In Vitro Screening for Regulated Transcription Factors with Differential Display of DNA-Binding Proteins (DDDP)

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INTRODUCTION

Strict regulation of transcription factor activity is essential to establish and maintain gene expression. Eukaryotic cells control transcription factors at many different levels. Post-translational regulatory mechanisms (e.g., phosphorylation, nuclear translocation, multimerization, regulated degradation, etc.) play particularly important roles because they enable cells to respond to various intra- and extracellular stimuli quickly and without prior protein synthesis. However, extensive post-translational changes can make it difficult to identify differentially regulated transcription factors. Common genomic screening techniques such as DNA microarray analysis are unable to detect any mode of regulation beyond that of mRNA stability. This protocol describes the differential display of DNA-binding proteins (DDDP), which is based on the electrophoretic mobility shift assay (EMSA) and detects DNA-binding transcription factors, independent of the number or nature of regulatory steps required for activation. DDDP is an unbiased screening technique that can be used in any experimental system that uses concentrated protein extracts. A plasmid library containing random DNA sequences is constructed. This library is then used to generate radioactive DNA probes to test protein extracts from different sources in parallel for differentially regulated DNA-binding proteins. Plasmids corresponding to probes that display differential DNA-binding activity can be sequenced, and the binding sequence can be narrowed down in a two-step procedure. The corresponding transcription factors can then be identified by bioinformatic and/or biochemical methods.

RELATED INFORMATION

Reinke et al. (2008) provides more information about the DDDP technique and explains in detail the rationale for using a random DNA library, including the underlying calculations.

MATERIALS

Reagents

$[\alpha^{32}P]dCTP$ (3000 Ci/mmol)
Agarose gels

Alternatively, a QiaQuick PCR-purification kit (QIAGEN) can be used.

Cloning vector, pUC19

dATP, dGTP, and dTTP (individual solutions at 5 mM each)

DNA oligonucleotides, synthetic:

P-5'-TCGAGGCGCGCC-3' (AscI-linker)

5'-AAGGGATCCCTT-3' (BamHI-linker)

5'-AATTCGAGCTCGGTACCC-3' (Rand100-A)

5'-ACCATGATTACGCCAAGC-3' (Rand100-B)

5'-ATTGGCGCGCC-[N]_{50}-AAGGGATCCCTT-3' (Random)

dNTP mix (25 mM each of dATP, dTTP, dCTP, and dGTP)

EMSA binding buffer (5X)

EMSA loading buffer

Ethanol (75%)

Klenow fill-in buffer (10X)

Klenow fragment (from DNA polymerase I; 5 U/µL)

NaCl (100 mM)

Polyacrylamide gel (4%)

Protein extracts

Prepare concentrated protein (preferably nuclear) extracts from different cell/tissue sources as required for comparative studies (e.g., wild-type vs. mutant cell lines, normal vs. cancer cells, cells at different stages of development, tissues harvested from subjects at different time points for circadian studies, etc.).

Reagents for sequencing plasmids

Restriction enzyme incubation buffers (10X)

Restriction enzymes: SalI, Ascl and BamHI

Equipment
Beaker (glass) containing 100 mL of boiling H2O

Equipment for running agarose and polyacrylamide gels

Equipment for sequencing plasmids

Filter paper (Whatman)

Gel dryer

Heating block preset to 75°C

Ice

Microcentrifuge

PCR machine

Pipettors and tips

Tubes (microcentrifuge, normal and screw-top)

Vortex mixer

X-ray film and cassettes (or PhosphorImager system)

METHOD

Generation of an Ordered Random DNA Library

See Figure 1 for an outline of the cloning procedure.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Construction of the random DNA library. Oligonucleotides with 50 random nucleotide positions ([N]50), a 5′-flanking region containing an AscI restriction site (blue), and a palindromic 3′-flanking region containing a BamHI restriction site (red), are self-annealed, rendered double-stranded, and cloned into the AscI site of a bacterial plasmid vector.

1. Construct the vector pUC19-AscI by cloning the 5′-phosphorylated oligonucleotide AscI-linker into the SalI site of the cloning vector pUC19.

2. Resuspend the DNA oligonucleotide Random to a concentration of 100 µM in 100 mM NaCl in a
screw-top microcentrifuge tube. Place the tube into a beaker containing 100 mL of boiling H2O. Cool the H2O in the cold room over the course of several hours to 4°C.

*Store the self-annealed oligonucleotide Random at -20°C. Always keep it on ice during future manipulations to prevent dissociation and imperfect reannealing.*

3. Working on ice, prepare the following reaction mixture:
   
   - 124 µL of H2O
   - 15 µL of 10X Klenow fill-in buffer
   - 1.2 µL of dNTP mix
   - 5 µL of self-annealed Random DNA oligonucleotide
   - 5 µL of Klenow fragment.

4. Incubate for 30 min at room temperature.

5. Incubate for 20 min at 75°C to inactivate the Klenow fragment.

6. Cut the double-stranded DNA fragment with the restriction enzyme AscI in the corresponding restriction buffer.

7. Purify the cut DNA fragment on an agarose gel.

8. Ligate the purified DNA fragment into the bacterial vector pUC19-AscI.

9. Prepare DNA minipreps:
   - i. Prepare at least 110 clones to perform a screen in a eukaryotic system. Number clones serially.
   - ii. Sequence the inserts from five to 10 clones to verify that they all contain different inserts of the correct size.
     
     *See Troubleshooting.*
   - iii. Keep only clones with a single insert. Store at -20°C for later use.

