

Targeted Display: Identifying Differentially Expressed mRNAs

Application of a Rapid Method (Targeted Display) for the Identification of Differentially Expressed mRNAs Following NGF-Induced Neuronal Differentiation in PC12 Cells (1999) *Mol. Cell. Neurosci.* **13**, 119.

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In the following methods application, Brown et al. offer a relatively quick and easy approach to genomic profiling of cell populations. Based upon the technique called "differential display," Brown et al. propose "targeted display," a technique that incorporates a modified subset of oligonucleotide primers and applies the technique to the study of differentiation of PC12 cells.

Introduction

A technique commonly referred to as differential display (DDRT-PCR) was developed seven years ago (1) as a means to compare two or more populations of mRNAs in a non-specific amplification process, which theoretically can amplify all the poly(A)⁺ RNA in a given cell using multiple primer combinations. The RT-PCR-based technique presented great advantages over subtractive hybridization, for example, as the procedure was much faster and less laborious. However, a disadvantage of the technique, presumably resulting from the degenerate oligonucleotides used for priming the reverse transcription reactions, is the high number of false positives.

Brown *et al.* call their technique "targeted display," and their example consists of applying NGF, as well as EGF and FGF, to PC12 cells for 2, 4 or 6 hours and profiling actively transcribed mRNA species. (The 2–6-hour time course of NGF induction of PC12 cells had not been reported previously.)

One improvement of Brown *et al.* to the original DDRT-PCR method consisted of utilizing octadecanucleotide sequences overrepresented, by statistical analysis, in human protein-encoding DNA strands. Using these sequences for constructing the primers, in lieu of the 3' UTR-hybridizing random poly(dT) primers in the original method, results in amplified gene fragments that represent approximately 70% of the gene coding sequences.

PC12 cells, a rat pheochromocytoma cell line, is a widely used model in neurobiology. Specifically, PC12 cells are induced to differentiate into neuronal cell types by administration of NGF, for example. In this report, Brown *et al.* apply targeted display to differentiated and undifferentiated PC12 cells.

Table 1. Modified Oligonucleotide Primers Used in Targeted Display by Brown *et al.*

Sense		Antisense	
6s	tgatga agaaggcc	6a	tggtga ggccttct
7s	tggtga catgaagg	8a	tggtga tttccagg
11s	tgatga agaccctg	10a	tgatga gtccagga
12s	tggtga ccaagaag	11a	tgatga cagggtct
13s	tgatga cctggaga	14a	tgatga tccaggtc
15s	tgatga tgctgagc	15a	tgatga gctcagca
16s	tgatga agaggagc	17a	tggtga tcatctgg
17s	tggtga ccagatga	18a	tgatga ccaggtag
		19a	tggtga ttcagctc
		20a	tggtga cttgtcca

Methods and Materials

The following methods, taken from the original reference, should be applicable to individual culture conditions as well as treatment schemes (e.g., choice of serum and growth factor/treatment duration). Additional methods and materials can be found in the original report.

Cell Culture

Culture PC12 cells in DMEM medium (Life Technologies, Inc.) with 10% donor horse serum and 5% fetal horse serum at 37°C in a humidified atmosphere with 5% CO₂. Maintain cells at 2 × 10⁵ cells/ml. Add NGF (50ng/ml), EGF (50ng/ml) or FGF (20ng/ml) (all available from Promega) for indicated times and harvest cells.

Primer Design

Oligonucleotide primers are based on a published subset (2). See Table 1 for a list of the oligonucleotide primers. The addition of 6bp to the 5' ends of the published octanucleotides greatly enhances primer annealing and hence, priming of transcription. Bases were added in such a manner to generate an equal %GC across the resulting 14mers.

cDNA Synthesis

After treating cells with growth factor, isolate total RNA from approximately 1 × 10⁶ cells. Lyse the cells in 400μl of RNA lysis buffer (0.15M NaCl, 10mM Tris-HCl [pH 7.4], 1mM MgCl₂, 0.5% [v/v] NP-40 with 1μl RNase inhibitor (Recombinant RNasin[®] Ribonuclease Inhibitor^(a), Cat.# N2511) on ice and remove nuclei by centrifugation. Add 50μl of 10% SDS to the supernatant and extract using phenol/chloroform. Precipitate RNA with ethanol and resuspend in 100μl of RNase-free water. Determine the integrity of the RNA by agarose gel electrophoresis.

Prior to cDNA synthesis, treat total RNA with DNase I to remove contaminating genomic DNA. This step is essential to maximize reproducibility of subsequent PCR amplifications. Add aliquots of total RNA (10–100μg) in 99μl of water to 1μl of RNase-free DNase I (10 units), 10μl of 50mM MgCl₂, 10μl of 100mM DTT, 3.3μl 3M sodium acetate (pH 5.3) and 0.5μl RNase inhibitor. Incubate at 37°C for 15 minutes. Inactivate DNase I by phenol/chloroform extraction and precipitate RNA.

The SV Total RNA Isolation System^(b) (Cat.# Z3100) is a suitable alternative for this application, and if used, it is not necessary to treat the total RNA with DNase I.

Prepare first-strand cDNA using 10µg aliquots of the RNA and SuperScript® II reverse transcriptase (or M-MLV Reverse Transcriptase, RNase H⁻); Promega, Cat.# M5301) in a total volume of 20µl. Incubate at 37°C for 20 minutes and then at 42°C for 1 hour. Terminate the reaction by heating the mixture at 70°C for 10 minutes. Add 9µl of sterile water and 1µl of RNase H (2 units, Cat.# M4281) and incubate an additional 30 minutes at 37°C. As a control for possible genomic DNA contamination, perform reactions with or without the addition of the reverse transcriptase or total RNA in the PCR amplification.

Targeted Display—PCR Conditions


Single-stranded cDNA is used directly in PCR amplifications. The reaction mixture consists of a volume of cDNA from the RT reaction corresponding to approximately 100ng of starting RNA, 2.5µM dNTP, 10pmol of each oligonucleotide primer and 1 unit

of KlenTaq DNA polymerase. Reaction profile consists of an initial denaturation of 94°C for 2 minutes, followed by a single cycle of 94°C for 1 minute, 42°C for 1 minute and 72°C for 3 minutes. This is followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, with a final extension of 72°C for 10 minutes.

Summary

This study validated the use of targeted display as a genomic profiling technique in PC12 cells. The advantage of this technique over DDRT-PCR, as pointed out by the authors, is that targeted display is potentially selective for protein-encoding DNA by nature of the oligonucleotide primer design.

Nevertheless, targeted display, like all PCR-based amplification procedures, is susceptible to false positives due to background amplification, and concerns over reproducibility are the same. An additional and more important concern, though, is whether identified clones are truly expressed. That is, are the mRNA species transcribed into mature proteins. Targeted display may prove to be a viable first step in profiling a specific genome, with potential unique transcripts being pursued by more traditional expression studies.

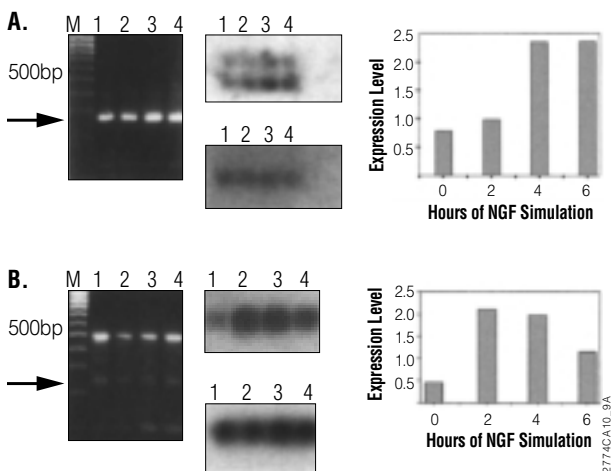
Importantly, most if not all improvements to the original DD RT-PCR technique should prove applicable to targeted display technique as well. 

References

- Liang, P. and Pardee, A.B. (1992) *Science* **257**, 967.
- López-Nieto, C.E. and Nigam, S.K. (1996) *Nat. Biotechnol.* **14**, 857.

Ordering Information

Product	Size	Cat.#
mNGF, 2.5S	100µg	G5141
rhEGF	100µg	G5021
rhFGF, basic	25µg	G5071
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SV Total RNA Isolation System ^(b)	50 preps	Z3100
SV Total RNA Isolation System, Trial Size ^(b)	10 preps	Z3101
M-MLV Reverse Transcriptase, RNase H ⁻ (c)	10,000 units	M5301
Recombinant RNasin® Ribonuclease Inhibitor ^(a)	2,500 units	N2511
	10,000 units	N2515
Ribonuclease H	50 units	M4281
	250 units	M4285



▲ **Figure 1.**

Isolation and characterization of two products identified by targeted display. Panels A and B: MARK1. Panels C and D: MAPKK3.

Panels A and C: Following PCR amplification with individual primer pairs, products were resolved on agarose gels and differentially expressed DNA bands were excised, cloned and sequenced. Arrows indicate the position of the selected bands. Individual cloned inserts were used for Northern analysis. Northern blots were reprobed for GAPDH to control for differences in RNA loading. DNA inserts were sequenced and identified by comparison to public databases. Lane 1, undifferentiated PC12 cells; lanes 2, 3 and 4, NGF for 2, 4 and 6 hours, respectively. Panels B and D: Expression pattern following NGF treatment. Densitometer readings from Northern hybridization signals generated using sequences isolated by targeted display were normalized with respect to levels of GAPDH and plotted against time of exposure to NGF.

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^(b)Patent Pending.

^(c)Product is available only in certain countries. Please contact your local Promega Branch Office or Distributor for ordering information.