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Rapid determination of the complexity of cDNA bands extracted from DDRT–PCR polyacrylamide gels

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ABSTRACT

A band extracted from a differential display polyacrylamide gel often represents a composite of heterogeneous products. We have developed a non-radioactive method to simply and rapidly analyse its complexity. A fluorescent restriction enzyme fingerprint of the composite mixture is generated. The number of individual bands observed in this fingerprint indicates the complexity of the re-amplified cDNA mixture. Restriction fingerprints of the inserts of cDNA subclones derived from the re-amplified cDNA mixture are compared to the composite fingerprint to select those representing the most intense bands in the composite. This dramatically reduces the number of clones required for further characterisation.

Differential display reverse transcription polymerase chain reaction (DDRT–PCR) is one of the most widely employed techniques to identify differential gene expression (1,2). However, a major problem is often encountered when extracting PCR products of interest from denaturing polyacrylamide gels for further characterisation; an apparently single band on a DDRT–PCR gel is frequently a composite of identically sized but distinct cDNA sequences. These 'contaminating cDNAs' are co-purified with the differentially transcribed cDNA and are likely to be responsible for the large frequency of false positives associated with DDRT–PCR. It is essential, therefore, that the moiety of interest is separated from the contaminating cDNAs before further characterisation is undertaken.

Generally, in order to overcome this problem the gel eluted material is subcloned and the insert corresponding to the differentially transcribed gene identified by northern blot analysis. At this stage, however, the complexity of the eluted cDNA is not known. Consequently, neither is the number of colonies that need to be screened before the clone containing the differentially expressed moiety is identified. Individual clones can be distinguished by sequencing, the most abundant species can then be taken for northern analysis. But again, as the complexity of the eluted cDNA is unknown then the number of clones that need to be sequenced before the most frequently occuring cDNA is identified cannot be predicted. These approaches are, therefore, time consuming and require large amounts of RNA.

One strategy to assess the complexity of DDRT–PCR bands employs single strand conformation polymorphism (SSCP) gels to resolve the individual cDNAs constituting the mixture (3). However, as the size and complexity of the DDRT–PCR band increases it becomes increasingly difficult to clearly establish the number and abundance of individual cDNAs in the composite due to products occupying the same positions on the SSCP gel. Alternative approaches employ radiolabelled cDNA from the excised band (4) or DDRT–PCR reaction (5) as probes to screen for the differentially amplified moiety. Only one differential can be screened at a time by these approaches. In addition, isolated bacterial colonies can be screened with labelled cDNA probe prepared from total RNA extracted from the two populations of cells under investigation (6). The major drawback of this approach is that it does not detect transcripts of low abundance and is time consuming, requiring the screening of duplicate colony lifts.

We have developed a non-radioactive strategy to circumvent these problems based on our previously described method of fluorescent DDRT–PCR or FDDRT–PCR (7). In FDDRT–PCR, modified 3′-anchoring oligo (dT) primers are employed to reverse transcribe total RNA such that a common 20mer sequence (dTGGTCTCACGGATCCGTCGA) is introduced at the 5′-end of every cDNA. A fluorescently-labelled (FAM-5-carboxyfluorosceinblue) universal primer (FAM-dCTCACGGATCCGTCGATTTT) is then used in every PCR together with an arbitrary 10mer to generate 3′ fluorescently-labelled cDNA which is analysed on an ABI Automated Sequencer (Perkin-Elmer). When a differential is identified by FDDRT–PCR the appropriate PCR is repeated in the presence of radioactivity and the segment of denaturing polyacrylamide gel containing the band of interest is cut out and cDNA eluted.

The complexity is assessed by first re-amplifying the eluted cDNA using a combination of the fluorescently-labelled universal primer and random 10mer used in the original amplification procedure. The amplified product is then divided such that one aliquot is taken for restriction enzyme analysis and the other is subcloned into an appropriate vector. Individual colonies are selected at random from the subcloned material and the inserts amplified using the same combination of primers used for the first

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round of amplification. Both the non-cloned and cloned amplicons are then digested with a series of restriction enzymes.

