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Journal of Chromatography B, 695 (1997) 93–102

JOURNAL OF CHROMATOGRAPHY B

Capillary electrophoresis methodology for identification of cancer related gene expression patterns of fluorescent differential display polymerase chain reaction

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Abstract

The mRNA differential display technique is a method for molecular survey and analysis of differential gene expression in eukaryotic cells and tissues. We have previously described the use of ABI Prism fluorescent technology to specifically amplify expressed sequence tags (ESTs) from several different biological paradigms. High throughput, fluorescent differential display performed on an automated sequencer (ABI 377) has proven to have significant cost cutting and time saving attributes compared to that of the radioactive differential display. Additionally, fluorescent tagged mRNA specific reverse transcription and PCR decreases the number of the inherent artifacts associated with radioactive differential display. We report here the application of a capillary electrophoresis system (ABI 310) to the identification of fluorescent differential display generated EST patterns. RNA samples from human and animal breast cancer paradigms were exposed to this technique and analyzed by the ABI 310 and the ABI 377. GeneScan and Genotyper software applications were used for rapid and semi-automated characterization of fluorescently labeled ESTs. Automated sample loading and uniform sample electrophoresis are among the main advantages of this system which significantly increase the precision and reproducibility of fluorescent differential display.

Keywords: Fluorescent differential display polymerase chain reaction; RNA

1. Introduction

Differential display PCR, originally described by Liang and Pardee [1], has been employed as a methodology for the study of altered gene expression events in a variety of cell types and tissues. In this technique, RNA from two or more samples is reverse transcribed using a 3′ anchored oligo-dT primer and PCR amplified using the same oligo-dT primer and a random arbitrary primer. Incorporation of radio-labeled nucleotides in the PCR reaction allows the resulting PCR products, representing a subset of the total mRNA, to be separated by polyacrylamide gel electrophoresis and visualized by autoradiography. Differential display has proven useful in the identification of genes involved in an array of biological processes including differentiation, hormonal regulation, apopotosis and carcinogenesis [2–11].

The traditional isotopic methods for differential display are both time consuming and labor intensive. As a result, numerous technical modifications and improvements including nonisotopic and fluorescent

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PII S0378-4347(97)00115-1

methodologies have been reported [12–20]. We have recently reported a fluorescent adaptation to the standard reaction that takes advantage of the specificity, selectivity and differential fluorescence of three dye labeled oligo-dT primers [21]. The advantages of this high throughput technique include decreased time and expense as compared to traditional isotopic methods and the ability to simultaneously screen and compare banding patterns from three different primer combinations. We have utilized this approach to identify expression events in a wide range of samples including bacteria, breast cancer cell lines, human cells and tumor samples.

In this report, we describe the use of the Perkin-Elmer ABI 310 Genetic Analyzer (capillary electrophoresis system) for the detection and identification of EST patterns generated using the fluorescent differential display technique. The ABI 310 uses a flowable polymer for uniform separation and automatically loads the samples. Using a human breast cancer cell line and a mouse mammary tumor model system, we have compared the patterns generated on both the ABI 377 and the ABI 310 instruments. The data generated on the ABI 310 was further analyzed using the ABI GeneScan and Genotyper software for semi-automated pattern recognition and comparison.

2. Experimental

2.1. Tissue culture and RNA isolation

MCF7 breast adenocarcinoma cells (ATCC HTB-22) were grown in a humidity controlled incubator at 37°C and 5% CO_2 in Dulbeccos modified Eagles medium with 1 m*M* sodium pyruvate, 10 μg/ml bovine insulin and 10% fetal bovine serum. Total RNA was isolated from cell monolayers using Trizol LS (Life Technologies, Gaithersburg, MD, USA) according to the manufacturers protocol. Mouse mammary tumor samples A and B were homogenized and total RNA extracted as above. The isolated RNA was treated with amplification grade DNase I (Life Technologies) according to the manufacturers protocol and was subsequently reextracted using Trizol LS.

2.2. Primers

HPLC purified 5′-6-carboxyfluorescein (6-FAM) labeled oligo-dT_{12}VA, 5′-4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX) labeled oligo-dT_{12}VC and 5′-4,7′,2′,7′-tetrachloro-6-carboxyfluorescein (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). The sequences of these primers can be found in Table 1.

2.3. Reverse transcription and fluorescent differential display conditions

All RNA samples were reverse transcribed using 300 ng of denatured total RNA in a 20-μl reaction containing 50 m*M* Tris–HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* DTT, 500 μ*M* dNTPs, 2.5

Table 1
Sequences of the arbitrary decamers

OP-01	5′-TACAACGAGG-3′
OP-02	5′-TGGATTGGTC-3′
OP-03	5′-CTTTCTACCC-3′
OP-04	5′-TTTTGGCTCC-3′
OP-05	5′-GGAACCAATC-3′
OP-06	5′-AAACTCCGTC-3′
OP-07	5′-TCGATACAGG-3′
OP-08	5′-TGGTAAAGGG-3′
OP-09	5′-TCGGTCATAG-3′
OP-10	5′-GGTACTAAGG-3′
OP-11	5′-TACCTAAGCG-3′
OP-12	5′-CTGCTTGATG-3′
OP-13	5′-GTTTTCGCAG-3′
OP-14	5′-GATCAAGTCC-3′
OP-15	5′-GATCCAGTAC-3′
OP-16	5′-GATCACGTAC-3′
OP-17	5′-GATCTGACAC-3′
OP-18	5′-GATCTCAGAC-3′
OP-19	5′-GATCATAGCC-3′
OP-20	5′-GATCAATCGC-3′
OP-21	5′-GATCTAACCG3′
OP-22	5′-GATCGCATTG-3′
OP-23	5′-GATCTGACTG-3′
OP-24	5′-GATCATGGTC-3′
OP-25	5′-GATCATAGCG-3′
OP-26	5′-GATCTAAGGC-3′

μ*M* fluorescently labeled oligo-dT and 300 units of Superscript RNase H reverse transcriptase (Life Technologies) at 42°C for 60 min followed by 5 min at 95°C and a 4°C hold in a Perkin-Elmer Model 9600 thermocycler (Perkin-Elmer ABI, Foster City, CA, USA). Reverse transcription was followed by PCR amplification of 2 μl of the cDNA in a 20-μl reaction containing 20 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 1.7 m*M* $MgCl_2$, 3.35 μ*M* dNTPs, 2.5 μ*M* of the same fluorescently labeled oligo-dT, 0.5 μ*M* arbitrary decamer and 2.5 units of Amplitaq (Perkin-Elmer). Reactions were cycled in a Perkin-Elmer Model 9600 thermocycler for 40 cycles of 94°C for 30 s, 40°C for 2 min and 72°C for 30 s, followed by a 5-min extension at 72°C. Equal volumes of the three differentially labeled fluorescent PCR products were mixed and purified using Centrisep columns (Princeton Separations, Adelphi, NJ, USA). All samples were dried down in a Speed Vac and resuspended in 6 μl of sterile distilled water.

2.4. Gel electrophoresis – ABI 377

An ABI 377 automated sequencer (Perkin-Elmer ABI) was utilized for polyacrylamide slab gel electrophoresis. A 2.0-μl aliquot of the FDDPCR product was combined with 2.5 μl of loading dye mix [formamide–blue dextran (5:1)] and 0.5 μl of GeneScan TAMRA-500 molecular mass standard (Perkin-Elmer ABI). The mixture was denatured for 2 min at 90°C, placed on ice, and 1.5 μl of the mixture was loaded onto a 0.2 mm thick, 36 cm, 4.5% polyacrylamide gel containing 6 *M* urea and 1X TBE. The samples were electrophoresed at 3000 V for 3 h at 51°C using GeneScan run module GS-36C. The ABI Prism dyes were excited by an

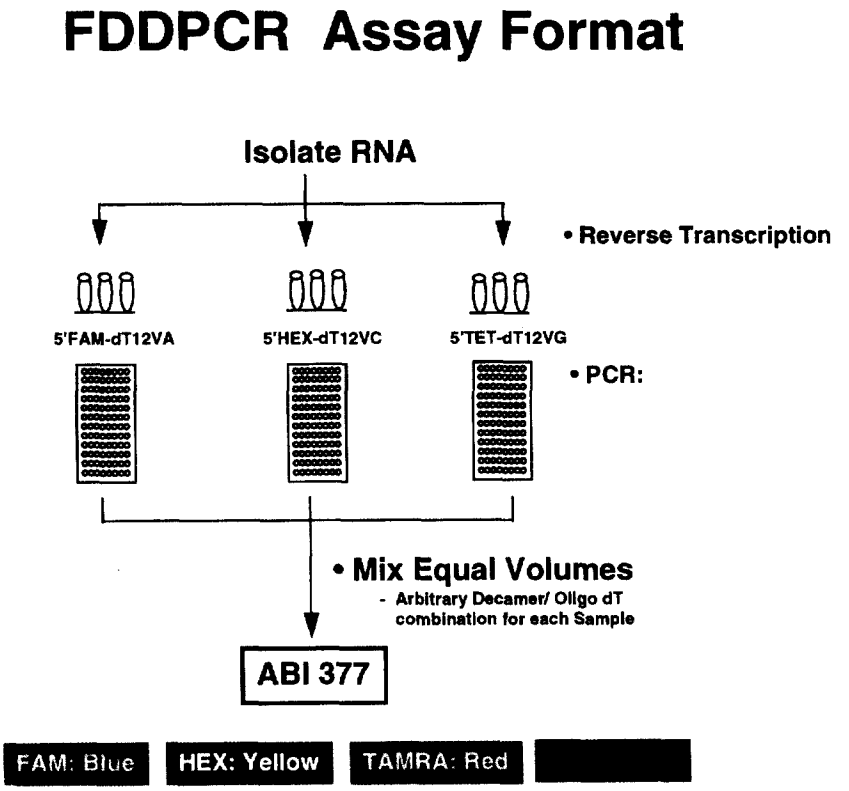


Fig. 1. Flow chart of the scheme used for Fluorescence Differential Display PCR (FDDPCR).

argon ion laser and detected using virtual filter C. Data was analyzed using the ABI Genescan analysis software (Perkin-Elmer ABI).

2.5. Capillary electrophoresis – ABI 310

The ABI 310 Genetic analyzer (Perkin-Elmer ABI) was utilized for capillary electrophoresis. Samples were prepared by mixing a 0.5-μl aliquot of the FDDPCR product with 8 μl of a formamide–TAMRA-500 (10:1) molecular mass standard mix. The samples were denatured for 2 min at 90 C, placed on ice, loaded into a sample rack and placed onto the Perkin-Elmer ABI model 310 Genetic

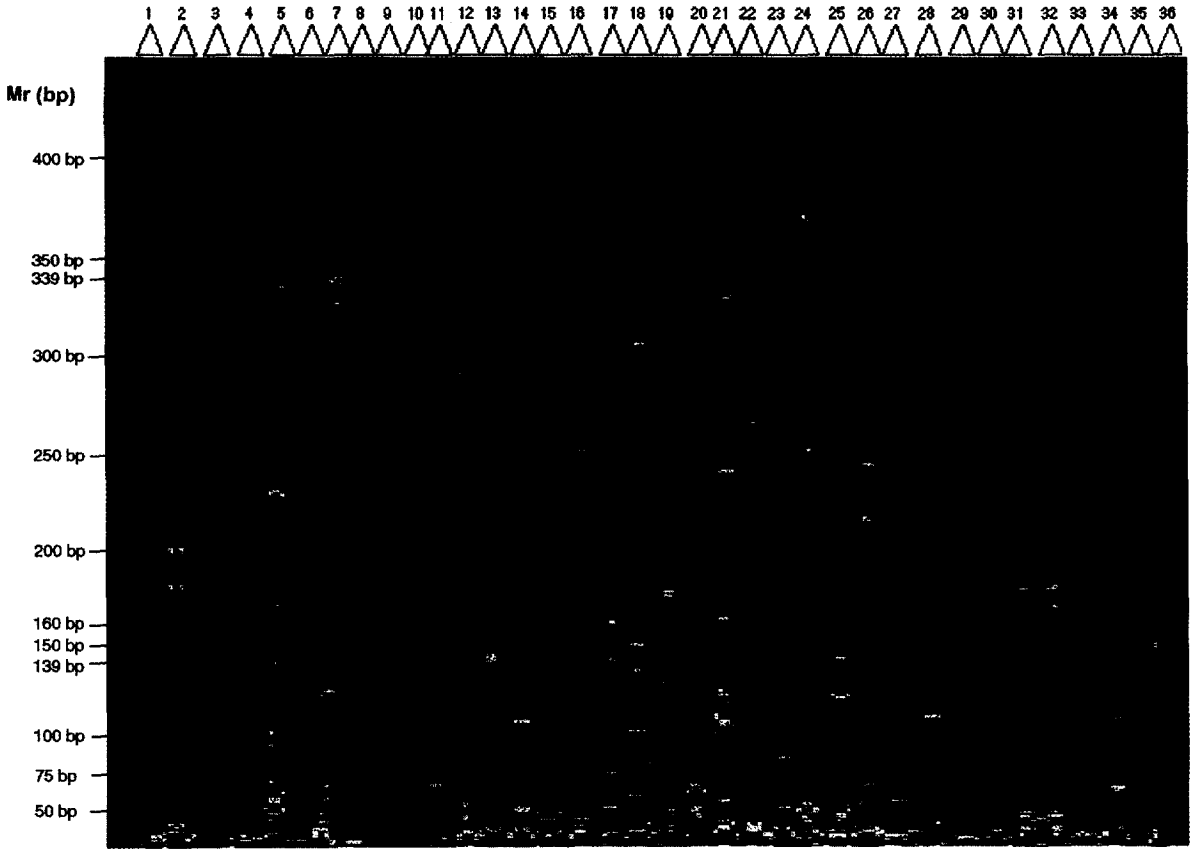
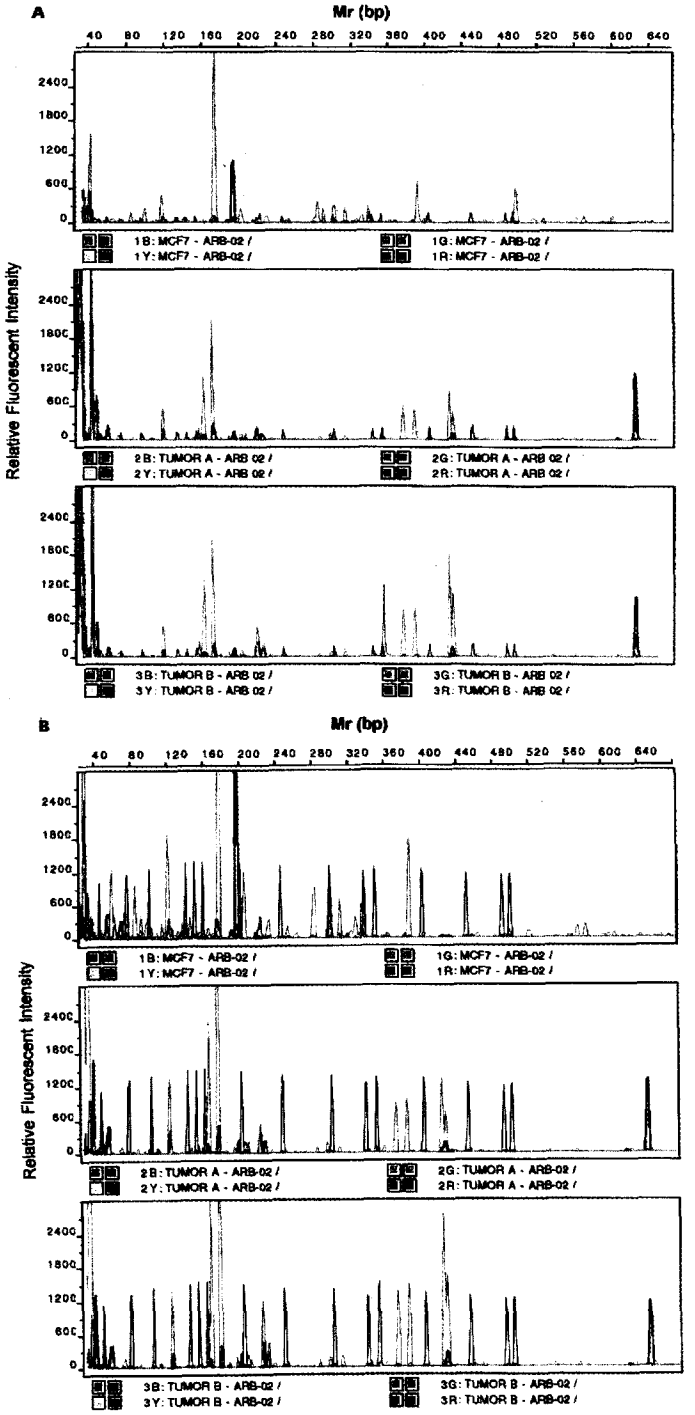


Fig. 2. Representative Fluorescent Differential Display gel. Total RNA was reverse transcribed using fluorescently labeled oligo-dT primers and PCR amplified with different upstream arbitrary decamers. Lanes 1–8 (OP-01–OP-08) and 12–28 (OP-10–OP26) and 35 (OP-09) MCF7 human breast adenocarcinoma cells, odd lanes 29–33 mouse mammary tumor A and even lanes 30–34 mouse mammary tumor B. Each two lanes of lanes 29–34 show the banding patterns for the comparison of highly related mouse mammary tumor samples. Lanes 29, 30 (OP-01), lanes 31, 32 (OP-02) and lanes 33, 34 (OP-05). Differential banding was observed for arbitrary OP-02 when comparing the fluorescent differential display patterns of these two RNA samples.

Fig. 3. GeneScan electropherograms of FDDPCR samples. (A) GeneScan electropherogram of samples lanes run on the ABI 377. The electropherograms show the fluorescent peaks corresponding to differentially labeled fluorescent bands observed in the representative gel (Fig. 2, lanes 2, 29 and 30). Patterns generated using arbitrary decamer OP-02 and RNA from MCF7 cells (top panel) and mouse mammary tumor samples A (middle panel) and B (bottom panel) are shown. The molecular mass in base pairs is displayed across the horizontal axis at the top of the electropherogram and the relative fluorescent intensity is along the vertical axis. (B) GeneScan electropherogram of samples run on capillary gel electrophoresis (ABI 310). These electropherograms show the fluorescent peaks corresponding to differentially labeled fluorescent bands from MCF7 (top panel) and mouse mammary tumor samples A (middle panel) and B (bottom panel), respectively, amplified with arbitrary decamer OP-02. The molecular mass in base pairs is displayed across the horizontal axis at the top of the electropherogram and the relative peak fluorescent intensity is along the vertical axis.


