

## Isolation of Song-Regulated Genes in the Brain of Songbirds

Claudio V. Mello, Erich D. Jarvis,  
Natalia Denisenko, and Miriam Rivas

### 1. Introduction

We have applied the differential display (DD) technique (1) to isolate genes whose expression is regulated in the brain of songbirds when they hear song of their own species. Song is known to cause a marked increase in mRNA levels of two immediate early genes, *ZENK* and *c-jun*, in the auditory forebrain of songbirds, the most pronounced induction occurring in the caudomedial neostriatum, or NCM (2–4). Several studies suggest that this gene regulatory event may be related to song processing, discrimination, and the formation of song auditory memories. For example, *ZENK* induction in NCM is highest for songs of the same species, lower for songs of other species, and lowest or absent for nonsong auditory stimuli (2). *ZENK* induction habituates to repeated presentations of the same song, but can be re-elicited by a novel song (5). Similarly, the electrophysiological response of NCM neurons habituates when the same song is presented repeatedly, but is high again when a novel song is then presented (6); this song-specific electrophysiological habituation is long-lasting and its long-term maintenance depends on local protein and RNA synthesis (6). Finally, behavioral studies indicate that *ZENK* induction in NCM correlates with associative learning when song is used as a stimulus (7). The studies above suggest that a cascade of gene regulatory events triggered by song in NCM is involved in the formation of song auditory memories. The *ZENK* and *c-jun* genes encode transcriptional regulators (8,9) and could play a role in coordinating this cascade.

To begin to decipher such a cascade, we have used DD in the isolation of a cohort of genes induced by song in NCM. We have studied two time-points,

30 min and 2 h after the start of song presentation. The first time aimed at identifying genes coregulated with *ZENK* and *c-jun*. The second represents a time when *ZENK* protein levels are high (10) and downstream genes are presumably being regulated. We describe here the strategy we developed and the optimization of the methods for secondary screening necessary to apply DD to our experimental system.

## 2. Materials

For items 2.2–2.4, 2.8, and 2.9, use RNase-free salts and reagents and DEPC-treated water (add 0.1% DEPC to distilled water, incubate for 12 h at 37°C and autoclave for 15 min), and work under RNase-free conditions (wear gloves and use sterile, disposable plasticware; see Note 1). Store solutions at –20°C unless otherwise stated.

### 2.1. Song Stimulation

Animals: adult male and female zebra finches (*Taeniopygia guttata*).

### 2.2. RNA Extraction

1. Solution D: 4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.72%  $\beta$ -mercaptoethanol ( $\beta$ -ME); stable for 1 mo at 4°C.
2. 2M NaOAc, pH 4.0; store at room temperature.
3. Phenol: saturated with distilled water (without any buffer); store at 4°C.
4. Chloroform-isoamyl alcohol (49:1).

### 2.3. DNase Treatment

1. RNase-free DNase I (Boehringer-Mannheim, Indianapolis, IN), 20 U/ $\mu$ L, and 10X DNase buffer.
2. RNase inhibitor (RNasin; Promega, Madison, WI), 40 U/ $\mu$ L.
3. Phenol:chloroform:isoamyl alcohol (25:24:1): saturated with 100 mM Tris-HCl, pH 8.0; stable for 1 mo at 4°C protected from light.
4. 3M NaOAc, pH 5.2; store at room temperature.

### 2.4. cDNA Synthesis

1. Superscript II reverse transcriptase (RT; Gibco-BRL, Grand Island, NY), 200 U/ $\mu$ L, and 5X RT buffer.
2. 100 mM DTT.
3. dNTP Stock: 250  $\mu$ M each of dATP, dCTP, dGTP, and dTTP.
4. 10  $\mu$ M T<sub>12</sub>MN primers: separate stocks of the following: T<sub>12</sub>MA, T<sub>12</sub>MC, T<sub>12</sub>MG, and T<sub>12</sub>MT (where M = A, C, or G), T<sub>12</sub>GC and T<sub>12</sub>CG.

### 2.5. PCR Amplification

1. 2  $\mu$ M Stocks of arbitrary 10-mers of random sequence (AP-primers 1-20; GenHunter, Brookline, MA).

2. AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT), 5 U/ $\mu$ L, and 10X PCR buffer I.
3. 25  $\mu$ M dNTP: 1:10 dilution of stock described in Section 2.4., item 3.
4.  $^{35}$ S-dATP (NEN, Boston, MA).
5. Loading buffer: 98% deionized formamide, 10 mM EDTA, pH 8.0, and 0.025% each of xylene cyanol FF and bromophenol blue.
6. 5X TBE buffer: combine 54 g of Tris base, 27.5 g of boric acid and 20 mL of 0.5M EDTA, pH 8.0, and add water to 1 L; store at room temperature.
7. 6% Polyacrylamide gel mix: to 230 g of urea add 75 mL of 40% polyacrylamide mix (Fisher), 100 mL of 5X TBE and water to 500 mL; filter (0.45- $\mu$ m pore) and store for several weeks at 4°C.
8. 100-bp DNA ladder (Gibco-BRL).
9. Klenow DNA polymerase (New England BioLabs, Beverly, MA), 2 U/ $\mu$ L, and Klenow buffer.

## **2.6. Fragment Excision and Reamplification**

1. 2% Nusieve (Sigma, St. Louis, MO) agarose gels (*see* Note 1).
2. Qiaex II gel extraction kit (Qiagen, Chatsworth, CA).
3. 10 mg/mL glycogen.

## **2.7. Cloning into PCR Plasmid Vectors**

1. pCRScript (Stratagene, La Jolla, CA) or TA pCR II (Invitrogen, San Diego, CA) cloning kits.
2. *Eco*RI and *Bss*HII restriction enzymes (Boehringer Mannheim), 10 U/ $\mu$ L.
3. Random primer labeling kit (Boehringer Mannheim).
4.  $^{32}$ P-dCTP (NEN).

## **2.8. Secondary Screening by Northern Hybridization**

1. 10X MOPS buffer: to 20.9 g of MOPS (3-[*N*-morpholino]propanesulfonic acid) add 300 mL of water, 50 mL of 0.8M NaOAc, and 50 mL of 0.5M EDTA; adjust the pH to 7.0 with NaOH, add water to 500 mL, filter (0.22- $\mu$ m pore), and store at 4°C protected from light.
2. 1% MOPS/formaldehyde agarose gel: 1% agarose in 1X MOPS buffer containing 2.2% formaldehyde; run in freshly diluted 1X MOPS buffer.
3. MOPS sample buffer: combine 4.0  $\mu$ L of the RNA in water, 2  $\mu$ L of 10X MOPS buffer, 10  $\mu$ L of formamide, 3.7  $\mu$ L of formaldehyde, 2  $\mu$ L of dye mix (50% glycerol, 1 mM EDTA, pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol FF) and 0.025% ethidium bromide. Prepare fresh and heat to 65°C for 10 min before loading.
4. 20X SSPE: dissolve 175.3 g of NaCl, 27.6 g of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O and 7.4 g of EDTA in 800 mL of water, adjust pH to 7.4 with NaOH and add water to 1 L. Store at room temperature.
5. Nylon membranes (NEN's GeneScreen Plus or Amersham's Hybond Plus).
6.  $^{32}$ P-UTP (NEN).

7. 5X Transcription buffer: 2.5 mM ATP, CTP and GTP each, 60  $\mu$ M UTP, 50 mM DTT, 200 mM Tris, pH 7.5, 30 mM MgCl<sub>2</sub>, 10 mM spermidine and 50 mM NaCl.
8. 20 mg/mL RNase-free BSA.
9. T7, T3, and SP6 RNA polymerases (Promega); 20 U/ $\mu$ L (T7 and T3); and 80 U/ $\mu$ L (SP6).
10. BamHI, XhoI, and HindIII restriction enzymes (Boehringer Mannheim), 10 U/ $\mu$ L.
11. Sephadex G-50 spun-columns (*see* Note 1).
12. Hybridization solution: 50% formamide, 10% PEG-8000, 0.25M sodium phosphate buffer, pH 7.2, 25 mM NaCl, 1 mM EDTA, 20  $\mu$ g/mL polyA, and 7% SDS. Prepare fresh from stocks kept at room temperature, except polyA ( $-20^{\circ}$ C) and formamide ( $4^{\circ}$ C). Mix all other components and heat to  $55^{\circ}$ C before adding the SDS.
13. Washing solution I: 1X SSPE and 0.1% SDS; store at room temperature.
14. Washing solution II: 0.1X SSPE and 0.1% SDS; store at room temperature.

## 2.9. Secondary Screening by In Situ Hybridization

1. TissueTek (Miles, Elkhart, IN).
2. Slide preparation: dip precleaned slides for 20 min in 1M HCl, rinse in water, dehydrate for 20 min in 100% ethanol, and air-dry. Dip slides in freshly prepared 2% TESPA (3-aminopropyl triethoxysilane; Aldrich, Milwaukee, WI) in dry acetone for 10 s, wash twice in dry acetone, and once in water, and air-dry. To minimize organic waste, prepare as many slides as practical in one batch and store coated slides for at least 6 mo.
3. Fixative: 3% paraformaldehyde in 0.1M sodium phosphate buffer (PB), pH 7.4. Prepare fresh as follows: to 30 g of paraformaldehyde add 400 mL of water, heat to  $55^{\circ}$ C for 5 min under agitation, and add a few drops of 10N NaOH. After solution clears add 500 mL of 0.2M PB, pH 7.4 and water to 1 L.
4. Washing buffer: fresh 0.1M PB with 0.14M NaCl and 5 mM KCl, pH 7.2.
5. Acetylation solution: 1.4% triethanolamine and 0.3% acetic anhydride in water. Very unstable: use within 10 s of mixing components.
6. <sup>35</sup>S- or <sup>33</sup>P-UTP (NEN).
7. Hybridization solution: 50% formamide, 2X SSPE, 2  $\mu$ g/mL tRNA, 1  $\mu$ g/mL BSA, 400 ng/mL polyA, and 100 mM DTT; prepare fresh from concentrated stocks kept at  $-20^{\circ}$ C.
8. Decoverslipping solution: 2X SSPE and 0.1%  $\beta$ -ME (added just before use).
9. Washing solution I: 50% formamide, 2X SSPE and 0.1%  $\beta$ -ME (added after heating to washing temperature and just before use).
10. Washing solution II: 0.1X SSPE.

## 2.10. Sequencing

1. CircumVent Thermocycler Sequencing kit (New England BioLabs).

## 3. Methods

### 3.1. Song Stimulation

1. Isolate nine birds (*see* Note 2) acoustically for 1 d to minimize basal expression of inducible genes.

2. Play tape-recorded song to six birds for 30 min; decapitate three birds immediately and three birds 2 h after start of the playback; use the three other birds that do not hear song as unstimulated controls.
3. Treat another nine birds as above (three per group) and use their brains for *in situ* hybridization (Section 3.9.).

### 3.2. Tissue Dissection and RNA Extraction

We base our protocol on Chomczynski and Sacchi (*see* Note 3), with minor modifications, as below:

1. Dissect out NCM brain regions quickly and freeze.
2. Homogenize tissues in solution D (1 mL/30–40 mg of tissue) in an all-glass homogenizer at 4°C.
3. Add 0.1 mL of 2M NaOAc, 1 mL of water-saturated phenol and 0.2 mL of chloroform-isoamyl alcohol (49:1), with vortexing after each reagent.
4. Keep mixture on ice for 15 min and centrifuge at 10,000g for 20 min at 4°C.
5. Transfer the aqueous phase to a new tube and add 1 mL of isopropanol.
6. Precipitate for 1 h at –20°C and centrifuge at 10,000g for 20 min at 4°C.
7. Dissolve pellet in 0.3 mL of solution D and reprecipitate for 1 h at –20°C with 1 vol of isopropanol.
8. Wash the final pellet with 70% ethanol, dry in a speedvac, and resuspend in 10 µL of water. Typical yields per bird (right and left NCMs combined) are 3–5 µg of total RNA, as estimated by spectrophotometry (*see* Note 1).

### 3.3. DNase Treatment

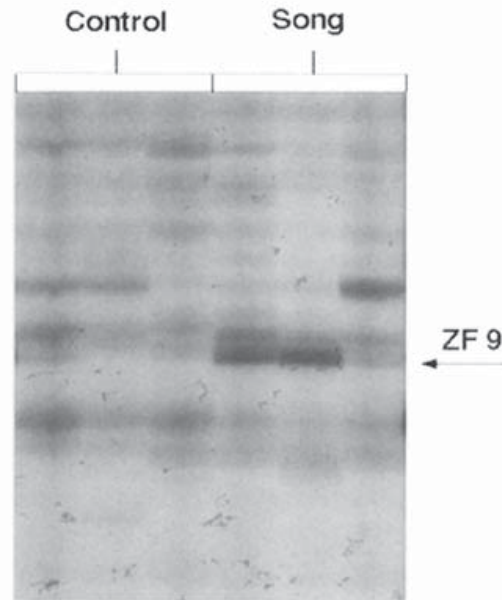
1. Incubate RNAs in 50-µL reactions with 1X DNase buffer, 20 U of DNase I and 20 U of RNasin (*see* Note 4) for 30 min at 37°C.
2. Inactivate DNase by heating samples to 65°C for 10 min and extract RNAs once with 1 vol of phenol:chloroform.
3. Precipitate RNAs at –20°C for 30 min after adding 1/10 vol of 3M NaOAc and 2 vol of 100% ethanol to the aqueous phase.
4. Pellet RNAs by centrifugation, wash in 70% ethanol, dry, and resuspend in 10 µL of water.

### 3.4. cDNA Synthesis

Incubate RNAs in 20-µL reactions containing 1X RT buffer, 5 µM DTT, 20 µM dNTPs, 1 µM of one T<sub>12</sub>MN oligonucleotide primer (*see* Note 5), 100 ng of DNase-treated RNA (preheated at 65°C for 10 min), 20 U of RNasin and 100 U of Superscript II RT (*see* Note 6) at 37°C for 30 min (*see* Notes 7 and 8 for further comments). Run control reactions without adding RT.

### 3.5. PCR Amplification

1. Amplify each cDNA in a 10-µL reaction containing 1X PCR buffer I, 2 µM dNTPs, 0.2 µM of one AP-primer, 1 µM of the T<sub>12</sub>MN primer used in Section 3.4., 1 µL of the cDNA synthesis reaction, 5 µCi of <sup>35</sup>S-dATP, and 0.4 U of



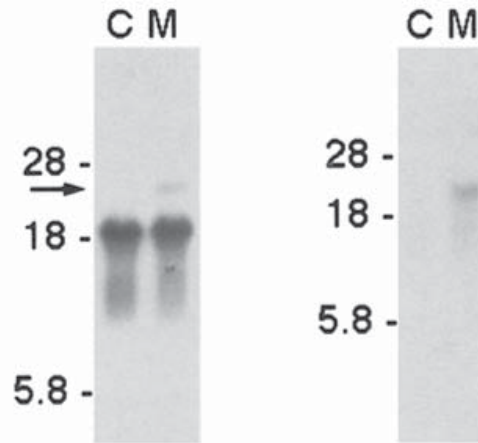
**Fig. 1.** DD gel comparing silent controls and song-stimulated zebra finches (30-min group). RNAs were isolated from NCM brain regions and cDNAs synthesized and PCR-amplified using T<sub>12</sub>MC and AP-3 primers. The band indicated by the arrow (ZF9) is high in two out of three stimulated birds and in none of the controls.

AmpliTaq (*see* Note 8). Program the thermocycler for 40 cycles of 94°C for 30 s, 40°C for 2 min and 72°C for 30 s (*see* Note 9), followed by 72°C for 5 min, and then hold at 4°C. Run control reactions without adding cDNAs.

2. Add loading buffer (5  $\mu$ L per reaction) and run 7  $\mu$ L of each final mix on a 6% polyacrylamide gel (*see* Note 1).
3. In parallel, prepare a radiolabeled DNA ladder in a 20- $\mu$ L reaction with 25 ng of the 100-bp DNA ladder, 0.5 mM dCTP, dGTP, and dTTP each, 1  $\mu$ Ci <sup>35</sup>S-dATP, 1X Klenow buffer and 1 U Klenow DNA polymerase, incubated at room temperature for 10 min. Add 10  $\mu$ L of loading buffer and run 3  $\mu$ L of the mix on the DD gel for size determination.
4. Dry the gel without fixing and expose to X-ray film for 1–3 d.

### 3.6. Fragment Excision and Reamplification

1. Cut from gel the PCR fragments present in at least two of three birds of one song-stimulated group and absent in all three birds of the control group, or vice-versa (*see* Fig. 1 and Note 10).
2. Elute by boiling fragments in 100  $\mu$ L of water for 15 min and transfer supernatant to a new tube.
3. Precipitate at –70°C for 30 min after adding 10  $\mu$ L of 3M NaOAc, 5  $\mu$ L of 10 mg/mL glycogen, and 450  $\mu$ L of 100% ethanol.
4. Pellet fragments by centrifugation, wash in 85% ethanol, dry, and resuspend in 10  $\mu$ L of water.



**Fig. 2.** Northern analysis of a differentially expressed PCR fragment. Single-stranded DNA probe (*see* Note 13) synthesized from a candidate song-induced fragment was used to hybridize a blot containing total brain RNA from birds killed 30 min after injection of metrazole (M; *see* Note 12), or from untreated controls (C). Differential expression of the upper band (left panel, arrow) confirms its activation by neuronal depolarization; a second, nonregulated, band illustrates the issue of heterogeneity of DD fragments. The experiment was repeated and, after a more careful isolation of the fragment from the DD gel, the contaminating band could be eliminated (right panel).

5. Reamplify 4  $\mu$ L of eluted fragments as in Section 3.5., step 1, but scale up the reaction to 40  $\mu$ L, use 20  $\mu$ M of dNTPs, and add no isotope or cDNA.
6. Use half of the reaction for plasmid cloning (*see* Section 3.7.). Analyze the other half on a 2% Nusieve agarose gel using the 100-bp ladder for size determination.
7. Cut fragments from the gel, extract with the Qiaex II kit and use for generating probes to confirm cloning into plasmid vectors.

### 3.7. Cloning into PCR Plasmid Vectors

1. Clone reamplified PCR products of the correct size into pCRScript or TA pCR II vectors (*see* Note 11).
2. Check transformants for insert size by running plasmids restricted with *Eco*RI or *Bss*HIII, respectively, on 1% agarose gels next to the 100-bp ladder.
3. Blot the gels (*see* Note 1) and confirm identity of transformants by hybridizing blots with  $^{32}$ P-labeled random-primed probes generated from the corresponding Qiaex II-purified PCR reamplification products (*see* Note 11).

### 3.8. Secondary Screening by Northern Hybridization

1. Run 10  $\mu$ g of total RNA from control and experimental birds on 1% MOPS/formaldehyde agarose gels (*see* Notes 1 and 12).
2. Soak gels for 1 hr in 20X SSPE and blot onto nylon membranes.
3. Synthesize sense and antisense riboprobes (or single-stranded DNA probes; *see* Note 13 and Fig. 2) in a 10- $\mu$ L reaction containing 50  $\mu$ Ci  $^{32}$ P-UTP, 1X transcrip-

tion buffer, 12 U of RNasin, 5–10  $\mu\text{g}/\text{mL}$  of BSA, 0.5 to 1  $\mu\text{g}$  of restricted plasmid DNA (from transformants obtained in Section 3.7., step 1) and 20 U of the appropriate RNA polymerase (T7 for *Bam*HI- and SP6 for *Xho*I-restricted TA plasmids, and T7 for *Bam*HI- and T3 for *Hind*III-restricted pCRScript plasmids), incubated at 42°C for 1 h (see Note 14).

4. Prehybridize filters for 15 min at 55°C in hybridization solution (2 mL/10 cm<sup>2</sup>).
5. Add riboprobes purified through G-50 columns (10<sup>6</sup> counts/min/mL) and hybridize overnight at 55°C.
6. Wash filters twice for 5 min at room temperature in washing solution I, twice for 30 min at 55°C in washing solution II, and expose to X-ray film for 1 to several days.

### 3.9. Secondary Screening by In Situ Hybridization (see Note 12)

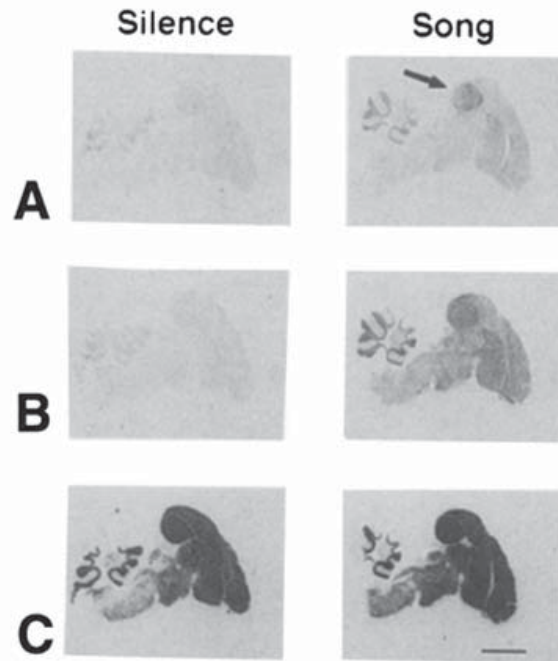
1. Dissect out brains, place in plastic molds with TissueTek and freeze on dry ice.
2. Cut 10- $\mu\text{m}$  sections on a cryostat and mount onto TESPA-coated slides.
3. Incubate sections in fixative for 5 min at room temperature, rinse twice in washing buffer, dehydrate in an ethanol series (70, 95, and 100%, 2 min each), air-dry, and store at -70°C.
4. On the day of hybridization, incubate sections for 10 min in acetylation solution, rinse three times in 2X SSPE, dehydrate in ethanol, and air-dry.
5. For each cloned fragment, make riboprobes in both orientations as described for Northern blots (Section 3.8., step 3), except use <sup>35</sup>S- or <sup>33</sup>P-UTP (see Note 15); purify probes through G-50 columns.
6. Cover acetylated sections with 16  $\mu\text{L}$  of hybridization solution containing riboprobe (0.5–1  $\times 10^6$  counts/min), add cover slips, and incubate under mineral oil in a 50–55°C water bath for 3 h.
7. Remove the oil with two washes in chloroform and the coverslips by dipping in “decoverslipping” solution.
8. Wash slides for 1 h at room temperature in fresh decoverslipping solution, followed by 1 h at 50–55°C in washing solution I and 2  $\times$  30 min at 50–55°C in washing solution II.
9. Dehydrate slides in the ethanol series, air-dry, and expose to X-ray films for at least a week (see Note 16).

An example of *in situ* hybridization of a differentially expressed clone is shown in Fig. 3. Fragments that show no differential expression (no difference in signal between song-induced and control brains) for either riboprobe strand should be discarded as false positives. For fragments that show no signal for either strand, or similar signal for both strands, repeat hybridization under different conditions (see Note 17).

### 3.10. Sequencing

Analyze clones verified to be differentially expressed by sequencing and subject results to homology searches against GenBank database (see Note 18).





**Fig. 3.** *In situ* hybridization analysis of a song-induced DD fragment. Parasagittal brain sections from song-stimulated (right column) or unstimulated control (left column) finches were hybridized with  $^{35}\text{S}$ -labeled riboprobes and exposed to X-ray film. (A) The *ZENK* gene is induced by song with a restricted pattern, in particular in NCM (oval area shown by arrow); (B) the candidate ZF9 DD fragment is induced by song in a less restricted fashion than *ZENK*; (C) pCF-2, a cDNA that is not regulated by neuronal depolarization, gives the same signal in both groups and serves as a negative control. Only antisense strands are shown. Orientation: rostral is to the right and dorsal to the top. Bar = 1.5 mm.

#### 4. Notes

1. See Sambrook et al. (11) for details on basic techniques, such as work under RNase-free conditions, spectrophotometry, preparation of agarose and polyacrylamide gels, DNA blots, and G-50 spun columns.
2. Interanimal difference is a main source of PCR variability and false positives in DD. To address it, we use multiple animals per condition and pick fragments that are consistent across animals (see Note 10). We recommend three animals per condition as a minimum; a higher number may be disadvantageous, considering the work involved in performing several primer combinations.
3. Chomczynski and Sacchi's method (12) gives, in our hands, reliable preparations of intact brain RNA. However, resuspension of the final RNA pellet in 0.5% SDS is not recommended, since traces of SDS can partially inhibit RT and DNase enzymes and increase PCR variability. For complete resuspension of RNAs in water, heat samples to 80°C for 10 min. For tissue samples with very high RNase content, or when a long time is required for the precise dissection of small brain nuclei from fresh slices, partial degradation of RNAs may be unavoidable. Two

studies used the protocols described in this chapter successfully under such conditions: the isolation of cDNAs enriched in song control nucleus HVC, or high vocal center (13), and the use of human skin biopsies for the isolation of cDNAs differentially expressed in psoriasis (14). Partially degraded samples can yield differentially expressed bands, but signals are weaker and fragments of >400 bp tend to be lost.

4. RNasin helps prevent RNA degradation owing to contaminating protein carried over from the RNA isolation. If too little material is available ( $\leq 1 \mu\text{g}$  of total RNA per sample), it may be wise to skip the DNase step in order to prevent RNA loss during the subsequent precipitation.
5. In our hands, using  $T_{12}GC$  or  $T_{12}MC$  (where  $M = A, G, \text{ or } C$ ) for cDNA synthesis and PCR amplification results in similar number of PCR fragments. Thus, the number of potential fragments is underestimated when the degenerate primer is used. We suggest that each one of the 12 possible  $T_{12}MN$  primers (where  $M = A, G, \text{ or } C$  and  $N = A, G, C, \text{ or } T$ ) be used separately.
6. Preheating of RNAs, addition of RNasin and the use of Superscript II RT are recommended in order to maximize yield of long transcripts.
7. Reducing the amount of RT enzyme (to 5 U) and/or duration of the RT reaction (10 min) improves the resolution of PCR fragments in DD gels in case of smearing. In our experience, use of >500 ng of total RNA for cDNA synthesis also leads to loss of resolution of fragments in DD gels; 50–200 ng of total RNA are sufficient to generate reliable DD products and is the range we recommend.
8. As in Liang et al. (15), we find that there can be some variability among repeated PCR amplifications with the same sample. This could be owing to factors such as contamination of tubes or pipet tips, differences in pipeting, false priming, and so on. We also find that slight variations in salt concentration or spurious DNA contaminations of different solutions or preparations of DEPC-treated water can affect the resulting display pattern. In addition to using multiple independent samples (see Notes 2 and 10), it is essential to process all samples simultaneously and consistently, with the same stock solutions and reagents. It is also advisable to perform pipeting by pooling common reagents and then dividing them into individual tubes, before adding the specific RNAs and primers to each reaction.
9. If using Stratagene's robocycler or a similar PCR machine, it is advisable to adjust the cycles to  $94^{\circ}\text{C}$  for 45 s,  $40^{\circ}\text{C}$  for 3 min, and  $72^{\circ}\text{C}$  for 45 s to compensate for faster temperature transitions.
10. When performing repeated DD reactions on the same set of multiple independent samples we noticed that some PCR products consistently appear in some individual animals, but their occurrence does not correlate with any particular group. Such fragments could represent genetic differences among individuals, or differences in gene expression levels in the brain due to variables difficult to control for, such as stress, arousal state, hormone levels, or differences in the precise anatomical dissection. To avoid spurious fragments due to these factors and to those discussed in Note 8, we use multiple animals per group and pick fragments that are consistent across a given group.

11. Protocols as provided by suppliers. The TA pCR II vector is straightforward to use after *Taq* amplification, but its SP6 promoter is not very efficient for riboprobe synthesis. Stratagene's pCRScript is our vector of choice, although treatment with pfu enzyme (as described by supplier) is necessary to clone fragments amplified with *Taq*. Fragments obtained in Section 3.6. are sometimes heterogeneous, due to contamination with adjacent bands from DD gels (Fig. 2) or to intrinsic heterogeneity of DD bands (16). It is thus advisable to pick several independent transformants for sequence and expression analysis.
12. Northern blots have limited use when tissue availability is restricted. Furthermore, for tissues with great diversity of cell types and mRNA species as the brain, differential expression in a discrete cell population might go unnoticed on blots. To address tissue availability in our system, we have used brain RNAs from birds treated with metrazole, a potent depolarizing agent that leads to widespread induction of activity-dependent genes in the brain (Fig. 2), including *c-jun* (3) and *ZENK* (17). *In situ* hybridization, however, is our method of choice for secondary screening, since small amounts of tissue are required and expression easily detected at the single cell level.
13. As in Liang et al. (15), only 40–60% of our DD fragments result in clear signal when random-primed DNA probes are used for Northern blots. In contrast, when we use riboprobes and the protocol in Section 3.8., modified from Clayton et al. (18), >90% of the fragments give detectable signal (even fragments of ~100 bp). Alternatively, single-stranded DNA probes are also satisfactory and avoid the need to clone PCR products. Sense and antisense DNA strands are synthesized each in a 20  $\mu$ L reaction containing 1  $\mu$ L of reamplified PCR product, 1  $\mu$ L of one of the primers used in Sections 3.4. and 3.5. ( $T_{12}$ MN for one strand or AP-primer for the other strand), 25 mM dATP, dGTP, and dTTP each, 50  $\mu$ Ci  $^{32}$ P-dCTP, 1X Klenow buffer and 1 U of Klenow DNA polymerase, incubated at 37°C for 1 h. After purification through G-50, probes are hybridized (in 50% formamide, 2X SSPE, 1% SDS, and 10% dextran sulfate) to Northern gels blotted onto nylon membranes; the recommended temperature for hybridization and washes (in 0.2X SSPE and 1% SDS) is 42°C. Figure 2 illustrates the use of such probes and the issue of heterogeneity of amplified fragments.
14. To maximize riboprobe synthesis, add 0.5  $\mu$ L of the appropriate RNA polymerase at the start of incubation and 0.5  $\mu$ L after 30 min. For SP6, the total amount added per reaction corresponds to 80 U.
15. When using  $^{35}$ S-UTP, it is essential to keep probes reduced to minimize background. This is done by adding 0.5  $\mu$ L of 1M DTT to the riboprobe synthesis reaction, in addition to the DTT in the buffer stock. Also add 1  $\mu$ L of 1M DTT to each probe after G-50 purification and after each freeze/thaw cycle, if storing probes for multiple experiments. We have lately turned to  $^{33}$ P-UTP, which reduces exposure time and avoids probe oxidation. Use 5  $\mu$ L of a 1:5 dilution of  $^{33}$ P-UTP (as provided by supplier) in the 10- $\mu$ L riboprobe synthesis reaction.
16. If accessible, a Phosphorimaging system cuts down on *in situ* exposure times significantly. Its resolution is not nearly as good as that of X-ray film autoradiography, but it

- allows a fast quality assessment of the hybridization and an initial evaluation of the differential expression of probes tested. For fine analysis at the cellular level, dip slides in autoradiographic emulsion (NTB-2, Kodak) and counterstain with cresyl violet (4).
17. The optimal conditions for *in situ* vary from probe to probe, owing to differences in size and GC content. We have modified our basic *in situ* protocol (17) by lowering the temperature of hybridization and washes to compensate for the small size of DD fragments relative to the large riboprobes (1–2 kb) for which the protocol was originally designed. A tight control over the temperature and timing of washes is essential, since a small difference (2–3°) can vastly affect the ability to detect signal. Washing solutions should be preheated and times counted after temperatures (measured inside washing solutions and after addition of slides) reach the appropriate level. For probes in the 250–500-bp range, we recommend 55°C for hybridization and washes. For smaller probes, 45–55°C is recommended. When no signal is detected for either strand, repeat the *in situ* lowering the temperature in 5° steps, until signal appears for only one strand. The differentially expressed clone shown in Fig. 3 (19), for example, showed no signal with either strand in the 60–65°C range, specific hybridization (signal with only one strand) at 55°C, and background (hybridization with both strands) at 50°C. Fragments with no detectable signal even at low stringency are not processed further. When signal is equally detected for both strands, repeat hybridization at higher temperatures, until signal remains for only one strand. We try to avoid RNase treatment because of the poor resulting histology; however, an alternative protocol using RNase is very effective at eliminating residual background that may persist after high temperature washes (3).
  18. GenBank homology searches with sequences from DD fragments obtained in zebra finches have not been very informative. This is particularly the case because DD fragments are typically (but not necessarily) representative of 3' untranslated regions. When at least a partial open reading frame is present, homologs from other species are more readily identified (13). In most cases, though, establishing the identity of the differentially expressed PCR fragments will require screening cDNA libraries for larger clones.

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