Restriction enzyme digestion of a fluorescently-labelled cDNA moiety will yield a single fluorescently-labelled product corresponding to the 3′-end of that mRNA. Thus, a heterogeneous mixture of end-labelled cDNAs of this type, produced by the re-amplification of cDNA eluted from a DDRT–PCR band, will yield a mixture of different sized fluorescently-labelled digestion products. These are separated and visualised by electrophoresis using an ABI Automated Sequencer thereby generating a restriction enzyme fingerprint of the gel eluted cDNA mixture. Each band size generated with a restriction enzyme will be characteristic of an individual cDNA. Hence, the number of different bands observed in the fingerprint will indicate the maximum number of cDNAs originally present in the eluate. Employing 4 bp cutting restriction enzymes will increase the chance of cutting a small differential display band. Upon cloning of the re-amplified cDNA mixture the same number of different clones would be expected. Their individual fingerprints can then be aligned with the composite fingerprint produced from the uncloned material. In this way the maximum number of different clones that need to be analysed can be immediately established. This procedure vastly decreases the number of amplified false positive inserts that need to be taken as likely candidates for confirmatory RNA studies such as northern blotting.

As part of a study investigating the differences in gene transcription between normal, pre-malignant and malignant oral tissue biopsies we identified seven putative differentially expressed bands (from 253 to 618 bp in length) by FDDRT–PCR using the anchoring primer dTGGTCTCACGGATCCGTCGA$(T)_{12}$CA for reverse transcription and the fluorescently-labelled universal primer (see above) and a selection of arbitrary 10mer primers for PCR. After repeating the PCR in the presence of radioactive deoxynucleotides, the seven bands of interest were excised from the polyacrylamide gel into 100 µl water and the cDNAs were eluted by boiling for 10 min. cDNA eluates (5 µl) were re-amplified in 50 µl PCR amplification buffer [10 mM Tris–HCl buffer pH 9.0, containing 50 mM potassium chloride, 0.1% Triton, 1.5 mM magnesium chloride, 50 µM deoxynucleoside triphosphates, 1.0 µM FAM-labelled universal primer, 0.2 µM 5′-arbitrary 10mer and 2.5 U Taq DNA polymerase (Promega Ltd, Southampton, UK)], using 30 cycles of 94°C for 1 min, 40°C for 1 min and 72°C for 1 min and then 72°C for 5 min. One aliquot (5 µl) of amplified cDNA was taken for immediate restriction enzyme analysis and the other for cloning into the pCR™II vector (Invitrogen BV, Leek, The Netherlands).

Thirty white colonies were randomly selected from plates of each of the cloned bands and dispersed into 20 µl water in wells of a 8 × 12 multi-well plate. Aliquots (15 µl) of each colony suspension were removed and used to prepare plasmid stocks. The residual 5 µl colony suspensions were lysed by boiling for 10 min. Subcloned cDNA fragments were PCR amplified from the colony lysates as described above using the fluorescently-labelled universal primer and the appropriate arbitrary 10mer primers.

Amplified cDNAs (5 µl) (both non-cloned and cloned inserts) were digested in 15 µl reaction volumes with the 4 bp cutting restriction enzymes, *Alu*I, *Hpa*II and *Rsa*I at 37°C for 1 h and then diluted 3-fold with water. Aliquots (4 µl) of these dilutions were mixed with 0.5 µl Genescan-500 TAMRA internal size standard (Perkin-Elmer) and 3.5 µl formamide loading dye and denatured at 90°C for 2 min. Each 4 µl aliquot was electrophoresed on a 4% polyacrylamide–6 M urea gel using an ABI 377 PRISM DNA Sequencer (Perkin-Elmer). ABI Genescan Software (Perkin-Elmer) was then employed to analyse the electrophoresis data using the Perkin-Elmer GS500 size standards to size the restriction fragments.

