

## [30] Identification and Evaluation of NO-Regulated Genes by Differential Analysis of Primary cDNA Library Expression (DAZLE)

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### Abstract

Nitric oxide (NO) has numerous physiological roles in the cell. One of the actions of NO is gene regulation through protein modification and signal transduction. In neurons, NO can be produced from neuronal NO synthase, which is activated by calcium following *N*-methyl-D-aspartate (NMDA) receptor activation. Differential analysis of cDNA library expression (DAZLE) was used to identify differentially expressed genes by NO. Fundamentally, this technique combines differential hybridization to isolate genes whose expression is differentially regulated with microarray to analyze the expression of the isolated genes. The expression of genes identified by the DAZLE method is verified further by quantitative real-time polymerase chain reaction (RT-PCR) and/or Northern blot analysis. The high selectivity and sensitivity of this technique for detecting differentially expressed gene transcripts enable the investigation and identification of a panel of genes that are regulated by NO.

### Introduction

Nitric oxide (NO) is a reactive free radical species that is a neuronal messenger. It can also mediate nitrosative and oxidative stress through nitrosation, nitration, or nitrosylation of various molecules, including lipid, DNA, and proteins. It is evident that NO plays many roles in the physiology and pathophysiology of the central nervous system (CNS), such as CNS development, long-term potentiation, trauma, ischemia, and neurodegenerative diseases ([Contestabile et al., 2003](#)). Although physiological levels of NO subserve signaling and neuromodulatory roles, excessive creation of NO leads to neuronal death.

In neurons, NO produced from neuronal NO synthase (nNOS) can diffuse into glial cells or other neurons and act as a signaling molecule. As a downstream event of NO-mediated signaling, the modulation of gene expression by NO may play a key role in long-term changes of neurons, such as synaptic plasticity and neuronal development ([Gibbs, 2003](#); [Pilz and Casteel, 2003](#)).

NO is known to affect gene regulation in cells, not only by activating NO-dependent signaling pathways (Schafer *et al.*, 2000), but also by direct posttranslational modification of transcription factors (Reynaert *et al.*, 2004). In this regard, it is not surprising that many transcription factors show redox sensitivity through cysteine residues in DNA binding domains under both nitrosative and oxidative stress (Marshall *et al.*, 2000).

The DAZLE method described in this chapter is designed to identify differentially regulated genes (Fig. 1). This method has several advantages: (1) Because DAZLE is based on the screening of a primary nonamplified cDNA library with the probes containing poly(dA/dT) tailless cDNAs that limit cross-hybridization between the 3' end of the sequences, it can detect low-abundant transcripts and medium- to high-abundant transcripts with minimal redundancy in differentially expressed genes. (2) There is no limit in the number of genes for cDNA library differential screening, which

