Use of Fluorescently Labeled DNA and a Scanner for Electrophoretic Mobility Shift Assays

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The electrophoretic mobility shift assay (EMSA) is commonly used to determine the presence of specific transcription factors within the nucleus. In this assay, nuclear extracts from cells are mixed with a radioactively labeled oligonucleotide or DNA fragment containing a sequence known to bind to the transcription factor. This mixture is then electrophoresed through a non-denaturing acrylamide gel, and the gel is subjected to autoradiography. Binding of the oligonucleotide, and hence the presence of the transcription factor, is evidenced by a change or shift in the migration of the oligonucleotide. This method is quite sensitive but has the drawback that it relies on the labeling of DNA with a radioisotope. These probes are short lived, require special precautions for handling and disposal, and pose the health risk associated with the use of radioisotopes. To overcome these problems, we wanted to determine if a fluorescently labeled oligonucleotide could be used in an EMSA.

To determine if non-isotopic methods could be used to detect the binding of NF-κB, several oligonucleotides were obtained from commercial sources (Research Genetics, Huntsville, AL, USA) (Table 1). Oligo 1 contained the fluorescent dye, carboxyfluorescein (FAM) at the 5’ end, while Oligo 1 unlabelled, which had the same sequence and therefore serves as a competitor for Oligo 1, was not fluorescently tagged. Oligo 1 complement binds to Oligo 1 and Oligo 1 unlabelled. To create an oligonucleotide that would not serve as a competitive inhibitor for NF-κB binding to Oligo 1, we synthesized a mutated version (and its complement) of the unlabeled oligonucleotide. Mutated Oligo 1 contained two point mutations that have been previously shown to abolish NF-κB binding (1).

Nuclear extracts were prepared as described previously (2). U-87 MG cells were seeded onto 150-mm culture plates and allowed to become approximately 80% confluent. Cells were removed from the plates by trypsinization and recovered by centrifugation at 2000 ×g for 5 min. The supernatant was discarded, and cells were washed in 1 mL buffer A [10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.1% Nonidet P-40 (NP-40), and 0.5 mM dithiothreitol (DTT)] and pelleted at 2000 ×g for 5 min. The supernatant was again discarded, and cells were resuspended by gentle pipetting in 80 μL buffer A containing 0.1% Triton X-100. After incubation at 4°C for 10 min, the homogenate was centrifuged at 2000 ×g for 5 min, and the nuclear pellet was washed in 60 μL 20 mM HEPES, pH 7.9, 0.42 M NaCl, 25% (v/v) glycerol, 1.5 mM MgCl2, and 0.2 mM EDTA. This suspension was incubated on ice for 30 min and then centrifuged at 16,000 ×g for 20 min at 4°C. The supernatant, which represented the nuclear extract, was stored at -80°C until use. Total protein concentrations of the extracts were determined using the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard.

Assays were performed in 40-μL volumes containing 20 μg nuclear protein incubated with 1 pmol Oligo 1 and its complement in binding buffer (10 mM HEPES, 50 mM KCl, 2.5 mM DTT, 0.2 mM, 0.05% NP-40, 10% glycerol, pH 7.9) for 30 min at room temperature. Competition experiments were performed by mixing Oligo 1 and its complement and 50 pmol of the oligonucleotides used for competition. The supershift assays were performed by adding 0.5 pmol antibody to the p65 subunit of NF-κB (Upstate Biotechnology, Lake Placid, NY, USA) to reactions that contained Oligo 1 and its complement as well as nuclear extract. Samples were loaded on a 7% native polyacrylamide gel, and electrophoresis was performed in 50 mM Tris, pH 8.3, 380 mM glycine. Following electrophoresis, the gel was analyzed by blue-excited fluorescence scanning with an 860 Storm FluorImager (Amersham Pharmacia Biotech, Piscataway, NJ, USA).
Benchmarks

Table 1. Sequences of the Oligonucleotides Used

<table>
<thead>
<tr>
<th>Oligo 1</th>
<th>5'-FAM-CATGAACCGGAATTTCGACATG-Dabcyl-3'</th>
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<tbody>
<tr>
<td>Complement Oligo 1</td>
<td>5'-AATTGGAAATTCGCGTTCC-3'</td>
</tr>
<tr>
<td>Oligo 1 Unlabeled</td>
<td>5'-CATGAACCGGAATTTCGACATG-3'</td>
</tr>
<tr>
<td>Mutated Oligo 1</td>
<td>5'-CATGAACGGCGAATTTCGACATG-3'</td>
</tr>
<tr>
<td>Mutated Oligo 1 Complement</td>
<td>5'-AATTGGAAATTGCGGTCC-3'</td>
</tr>
<tr>
<td>AP-1</td>
<td>BODIPY 630/650-5'-CGCTTGATGAGTCAGCGAA-3'</td>
</tr>
<tr>
<td>AP-1 Complement</td>
<td>5'-CTCCGCTGACTCATCAAGCG-3'</td>
</tr>
</tbody>
</table>

The underlined bases in the mutated oligonucleotides indicate the point mutations.

As can be seen in Figure 1, protein from the extract was able to cause a shift in the migration of the oligonucleotide, and this shift could be inhibited by excess unlabeled oligonucleotide but not by the oligonucleotide that contained the two point mutations. This shift was due to the association of NF-kB proteins with the oligonucleotide because an increase in the apparent molecular weight of the complex occurred when the antibody to p65 was present. There was no shifted band without the addition of the extract (data not shown). This data demonstrated that these fluorescently labeled oligonucleo-

Figure 1. EMSA performed with extracts of U-87 MG tumor cells. Extracts were prepared from U-87MG glioblastomas as described in the text, and 20 μg were used for EMSAs. All assays contained nuclear extract and the NF-κB binding oligonucleotides Oligo 1 and its complement. The diamonds indicate the unshifted oligonucleotides, while the arrows indicate the shifted oligonucleotides and the circle indicates the position of the supershifted oligonucleotides.
As EMSAs are commonly performed with radioactively labeled oligonucleotides, we were interested in the relative sensitivity of a fluorescently labeled oligonucleotide as compared to a radioactively labeled oligonucleotide. We obtained an oligonucleotide that contained the binding site for the transcription factor AP-1 and the complement of this oligonucleotide used for detection. The upper panel shows the image from the isotope detection, while the lower panel shows the image from the fluorescent detection. (B) Multiplex analysis using both the NF-κB oligonucleotides and the AP-1 oligonucleotides. The upper panel is the image from the red excited fluorescence (BODIPY630/650, AP-1)-labeled oligonucleotide, while the lower panel is from the scan performed with the blue excited fluorescence (FAM, NF-κB).

In summary, we have demonstrated that the technique works quite well in a polyacrylamide gel format, which is more commonly used, and that multiplex analysis can be performed on a single sample.

REFERENCES


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