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volume of 50 μ L. The optimal ratio of primers (M13Fw vs. the specific oligonucleotide) was found to be 1:10, which is in agreement with previously reported data (2). The inserts were amplified by denaturation at 92°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 3 min for 35 cycles in a Crocodile™ II Thermal Cycler (Appligene-ONCOR, Illkirch, France). The first cycle was preceded by an initial step at 94°C for 3 min, and the last cycle was followed by a final extension step at 72°C for 3 min. Aliquots (5 μ L) of each amplification reaction were analyzed by agarose gel electrophoresis stained with ethidium bromide. Figure 2 shows an illustration of a typical walk, corresponding to the fourth walk of Figure 1. The PCR products were purified and sequenced with the primers used for the amplification.

Applying this protocol, we performed nine different walks. All of them were successful, indicating that the reliability of this procedure is high. When applied to the promoter region of the *Tage4* gene, four successive walks allowed us to get sequence information on approximately 2 kb of DNA (Figure 1). The length of the walks we performed ranged from approximately 100–800 bp. This is in agreement with what can be expected using these frequent cutter enzymes. We have not observed any false positive amplification (i.e., PCR products that would not partially overlap the known sequence). Limited background amplifications were seen in very few reactions. Figure 2, lane 3 presents one such reaction. In such cases, the major band was gel purified before sequencing.

A single day is sufficient to prepare the ligation reactions. Then, the same digestion/ligation can be used repeatedly to walk in both directions from a known sequence. Indeed, the use of frequent cutter restriction enzymes results in the identification of short sequences. Therefore, in most cases, several walks will be necessary to overcome the limitation of this approach.

In summary, the method we report is simple and versatile for short genomic walks from any known sequence. We found it most convenient to quickly obtain information on the promoter and exon-intron boundaries of a given gene.

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Improved Band Resolution, Loading Reliability and Reduced ³²P Contamination in Mobility Shift Assays by Retention of Unbound Probe

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Electrophoretic mobility shift assays (EMSAs) are widely used to study DNA-protein interactions (1–4). EMSA detects the binding of small amounts of transcription factors to gene promoters. Multiple proteins of similar molecular weight can bind the same DNA probe, so it is important to achieve good band resolution. One problem we encountered while separating higher molecular weight complexes and supershifted proteins is the loss of unbound probe into the tank buffer because of long running times. This run-off prevents comparison of unbound probe loads and produces

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large volumes of aqueous radioactive contamination.

We describe a simple technique that can greatly improve the separation of higher molecular weight complexes without losing the unbound DNA probe. In addition, by retaining the unbound probe in the gel, we found this technique greatly reduces the amount of aqueous radioactive waste produced.

The EMSA technique was used to study the interaction between the human IRF-1 gene promoter (3) and nuclear proteins from lymphocytes. Two DNA probes, IRF-1 (45 bp) and GAS (24 bp), were labeled on the 5' end with [γ - 32 P]ATP (ICN Biomedicals, Costa Mesa, CA, USA) using T4 Polynucleotide Kinase (Promega, Madison, WI, USA) (4). The sample preparation includes incubation of 4 μ g of nuclear extract in a master mixture of a binding solution containing 10 mM Tris (pH

7.5), 0.5 mM EDTA, 5% Ficoll® (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.5 mM dithiothreitol (DTT), 37.5 mM KCl and 2 μ g of double-stranded Poly(dI-dC) (Amersham Pharmacia Biotech). In addition, 47.5 μ g/mL of leupeptin, 0.7 μ g/mL of pepstatin, 2.0 μ g/mL of aprotinin and 1 mM of pervanadate are added to inhibit protease and phosphatase activity. The master mixtures for IRF-1 or GAS probes were incubated at 4°C for 1 h with 20 fmol of labeled probe (4).

Five-percent polyacrylamide gels (19:1) are cast using the PROTEAN II® system (Bio-Rad Laboratories, Hercules, CA, USA). Gels are pre-run with 1× TBE buffer for 30 min at 150 V constant voltage, with a current of approximately 15 mA. Samples are run for 5 h at the same voltage and current. The finished gels are dried at 80°C for 40 min and exposed to Hyperfilm™-ECL H; (Amersham Pharmacia Biotech) with intensifying screens.

To increase the resolution of higher weight molecular complexes while retaining the unbound probe in the gel, we first cast a 2 cm, 15% acrylamide layer at the bottom of the EMSA gel

and overlaid this with 0.5 mL n-butanol. After polymerization, the n-butanol was washed out, and a 5% gel was added on top of the 15% layer.

Figure 1 demonstrates that at shorter running times, the unbound probe is not run off, but band resolution is poor (lanes 1, and 2 and 3). Lanes 4 and 5 were run longer to resolve the DNA binding complexes. However, without the retaining layer, there is a loss of all unbound probe. Lane 6 was poured with the 15% acrylamide retaining wall and contains all unbound probe. There is no difference in the appearance of the higher weight complexes between lanes 5 and 6 that have equal running times, regardless of the presence of the retaining layer. Analysis of Figure 1 reveals that the thick protein band in lanes 1, 2 and 3 was actually multiple complexes of nearly identical weight (lanes 5 and 6), which could not be distinguished under the standard running conditions needed to retain the probe. In contrast, the high percentage layer retains the small probe and provides an internal signal for the amount of probe loaded.

This technique can also be used to retain probes of different sizes on the same gel. Figure 2 shows that free probes (45 bp in lane 1 and 24 bp in lanes 2 and 3) were retained in the 15% gel layer. This step facilitated comparison of the relevant transcription factor-probe bands after antibody supershifting (lane 3, upper arrowhead). In contrast, without the retaining layer, the free probes ran out into the running buffer (lanes 4 and 5). The band at 182 kDa, now missing because of antibody supershift, could have been considered an artifact from insufficient sample loading.

Protein bands of nearly identical molecular weight were clearly seen. The retaining layer also contained small probes of different sizes that acted as internal loading controls for competition or supershift assays. Antibody supershift or cold competitors displace transcription factors and often reduce protein band intensity; this fact could be confused with insufficient sample loading without the retaining wall. In comparison with gradient gels, these gels are easy to prepare, require no special equipment and are more consistent from gel to gel. The resolution is comparable, and it is simple to determine

Figure 1. Comparison of band resolution after different run times, with and without retaining layer. Lanes 1, 2, 3, 4 and 5 were run on 5% gel for 3, 3.5, 4, 4.5 and 5 h, respectively. Lane 6 was run for 5 h on a 5% upper gel with a 15% retaining gel. The resolution gained in lanes 4, 5 and 6 shows the presence of a second band (arrowheads) that is not visible in lanes 1 and 2. The retaining technique in lane 6 allows separation of higher molecular weight proteins, as in lanes 4 and 5, yet retains the unbound probe to check sample loading and reduces radioactive contamination of running buffer. Solid line marks top of retaining layer.

Figure 2. EMSA supershift with different-sized probes. Lane 1 contains a 45 bp probe; lanes 2 through 5 contain 24 bp probes. Antibodies are added to samples 3 and 5 for supershift. Running times are lane 1 for 4 h, lane 2 for 4.5 h, lane 3 for 5 h and lane 4 for 5 h. Solid line marks top of retaining layer. Probe runoff in lanes 4 and 5 could be confused with insufficient loading. Bands and probes are easily seen in lanes 1, 2 and 3 with retaining wall.

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molecular weights of unknown complexes. In addition, it prevents contamination of the EMSA running buffer with a radioactive probe and reduces aqueous waste. This method can be modified with various acrylamide percentages in the retaining and running layers to optimally isolate the desired range of proteins.

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Efficient and Rapid Procedure for Blue-White Screening of Recombinant Bacterial Clones

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Many plasmid cloning vectors allow blue-white screening for recombinant bacterial clones by α -complementation (5). The regulatory sequences and part of the coding information of the β -galactosidase (β -gal) gene (*LacZ*) carry embedded the multiple cloning site (MCS) of the plasmid vector without interrupting the reading frame for *LacZ*. In bacterial host cells that express the carboxy-terminal part of β -gal, the two fragments associate to yield an enzymatically active protein.

As an indicator for galactosidase activity, the chromogenic substrate X-gal is used (2) and usually added at 40–100 μ g/mL concentration in agar plates producing blue colonies in the case of *Lac*⁺ bacteria. Insertion of a fragment of foreign DNA into the MCS almost invariably leads to the production of an amino-terminal fragment that is incapable of α -complementation resulting in a white colony.

The development of this simple color test has greatly simplified the identification of recombinants with plasmid vectors of this type. However, X-gal is expensive and decomposes over time in pre-poured and stored agar plates due to light sensitivity. Costs can be partially reduced by spreading a concentrated solution of X-gal on the surface of a pre-made agar plate instead of incorporating the sugar throughout the entire volume of the agar medium (4). However, because of the low volatility of the solvent dimethylformamide (DMF) it can take up to 3 or 4 h before the plates are usable for spreading the bacteria (4).

Here, I present an easy, rapid and minimal-cost procedure for blue-white screening with X-gal. It is performed on the grown plates, which allows for a preselection of plates that are worth assaying for β -gal activity. The protocol requires a minimal amount of hands-on time, and results are readily available within minutes.

Agar plates are prepared from LB

Table 1. Required Incubation Times for Colony Staining in Relation to X-Gal Concentration Used

X-Gal Concentration μ g/mL	Incubation Time min
1280	4
640	9
320	20
160	40
80	90

media (4), supplemented with 1.2% bacto-agar (Becton Dickinson Microbiology Systems, Sparks, MD, USA) and 100 μ g/mL ampicillin (Sigma, St. Louis, MO, USA). Preparation of competent bacteria and DNA transfections follow standard protocols (1,4). The agar plates to be analyzed with the overnight grown bacterial colonies are removed from the 37°C incubator. An X-gal (Ambion, Austin, TX, USA) stock solution of 20 mg/mL in DMF is diluted to the desired concentration with sterile distilled water (see Table 1) and applied to the plate surface by spraying as shown in Figure 1. Approx-

Figure 1. Spraying procedure of agar plates with dilute X-gal solution. The nozzle assembly of an adjustable spray wash bottle (VWR Scientific Products, W. Chester, PA, USA) is placed into a 15 mL Falcon® tube (VWR Scientific Products) containing the X-gal solution of the desired concentration. By pushing down the pump trigger once, approximately 500 μ L of the substrate solution is dispersed uniformly over the agar plate surface without danger of dislodging bacterial colonies. Sterility during X-gal application is generally not required, but can be achieved by working under a laminar flow hood if desired.