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AN IMPROVED METHOD FOR SOUTHWESTERN BLOTTING

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1. ABSTRACT

We have developed a modified Southwestern blotting technique which utilizes broad-spectrum protease inhibitors during nuclear protein extraction and a procedure for radiolabelling an oligonucleotide probe to a high specific activity. These modifications have resulted in minimal protein degradation during nuclear protein isolation and have permitted room temperature hybridizations, improving both the facility and sensitivity of the standard Southwestern assay. This technique was used in our laboratory to visualize NFAT-1 consensus sequence binding proteins in nuclear extracts of human promyelocytic HL-60 cells.

2. INTRODUCTION

Southwestern blotting, first described by Bowen and colleagues (1), is a powerful technique for identifying and characterizing DNA-binding proteins by their ability to bind to specific oligonucleotide probes. Nuclear protein extracts are typically separated electrophoretically on an SDS-polyacrylamide gel and then transferred to nitrocellulose for screening with oligonucleotide probes. The usefulness of this technique is hampered by its requirement for relatively large amounts of nuclear proteins (typically 50-100 mg), problems with protein degradation during isolation, and the difficulties in achieving efficient electrophoretic separation and transfer of a wide molecular size range of proteins.

Here, we describe a modified Southwestern technique that takes advantage of a highly efficiently labeled oligonucleotide probe for screening. In addition, the technique uses a wide-spectrum protease inhibitor that minimizes protein degradation and permits room temperature hybridization, and optimizes electrophoretic parameters for separation of a wide molecular size range of proteins. This technique was used in this laboratory to visualize NFAT-1 consensus sequence binding proteins in nuclear extracts of human promyelocytic HL-60 cells.

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3. MATERIALS AND METHODS

Nuclear protein fractions were obtained from cells of the HL-60 human promyelocytic leukemia cell line as described below:

I. Cells (1 x 10^8 in log phase growth) were harvested centrifuged (800x g for 5 min) and washed once in sterile, ice-cold phosphate buffered saline without calcium or magnesium (PBS).

II. The washed cells were resuspended in 1.25 ml of cold buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.3 M sucrose, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride (pmsf) (Sigma Chemical Co., St. Louis, MO) and protease inhibitors as follows (Boehringer Mannheim Indianapolis, IN): 50 mg/ml antipain dihydrochloride, 0.7 mg/ml pepstatin, 330 pg/ml phosphoramidon, 2 mg/ml aprotinin, 60 mg/ml chymostatin, 0.5 mg/ ml leupeptin, 40 mg/ml bestatin, 10 mg/ml E-64, and 1 mg/ml pefabloc.

III. Resuspended cells were harvested and resuspended in 0.5 ml of the same buffer, and lysed with 15 strokes with a B type pestle in a Dounce homogenizer.

IV. The supernatant containing the cytosolic contents from the lysed cells was removed, and the pelleted nuclei were resuspended in 0.3 ml ice-cold buffer as described above, except that 25% glycerol and 0.2 mM EDTA was added.

V. The resuspended nuclei were gently agitated for 30 min at 4^{o}C, and the result supernatant harvest was centrifuged (17000x g for 15 min at 4^{o}C) and then dialyzed in a 10 KDa pore size membrane (Spectrum, Houston, TX) at 4^{o}C against more than 100 volumes of buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 10 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, and 0.5 mM PMSF for 3-5 hrs.

Southwestern blotting

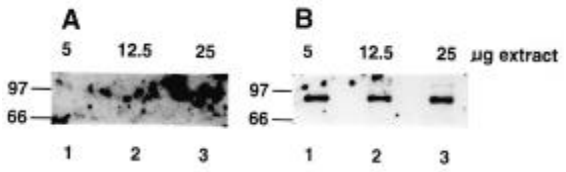

Figure 1: (A) Southwestern blot of HL-60 nuclear extract probed with a double-stranded oligonucleotide containing an NFAT-1 consensus binding site that was end-labeled by standard techniques (7). (B) Southwestern blot of HL-60 nuclear extract probed with the same double-stranded oligonucleotide end-labeled to a 10-fold greater specific activity. Lane 1, 5 µg nuclear protein extract; Lane 2, 12.5 µg nuclear protein extract; Lane 3, 25 µg nuclear protein extract. Arrow points to an additional minor band (100 kDa).

