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Non-radioactive electrophoretic mobility shift assay using digoxigenin-ddUTP labeled probes.

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Electrophoretic mobility shift assay (EMSA) is a powerful technique used to quantitatively analyze sequence specific DNA binding proteins. Traditionally a radiolabeled DNA probe is added to a candidate protein and then the mixture is separated on an acrylamide gel. When a DNA-protein complex forms, it is retarded by the gel and appears "shifted" in comparison to the mobility of the free probe. This apparent complex can then be challenged with an unlabeled probe of the same or different sequence to establish specificity. Since this method uses a radiolabeled probe, it not only requires the safety concerns associated with proper storage, usage and disposal of radioactivity, but also limits the lifespan of the probe. Here we present the use of Digoxigenin labeled probes as an alternative to radioactivity in EMSA. In this assay the shift reaction is resolved on an acrylamide gel and then transferred to a nylon membrane for detection using an antibody against Digoxigenin. It offers the advantages of high resolution and comparable sensitivity of ^{32}P labeled probes. In addition this method is well suited for teaching students new biochemical techniques.

To demonstrate the effectiveness of this method we tested the ability for Twist, a basic Helix-Loop-Helix transcription factor, to bind a 20 bp double-stranded DNA fragment from the *rhomboid* promoter of *D. melanogaster*. This fragment contains a canonical E-box (CATATG) to which Twist has been previously shown to bind (Ip *et al*., 1992). Twist binds DNA by forming homodimers with itself or heterodimers with other bHLH proteins to either activate or repress myogenesis (Castañon *et al*., manuscript submitted).

Labeling Probe with Digoxigenin: 1 nmol of each oligonucleotide (5′-GATCCCTCG**CATATG**TTGAA-3′ Top, 5′-GATCTTCAACATATGCGAGG-3′ Bottom) was annealed by heating at 95ºC for ten minutes and slowly cooled to room temperature in a buffer of Tris (10 mM, pH 8), EDTA (1 mM, pH 8) and NaCl (100 mM). The double-stranded oligonucleotide was then labeled using the reagents provided in the DIG Oligonucleotide 3′-End Labeling Kit (Roche Cat. No. 1 362 372). The labeling protocol was modified only in the incubation and precipitation times. 100 pmol of the fragment was added (on ice) to 4 µl 5× labeling buffer, 4 µl, 25 mM $CoCl_2$, 1 µl 1 mM DIG-11-ddUTP and 2.5 U Terminal Transferase in a final volume of 20 µl and incubated at 37ºC for 1 hour. The DNA was then precipitated with 2 µl 4 M LiCl and 60 µl 100% ice-cold ethanol and incubated at –70ºC for 2 hours. The precipitated DNA was then pelleted by centrifugation for 15 minutes at 4ºC, 13,000 × g, washed once with 100 µl ice-cold 70% ethanol and recentrifuged for an additional 15 minutes. After air drying the pellets, the DNA was resuspended in ddH_2O to a final concentration of 2.5 pmol/µl. Generating a dot blot, as shown in Figure 1A, tested the efficiency of the labeling reaction. Serial dilutions were spotted on a nylon membrane and detected as described below. The resulting intensity was then directly compared to the control labeled oligonucleotide provided with the labeling kit. The probe was then stored at –20ºC.

Shift Reaction: Full-length *twist*, *bHLH twist* and tethered *twist-twist* DNA constructs were used as templates in a TnT Coupled Reticulocyte Lysate System (Promega Cat. No. L4610, Castañon *et al.*, manuscript submitted). The *bHLH twist* construct is a truncated form missing amino acids 141-331 yet retains both the dimerization and DNA binding domains. The *twist-twist* tethered dimer has two full length *twist* monomers connected by a serine and glycine flexible linker. In vitro translations were performed using 150 ng of input DNA in a 7.5 µl reaction containing 3.75 µl of rabbit reticulocyte lysate. The protein products were incubated in 2 mM DTT at 37ºC for 10 minutes (Marcus, 2000). After a 5-minute equilibration to 25ºC, 5 µl of the protein products were added to 50 fmol of the Digoxigenin labeled probe. The 25 µl reaction mixture contained 1 µg poly d(I-C) (Roche Cat. No. 108 812), 2 mM $MgCl_2$, 25 mM HEPES pH 7.5, 100 mM NaCl, 0.1% Igepal CA-630 (Sigma Cat. No. I-3021), 12% glycerol (v/v). The mixture was electrophoresed at 10 volts/cm through a 0.25× TBE – 5 % polyacrylamide gel (acrylamide-bisacrylamide, 29:1 - 3.3 % C). The 15 cm × 15 cm gel was then transferred to a positively charged nylon membrane (Roche Cat. No. 1 209 272) using a semi-dry transfer cell (Bio Rad) under a constant amperage of 0.56 mA (25 V max) for 15 minutes. The DNA was then cross-linked to the membrane using a UV stratalinker (Stratagene).