**Generation of Radioactive EMSA Probes**

*See Figure 2 for an outline of the procedure for generating the EMSA probes.*

![Figure 2](http://example.com/figure2.png)

**Figure 2.** Generation of EMSA probes from individual clones of the random DNA library. Random DNA inserts are amplified by PCR, digested with AscI and BamHI, and radioactively labeled by the incorporation of [α-32P]dCTP (asterisks).

*View larger version (7K):*
10. Amplify each clone of the DNA library in a 100-µL standard PCR reaction with the primers Rand100-A and Rand100-B using the following program:

<table>
<thead>
<tr>
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<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
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<td>30 sec</td>
</tr>
<tr>
<td>1</td>
<td>72°C</td>
<td>5 min</td>
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</tbody>
</table>

11. Purify at least 500 ng of each PCR product on an agarose gel.

12. Digest the purified PCR products with the restriction enzymes AscI and BamHI in their corresponding restriction buffers.

13. Adjust the concentrations of digested DNA fragments to ~10 ng/µL with H2O. *Concentrate the samples if necessary.*

14. Chill 3 µL each of the digested DNA fragments in microcentrifuge tubes on ice.

15. For each set of six probes to be labeled, mix (on ice) the following:
   - 3 µL of Klenow fill-in buffer
   - 0.5 µL each of 5 mM dATP, dGTP, and dTTP
   - 1.5 µL of [α-32P]dCTP
   - 1.5 µL of Klenow fragment

   *This amount can be scaled up. However, label at least six probes at the same time to avoid pipetting of extremely small volumes.*

16. Add 1 µL of the Klenow/nucleotide solution to each tube containing the DNA fragments. Incubate for 15 min at room temperature.

17. Add 95 µL of 75% ethanol at room temperature to each tube. Vortex.

18. Centrifuge for 5 min at room temperature in a microcentrifuge to separate unincorporated radioactivity from the probes.

19. Discard the supernatant. Dry the pellets for 5 min at room temperature.

20. Resuspend the pellets in 10 µL of H2O. *The specific activity of the probe should be ~10^7 dpm/µg DNA. Radioactively labeled probes can be stored at -20°C.*
Differential Display

21. Prepare a set of tubes such that each protein extract to be tested can be incubated with each of the radiolabeled probes from Step 20.

22. Working on ice, mix the following:
   - 5 µg of protein extract (typically in a volume of 0.7-2.5 µL)
   - 2 µL of 5X EMSA binding buffer
   - 1 µL of a radiolabeled probe from Step 20
   - Add H2O to a total volume of 10 µL.

Depending on the type of transcription factors expected, or if a screen for proteins with specific cofactor requirements has been performed, extra salts, cofactors, energy, etc., can be added to the binding reaction. The concentrations of nonspecific competitors (salmon sperm DNA and poly[dI-dC]) used here are only approximate values and should be optimized for each set of extracts and/or binding conditions. Adjust the amount of competitor such that each lane displays several readily distinguishable protein-DNA complexes (e.g., Fig. 3).

23. Incubate for 15 min at room temperature. Add 1 µL of EMSA loading buffer.

24. Load the reactions containing the same radioactive probe but different protein extracts next to each other on a 4% polyacrylamide gel (see Fig. 3).

25. Run the gel at 7.5 V/cm for 2.5-3 h at room temperature.

26. Vacuum-dry the gel on Whatman paper. Expose to X-ray film or a PhosphorImager screen.

27. Identify differentially binding transcription factors on the images. Sequence the corresponding plasmids to obtain the sequences of their binding sites.

See Troubleshooting.

Narrowing Down Binding Sites

To facilitate further sequence analysis, this two-step procedure can be used to narrow down the exact position of a binding site within a random probe from 100 bp to 10-30 bp. It is usually sufficient to use only one protein extract for these procedures. Only use other extracts for side-by-side comparisons if in...
doubt as to the identity of the band showing differential binding activity

28. For a given insert of interest, separately amplify by PCR the two 50-bp random DNA regions, using the oligonucleotide BamHI-linker in combination with Rand100-A and Rand100-B, respectively. Use the following program:

<table>
<thead>
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<th>1.5 min at 95°C</th>
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<tbody>
<tr>
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<td>3 cycles</td>
<td>30 sec at 95°C</td>
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<tr>
<td>21 cycles</td>
<td>30 sec at 95°C</td>
</tr>
<tr>
<td>1 cycle</td>
<td>5 min at 72°C</td>
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</table>

29. Working on ice, mix the following:

- 5 µg of protein extract
- 2 µL of 5X EMSA binding buffer
- 500 ng of nonradiolabeled PCR product (as competitor DNA)

Add H2O to a total volume of 10 µL. Assay each PCR product separately.

30. Incubate for 10 min at room temperature. Add 1 µL of the radiolabeled probe of interest. Perform EMSAs as described in Steps 23-27.

31. Design six 20-bp oligonucleotides spanning the sequence of the competing fragment such that each oligonucleotide overlaps its counterpart on the opposite strand by 10 bp (Fig. 4A).

32. Anneal the oligonucleotides with each other in various combinations. Generate double-stranded oligonucleotides by fill-in with Klenow polymerase (Fig. 4B).

33. Use the double-stranded oligonucleotides as competitor DNA in EMSAs as described in Steps 29 and 30 to identify the minimal double-stranded sequence needed to compete with the radiolabeled probe.
TROUBLESHOOTING

Problem: Many clones of the random DNA library contain inserts that are too small and contain less than 100 bp of random DNA.

[Step 9.ii]

Solution: The Random DNA oligonucleotide might not have self-annealed correctly. