the LC/LINK-D assembly. The sluggishness of the VH/CH1-LINK-D reaction may be caused by formation of strong secondary structures within the coding region of CH1, which would also account for the relatively weaker amplification of the Fd gene (as compared to the VH gene) as observed previously (9).

Although the PCR assembly strategy has been described as somewhat difficult (4), we find it an attractive alternative to the cloning of heavy- and light-chain genes separately. Furthermore, the rare cutting-restriction enzymes SfiI and NotI are the only enzymes used in the entire cloning procedure. Use of more enzymes inevitably increases the danger of cleavage within the Fab coding regions, which would lead to less comprehensive libraries.

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Peter Sejer Andersen, Henrik Ørum and Jan Engberg
The Royal Danish School of Pharmacy
Copenhagen, Denmark

Sensitive, Nonradioactive Differential Display Method Using Chemiluminescent Detection

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Isolation of genes that are differentially expressed in closely related but different cell types, such as normal and cancer cells, is of great interest in modern molecular biology. The classical approaches that have been used widely for isolation of differentially expressed genes are subtractive hybridization and differential hybridization (7). Although many successful applications have been reported in different systems, these methods are both time-consuming and technically difficult. The recently described mRNA differential display method provides an attractive, alternative tool for cloning differentially expressed genes and has gained considerable attention (3). In the differential display method, mRNAs from two or more cell populations are reverse-transcribed and amplified by polymerase chain reactions (PCR) using one 3’ anchored primer and one 5’ arbitrary primer. The radioactive cDNA products from PCR are then resolved on a DNA sequencing gel in adjacent lanes. Differentially expressed mRNA species are identified by comparing the intensities of cDNA bands on the gel.

Currently, radioactive detection methods are used by most researchers to identify band patterns on a sequencing gel in a differential display experiment (3,4). The disadvantages of the standard procedure include the handling of hazardous radioisotopes, potential contamination of the working area and thermal cycler, and cost and burden of waste disposal of the radioactive materials. Recently, a silver staining-based differential display protocol has been described (5). Although valuable, this method has some drawbacks. The detection sensitivity by silver staining is generally much lower than that obtained by using radioisotopes. This limitation leads to much fewer bands being displayed per reaction (5) and causes low-abundance messages to be undetectable. Furthermore, silver-
staining procedures are generally difficult to control (1), and the results obtained are often inconsistent. On the other hand, the chemiluminescence-based sequencing protocol has been used widely and has proven to be a sensitive and reliable alternative to radioactive sequencing (6). We developed and describe here a nonradioactive differential display method using chemiluminescent detection. Our method has all the benefits of nonradioactive procedures without sacrificing the sensitivity of radioactive detection. We have successfully used this method to identify and isolate potential metastatic cancer cells.

Four prostate cancer cell lines (LNCaP, PC3-PF, PC3-MF and DU-145) (ATCC, Rockville, MD, USA) were used for the study. PC3-PF, PC3-MF and DU145 have been shown previously, both in vitro and in nude mouse animal models, to have high tumorigenic and metastatic potential (2,8). The LnCaP cell line has been described as being less tumorigenic and less metastatic in the nude mouse model (2,8). The cells were cultured in RPMI-1640 (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Life Technologies). Total RNAs were isolated from the four cell lines and reverse-transcribed into cDNAs as described (3), except that the four degenerate, anchored oligo(dT) primers (T12MN) used were 5' end-labeled with a biotin during primer synthesis. The four anchored primers used were 5'-biotin-TTTTTTTTTTTTMA-3', 5'-biotin-TTTTTTTTTTTTMG-3', 5'-biotin-TTTTTTTTTTTTMN-3' and 5'-biotin-TTTTTTTTTTTTMC-3'. Subsequent PCR amplification of the cDNAs was performed with the appropriate biotin-labeled T12MN in combination with different arbitrary decamers (Kit A; Operon Biotechnology, Alameda, CA, USA). Four arbitrary decamers were used: 5'-CAGGCCCTTC-3', 5'-TGCCGAGCTG-3', 5'-AGTCAGC-CAC-3' and 5'-AATCGGGGCTG-3'. The conditions used for PCR were as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 20 μM of each deoxyribonucleoside triphosphate (dNTP), 0.2 μM of arbitrary decamer, 1 μM of biotin-labeled T12MN, 1/10 volume (2 μL) of the cDNA reverse-transcribed from 0.2 μg total RNA, 1 U of Taq DNA Polymerase (Boehringer Mannheim, Indianapolis, IN, USA) in a final 20-μL mixture. The amplification parameters included 40 cycles of reaction with 30 s denaturing at 94°C, 2 min annealing at 40°C and 30 s extension at 72°C. The amplified products were then separated on a 6% polyacrylamide sequencing gel.

The SEQ-Light™ chemiluminescent detection system (Tropix, Bedford, MA, USA) was used to detect amplified bands on the sequencing gel. DNA fragments on the gel were transferred to the Tropilon-Plus™ nylon membrane (Tropix) by capillary transfer following manufacturer's instructions. Approximately 20%–30% of the DNA was transferred to the membrane, which could easily be detected using the chemiluminescent detection procedure. Once a positive band was identified from the DNAs transferred to the membrane, the remaining DNAs (70%–80%) on the gel were recovered and served as templates for reamplification. The reamplified cDNAs were cloned into pCR™ II (TA Cloning™ Vector) (Invitrogen, La Jolla, CA, USA), and the expression pattern was confirmed.

Figure 1. Band patterns from a part of the sequencing gel in the mRNA differential display detected by chemiluminescent reaction. Primers used: 5'-biotin-TTTTTTTTTTTTMA-3' and 5'-TGCCGAGCTG-3'. Lane 1, RNA from LNCaP cells; lane 2, RNA from PC3-PF cells; lane 3, RNA from PC3-MF cells; lane 4, RNA from DU-145 cells. Arrow indicates an mRNA transcript (C1) that is expressed highly in PC3-PF, PC3-MF and DU-145 cell lines but very little in the LNCaP cell line.

Figure 2. Confirmation of differential expression of the gene by Northern hybridization. A sample of total RNAs (10 μg each) from LNCaP (lane 1), PC3-PF (lane 2), PC3-MF (lane 3) and DU145 (lane 4) were subjected to Northern blot analysis. C1 cDNA was labeled with biotin using the BioPrime™ DNA labeling system (Life Technologies), and the hybridization signals were detected by chemiluminescent detection using the Northern-Light™ system (Tropix). Upper panel shows the Northern result of the mRNA (C1) identified in Figure 1; lower panel shows the β-actin control.
by Northern blot analysis.

Figure 1 shows part of the sequencing gel from the differential display experiments using chemiluminescent detection. Generally, about 100–200 bands were seen from the whole gel for each primer set; this number is about the same as with radioisotope detection (data not shown); most of the bands showed identical intensity among the cells examined. The band indicated by the arrow (C1) shows much higher intensity in PC3-PF, PC3-MF and DU145 cell lines compared to the low tumorigenic and metastatic LNCaP cell line. The differential expression of this cDNA among the four cell lines was further confirmed by Northern hybridization (Figure 2). The confirmation rate of the differentially expressed cDNAs by Northern blots using our method is similar to that of differential display using radioactive detection (data not shown).

In conclusion, the method described here is a sensitive, reliable and safe alternative to the radioisotopic differential display technique; it has all the advantages of a nonradioactive method without sacrificing the sensitivity of radioactive detection. This method could be applied to any experiment where differential display technology might apply, particularly for those who wish to use nonradioactive procedures.

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Gang An, Guizhen Luo, Robert W. Veltri and S. Mark O'Hara
UroCor
Oklahoma City, OK, USA

Guanidine-HCl Extraction of Proteins Expressed in Escherichia coli Using Polycistronic Operons Based on the pUR Expression Vectors


Recently, we developed a new system for efficient expression of individual heterologous proteins in Escherichia coli cells using polycistronic operons that contain a gene of full-length β-galactosidase (β-gal) as the first cistron followed by one or two copies of a heterologous gene (4). Specifically, polycistronic constructs based on the pUR290 plasmid vector (8) that contain the β-gal gene followed by one or two copies of an individual NS1 gene of tick-borne encephalitis virus (TBEV; Flaviviridae family) were made. These constructs were called pUR290NS1 and pUR290 (NS1), respectively. Both plasmids provided expression of both β-gal (116 kDa) and NS1 (39 kDa). The pUR290 (NS1)2 plasmid provided at least a fivefold higher level of synthesis of NS1 (20 mg/L of culture) in comparison with pUR290NS1.

Our preliminary attempts to express the NS1 protein using conventional plasmid vectors [pUC (New England Biolabs, Beverly, MA, USA), pTTQ (9), pCEQ (3) and pCE (2) series] were unsuccessful, which suggested that the NS1 expression product was toxic to the bacteria. We encountered difficulties in construction of the recombinant plasmids that contained the NS1 gene in the correct orientation, poor growth of the plasmid-containing cells and alterations of the plasmids during expression (K.V. Pugachev, unpublished observations). In contrast, the pUR290NSΔβ plasmid (4), which contains the NS1 coding region fused inframe with the 3'-end of the gene of bacterial β-gal, provided abundant expression of the β-gal-NS1 fusion protein (155 kDa), indicating that β-gal sequences at the N terminus of the expression product reduced the deleterious effect of NS1 on bacteria. This

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