

Elimination of False Positives Generated Through PCR Re-amplification of Differential Display cDNA

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ABSTRACT

Differential display (DD) is a powerful molecular tool that allows the identification and subsequent isolation of transcripts differentially expressed between biological samples, for example, between undifferentiated and differentiated cells, between different tissues or in one tissue at different stages of development.

However, significantly high rates of apparent false positives have been reported using this technique. We suggest that the vast majority of false positives do not represent the originally selected transcript, but instead result from the re-amplification of cDNA species that co-migrate with the cDNA of interest in DD gels.

Here we describe the use of a procedure to resolve co-migrating cDNAs and to purify the candidate of interest before cloning. The use of this modified technique resolves downstream problems encountered during DD experiments.

INTRODUCTION

One of the most significant recent technical advances in molecular biology has been the description of the procedure of differential display reverse transcription polymerase chain reaction (DD-RT-PCR) (5). This technique allows the direct comparison of genes expressed in multiple samples by the creation of an "RNA fingerprint" for each sample. Total RNA is fractionated into specific subpopulations of cDNA using modified oligo(dT) primers for reverse transcription. This fractionated RNA, represented as cDNA, is used as a template in PCRs with the original 3' primer and an additional 5' 10-mer of arbitrary but defined sequence. Incorporation of a radiolabeled nucleotide into the display reactions allows the resulting PCR products (RNA fingerprints) to be visualized by autoradiography following standard polyacrylamide gel electrophoresis. Visual comparison of the banding pattern across gel lanes readily identifies any differentially expressed transcripts. Regions corresponding to bands of interest are excised from the dried gel, and the cDNA is recovered. Recovered cDNA is re-amplified using the original primer combination and cloned into an appropriate vector. Clones can then be used as probes to confirm differential expression using such techniques as Northern blot analysis, ribonuclease protection assay (RPA) or in situ hybridization.

The technique has several advantages over other methods of isolating differentially expressed genes: (i) the low quantity of starting material re-

quired (DD-RT-PCR can be performed with as little as 200 ng of total RNA per sample); (ii) the ability to simultaneously analyze multiple samples (this is limited only by the number of lanes on the electrophoresis apparatus used); (iii) sensitivity (being PCR-based, low-copy number transcripts are also included in the analysis); and (iv) the speed at which the process can be completed from RNA extraction to sequenced clones (as few as 8 working days).

However, significantly high false-positive rates have been reported (4,10) where expression studies using isolated clones have failed to replicate the differential expression patterns seen on the original display gel. Indeed, clones derived from an apparently single display band frequently represent a number of different sequences, making the identification of the real candidate a laborious task (2,4). We consider that there are three main sources of false positives: (i) artifactual differences created in the original RNA populations by nonstandardized extraction procedures, (ii) identical-sized DNA fragments that co-migrate with the band of interest on display gels and (iii) DNA contamination introduced into the re-amplification PCR. Therefore, the display autoradiographs represent a genuine picture of gene expression with the apparent false positives resulting from co-migrating cDNAs and/or contamination of the re-amplification process. Even under ideal conditions, several co-migrating cDNA species may be present in addition to the cDNA of interest. While originally present as only a very small proportion of the total DNA recovered,

after 40 cycles of PCR, these co-migrating species will be amplified to levels equivalent to the real candidate cDNA (5). In some instances, these co-migrating species can in fact clone with

greater efficiency than the cDNA of interest. Cases where multiple sequences are cloned have necessitated using methods such as reverse Northern blot procedures to identify the true differen-

tially expressed candidate. However, this procedure is not sensitive and requires the use of substantial quantities of poly(A) RNA (6,9). The problems with this are obvious in situations where large quantities of material are not available and where low-copy-number transcripts are involved.

We are using the DD-RT-PCR technique to investigate genes showing altered expression profiles in the developing mouse brain as a result of the targeted disruption of a single copy gene expressed throughout the central nervous system. As a result of problems experienced with co-migrating cDNA species amplifying to levels equivalent to the genuine candidate and frequently cloning with greater efficiency, we have inserted an additional purification step downstream of the DD-RT-PCR technique.

