manner. In this paper, we have presented a simple but efficient illumination system for photoactivation at the single-cell level. A standard 100-W mercury lamp can provide sufficient UV light for photoactivation when the light is critically focused on the specimen. A He-Cd laser, which can supply more intense (>10 times) UV light to the specimen, is critically focused on the specimen. The laser provides more rapid activation, and the light is more restricted in area (to minute subcellular structures) or in time (for more rapid kinetic phenomena).

REFERENCES


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INTRODUCTION

Many techniques have been developed to identify differences in transcription between two populations of cells or tissues such as the (±) differential hybridization and subtraction hybridization techniques (5,7,9). Major disadvantages of these approaches are that they are technically very demanding, time-consuming and permit the simultaneous comparison of only two RNA populations. Recent technologi-
cal developments have improved the probability of identifying differentially expressed genes of low abundance. Differential display reverse transcription polymerase chain reaction (DDRT-PCR) is currently the method of choice (1–4,6).

With DDRT-PCR, a set of 3′-anchoring (dT)_{12}VN primers are used to reverse-transcribe and subdivide mRNA populations (where N can be any deoxynucleoside and V can be any deoxynucleoside other than thymidine). Next, one of the many possible arbitrary 10-mers and the appropriate (dT)_{12}VN anchor primer are used in a low-stringency PCR in the presence of radioactively labeled dNTPs to randomly amplify fragments of the cDNA subpopulations. PCR products are separated on a denaturing polyacrylamide electrophoresis gel. The resulting “transcription profiles” or “RNA fingerprints” are compared to identify differentially expressed transcripts, which can then be rapidly cloned and sequenced. The advantages of this approach are that transcriptional levels in large numbers of mRNA populations from different samples can be compared simultaneously and also that the levels of the majority of polyadenylated RNA moieties (both low and high abundance) are investigated.

However, DDRT-PCR is not without its problems. Amplicons are produced that result from arbitrary 10-mer to arbitrary 10-mer amplification. These products may mask the amplicons generated from the 3′ ends of mRNAs on autoradiographs, and their levels and nature may vary between samples. Arbitrary 10-mer to arbitrary 10-mer priming can occur along the length of the cDNA transcripts, and therefore loss of the 5′ ends of transcripts in one sample as a result of tissue deterioration, handling or processing compared with a second sample may appear as differential gene expression after DDRT-PCR.

The use of fluorescently labeled oligonucleotides has a number of potential advantages in addition to the safety benefits of avoiding radioactivity. Analysis of amplified products using an automated gene sequencer facilitates a high throughput of samples, easier data capture and storage for future profile comparison. However, the use of fluorescently labeled arbitrary 10-mer primers would not overcome the problem of amplicons that result from arbitrary 10-mer to arbitrary 10-mer primer amplification. In this paper, we describe a modification of the original automated DDRT-PCR approach (1) such that only one fluorescently labeled (universal) primer is required for all PCRs. This approach eliminates problems associated with arbitrary 10-mer to arbitrary 10-mer amplicons.

**MATERIALS AND METHODS**

**Reverse Transcription**

Total RNA was isolated from oral biopsies using the TriZOL™ Reagent method of extraction (Life Technologies, Paisley, Scotland, UK). The twelve possible 3′-anchoring (dT)_{12}VN RT primers (4) were re-designed such that a 20-bp oligonucleotide (dTGGTCT-CACGGATCCGTCA) was attached to the 5′ end of each, to give the general structure, dTGGTCT-CACGGATCCGTCA-(dT)_{12}VN. Total RNA (1 µg) was reverse-transcribed in 50 µL 50 mM Tris-HCl buffer, pH 8.3, containing 75 mM potassium chloride, 3 mM magnesium chloride, 20 µM dNTPs, 2.5 µM anchoring 20-mer-(dT)_{12}VN primer and 300 U of Moloney murine leukemia virus (MMLV)-RT (Life Technologies) at 42°C for 1 h. For impure RNA samples, temperatures lower than 42°C can result in RT that is independent of added primer-adapter and that is variable between samples (8). MMLV-RT was then heat-denatured by incubation at 95°C for 5 min and the reverse transcripts stored at -20°C. Through the use of these modified anchoring primers, a common 20-mer sequence is introduced at the 5′ end of every cDNA moiety.

**PCR**

The universal primer, d[F]CTCAC-GGATCCGTCA, fluorescently labeled at its 5′ end, was used in every PCR together with a selected arbitrary 10-mer. cDNA (1 µL) was added to a 20-µL reaction buffer (10 mM Tris-HCl buffer, pH 9.0, containing 50 mM
potassium chloride, 0.1% Triton® X-100, 1.5 mM magnesium chloride and 10 µM dNTPs [see Optimization of PCR Conditions]) together with 1.0 µM fluorescently labeled universal primer, 0.2 µM 5′-arbitrary primer and 1 U Taq DNA polymerase (Promega, Southampton, England, UK). Amplification was then performed at 94°C for 1 min, 40°C for 5 min and 72°C for 1 min for 5 cycles, then 94°C for 1 min, 40°C for 1 min and 72°C for 1 min for 35 cycles and finally, 72°C for 5 min.

Analysis of Products

GENESCAN™-500 TAMRA internal size standard (0.5 µL; PE Applied Biosystems, Foster City, CA, USA), formamide loading dye (3.5 µL) and PCR sample (3.0 µL) were mixed and then denatured at 90°C for 2 min. An aliquot (4.0 µL) was applied to a 4% polyacrylamide-6 M urea gel, and electrophoresis was performed using a Model 377 PRISM™ DNA Sequencer (PE Applied Biosystems). Electrophoresis data were analyzed using the GENESCAN software and stored on optical discs. Transcription profiles were imported into the ABI Genotyper™ Software Application (PE Applied Biosystems) and compared using a modified version of the Segregating Populations Template (PE Applied Biosystems). The GS500 Size Standards (Perkin-Elmer, Norwalk, CT, USA) were then used to size amplicons.

Recovery of Bands from Radioactive Profiles

Sequencing and/or cloning of differentially expressed fluorescent products identified after comparison of large numbers of specimens can then be achieved by repeating single DDRT-PCRs using radioactivity for detection of the particular target band. For sequences up to 400 bp, DNA was eluted from gel slices by boiling for 10 min in 100 µL water. For sequences of between 400 and 800 bp, DNA was electroeluted using a Biometra HSB-Elutor (Biometra Ltd., Maidstone, England, UK). DNA was then concentrated by ethanol-precipitation and resuspended in 20 µL TE buffer (10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA). DNA can be re-amplified for sequencing or cloning using conditions described for the fluorescent DDRT-PCR, except that unlabeled universal primer is used, dNTP concentrations are increased to 20 µM, and the initial 5 cycles of annealing are omitted from the amplification process.

RESULTS AND DISCUSSION

Optimization of PCR Conditions

Raising the dNTPs concentration markedly increased the number and the intensity of peaks, with a 10 µM con-

Figure 1. Capture of the 3′ ends of transcripts. An aliquot of RNA extracted from an oral cancer biopsy was reverse-transcribed into cDNA using dTGGTCTACGGATCCGTCGA-(T)12 CG as primer. Four PCRs were then performed on identical aliquots of this cDNA using different oligonucleotide primer pairs: dF[TGGTCTACGGATCCGTCGA alone (Panel D), this latter primer with dTACAACGAGG (Panel A), with dTCGGTCATAG (Panel B) or with dGATCGCATTG (Panel C). Identical experiments were performed in which primer dF[TGGTCTACGGATCCGTCGA was replaced by the universal primer dF[CTCACCGATCCGTCGA]TTT (Panels I and F–H, respectively).
centration being optimal (data not shown). Above this concentration, no additional bands were observed; although, the intensity of individual peaks increased. Magnesium ion concentrations in the range of 1.0 to 2.0 mM consistently gave the strongest banding profiles with a dNTP concentration of 10 µM. At 40 cycles of PCR, a threshold is reached and the intensity of the banding profiles does not change. An annealing temperature of 40°C facilitated the amplification of the largest number of cDNA species.

We compared the use of different 5′-fluorescent tags so that we could consider multiplexing (running different dyes in the same lane of a sequencing gel). In our hands, FAM label (5-carboxyfluorescein - blue) was found to give a stronger fluorescent banding pattern than either ROX (6-carboxytetramethylrhodamine - red) or TAMRA (6-carboxytetramethylrhodamine - yellow), which gave similar profiles. Poor fingerprints were consistently obtained using HEX (6-carboxy-2′,4′,7′,4,7-hexachlorofluorescein - green) labeling. Under optimal conditions, reproducible transcription profiles of up to 600 bp could be regularly and consistently generated using FAM, ROX and TAMRA. However, we have observed slight but consistent differences between the banding profiles of replica DDRT-PCRs performed using different dye labels. Therefore, in our experience, only the transcription profiles generated using the same dye label should be compared, and multiplexing should be avoided.

