Generation of Multiple mRNA Fingerprints Using Fluorescence-Based Differential Display and an Automated DNA Sequencer

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ABSTRACT

Differential display is a method for the survey, analysis and comparison of gene expression in eukaryotic cells and tissues. Differential display involves isolation of high-quality nondegraded RNA, selective reverse transcription of polyadenylated mRNA using specific anchored oligopolydeoxythymidine [oligo(dT)] primers, and the subsequent PCR amplification of the cDNA with the same oligo(dT), an arbitrary upstream primer and radioisotopes for labeling the PCR products. The radioisotopically labeled products are then separated on a sequencing gel. In this report, we describe a rapid, specific, nonradioactive fluorescent differential display methodology in which fluorescently differentially labeled anchored oligo(dT) downstream primers are used in the reaction, with subsequent analysis of fluorescently labeled PCR products on an automated sequencer. Complete gene expression profiles, containing multiple mRNA fingerprints are possible by the simultaneous comparison of the multicolored banding patterns of the fluorescently differentially labeled products from several primer combinations. This modification of the differential display technique simplifies the assay and increases the throughput of high sample volumes required for comparative gene expression studies in various clinical applications.

INTRODUCTION

The differential display technique was originally developed as a rapid, sensitive polymerase chain reaction (PCR) methodology to specifically amplify, compare and characterize altered gene expression events in eukaryotic cells (16). The technique has been utilized in studies of gene expression and comparisons in several different tissues or cell types. Differential display is particularly useful in the study of carcinogenesis and hormonal regulation at the transcriptional level (4,6,14,19,22,23). Moreover, this technique can be used as a particularly powerful technology for the study and determination of genes regulated at the transcriptional level in most types of cells from which polyadenylated mRNA can be isolated (1,8,26). An excellent outline of the basic strategies, technical setup and considerations for differential display is presented by Bauer et al. (3).

Several technical modifications of differential display have evolved for improvement of banding patterns and their detection (2,5,7,10,15,17,18,20), and differential display kits that utilize radioactive isotopes for band detection are commercially available.

Powerful capabilities of differential display allow for the rapid and sensitive survey of gene expression using small amounts of mRNA. Additionally, differentially expressed genes, often referred to as expressed sequence tags (ESTs), can be further characterized by DNA sequencing, Northern blotting and protein expression studies (13,21). Several technical characteristics of the differential display procedure, using traditional isotopic methods, require further consideration. These include *(i)* the potential for re-representation of the same expressed genes, or portions thereof, in two different ESTs (due to arbitrary primers), *(ii)* limited sizes of PCR amplicon represented (currently averaging 100–500 bp) and *(iii)* the requirement to run numerous sequencing gels to be able to observe which of the many possible primer combinations produce optimal differential banding patterns for the expression events being experimentally examined.

In this report, we describe a strategy and technique for multicolored fluorescent differential display PCR (FDD-PCR) that takes advantage of the specificity and selectivity of the differential fluorescence of three dye-labeled oligopolydeoxythymidine [oligo(dT)] primers. The selectivity and increase in specificity of differential display using an isotopically labeled oligo(dT) primer has been described (25).

Our strategy allows for a high-throughput survey, the simultaneous screening and comparison of the banding patterns generated from several different primer combinations and significant decreases in the expense and time of running and processing the many sequencing gels that would be involved if traditional isotopic methods were used. Differential fluorescence of the oligo (dT) primers, combined with the facile use of the Model 377 Automated Sequencer and GENESCAN™ software (PE Applied Biosystems, Foster City, CA, USA) features, significantly increases the capabilities of the investigator in the analysis of banding patterns. The ability to obtain a more complete profile of mRNA expression will be useful in molecular carcinogenesis,

molecular toxicology or other studies of differential gene expression.

MATERIALS AND METHODS

Human Cell Cultures, RNA Purifications and Analyses

Human CD4(+) cells stimulated with phytohemagglutinin (PHA) and Human CD4(+) cells co-stimulated with anti-CD3/anti-CD28 monoclonal antibodies, as described by Levine et al. (12) and Smithgall et al. (24) were used for RNA isolation. Total cellular RNA was isolated by extraction using the TriZOL™ LS reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's procedure. The isolated RNA was then treated with amplification grade DNase I (Life Technologies) according to the manufacturer's procedure, then re-extracted using the TriZOL LS reagent to assure further removal of any residual contaminating chromosomal DNA. The twice-extracted RNA was then spectrophotometrically quantitated and subsequently analyzed for integrity (lack of degradation) of the 28S, 18S and 5S ribosomal RNA bands, by electrophoresis of 3 µg of total RNA per lane on a 0.8% agarose midi-gel in TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.3) containing 0.5 µg/mL ethidium bromide (EtdBr), run at 95 V for 1.5 h.

Primers Used in Fluorescent Differential Display

HPLC-purified 5′ FAM-labeled oligo(dT_{12}VA), 5′ HEX-labeled oligo (dT_{12}VC) and 5′ TET-labeled oligo (dT_{12}VG) primers (V = A, G or C degeneracy) were obtained from Genosys Biotechnologies (The Woodlands, TX, USA). Arbitrary random decamers (OP-01 through OP-26) were obtained from Operon Technologies (Alameda, CA, USA).

Reverse Transcription and Fluorescent Differential Display Conditions

The reverse transcription (RT) of 300 ng of denatured RNA in a 20-µL reaction mixture was performed by incubation of the reaction mixture for 60 min at 42°C, followed by 5 min at 95°C and a 4°C hold, in the Model 9600 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). The reaction mixture contained 300 U SuperScript™ RNase H$^-$ Reverse Transcriptase (Life Technologies), 2.5 µM fluorescently labeled anchored oligo(dT), 25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 1.5 mM $MgCl_2$, 500 µM dNTPs and 10 mM dithiothreitol (DTT). Subsequently, 2 µL of the RT reaction were utilized in a 20-µL, final volume PCR containing 2.5 µM of the same fluorescently labeled anchored oligo(dT), 0.5 µM arbitrary decamer, 10 µM dNTPs, 1.7 mM $MgCl_2$ and 2.5 U AmpliTaq® (Perkin-Elmer). The samples were then amplified in a Model 9600 Thermal Cycler using 40 cycles of 94°C for 30 s for denaturing, 40°C for 2 min for annealing and 72°C for 30 s for extension, followed by one cycle of extension for 72°C for 5 min. Equal volumes of the different fluorescently labeled amplified differential display products were then mixed and prepared for electrophoresis.

Gel Electrophoresis and Analyses

Fluorescently labeled differential display products were prepared for gel loading as follows: *(i)* 2 µL of product directly from the PCR were combined with 2.5 µL of loading dye mixture containing formamide/blue dextran (5:1 vol/vol) and 0.5 µL of TAMRA-500 molecular weight standard (PE Applied Biosystems); *(ii)* the PCR product/loading dye mixture was then denatured by incubation for 2 min at 90°C in a Model 9600 Thermal Cycler, and 1.5 µL of the denatured PCR mixture was loaded into the wells of a 36-cm, 4.5% acrylamide gel containing 6 M urea and 1× TBE buffer; and *(iii)* electrophoresis was carried out at 2500 V for 3 h at 51°C using GENESCAN for data collection and virtual filter set C on the Model 377 Automated Sequencing Apparatus (PE Applied Biosystems).

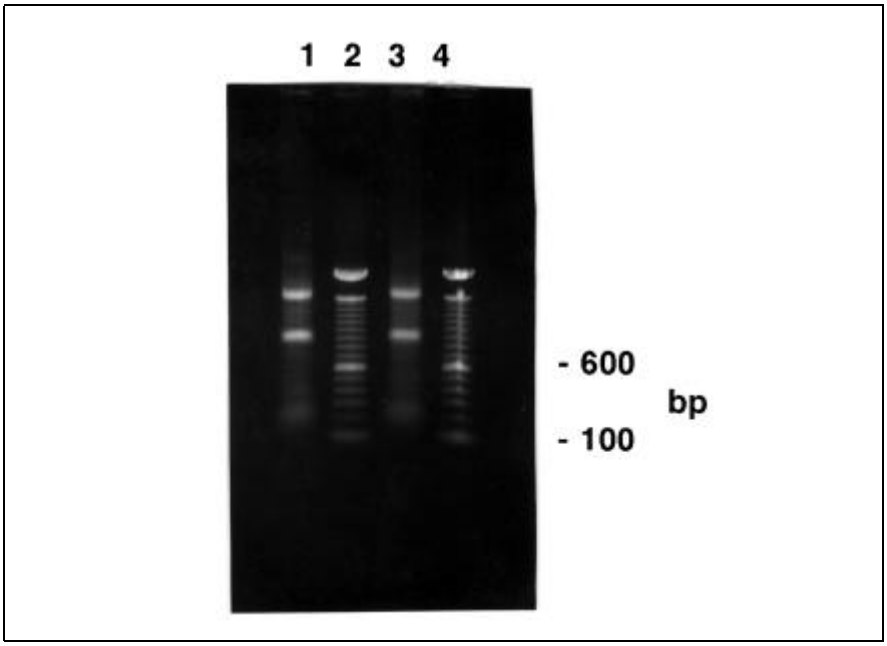

Figure 1. EtdBr-stained agarose gel. Each lane contains 3 µg of total RNA from the following: lane 1, CD4(+) PHA-stimulated cells and lane 3, CD4(+) cells co-stimulated with anti-CD3/CD28 antibodies. Lanes 2 and 4 contain 1 µg each of 100-bp DNA ladder.

RESULTS AND DISCUSSION

Differential display was carried out using high-quality RNA samples isolated from CD4(+) cells cultured and stimulated with PHA and from CD4(+) cells cultured and co-stimulated with anti-CD3/CD28 antibodies as described by Levine et al. (12). CD4(+) cells need to be stimulated for propagation (12). After the RNA was twice extracted with TriZOL LS to ensure removal of contaminating chromosomal DNA and RNases, the RNA was quantitated and electrophoresed to determine its integrity, as indicated by the

lack of degradation of the ribosomal RNA bands (Figure 1). The ribosomal bands are clearly distinct and would appear to be smeared if RNA degradation by RNase contamination had occurred. The RNA was then reverse-transcribed using separate RT reactions for each fluorescently labeled oligo(dT). The strategy for the fluorescent differential display setup is shown in Figure 2. After RT, 2 µL of cDNA product from each RT reaction were added to duplicate PCRs containing the same fluorescently labeled oligo(dT) and one of the 26 arbitrary decamers (Figure 2). PCR was performed with three differentially labeled oligo(dT)s, 26 different arbitrary decamers and two RNA samples, in duplicate, with appropriate experimental controls. Several negative controls were necessary: a reagent control fluorescent differential display (no RNA in RT-PCR mixture), fluorescent differential display using 300 ng of RNA in PCR (RNA only, no reverse transcription) and the reverse-transcribed RNA (no PCR amplification). All negative controls demonstrated no banding patterns (data not shown).

The fluorescent primers used in the RT and PCR were the same sequences as those previously described by Liang and Pardee (16) except that oligo-(dT_{12}VA) was 5′-labeled with 6-FAM (6-carboxyfluorescein), oligo(dT_{12}VC) was 5′-labeled with HEX (4,5,2′,4′, 5′,7′-hexachloro-6-carboxyfluorescein) and oligo(dT_{12}VG) was 5′-labeled with TET (4,7,2′,7′-tetrachloro-6-carboxyfluorescein). These are dyes compatible with the Model 377 Automated Sequencer. Oligo(dT_{12}VT) was not used due to the lack of availability of a fourth dye for use in simultaneous analyses (the other, TAMRA is used as a label for the molecular weight marker) and the potential for smearing, which is due to lack of specificity of oligo(dT_{12}VT), as previously described (15).

Figure 3A shows a gel file from the Model 377 demonstrating fluorescent differential display products. Each two lanes represents a PHA-stimulated and an anti-CD3/CD28 antibody co-stimulated CD4(+) cellular RNA sample, which is reverse-transcribed with each differentially labeled oligo(dT) and then amplified with the same oligo(dT) and an arbitrary decamer. Only arbitrary decamers OP-01 through OP-18 were shown in this gel picture; however, the presence of the TAMRA-500 molecular weight standard in all lanes permits comparisons across gels. Similarly, application of a fluorescent dye matrix to all gels controls for quantitation and standardization of the dye labels, 6-FAM, HEX and TET, throughout the analyses. The differential fluorescence and application of dye matrix as a control allows for the simultaneous or individual comparison of the multicolored fluorescent dye labels involved in the differential banding and peak heights using the GENESCAN software (Figure 3, A and B).

Comparison of gene expression from CD4(+) cells stimulated with PHA and CD4(+) cells that were co-stimulated with anti-CD3/CD28 antibodies has indicated that one treatment, the co-stimulated, yielded more bands with the HEX-labeled primer, [oligo (dT_{12}VC)] than the other treatment. This is indicative of a potentially more C-rich message. These subtle differ-

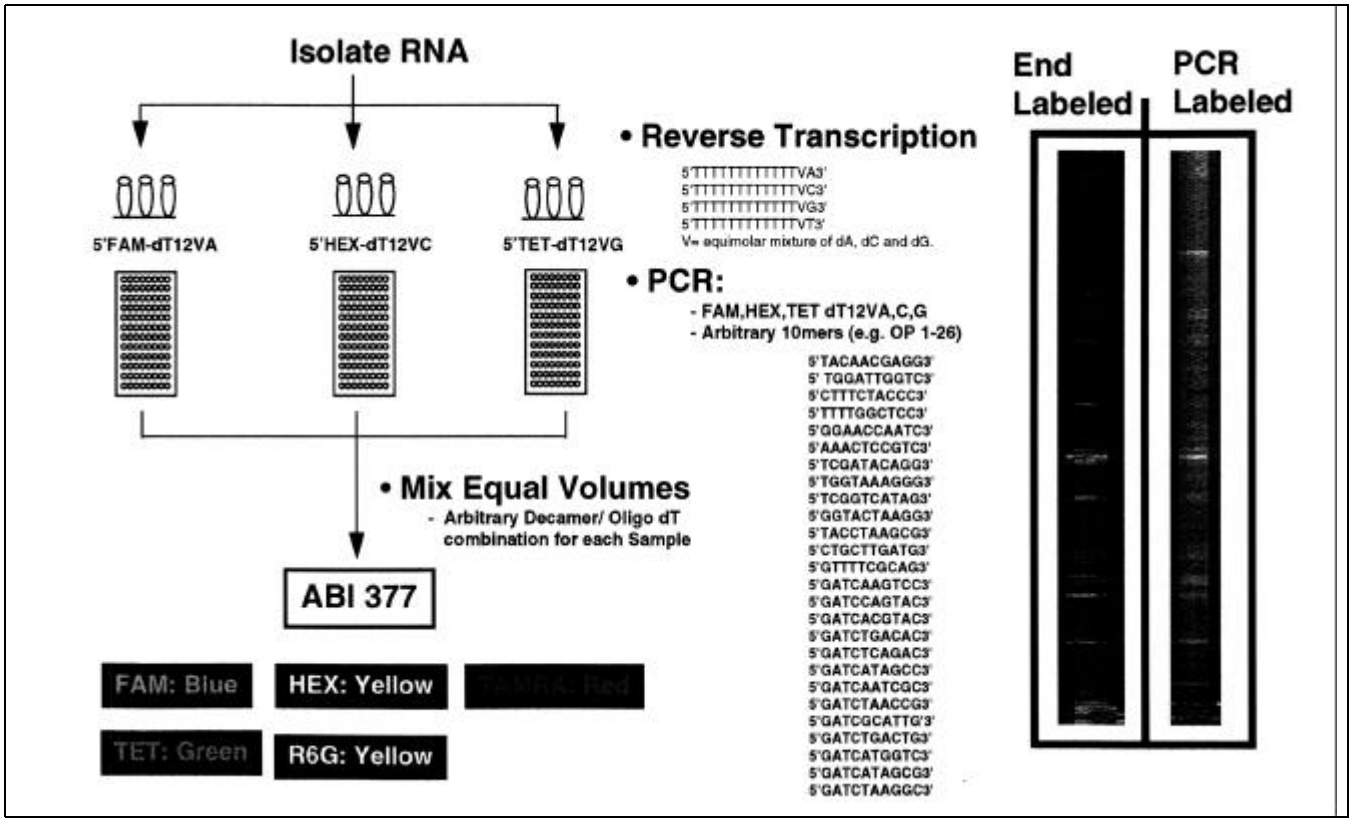

Figure 2. Schematic diagram of strategy used in fluorescent differential display.

ences may be quantitated at a secondary level with the appropriate software to determine the percentage of bands that are detected with this label, thus giving additional information to add to the mRNA profile. The fluorescent differential display technique is not quantitative, due to the different relative efficiences of priming of the arbitrary decamers and the need for an internal amplification standard.

Nonisotopic differential display methodologies have been reported by other investigators (2,19). Ito et al. (11) described a fluorescent differential display technique in which fluorescein isothiocyanate (FITC)-labeled oligo(dT)s were utilized with random arbitrary primers in the reaction. Differential gene expression was detectable; however, fewer messages would be represented with the use of single oligo(dT)s and single fluorescent label. Bauer et al. (2) described a procedure in which the arbitrary upstream primers (decamers) were labeled with PE Applied Biosystems' dyes on a Model 373 apparatus. This technique presents the possibility of detecting nonspecific fluorescently labeled PCR amplification products generated from two fluorescently labeled arbitrary upstream primers. These products would not be detected in the strategy presented in this report, as the arbitrary decamers are not fluorescently labeled. Neither of the strategies mentioned above, however, involved multiple differentially labeled oligo(dT)s, amplified in a PCR array representing all possible combinations of oligo(dT) and arbitrary decamers, with the simultaneous detection of differentially labeled fluorescent differential display bands.

We have presented a novel application of the PE Applied Biosystems fluorescent dye labels for use in a rapid, enhanced and specific technique useful for high-throughput screening of differential gene expression. Using these techniques, more complete gene expression profiles can be generated using multiple primer-pair combinations to provide optimal differential gene expression banding patterns, which are detected by using PE Applied Biosystems' automated fluorescent sequencing technology. These techniques will be very useful in large clinical molecular genetic studies for the elucidation of new diagnostic and therapeutic markers relevant to diseases, such as cancer, or for isolating differentially expressed genes in genome research or in determining the effect of specific toxicologic chemical compounds on gene expression.

Finally, we have successfully isolated and sequenced fluorescently labeled EST's directly from FDDPCRs. Of the several experimental approaches, prototype capillary electrophoresis and