VI. The dialyzed supernatant was clarified by centrifugation, and the protein concentration measured using the BCA protein assay reagent system (Pierce, Rockford, IL). Nuclear extracts were aliquoted and stored at -80^{o}C.

The nuclear protein fraction was electrophoretically separated on a Tris-glycine gel and then transferred to a nitrocellulose membrane.

I. Five to 25 µg of the nuclear protein extract (1 µg/µl) was diluted with an equal volume of 2x reducing sample buffer containing 25% 0.5 M Tris-HCl, pH 6.8, 4% SDS, 0.1% bromophenol blue, 20% glycerol, and 5% ß-mercaptoethanol.

II. The sample was then denatured at 95^{o}C for 5 minutes prior to loading onto the gel, and subjected to electrophoresis (160V) for 1.5 hours at room temperature on a precast 14% Tris-glycine gel (Novex, San Diego, CA) in running buffer containing 2.9 g/L Tris base, 14.4 g/L glycine, and 1g/L SDS.

III. Proteins were transferred to a nitrocellulose membrane overnight at 15V (2). The nitrocellulose membrane was prehybridized for 30 minutes at room temperature with buffer containing 10 mM HEPES and 5% non-fat dry milk, and then hybridized with binding buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 1mM EDTA, 1mM DTT, 0.25% non-fat dry milk, 5 µg/µl heat denatured sheared salmon sperm DNA, and 10 ng radioabelled probe ($6x10^5$ cpm/ng) for one hour at room temperature. The blot was then washed 3 times for 20 minutes each at room temperature with the same binding buffer with 300 mM NaCl, air-dried and exposed for autoradiography.

The oligonucleotide probe was end-labeled to a high specific activity and purified.

I. A double stranded oligonucleotide probe containing a tandem repeat of the NFAT-1 consensus recognition site, GGAGAA (3), was prepared by heating the two strands to 65^{o}C followed by gradual cooling to room temperature (approximately 30 minutes).

II. The duplex was then end-labeled with [γ-^{32}P]ATP. One µl of 10 ng/µl oligonucleotide probe was combined with 10 units T4 polynucleotide kinase (New England Biolabs, Beverly, MA), 2 µl 10x kinase buffer, and 16 µl [γ-^{32}P]ATP [150 mCi (6000Ci/mmol) (DuPont NEN, Boston, MA).

III. The radiolabeled probe was then phenol-chloroform purified, and redissolved in 1x Tris-EDTA buffer (Biofluids, Rockville, MD).

The probe was efficiently labeled at $6x10^5$ cpm/ng, representing an order of magnitude increase over the specific activity of the same probe prepared using standard end-labeling with 1 µl of [γ-^{32}P]ATP (4).

4. RESULTS AND DISCUSSION

A number of modifications to the Southwestern blotting was done to increase the sensitivity of detection without excessive background and with the improved ease of room temperature hybridization. End-labeling with a 15 fold excess of [γ-^{32}P]ATP followed by size-selection created a radiolabeled probe with high specific activity that yielded little background into the room temperature-hybridization step. Room temperature hybridization steps were made possible by the inclusion of the protease inhibitor cocktail in the nuclear sample preparation. We found this method allowed detection of an approximately 90 kDa DNA binding protein on a nitrocellulose blot of HL-60 nuclear proteins that could not be detected using the less efficiently labeled probe (Figures 1A and 1B). Additionally, a faint band of approximately 100 kDa was also detected. Using this method, we were able to detect strong signals from as little as 5 µg of protein from a crude nuclear extract after an overnight exposure to X-ray film.

5. ACKNOWLEDGMENT

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