Here we report the use of a modification of a single-strand conformation polymorphism (SSCP) protocol to purify the excised display candidate of interest from potential co-migrating cDNA species before re-amplification and cloning (3,7) (Figure 1). SSCP is an electrophoresis protocol designed to separate single-stranded DNA fragments on the basis of conformation rather than size. Under standard conditions (3), SSCP will separate individual strands of a DNA duplex. However, the conditions used here are designed to differentiate between similarly sized fragments of completely different sequence. While similar in concept to the approach published by Mathieu-Daude et al. (7), the procedure reported here differs significantly in detail and is specifically designed to circumvent re-amplification contamination. We now routinely subject all recovered cDNAs to this procedure before re-isolating the cDNA of interest and have reduced the final re-amplification step to 5 cycles to minimize the possibility of cloning contaminating DNA. Following a combination of modified SSCP and 5-cycle PCR re-amplification, we have been able to reconfirm patterns of expression observed on display gels for all DD-RT-PCR candidates detectable by Northern blot and/or ribonuclease protection assay (RPA). This paper fully documents the protocols used in our laboratory to obtain DD gels of high

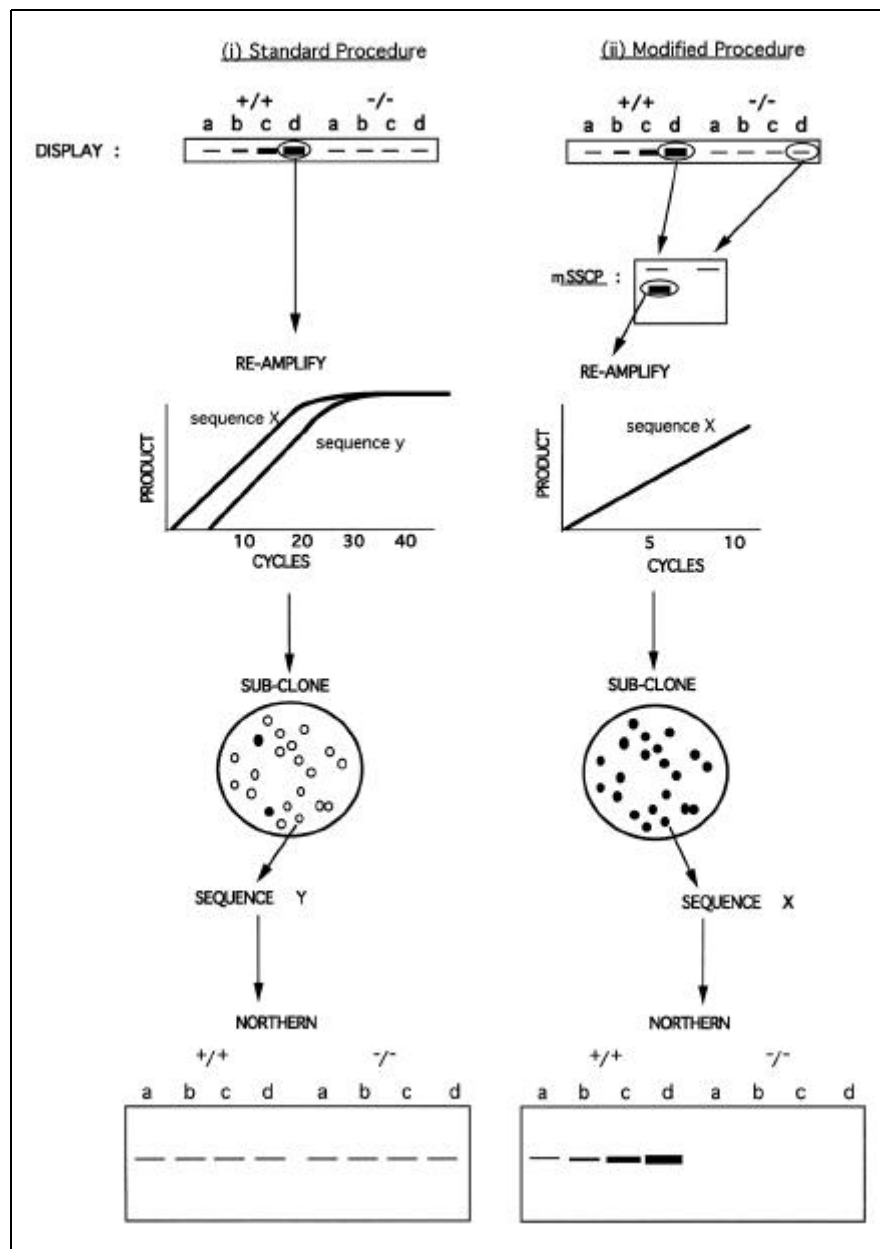


Figure 1. Rationale for incorporation of mSSCP step into DD-RT-PCR procedures. Standard (i) and modified procedure (ii). Under the standard procedure (i), the eluent recovered from an apparently single band on a display gel may in fact contain co-migrating species of cDNA. When re-amplified by PCR for 40 cycles, the quantity of co-migrating sequence Y can be equivalent to sequence X, the cDNA of interest. Depending on cloning efficiency, the co-migrating sequence may be overrepresented in the subclone population. While examination of a number of subclones may indicate the presence of only one cDNA sequence, Northern blot analysis fails to replicate the original differential expression pattern. Incorporation of mSSCP into the modified procedure (ii) allows the purification of the candidate cDNA of interest from any co-migrating species by separation based on sequence. Re-amplification of candidates from mSSCP gels results in a population of subclones all containing the cDNA of interest, which when used as a probe in expression studies, reproduces the original DD expression profile.

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reproducibility and the modified downstream procedures required to exclude co-migrating cDNA species from further analysis.

MATERIALS AND METHODS

DD-RT-PCR

Total RNA was extracted from wild-type and null neonate, post-natal day 10, post-natal day 20 and adult whole brains using the RNeasy[®] B method (AMS Biotechnology, Whitney, Oxon, England, UK) and stored as ethanol precipitates at -20°C. Following centrifugation (10 000× *g*, 25 min, 4°C) of a volume containing approximately 8 µg RNA, the pellet is washed with 85% ethanol, dried at 45°C for 2 min and resuspended in 7 µL of RNase-free distilled (d)H₂O. A small portion (2 µL) of each sample was used for spectrophotometric quantitation (average of three readings taken), and 5 µg total RNA (in 5 µL volume) were used to synthesize first-strand cDNA (First-Strand Synthesis Kit; Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK). Reactions contained 1 µL dithiothreitol (DTT; 200 mM), 5 µL of bulk first-strand mixture and 4 µL of either (dT)₁₂VA, (dT)₁₂VG, (dT)₁₂VC or (dT)₁₂VT primer (24 µM; V = A, G or C). Reactions were incubated at 37°C for 1 h and heated to 95°C for 10 min to inactivate reverse transcriptase. Reactions were dispensed into 1-µL aliquots and stored at -20°C. For use, 1 µL of aliquoted cDNA was diluted to 133 µL with dH₂O, and 10 µL of this solution were used for each display PCR (equivalent to the amount of cDNA produced from 25 ng RNA). To each 10 µL of cDNA on ice, 2 µL of random primer (5 µM) were added and overlaid with 30 µL mineral oil (Sigma Chemical, Dorset, England, UK). Master mixture (8 µL) containing 2 µL 10× PCR buffer (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3), 2 µL dNTPs (20 µM; Amersham Pharmacia Biotech), 0.3 µL *Taq* DNA Polymerase (1.5 U; Boehringer Mannheim, Sussex, England, UK), 2 µL of (dT)₁₂VN (25 µM), 1 µL [α -³⁵S]dATP (1000 Ci/mmol; Amersham Pharmacia Biotech), and 0.7 µL dH₂O was added

to each tube. Tubes were centrifuged briefly and incubated in a UNO Thermoblock[™] (Biometra GmbH, Maidstone, Kent, UK) at 94°C (2 min), followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 2 min and extension at 72°C for 30 s, followed by a final extension step at 72°C for 5 min. Loading dye (4 µL of 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to each tube, and 8 µL of each sample were loaded onto a 6% Non-Denaturing HR-1000 GenomixLR[™] Polyacrylamide Gel (Genomix, Foster City, CA, USA). Samples were run for 2 h 15 min at 2700 V (50°C) on a GenomixLR DNA Analyzer (Genomix). The gel was transferred to blotting paper (3 MM; Whatmann, Clifton, NJ, USA), dried and exposed overnight to BioMaxMR film (Sigma Chemical).