Detection: 10× blocking reagent (Roche Cat. No. 1 096 176) was prepared in a maleic acid buffer [0.1 M maleic acid (pH 7.5), 0.15 M NaCl] according to the manufacturer. The nylon membrane was blocked for one hour at room temperature in 2× blocking reagent and then incubated with Anti-Digoxigenin coupled to Alkaline Phosphatase (Roche Cat. No. 1 093 274) diluted 1:20,000 for 30 minutes. The membrane was washed twice for 20 minutes each with 200 ml of maleic acid buffer with 0.3% Tween 20 added. After a 5 minute equilibration to pH 9.5 in 50 ml of the detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl) the membrane was carefully placed on a plastic sheet protector. The detection substrate, CDP-Star (Roche Cat. No. 1 685 627) was diluted 1:100 in detection buffer. 2 ml of diluted substrate was used to cover the membrane. As the CDP-Star can precipitate when stored at 4ºC, it was first warmed to room temperature and briefly vortexed. To ensure low background, care was taken to add the substrate dropwise around the edges of the membrane before covering the entire membrane by tilting. After incubating the membrane for 5 minutes, it was briefly blotted on Whatman paper and placed between two pieces of plastic. The chemiluminescence required 15 minutes to peak and the membrane was then exposed for 5 minutes using Hyperfilm-MP (Amersham Cat. No. RPN1675H).

Results and Discussion

As demonstrated in Figure 1B, both the full length Twist (Lane 2) and truncated bHLH Twist (Lane 3) independently form strong homodimers, shown by their intense, sharp bands. When co-translated, a mixture of three distinct complexes can form. These include the individual homodimers and a Twist/bHLH Twist heterodimer of intermediate mobility. Lane 4 shows that all three complexes can be resolved very clearly using this technique. Digoxigenin-labeled shifts provide excellent resolution that is useful when identifying distinct species that migrate closely.

Non-radioactive EMSA has several advantages over the traditional radioactive approach. Being non-radioactive, the labeled probes are safer to handle in the laboratory. Although the labeling reaction buffer contains Potassium cacodylate, a toxic chemical, once labeled the DNA probes require no additional handling precautions or disposal methods unlike ^{32}P waste. Digoxigenin labeled probes are also more stable than radioactive probes. Whereas ^{32}P labeled probes have a half-life of two weeks, Digoxigenin labeled probes are stable for much longer periods of time. We have recently used probes that were labeled over a year earlier.

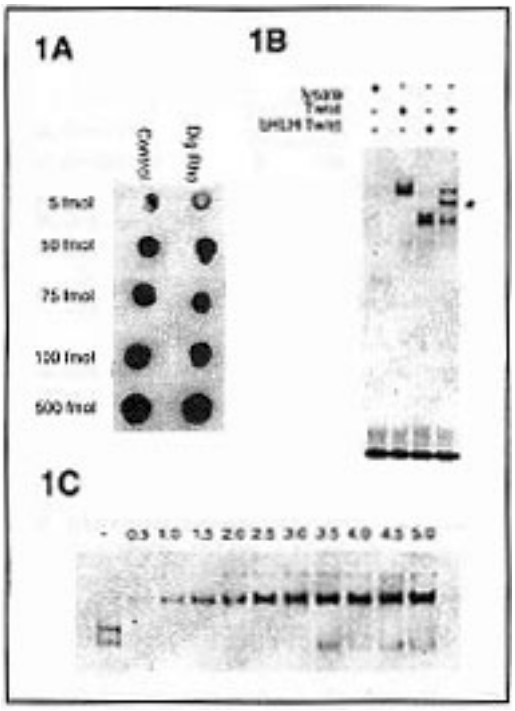

Figure 1. Digoxigenin labeled oligonucleotide from the rhomboid promoter used in EMSA. (A) Testing efficiency of the labeling reaction using a dot blot. 1 µl of 5 serial dilutions were spotted next to dilutions of a control labeled oligonucleotide. (B) In vitro translated products of Twist and bHLH Twist were assayed for binding to the rhomboid promoter in an EMSA using Digoxigenin labeled oligonucleotide as probe. 5 µl of the following products were added to each lane: 1, unprogrammed lysate; 2, Twist; 3, bHLH Twist; 4, co-translated Twist and bHLH Twist. Asterisk shows heterodimer. Free probe is at bottom of gel. (C) The linked dimer Twist-Twist was added in 0.5 µl increments to show the range of sensitivity of the non-radioactive EMSA. Lane 1 contains 3 µl of unprogrammed lysate. The free Digoxigenin probe was in excess for each lane.

This technique is also competitive in terms of sensitivity, time commitment and cost. The range of input DNA (50 fmol, 0.6ng) and in vitro translated protein (5µl) is consistent with radioactive shifts of other Helix-Loop-Helix family members (Benezra *et al.*, 1990 and Murre *et al.*, 1989). As seen in Figure 1C the Twist-Twist tethered homodimer can be detected using as little as 1.0 µl of in vitro translated product. Similar results were achieved with a number of other bHLH proteins. This titration illustrates the range of sensitivity as well as the ability to use this technique to calculate dissociation constants. In terms of time commitment the transfer and development can be consistently completed in 3 hours and generally requires only 5-10 minute exposures. Similar time requirements are used to develop radioactive shifts depending on the age of the probe and visualization equipment. Finally we have found this technique to be cost competitive with ^{32}P labeled shifts. The initial oligonucleotide labeling costs are equivalent to that of ^{32}P probes and while the membrane and development reagents add approximately $19 (Roche 2000 price list) to each experiment, the savings in multiple radioactive purchases may compensate for these costs.

We have found non-radioactive EMSA to be a sensitive, efficient, safe and durable technique. Most importantly the shift complexes give excellent resolution and are easily identifiable. This robust technique can also serve as an excellent teaching tool for undergraduate laboratory classes.

References: Benezra, *et al.*, 1990, Cell 61: 49-59; Castañon, *et al.* (manuscript submitted). Dimerization partners determine the activity of the Twist bHLH protein during *Drosophila* mesoderm development; Ip, *et al.*, 1992, Genes Dev. 9: 1728-1739; Murre, *et al.*, 1989, Cell 58: 537-544.