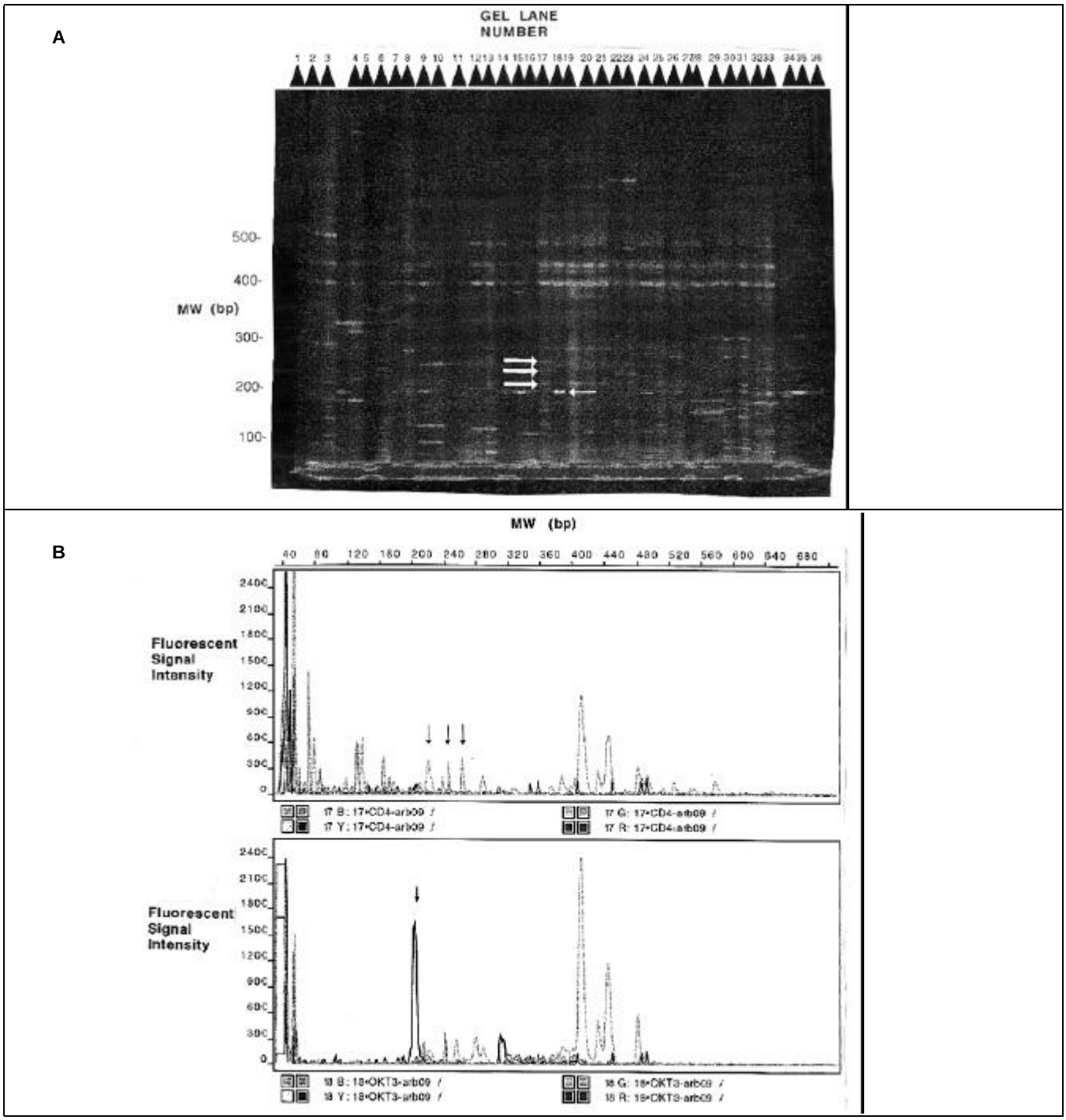

Figure 3. Sample gel and electropherogram of sample lanes. (A) Sample fluorescent differential display gel. RNA isolated was reverse-transcribed using fluorescently labeled oligo(dT) primers and amplified with different upstream arbitrary decamers by PCR. Odd lanes 1–35 differential display of CD4(+) PHA-stimulated cells; even lanes 2–36 differential display of CD4(+) cells co-stimulated with CD3/CD28 antibodies. Arbitrary upstream decamers were amplified with each fluorescently labeled oligo(dT), for each RNA sample tested, as described in the text. Each two lanes on this gel shows the banding patterns for the comparison of PHA-stimulated and anti-CD3/CD28 co-stimulated CD4(+) RNA, respectively, for each consecutive arbitrary decamer represented on this gel. Such as, lanes 1 and 2 (OP-01), lanes 3 and 4 (OP-02), lanes 5 and 6 (OP-03), lanes 7 and 8 (OP-04), lanes 9 and 10 (OP-05), lanes 11 and 12 (OP-06), lanes 13 and 14 (OP-07), lanes 15 and 16 (OP-08), lanes 17 and 18 (OP-09) ... lanes 35 and 36 (OP-18). Only arbitrary decamers OP-01 through OP-18, for both RNA samples tested, are shown on this gel. When comparing the differential display for these two RNA samples (see arrows, lanes 17 and 18, respectively), differential banding was particularly noticeable for arbitrary primer OP-09. (B) GENESCAN electropherogram of sample lanes in differential display gel. This is an electropherogram showing the fluorescent banding peaks corresponding to differentially labeled fluorescent bands from the gel, in lanes 17 and 18, with arbitrary decamer OP-09 and RNA from CD4(+) PHA-stimulated cells and CD4(+) cells co-stimulated with anti-CD3/CD28 antibodies. The molecular weight in base pairs is demonstrated across horizontal axis at the top of the electropherogram, and the relative peak fluorescent intensity is along the vertical axis. Arrows demonstrate fluorescent peaks of differential banding corresponding to the bands shown (see arrows) on the gel file, Panel A.

fraction collection have proven most useful because of ease of automation. Our findings for full automation of this high-throughput gene expression discovery system (GEDS^{SM}) as well as its application to the discovery of novel genes of extremely low, medium and high abundance levels will be reported elsewhere (9).

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