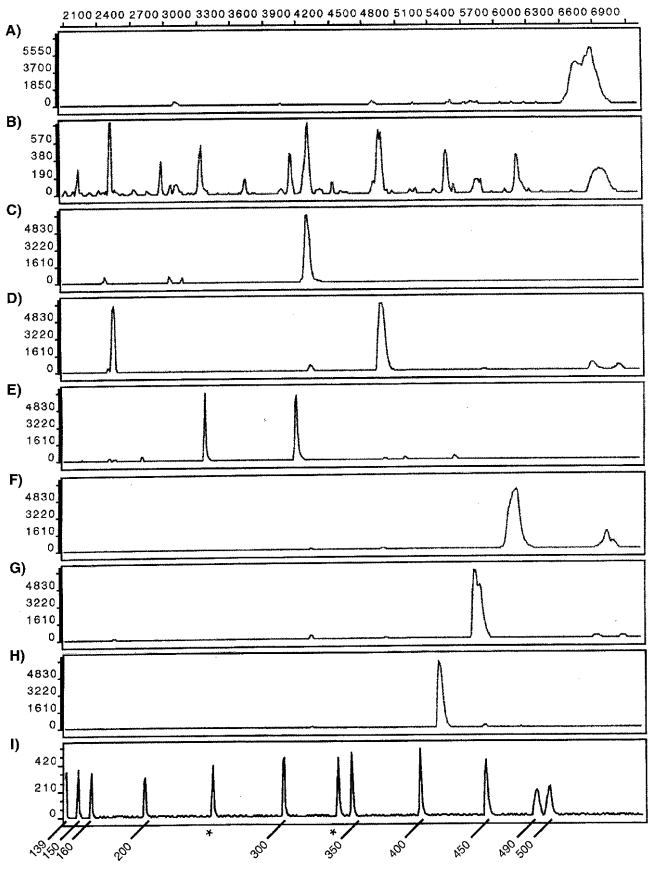

Figure 1. Analysis of the complexity of a cDNA mixture re-amplified from an eluted DDRT–PCR electrophoresis gel band. An eluted cDNA fragment (537 bp) was re-amplified using FAM-labelled universal primer in combination with arbitrary 10mer primer, dTACAACGAGG. Re-amplified cDNA was analysed on the ABI 377 PRISM DNA Sequencer before and after restriction enzyme digestion with *Alu*I (**A** and **B**, respectively). An aliquot of this re-amplified cDNA mixture was subcloned in the pCR™II vector, 30 clones were picked at random, re-amplified and then analysed with *Alu*I as described above. Examples of profiles of individual products are shown (**C**–**H**). (**I**) Molecular weight markers (*, not sized).

The complexity of the seven re-amplified cDNA mixtures was indicated by the number of peaks in each of the composite fingerprints. The complexity of individual bands ranged from six peaks for the simplest composite (287 bp band) up to 11 peaks for the most complex (537 bp band). Figure 1 demonstrates the results obtained from the analysis of the most heavily contaminated band of 537 bp (compare Fig. 1A and B, pre and post digestion, respectively). Eleven distinct major peaks were observed when the amplified cDNA was digested with the restriction enzyme *Alu*I (Fig. 1B). This graphically illustrates the extent to which a sequence of interest can be contaminated and explains why false positives are frequently reported in DDRT–PCR. *Alu*I was the most efficient in digesting the majority of composite mixtures to completion although all 4 bp cutting restriction enzymes tested digested sequences in the re-amplified cDNA mixture.

Independent clones generated from the sub-cloned cDNA mixtures produced characteristic profiles that could be aligned to peaks in the composite profile. This is clearly demonstrated by the clones derived from the 573 bp cDNA (compare Fig. 1B with C–H). By aligning these profiles with that of the composite profile (Fig. 1B) six distinct cloned sequences were identified. The relative number of each individual subclone varied. Ten colonies gave the profile observed in Figure 1C, six in D, two in E, three in F, two in G and two in H. These frequencies approximate the relative intensities of the peaks in the composite fingerprint (Fig. 1B). Two peaks were observed in Figure 1D and E. These result from fluorescent universal primer amplification at both ends of the cDNA moiety; both restriction fragments were FAM-labelled. Five amplification products gave no peaks on the automated gene sequencer. These moieties were demonstrated to represent arbitrary 10mer–arbitrary 10mer amplification products which do not carry a fluorescent-label and therefore cannot be visualised on the automated sequencer (data not shown).

Amplicons produced from either universal primer–universal primer or arbitrary 10mer–arbitrary 10mer priming are potentially artefactual. This approach, therefore, allows the exclusion of such spurious products from further analysis. Since the most intense peaks in the restriction digestion fingerprint are likely to represent the differentially amplified cDNA of interest then clones harbouring this sequence can be immediately identified. Differential expression of these moieties can subsequently be confirmed by northern blot analysis.

In conclusion, this non-radioactive approach represents a simple and rapid way to assess the complexity of large numbers of differentially amplified cDNA mixtures. The method has the following advantages: (i) the distinct cDNA sequences that constitute a re-amplified DDRT–PCR band can be clearly resolved; (ii) large numbers of cDNA clones, derived from the re-amplified composite, can be quickly characterised; (iii) the relative abundance of individual clones can be established; and (iv) the likely candidates for the differentially expressed cDNA can be identified reducing the number of subclones that need to be screened by northern blot analysis.

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