Sequences of the random 5' primers were as followed: P1: 5'-GGAACCAATC-3'; P2: 5'-ACAGAGCACA-3'; P3: 5'-ACGTATCCAG-3'; and P4: 5'-CTTCTACCC-3'.

Modified-SSCP (mSSCP)

Gel regions corresponding to bands representing candidate cDNAs were excised using sterile scalpels and transferred to sterile 0.5-mL microcentrifuge tubes. Glogos[™] Autoradiograph Markers (Stratagene, Cambridge, England, UK) were used to align the gel with the autoradiograph, and identical regions were excised from all 4 developmental time points. The gel fragments were rehydrated by incubation at room temperature for 15 min in 100 µL dH₂O, and cDNA was eluted at 99°C for 15 min before transfer of the liquid phase to fresh 0.5-mL microcentrifuge tubes. DNA was precipitated by the addition of 1 µL See-DNA (Amersham Pharmacia Biotech), 2.5 vol ethanol, 1/10th vol 3 M sodium acetate (pH 5.2) and stored on dry ice for 1 h. Following centrifugation (10 000× *g*, 25 min, 4°C) and washing with 85% ethanol, the pellet was resuspended in 4 µL of dH₂O. For the mSSCP-PCR, 4 µL 10× PCR buffer (Boehringer Mannheim), 3.2 µL dNTPs (2.5 mM dGTP, dCTP, dTTP; 0.025 mM dATP; Amersham Pharmacia Biotech), 2.5 µL modified oligo(dT) primer (20 µM), 2.5 µL ran-

dom primer (20 µM), 0.3 µL *Taq* DNA Polymerase (1.5 U; Boehringer Mannheim) and 0.5 µL [α -³³P]dATP (1000 Ci/mmol) were added to the DNA, and the reaction volume was adjusted to 40 µL with sterile dH₂O. PCR conditions were similar to those used for display PCR, with the exception that an extension time of 1 min at 72°C was used, and only 5 cycles were performed. After removing the mineral oil, PCR products were precipitated for 1 h on dry ice as described above. Pellets were washed, resuspended in 8 µL loading buffer (80% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol, 1 mM EDTA, 10 mM sodium hydroxide) and denatured at 95°C for 10 min before loading onto a 0.5× MDE[™] gel (Flowgen, Staffs, England, UK). Samples were electrophoresed for 18 h at 8 W in 0.6× TBE buffer (0.054 M Tris-borate, 0.001 M EDTA). Following autoradiography, areas of the gel corresponding to candidate cDNAs were excised, cDNA was eluted and precipitated as previously described. A final re-amplification of the recovered cDNA was performed for 5 cycles using similar conditions as for mSSCP, with the exception that 3.2 µL of dNTPs (10 mM) were used. PCR products were precipitated as before and resuspended in 4 µL dH₂O.

Cloning SSCP Purified and Re-Amplified DNA

PCR products derived from cDNA recovered after mSSCP were cloned using the pGEM[®]-T Easy Vector System (Promega, Southampton, England, UK) according to the manufacturer's instructions, with the following exceptions: (i) 0.5-µL vector was used for ligation reaction, (ii) 25 µL JM109 cells (1 × 10⁸ colony-forming units [cfu]/µg) were used for transformation and (iii) the entire transformation volume was plated out.

Northern Blot Analysis

Probes were labeled using a Rediprime[™] Kit (Amersham Pharmacia Biotech). Gel-purified cDNA (20 ng in 45 µL) was denatured at 99°C for 10 min then placed on ice for 5 min. Following addition of 5 µL [α -³²P]dCTP (3000 Ci/mmol) to a Rediprime vial,

the denatured probe DNA was added, and the contents were mixed. The labeling reaction was incubated at 37°C for 15 min, and the labeled probe was then purified using Sephadex® G-50

Nick™ Columns (Amersham Pharmacia Biotech).

Denatured total RNA (10 µg) was loaded onto 1% agarose gels, containing formaldehyde, and electrophoresis

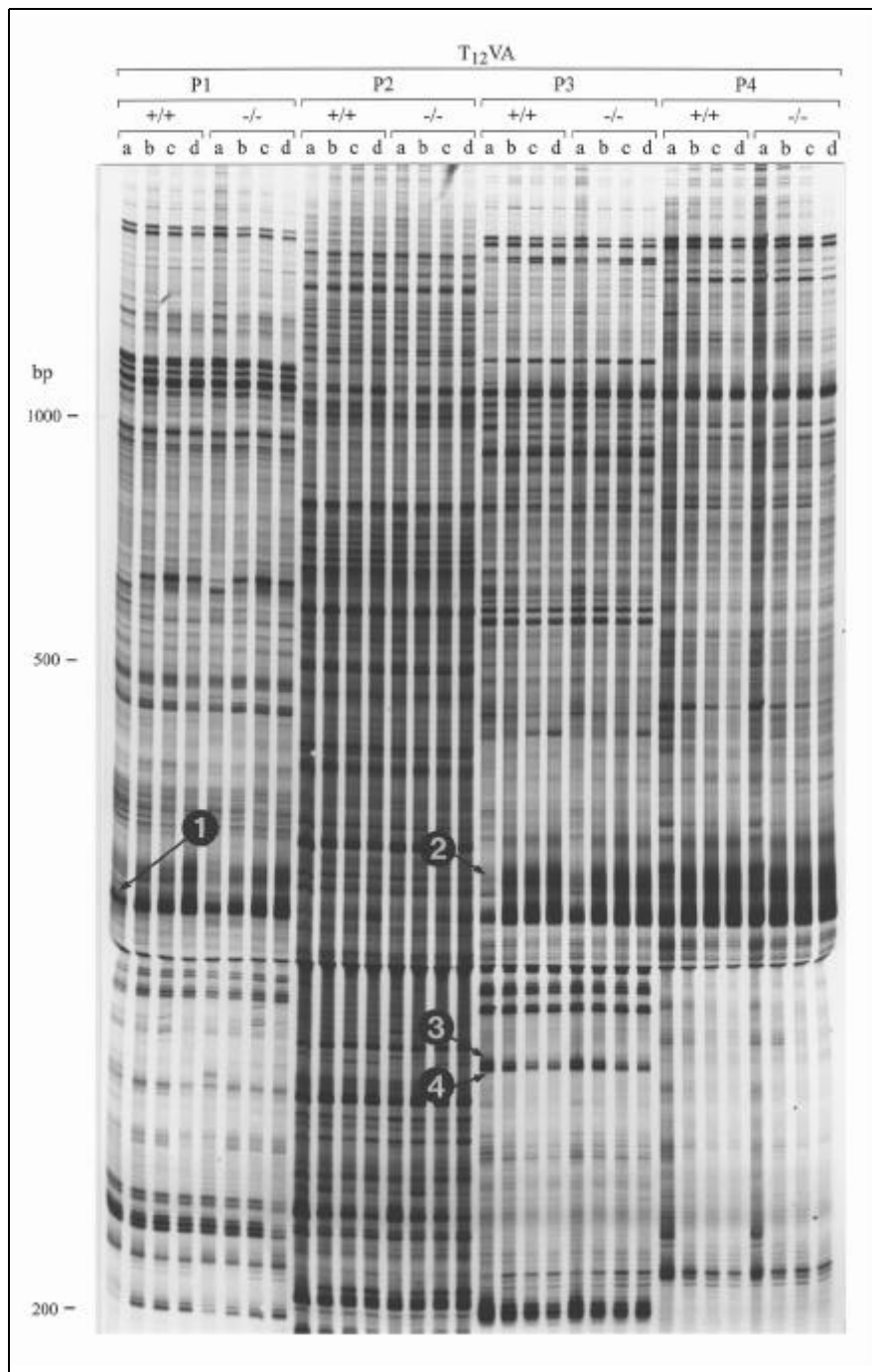


Figure 2. DD-RT-PCR gel. RNA fingerprints from neonatal, post-natal day 10, post-natal day 20 and adult brains from wild-type and null (targeted disruption of single-copy gene) mice were compared using (dT)₁₂VA 3' primer in combination with four random 5' primers (P1, P2, P3 and P4). Eight microliters of display PCR were loaded onto 6% non-denaturing HR-1000 gel and electrophoresed for 2 h 15 min at 2700 V (50°C) on GenomylxLR. The dried gel was exposed to BioMaxMR for 18 h. Arrows indicate positions of candidates chosen for further analysis.

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was performed at 80 V for 3 h. RNA was transferred to a Hybond®-N membrane (Amersham Pharmacia Biotech) overnight by capillary action. Membranes were rinsed briefly in 2× saline-sodium citrate (SSC) and baked at 80°C for 2 h. Blots were pre-hybridized in 20 mL of 0.25 M Na₂HPO₄/7% sodium dodecyl sulfate (SDS), containing 100 µg/mL of denatured salmon sperm DNA (Life Technologies, Paisley, Scotland, UK) for 1 h at 65°C. Denatured radiolabeled probe was added to the hybridization solution at a concentration of 1 × 10⁶ counts per minutes (cpm)/mL, and hybridization was performed overnight at 65°C. Blots were washed 1 × 15 min at room temperature and 1 × 20 min at 65°C in 25 mM Na₂HPO₄/1% SDS and exposed overnight at -80°C to BioMaxMS film. Comparison of ethidium bromide staining and hybridization to 18S rRNA was used to ensure equivalent loading of RNA. Three micrograms of a 255 bp 18S rRNA cDNA probe were radiolabeled to ensure an excess of probe to target RNA.

RESULTS

DD-RT-PCR creates “RNA fingerprints” representing a subpopulation of genes expressed in the tissues or cells under study. The resolving power of the GenomyxLR allows the separation of products from approximately 200 bp to greater than 1.5 kb, whereas a standard sequencing-sized apparatus would permit efficient resolution only up to approximately 350 bp. We routinely analyze all DD-RT-PCRs on standard-sized sequencing gel apparatus to resolve products less than 300 bp in size (at 220 V for approximately 18 h) and on the GenomyxLR to resolve products in the size range 200–1500 bp. The advantages in using the GenomyxLR include: (i) greater resolution of bands, (ii) 2-fold–3-fold increase in the number of transcripts displayed per primer combination and (iii) the majority of recovered cDNA fragments are of a larger size, providing more sequence information and superior probes. Figure 2 illustrates a typical GenomyxLR DD

gel as routinely produced in our laboratory. As Figure 2 shows, the consistency of banding patterns within a particular primer combination is extremely high, and the reproducibility between the developmental time points of the wild-type and null brain material is excellent for each primer combination. Display PCRs were performed on cDNA that had been stored, aliquoted and undiluted at -20°C for more than 3 months. We have observed that dilution of cDNA products before storage results in DD-RT-PCR gels of poor quality in terms of resolution and background. This particular display gel is an example in which no differences in gene expression were observed between the wild-type and null brains.