**Capture of the 3′ Ends of Transcripts**

PCR amplification of cDNA in the

![Figure 2. Comparison of fluorescent and radioactive displays. RNAs extracted from three oral squamous cell carcinoma samples were reverse-transcribed using dTGGTCTCACGGATCCGTCGA (T)_{12} GG anchor primer, then PCR-amplified using the universal primer and dTGGATTGGTC in the presence or absence of radioactive dNTPs to generate the radioactive and fluorescent displays, respectively. Electropherograms are compared to the autoradiograph for three transcription profiles (Panels B–D). Panel A contains GS-500 size standards. Arrows indicate examples of equivalent bands. Labels i) and ii) indicate peaks present in transcription profile C but not B and D.](#)
presence of the universal primer, d[F]CTCACGGATCCGTCGA TTTT, alone did not yield significant numbers of peaks (see Figure 1, Panel I). Amplification of cDNA using the universal primer in combination with different arbitrary 10-mers resulted in the generation of significantly different fingerprints (compare Figure 1, Panels F–H with Panel I). In contrast, when a modified universal primer, d(F)TGGTCTCACGGATCCGTCGA, that lacks the 3′oligo(dT) anchoring sequence was used for FDDRT-PCR, multiple peaks were generated in the absence of arbitrary primer (Figure 1, Panel D). In the presence of different arbitrary 10-mer, transcription profiles were largely independent of the added arbitrary 10-mer, and 20-mer–20-mer amplification products dominated (compare Figure 1, Panels A–C with Panel D). This demonstrates that under these low-stringency PCR conditions, unanchored 20-mers behave in an arbitrary manner by randomly amplifying related sequences and that 20-mer–20-mer priming is rare with the universal primer. Therefore, to amplify from the oligo(dT) end of cDNAs, a short 3′oligo(dT) anchoring sequence at the end of the universal primer is essential.

Comparison of Fluorescent and Radioactive Displays

The major purpose of differential display, other than the characterization of mRNA populations by their banding profile, is to isolate genes that are differentially expressed. Bands analyzed by the fluorescent approach are not recoverable. For those instances, where bands need to be excised, a match between radioactive and fluorescent displays is required. To demonstrate that this can be achieved, RNAs extracted from three oral squamous cell carcinoma (SCC) samples were reverse-transcribed, then PCR-amplified using the universal primer and an arbitrary 10-mer in the presence or absence of radioactive dNTPs to generate radioactive and fluorescent displays, respectively. When electropherograms were compared to the autoradiograph for the three transcription profiles, close similarities were observed between the two (Figure 2, Panels B–D). Arrows indicate examples of equivalent bands. Labels [i] and [ii] indicate peaks present in transcription profile C but not B and D. Thus, bands present in the electropherograms are also present in the radioactive display from which they can be recovered for cloning or sequencing. Artifacts that are the result of arbitrary

![Figure 3. Identification of putative differentially expressed sequences.](image)
Identification of Putative Differentially Expressed Sequences

We have developed the method of automated differential display to generate and analyze large numbers of transcription profiles from large numbers of specimens with a view to identifying genes which are differentially expressed between normal, premalignant and malignant oral tissues. These may therefore serve as molecular markers for oral premalignancy. This approach should be generally applicable for the identification of markers of malignant progression. Transcription profiles generated from two oral SCCs (Figure 3, Panels A and B) and two matched normal oral tissue samples from the same patients (Figure 3, Panels C and D) using one primer pair are compared. Peaks a–c indicate sequences potentially down-regulated in both SCCs. Some differences were observed between samples of the same lesion group, i.e., between individual normal (d) and between individual malignant (e) tissues. Differences were not the result of differences in intra- and inter-assay reproducibility, as consistent FDDRT-PCR banding profiles were observed when a series of replica FDDRT-PCR experiments were performed on the same and different days using the same sample of total RNA (data not shown). These observations highlight the necessity of comparing multiple samples of the same type of lesion to identify consistent changes between lesion groups as opposed to rare artifacts manifest only in specific samples.

REFERENCES


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