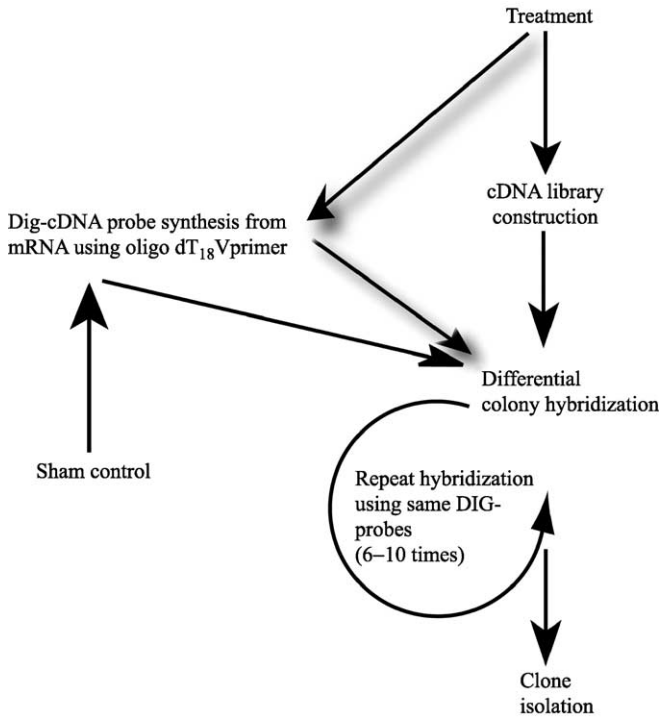


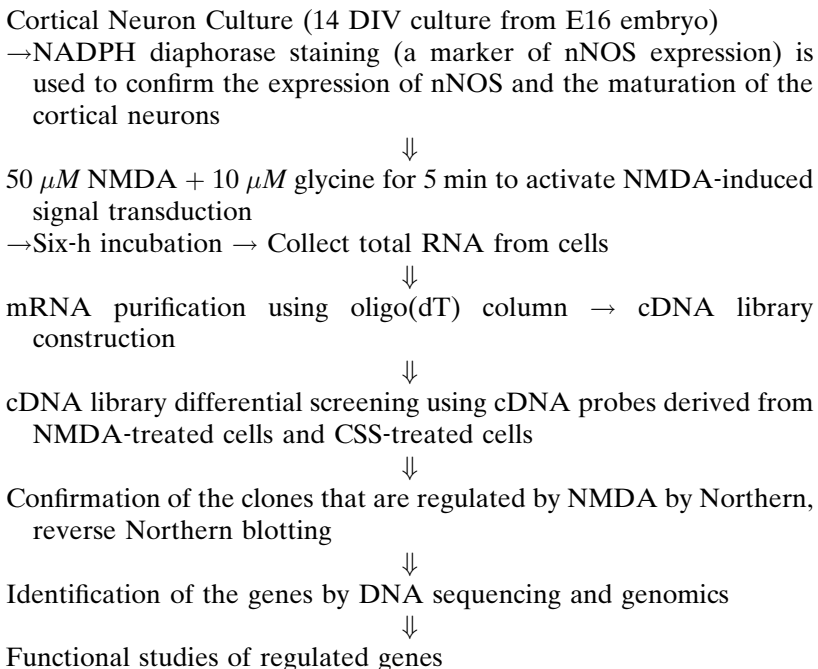
FIG. 1. Illustration of primary screening in differential analysis of cDNA library expression [differential analysis of cDNA library expression (DAZLE)].

allows one to investigate as many genes as possible. (3) A large number of genes from the primary screening can be analyzed using microarray simply and efficiently.

This technique can be applied not only to NO-mediated gene regulation (Li *et al.*, 2004), but also to other paradigms of gene modulation (Hong *et al.*, 2004).

## Procedures

### *Experimental Scheme (See Figure 2)*



### *Cell Culture*

Primary cortical neurons are prepared from gestational day 16 fetal C57Bl mice and from nNOS-null C57Bl mice (Dawson *et al.*, 1993). The dissected cortical brain regions are incubated for 15 min in 0.027% trypsin/saline solution [5% phosphate-buffered saline (PBS), 40 mM sucrose, 30 mM glucose, 10 mM HEPES, pH 7.4] and transferred to modified Eagle's medium (MEM) containing 10% horse serum, 10% fetal bovine serum, and 2 mM glutamine. Cells are dissociated by trituration, counted, and