A
Mr (bp)
40 80 120 160 200 240 280 320 360 400 440 480 520 560 600 640
2400
1800
1200
600
0
1B: MCF7 - ARB-02 /
1Y: MCF7 - ARB-02 /
1G: MCF7 - ARB-02 /
1R: MCF7 - ARB-02 /
Relative Fluorescent Intensity
2B: TUMOR A - ARB 02 /
2Y: TUMOR A - ARB 02 /
2G: TUMOR A - ARB 02 /
2R: TUMOR A - ARB 02 /
3B: TUMOR B - ARB 02 /
3Y: TUMOR B - ARB 02 /
3G: TUMOR B - ARB 02 /
3R: TUMOR B - ARB 02 /
B
Mr (bp)
40 80 120 160 200 240 280 320 360 400 440 480 520 560 600 640
2400
1800
1200
600
0
1B: MCF7 - ARB-02 /
1Y: MCF7 - ARB-02 /
1G: MCF7 - ARB-02 /
1R: MCF7 - ARB-02 /
Relative Fluorescent Intensity
2B: TUMOR A - ARB-02 /
2Y: TUMOR A - ARB-02 /
2G: TUMOR A - ARB-02 /
2R: TUMOR A - ARB-02 /
3B: TUMOR B - ARB-02 /
3Y: TUMOR B - ARB-02 /
3G: TUMOR B - ARB-02 /
3R: TUMOR B - ARB-02 /

Analyzer. Samples were injected at 15 000 V for 5 s onto a 47-cm capillary (36 cm to detector) containing POP-4 polymer (Perkin-Elmer ABI). Electrophoresis was performed at 15 000 V for 30 min at 60°C. The ABI Prism dyes were excited by an argon ion laser and detected using virtual filter C. Data was analyzed using the ABI Genescan analysis software (Perkin-Elmer ABI).

3. Results and discussion

Fluorescent differential display was performed using high quality RNA isolated from MCF7 human breast adenocarcinoma cell monolayers and from paired mouse mammary tumor samples A and B. As shown in Fig. 1, RNA was reverse transcribed using separate reactions for each of the three fluorescently labeled oligo-dT primers. These cDNAs were then PCR amplified with three different fluorescently labeled oligo-dTs and twenty-six different arbitrary decamers in duplicate. Appropriate experimental controls were performed including reagent controls for both the reverse transcription and PCR amplification, reverse transcription with no PCR amplification and PCR amplification of total RNA. All controls demonstrated no banding patterns (data not shown).

The fluorescently labeled differential display products were analyzed by both the ABI 377 (acrylamide gel electrophoresis) and the ABI 310 (capillary electrophoresis). A representative gel file, from the ABI 377, displaying fluorescent differential display patterns generated with MCF7 human breast adenocarcinoma cells and paired mouse mammary tumor samples is shown in Fig. 2. Unique patterns were

