expressed product.

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