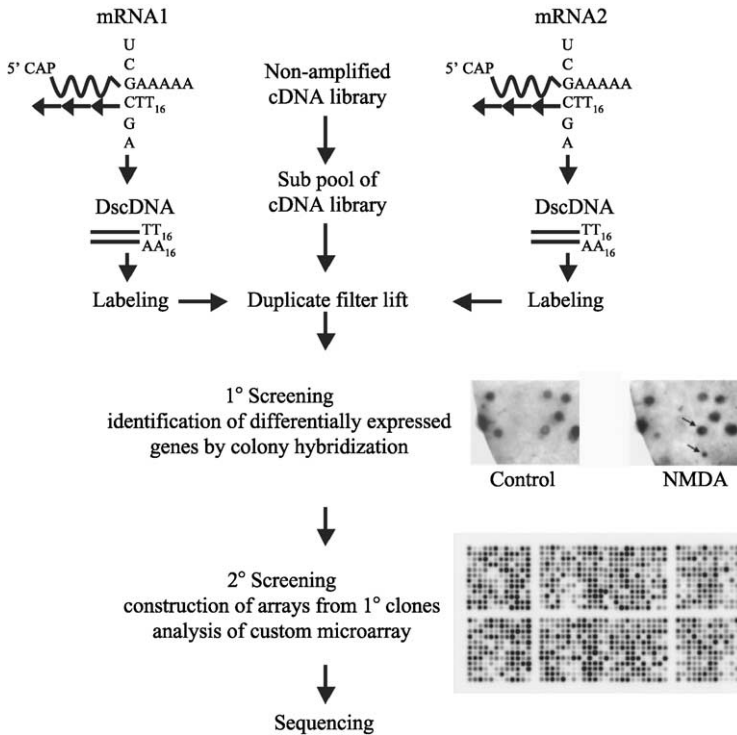


FIG. 2. Scheme displaying the use of differential analysis of cDNA library expression (DAZLE) in identification of *N*-methyl-D-aspartate receptor-mediated genes.  $1.2 \times 10^6$  bacterial cDNA library clones were used for primary screening. There were 1152 clones that were identified from primary screening and arrayed in nitrocellulose membranes. The microarray membranes were used for secondary screening and used for isolation of genes regulated by nitric oxide. Reproduced with permission (Hong *et al.*, 2004).

plated in 24-well plates (Nunc) coated with poly-ornithine at a density of  $4 \times 10^5$  cells/well. Four days after plating, the cells are treated with  $6.7 \mu\text{g/ml}$  of 5-fluoro-2'-deoxyuridine for 3 days to inhibit proliferation of nonneuronal cells. Cells are maintained in MEM, 10% horse serum, 2 mM glutamine and 25 mM glucose in 7%  $\text{CO}_2$  humidified, 37° atmosphere. The medium is changed twice a week. Mature neurons (>14 days in culture) are used. In the mature cultures, the percentage of neurons is approximately 70–90% of the total number of cells, as assessed by neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) immunocytochemical staining of neurons and astrocytes, respectively.

### *Diaphorase Staining*

NADPH diaphorase staining is used to detect NOS-containing neurons in cultures. Cells are washed three times with PBS and fixed for 30 min at 4° in a 4% paraformaldehyde (PF), 0.1 M phosphate buffer. The PF solution is washed away with Tris-buffered saline (TBS) containing 50 mM Tris-HCl, 1.5% NaCl, pH 7.4. The reaction solution containing 1 mM NADPH, 0.2 mM nitroblue tetrazolium, 0.2% Triton X-100, 1.2 mM sodium azide, 0.1 M Tris-HCl, pH 7.2, is applied to the fixed cell cultures for 1 h at 37°. The reaction is terminated by washing away the reaction solution with TBS. All diaphorase-positive cells in each well are counted using an inverted microscope. Generally, mature cortical cultures contain more than 200 diaphorase-positive cells per well.

### *Cell Treatment*

Mature neurons are washed with Tris-buffered control salt solution (CSS) containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 25 mM Tris-HCl pH 7.4, and 20 mM D-glucose. Next, 50 μM NMDA and 10 μM glycine in CSS is applied to the cells for 5 min to induce NMDA-dependent genes, and then the cells are washed and replaced with minimum essential medium containing 10% horse serum and incubated for 6 h in the incubator. Sham (control) treatment is performed as described earlier, except for 5-min treatment with CSS only. *N*-nitro-L-arginine (0.5 mM) is dissolved in CSS to make a stock solution. To inhibit NOS activity, *N*-nitro-L-Arg (100 μM) is added to neuronal cultures 15 min before NMDA treatment. Then, NMDA (50 μM), glycine (10 μM), and *N*-nitro-L-Arg (100 μM) are applied to the cultures for 5 min. Neuronal cultures are washed with culture medium three times. Using another set of neuronal cultures, NO donors (NOR3, 10 μM, Sigma; NOR3-NO<sup>-</sup>, 10 μM) are added to culture medium to deliver NO directly to neurons.

### *cDNA Library Construction*

The cDNA library should be large enough to contain representatives of all sequences of interest, some of which may be derived from low-abundance messenger RNAs (mRNAs). It includes a minimal number of clones that contain small (<500 bp) cDNA inserts, and it is composed of cDNA inserts that are near full-length copies of the mRNAs. Total RNA is extracted from neurons with TRIzol reagent (Invitrogen), and poly(A)<sup>+</sup> RNA is purified with oligo(dT) cellulose chromatography. A bacterial cDNA library from the mRNA of NMDA-treated neurons or NO-donors-treated neurons was constructed with CloneMiner cDNA library construction kit (Invitrogen).

### *Colony Hybridization*

To identify genes regulated by NO signaling pathway, bacteria containing the plasmid cDNA library are used for differential hybridization screening. Nylon membrane filters (137 mm) are laid on an agar plate (150 mm) taking care to avoid air bubbles between the membrane and agar surface. Up to 2000 bacterial colonies are applied onto the filters and incubated until they grow 0.1–0.2 mm in diameter. The template filter is peeled off and laid, colonies up, on a bed of sterile Whatman paper. A wetted sterile filter is held between two flat-bladed forceps and laid on the template filter. The sandwich is pressed firmly together with a velvet-covered replica-plating tool (or thick glass plate). The replica is peeled off the template and placed on a fresh agar plate (up to five filters). The replicas are incubated at 30–37° until the colonies develop to 0.5–1.0 mm. Filters are removed carefully from the plates and placed (colonies side up) on the prepared filter paper (on two layers) soaked with denaturation solution (0.5 N NaOH, 1.5 M NaCl) for 15 min. Filters are then placed for 15 min onto the prepared filter paper soaked with neutralization solution (1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl). Filters are transferred onto the prepared Whatman paper soaked with 2× SSC for 10 min and air-dried for 30 min. The transferred DNA is crosslinked with UV-crosslinker (1200 unit, two times). Then, each filter is placed on a clean piece of aluminum foil and treated with 2 ml of 2 mg/ml proteinase K. The solution is distributed evenly and incubated for 1–2 h at 37°. Using Whatman paper fully wetted with dH<sub>2</sub>O, the filters are blotted between the Whatman paper and pressure is applied by passing over the area with a bottle. Cellular and agar debris are removed by gently pulling off the upper filter paper (the debris will stick to this filter paper). If necessary, the blotting step is repeated with a fresh piece of paper.

### *Hybridization*

1. Place up to three membrane discs in hybridization bag and add 100 ml prehybridization solution [DIG Easy Hyb Granules (Roche) can be used].
2. Prehybridize for 2 h in a hybridization oven at 42°.
3. Radioactive first-strand cDNA probe is prepared as described in Reverse Northern Blotting procedure. Denature the labeled probe by incubating at 68° for 10 min (reuse only). If the probe is synthesized freshly, boil it for 5 min and cool it rapidly on ice.
4. Fresh probe only: Mix the denatured probe with 20 ml hybridization solution, prewarmed to 42°.

5. Remove the prehybridization solution and add the hybridization solution.
6. Incubate for 2 h to overnight at 42°.
7. At the end of the hybridization, pour the hybridization solution into a 50-ml tube.
8. Wash the membranes twice for 5 min in ample 2× SSC, 0.1% SDS at room temperature with gentle agitation.
9. Transfer the membranes to 0.5× SSC, 0.1% SDS, and wash twice for 15 min at 68° with gentle agitation.

#### *Immunological Detection*

- Washing buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween 20
  - Maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5
  - Detection buffer: 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5
  - Blocking solution: Dilute 10× blocking solution (Roche) 1:10 with maleic acid buffer (prepare fresh)
  - Antibody solution: Centrifuge anti-digoxigenin-AP (Roche) for 5 min at 10,000 rpm in the original vial before each use, and pipette the proper volume carefully from the surface. Dilute anti-digoxigenin-AP 1:10,000 in blocking solution (3  $\mu$ l in 30 ml).
1. After hybridization and stringency washes, rinse membranes briefly 1–5 min in washing buffer.
  2. Incubate for 30 min in 100 ml blocking solution.
  3. Incubate for 30 min in 30 ml antibody solution.
  4. Wash 2 × 15 min in 100 ml washing buffer.
  5. Equilibrate 2–5 min in 40 ml detection buffer.
  6. Place membranes with DNA side up on a development folder (big X-ray film can be used).
  7. Apply 1 ml CSPD ready-to-use (Roche) spread the substrate evenly and without air bubbles over the membrane (by covering Saran Wrap).
  8. Incubate for 10 min at 15–25°.
  9. Squeeze out excess liquid (do not dry the membrane).
  10. Incubate the damp membrane for 10 min at 37° to enhance the luminescent reaction.
  11. Expose to X-ray film for 25 min to 1 h at 15–25° (up to 48 h).
  12. The bacterial colonies that show higher intensity on X-ray film with NMDA-treated neuronal probe are picked up, cultured in LB broth containing ampicillin, and preserved at –80° in 50% glycerol.

### *Reverse Northern Blotting Procedure*

Plasmid DNAs from the positive bacterial clones are isolated, denatured, and spotted on a positively charged Nylon membrane (Amersham) with a 96-well vacuum manifold. The spotted DNA is crosslinked to the membrane with a UV crosslinker (Amersham Pharmacia).  $^{32}\text{P}$ -labeled first-strand cDNA is prepared by reverse transcription of total RNA. Next, 30  $\mu\text{g}$  of total RNA is mixed with 4  $\mu\text{g}$  of dT<sub>18</sub>V, incubated 10 min at 70°, and cooled on ice for 5 min. The mixture is added with 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (DTT), 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.02 mM dCTP, 100  $\mu\text{Ci}$   $\alpha$ - $^{32}\text{P}$ -dCTP, and 200 units of Superscript RT II (Invitrogen) in a final 25- $\mu\text{l}$  reaction solution. The reaction mixture is incubated at 42° for 1 h and at 42° for 30 min after addition of 200 units of Superscript RT II. The membrane is hybridized and washed as described earlier. The signal intensity of each spot is measured by ImageQuant software (Amersham Pharmacia), normalized, and compared between control and NMDA-treated neurons. Each image is overlaid with grids to compare signal intensities of individual spots.

### *Microarray Construction and Analysis*

Because the gene expression of a large number can be easily monitored by microarray analysis, it is beneficial to make an array of differentially expressed genes. The NMDA-induced gene-enriched microarray is constructed by arraying polymerase chain reaction (PCR)-amplified cDNA clones at high density on a nylon membrane. 1152 bacterial clones are selected from differential screening. The plasmids are purified from 96-well bacterial cultures (Edge Biosystems), and the cDNA inserts are amplified by PCR. Each PCR product is verified by agarose gel electrophoresis, and each product is printed onto nylon membrane by an array robot. Thirty micrograms of total RNA is used to label cDNA probes by reverse transcription for hybridizing to the microarrays. Total RNA (30  $\mu\text{g}$ ) and 3  $\mu\text{g}$  of oligo(dT)<sub>18</sub>V are mixed (adjust final volume with RNase-free water to 13  $\mu\text{l}$ ), incubated at 70° for 10 min, cooled down to 42°, and then placed on ice. Reverse transcription reaction buffer [5 $\times$  first-strand synthesis buffer (6  $\mu\text{l}$ ), 100 mM DTT (2.5  $\mu\text{l}$ ), 33 mM d(AGT)TP mix (0.6  $\mu\text{l}$ ), 100  $\mu\text{M}$  dCTP (1.5  $\mu\text{l}$ ),  $\alpha$ - $^{33}\text{P}$ -dCTP (5  $\mu\text{l}$ ), and SuperscriptII (1.5  $\mu\text{l}$ )] is added to RNA-oligo(dT) solution and incubated 42° for an hour. RNA is hydrolyzed at 65° for 30 min after adding 1  $\mu\text{l}$  of 1% SDS, 1  $\mu\text{l}$  of 0.5 M EDTA, and 3  $\mu\text{l}$  of 2 N NaOH. Sample is neutralized with 10  $\mu\text{l}$  of 1 M Tris-HCl (pH 7.5) and 3  $\mu\text{l}$  of 2 N HCl.  $^{33}\text{P}$ -labeled cDNA is purified with ProbeQuant G-50 microspin column (Amersham). And then cDNA sample