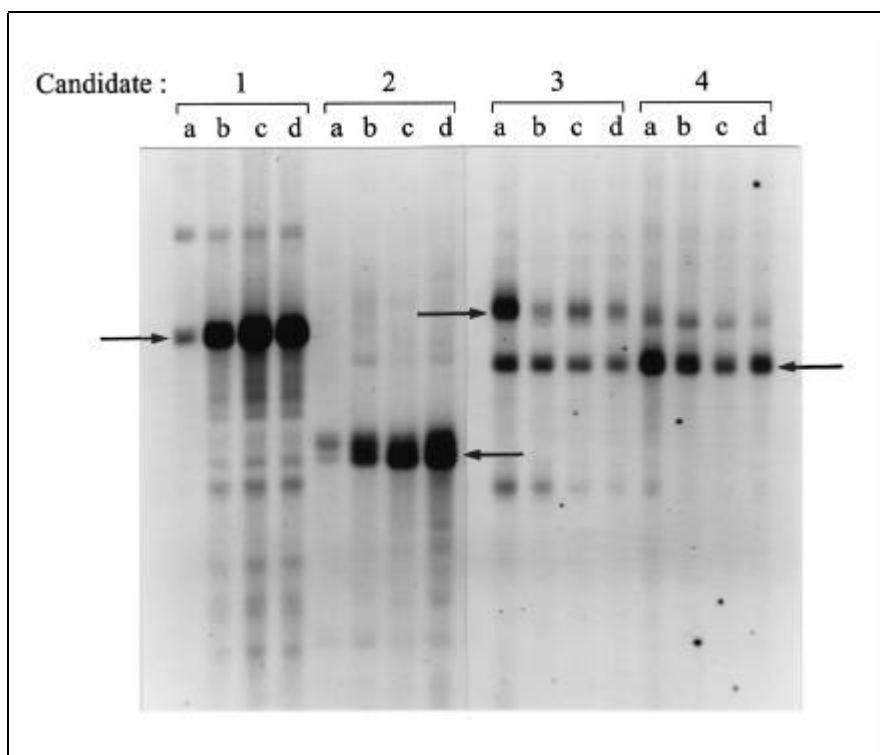


Figure 3. Purification of cDNA of interest by mSSCP. Candidate cDNAs recovered from identical regions from all four developmental time points were re-amplified for 5 PCR cycles and subjected to mSSCP electrophoresis as detailed in Materials and Methods. Samples were denatured at 95°C for 10 min before loading onto a 0.5× MDE gel and electrophoresed for 18 h at 8 W. The dried gel was exposed to BioMaxMR for 17 h. Arrows indicate candidate cDNAs replicating the original display profile.

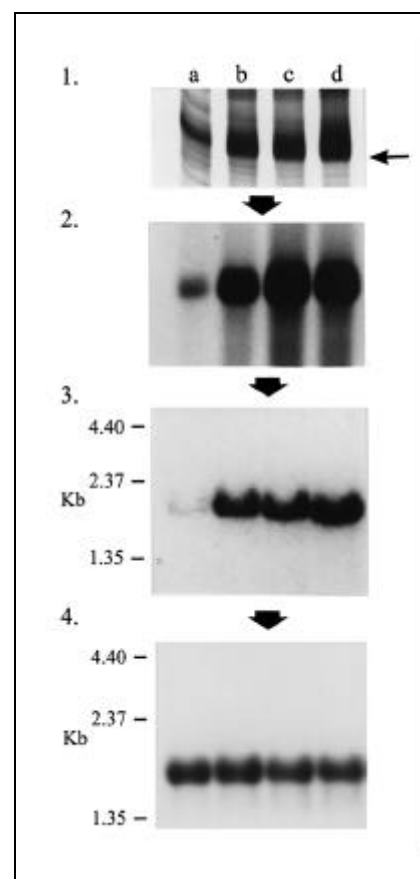


Figure 4. Demonstration of modified approach. cDNA (candidate 1) from the DD gel (1) was eluted, re-amplified through 5 PCR cycles and subjected to mSSCP analysis (2). Following recovery and a further 5 PCR cycles, the product was cloned. Gel-purified insert DNA was labeled and used as a probe for Northern blot analysis (3). Hybridization of a 255-bp radiolabeled probe to 18S rRNA was used to ensure equivalent loading of RNA in each lane (4).

However, a small number of transcripts do show changes in gene expression during brain development for both wild-type and null animals. Four such transcripts were selected to illustrate the potential of the mSSCP procedure. Arrows indicate the positions of the four candidates excised and processed for mSSCP.

The eluted cDNA was re-amplified for 5 cycles in reactions containing [α - ^{33}P]dATP and subjected to mSSCP analysis. Figure 3 shows an autoradiograph of the resulting mSSCP gel. As can be seen, the 5-cycle PCR procedure amplifies the material sufficiently to permit detection without distorting the relative levels of different cDNA species recovered from the display gel or the changing levels of expression at different time points of the candidate of interest. In addition to the fragments that replicate the profile of interest, it is apparent from the mSSCP pattern for candidate 1 that co-migrating cDNA species have also been recovered from the original display gel. The co-migrating cDNA is represented here by bands displaying a constant intensity across all developmental time points. Similar co-migrating species were seen with candidates 3 and 4. Arrows on the mSSCP gel indicate the bands that replicate the original expression profiles and that were selected for cloning as detailed in Materials and Methods.

Figure 4 represents the Northern blot analysis of whole-brain RNA at different developmental time points probed with a clone derived from cDNA recovered after mSSCP (band 1 on Figure 2). Following cloning of this cDNA into a plasmid vector, ten of the resulting clones were analyzed and found to be identical in sequence. Northern blot analysis of this transcript replicated the expression profile observed on the display gel, demonstrating the potential of the procedure.