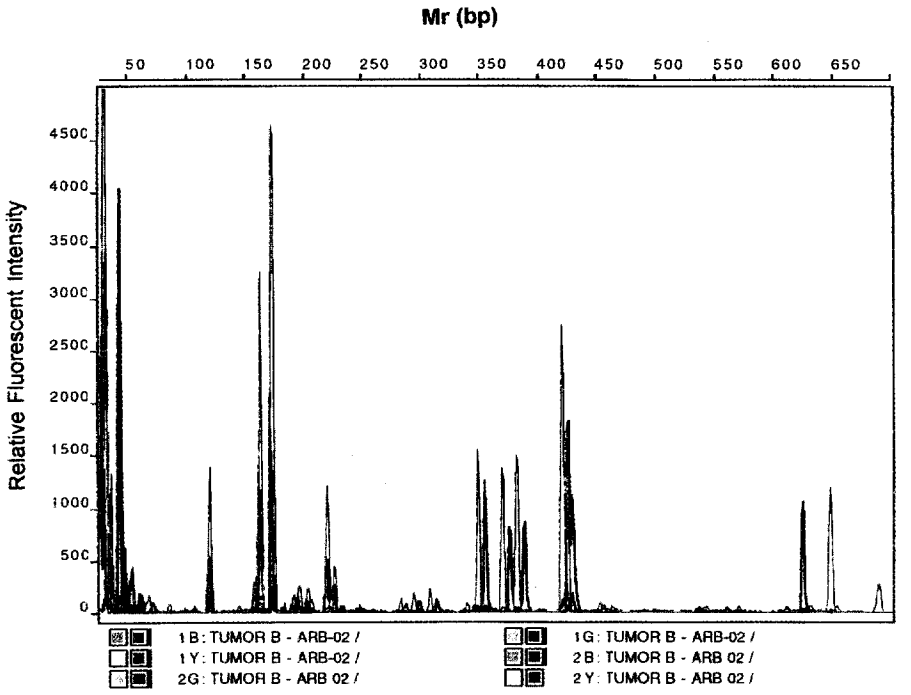


Fig. 4. Overlay of a sample run on the ABI 377 and ABI 310. Overlay of mouse mammary tumor sample B and arbitrary primer OP-02 patterns generated on the ABI 377, acrylamide gel electrophoresis and the ABI 310, capillary gel electrophoresis. The red peaks represent data generated on the ABI 310 and the black peaks represent data generated on the ABI 377. This figure demonstrates the importance of the internal size standard for comparisons between the two instruments. The molecular mass in base pairs is displayed across the horizontal axis at the top of the electropherogram and the relative peak fluorescent intensity is along the vertical axis.

observed for amplifications with each of the twenty-six arbitrary decamers and MCF7 cells. Comparison of the paired mouse mammary tumor samples revealed a unique differentially represented band in amplifications with arbitrary decamer OP-02 as observed in lanes 31 and 32.

An internal TAMRA-500 molecular mass standard was included in all samples run on both the ABI 377 and the ABI 310 to permit identification of unknown fragments based on the mobility of known fragments. Utilizing these internal mobility mass standards the GeneScan software is able to compensate for variations observed from run-to-run that may be due to multiple parameters, for example, variation due to the use of different polymers for electrophoresis.

Similarly, a fluorescent dye matrix was applied to all samples to control for quantitation and standardization of the dye labels, 6-FAM, HEX and TET, throughout the analyses. The differential fluorescence and application of a dye matrix allow for the simultaneous or individual comparison of the multi-colored fluorescent dye labels involved in the differential banding and peak heights using the ABI GeneScan analysis software.

The electropherograms of the fluorescent differential display products from human MCF7 cells and mouse mammary tumor samples A and B with arbitrary decamer OP-02 are compared in Fig. 3A. Capillary electrophoresis of the same samples revealed similar patterns (Fig. 3B). Comparisons of numerous samples run on both instruments revealed similar results suggesting that either instrument can be utilized for the analysis of fluorescent differential

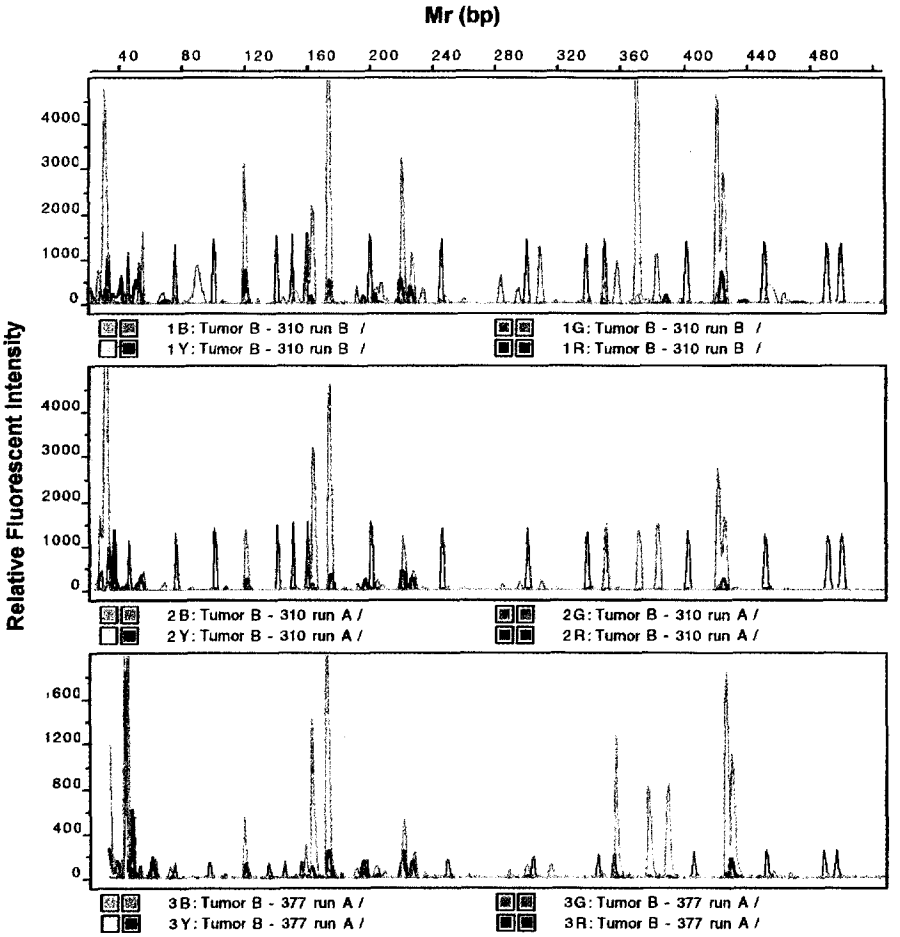
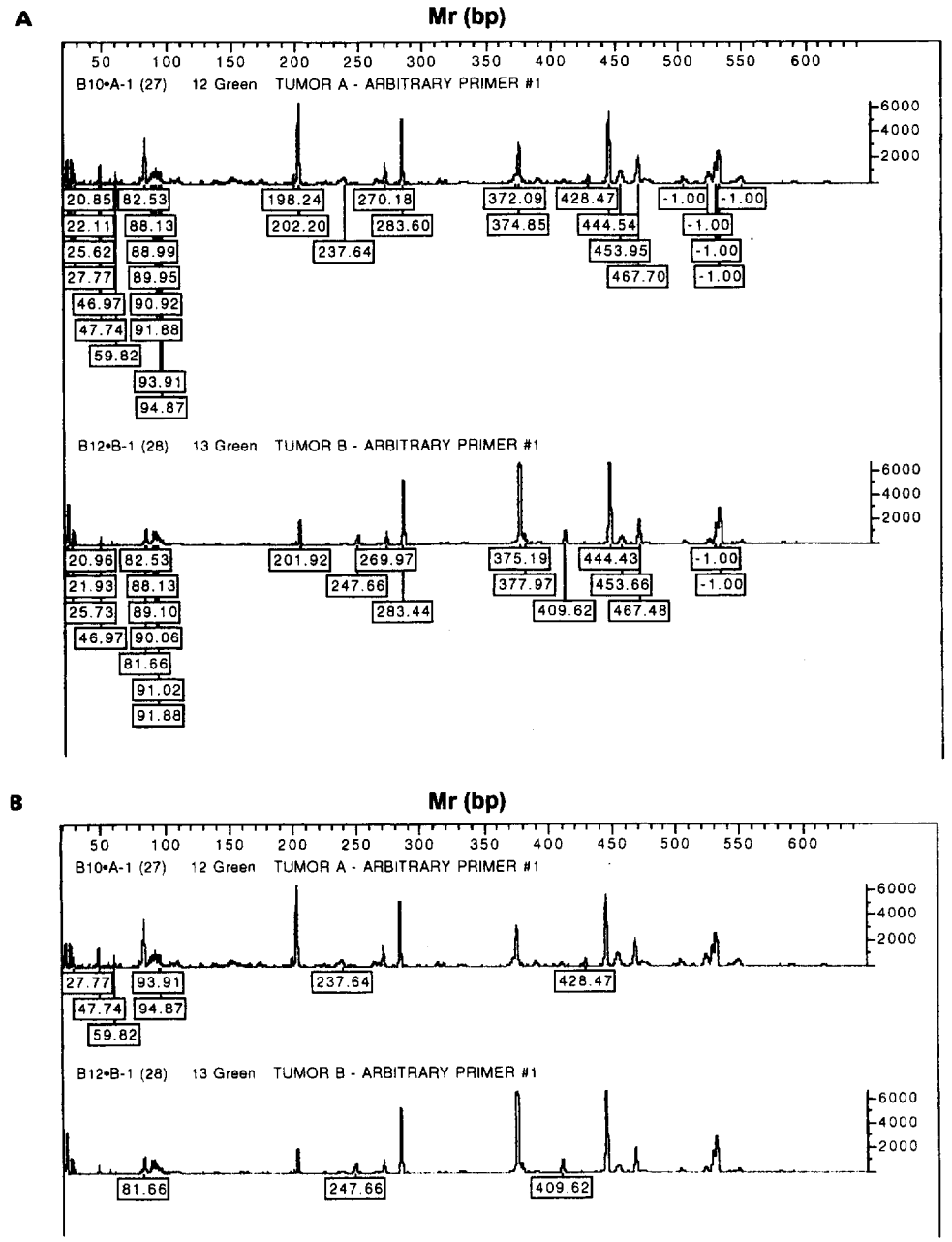


Fig. 5. Reproducibility of FDDPCR patterns. Electropherograms demonstrating the reproducibility of FDDPCR patterns from run-to-run. PCR products generated in reactions with tumor sample B and arbitrary primer OP-02 were run on two separate occasions on the ABI 310 capillary electrophoresis system (top and middle panels) and on the ABI 377 slab gel electrophoresis unit (bottom panel). The molecular mass in base pairs is displayed across the horizontal axis at the top of the electropherogram and the relative peak fluorescent intensity is along the vertical axis.



C

File Name	Dye	Sample Info	Label						
B10•A-1 (27)	B	TUMOR A - ARBITRARY PRIMER #1	25.53	39.04	54.74	82.31	95.94	202.34	388.11
B10•A-1 (27)	G	TUMOR A - ARBITRARY PRIMER #1	27.77	47.74	59.82	93.91	94.87	237.64	428.47
B12•B-1 (28)	G	TUMOR B - ARBITRARY PRIMER #1	81.66	247.66	409.62				

Fig. 6. Genotyper analysis of samples run on the ABI 310. Genotyper analysis of fluorescent differential display products from mouse mammary tumor samples A and B with arbitrary decamer OP-1 run on the ABI 310. (A) Identification and labeling of all peaks for TET labeled (green) fluorescent peaks using Genotyper. Labels reflect the molecular mass of each peak in base pairs. (B) Subtraction of labels for all common peaks identified above. Labeled peaks reflect the differences between tumor A and B. (C) Table representing all of the unique peaks identified in comparisons of the patterns from tumor A and B. Unique peaks with FAM (blue) and TET (G) fluorescent labels are represented in the table. No unique peaks were observed for HEX (yellow).

display products. Differences observed in the relative peak intensity among samples run on the ABI 377 and the ABI 310 (Fig. 3A and 3B) are most likely due to a difference in the amount of PCR product loaded. The capillary electrophoresis system loads samples based on injection time and voltage, therefore, the actual volume loaded may differ from that loaded onto the ABI 377 acrylamide gel.

Fig. 4 displays an overlay of the electropherogram data for mouse mammary tumor B with oligo-dT_{12}VA and arbitrary decamer OP-02. The patterns generated using both the ABI 377 and ABI 310 were identical, however, a slight difference in migration was observed. This shift is probably a result of subtle differences in the electrophoresis matrix, as 4.5% polyacrylamide was utilized on the ABI 377 and POP-4 polymer was used for the ABI 310. The reproducibility of the fluorescent differential display patterns was further examined by repeated analysis of identical samples on both instruments. Comparisons of the resulting electropherograms, using the internal molecular mass standards as a reference, revealed that the patterns were highly reproducible from run-to-run and from the ABI 310 to the ABI 377 (Fig. 5). The results indicate that analysis of fluorescent differential display products can be easily adapted to capillary electrophoresis on the ABI 310.

The data generated on the ABI 310 was subjected to further analysis using the Genotyper Genetic Analysis software (Perkin-Elmer ABI). This software has the capability to compare the electropherogram data for each individual dye, to identify and label the unique peaks, and place the data into a tabular format. The results of the analysis of mouse mammary tumor B with oligo-dT_{12}VG and arbitrary decamer OP-02 are demonstrated in Fig. 6. This group of figures demonstrates the stepwise process involved in the comparison of the data including (1) identification of all of the peaks, (2) subtraction of labels for all common peaks and (3) tabular display of the data including the fluorescent dye examined and molecular masses of the unique bands identified. The Genotyper software allows for rapid and semi-automated comparison of the fluorescent differential display patterns which eliminate the need for subjective visual comparisons. The use of genetic analysis software packages such as the ABIs Genotyper software will increase the throughput of this technique allowing for more rapid recognition and identification of unique ESTs.

We have presented data indicating that fluorescent differential display can be easily adapted to the ABI 310 and that analysis can be performed with equal precision and reproducibility on both the ABI 377 and the ABI 310. Both of these methodologies provide a higher throughput means for sample screening than the conventional radioactive differential display. The main advantages of using the ABI 310 are the automated sample loading and uniform sample electrophoresis. An additional benefit of capillary electrophoresis is the ability to adapt the system for fraction collection of bands of interest. Muller et al. [22] have reported the use of a prototype capillary electrophoresis system that allows for high precision fraction collection of microliter volumes of fluorescent PCR products using a sheath flow collection device. Modifications to current capillary electrophoresis systems allowing fraction collection of fluorescent PCR products would eliminate the need for gel based band isolation and increase the throughput of fluorescent differential display. Investigations are in progress to assess the feasibility of a variety of systems, including prototype fraction collectors for capillary electrophoresis and microchip based capillary electrophoresis, for fluorescent band isolation [23].

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