is boiled for 5 min and either directly added to the hybridization buffer or placed on ice before hybridization.  $^{33}\text{P}$ -labeled cDNAs from sham-treated and NMDA-treated cortical neurons (or NO-donor-treated neuron) are used as the reference probe and the sample probe, respectively, in all hybridizations. Ten micrograms of polydeoxyadenylic acid and 20  $\mu\text{g}$  of human CoT1 DNA (Invitrogen) are added to a DIG easy hybridization solution (Roche), and the microarray membrane is prehybridized at 42° for 1 h before the probe is added directly to the prehybridization solution. Denatured probe (95°, 5 min) is added to the solution and hybridized for 16–24 h. Unbound probe is removed by washing the membranes three times for 5 min each at 55° with 0.5 $\times$  SSC solution containing 0.01% SDS and once for 5 min at room temperature with 0.06 $\times$  SSC solution. The microarray membrane is exposed to a phosphoimage screen for 24 h. The screen is scanned in a phosphoimager at 50-micron resolution. Genes are selected as differentially expressed clones if their expression level deviated from that of sham-treated neurons by a factor of 2.5 in at least five of the samples from NMDA- (or NO-donor-) treated neurons or the standard deviation for the set of five values of z-ratios determined in the analysis of the time course of gene expression exceeded 0.8. The cDNAs displaying differential expression are selected, confirmed by Northern analysis or by RT-PCR. The sequences of differentially expressed genes are analyzed.

### *Quantitative RT-PCR Analysis*

*Preparation of Total RNA.* Total RNA (5  $\mu\text{g}$ ) is mixed with random hexamers (200 ng), incubated at 70° for 10 min and chilled on ice for 1 min. Reverse transcription buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10 mM DTT, 500  $\mu\text{M}$  each dNTP) is added, and reaction mixture is incubated at 25° for 5 min. Superscript RT II (200 units) is added to the mixture and incubated for 10 min at 25°, for 50 min at 42°, and for 15 min at 70°. After the mixture is chilled on ice, 2 units of RNase H is added and further incubated for 20 min at 37°. The reaction is stopped by incubation for 15 min at 70°.

*QPCR (SYBR Green Method).* Primers (22mers, 50% GC content, PCR product size between 100 and 150 bp) are designed and synthesized. cDNA (1  $\mu\text{l}$ ) is mixed with 24  $\mu\text{l}$  of reaction mixture (1 $\times$  SYBR PCR buffer, 3 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  dNTPs, 0.5 units AMP Erase UNG, 100 nM forward primer, 100 nM reverse primer, and 1.25 U AmpliTaq Gold polymerase). PCR conditions are initial incubation at 50° for 2 min and 95° for 10 min, then 40 cycles of 95° for 15 s and 60° for 1 min. The change in fluorescence during PCR is measured in ABI Prism 7700 system.

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## [31] Role of NO in Enhancing the Expression of HO-1 in LPS-Stimulated Macrophages

By KLAOKWAN SRISOOK, CHAEKYUN KIM, and YOUNG-NAM CHA

## Abstract

Macrophages serve as the first-line defense against invading pathogens by (a) overproducing  $O_2^-$  via activation of NADPH-oxidase localized in its plasma membrane, (b) inducing the expression of inducible nitric oxide synthase (iNOS) and overproducing NO, and (c) generating highly toxic peroxynitrite ( $ONOO^-$ ) to kill the invading pathogens without killing the macrophages themselves. Results show that this was due at least in part to the NO-derived induction of heme oxygenase-1 (HO-1) expression. The NO-derived induction of HO-1 caused (a) rapid elimination of toxic heme to inhibit lipid peroxidation and to prevent further induction of iNOS, (b) rapid production of bile pigment antioxidants to scavenge reactive