DISCUSSION

DD-RT-PCR is a powerful molecular technique that allows the comparison of gene expression between different cell types or tissues or between the same cells or tissues at different stages of development (5). The ability to iden-

tify and subsequently isolate even rare transcripts or transcripts exhibiting only subtle changes in expression gives this technique obvious advantages over alternative methods. Although representing a significant advance in molecular biology, there have been several reports documenting problems with the technique. The main criticism has been a frequent failure to confirm the original expression profile (10). This has led to the suggestion that DD-RT-PCR produces a high rate of artifactual differences. We believe that the majority of clones derived from DD-RT-PCR analyses that fail to replicate the original displayed expression profile do not in fact represent the original selected transcript. We suggest that the majority of false positives are derived from cDNA species of identical size, which co-migrate with the cDNA of interest. These co-migrating cDNAs may appear on display gels as bands of very low intensity or may not be visible if insufficient radiolabeled nucleotide has been incorporated during DD-RT-PCR. Although originally present at lower levels, after the standard 40 cycles of PCR re-amplification, these co-migrating cDNAs may be equal in concentration to the DNA of interest (8).

Here we present both the full protocol used in our laboratory to obtain high-quality DD gels and the protocols used to clone cDNA fragments that represent the originally selected candidates. As Figure 3 shows, separation of eluted cDNAs by mSSCP reveals the presence of co-migrating cDNA species in addition to the selected candidate of interest. Use of a low number of PCR cycles for mSSCP analysis produces sufficient product to allow detection by autoradiography without affecting the quantitative differences between different time points and different cDNA species. This allows the identification of the cDNA of interest by intensity level and expression profile. Cloning of mSSCP purified cDNA after re-amplification results in only one cDNA sequence, which represents the original displayed candidate. By incorporating mSSCP into the overall DD-RT-PCR procedure, the expression profiles of the majority of candidates were easily confirmed by Northern blot analysis. Figure 4 shows an example for candidate 1.

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In a differential display analysis, it is always necessary to confirm the original expression profile by other means such as Northern blot analysis, RPA or RT-PCR. During this stage of the procedure, we often find it necessary to use poly(A) RNA to confirm the expression profiles of transcripts that were obvious on DD-RT-PCR gels. This would indicate that this technique is not biased towards mRNAs of moderate to high abundance, as suggested by others (1).

Before the introduction of mSSCP, an analysis of cDNAs re-amplified from twenty different display bands revealed that 75% of the resulting PCR products contained more than one cDNA species. Since routinely including modified SSCP downstream of DD-RT-PCR, we have eliminated these false positives. For ten candidate DD-RT-PCR cDNAs processed through mSSCP in our laboratory, sequence analysis of the resulting clones has revealed only a single cDNA species isolated for each candidate. Nine of these candidate cDNAs have replicated the expression profile observed by DD-RT-PCR when used as probes in Northern blot analyses. The remaining candidate could not be detected by this procedure and, arguably, could still represent a false positive.

To eliminate the possibility of selecting artifactual differences in the original DD-RT-PCR, we routinely analyze multiple developmental time points from the tissue under study. The majority of transcripts displayed should appear identical in intensity in all lanes, while a small percentage, representing developmentally regulated genes, should show the same differential expression profile in the test and control groups (e.g., wild-type and null). Both types of expression pattern act as internal controls, and individual transcripts that differ between wild-type and null can be selected with confidence. We routinely use 1–5 µg of total RNA to produce cDNA for DD-RT-PCR. In these circumstances it is necessary to dilute the cDNA to a suitable concentration for use. We have found that attempts to store and re-use this diluted cDNA results in DD gels of much poorer quality in terms of resolution and background than when using freshly prepared cDNA. However, if aliquots of the original cDNA reaction

are stored undiluted at -20°C, we observed no compromise in display quality. This has obvious advantages in terms of time, cost and standardization between display gels. Once cDNA representing a particular subpopulation of mRNAs has been synthesized, this can be stored and will be of sufficient quantity to be used as a template in a large number of reactions.

In summary, we suggest that ideal conditions for a DD-RT-PCR analysis are as follows: (i) use of multiple developmental time points from the tissue under study, (ii) use of standard sequencing size gels and GenomyxLR electrophoresis apparatus to resolve products in the range 50–300 bp and 200–1500 bp, respectively, and (iii) purification of the cDNA of interest by mSSCP followed by 5-cycle re-amplification of purified cDNA and cloning into pGEM-T Easy. We believe that the use of this mSSCP technique to resolve true candidate cDNAs from co-migrating species should serve to resolve problems frequently encountered in confirming the expression pattern of differential display products.

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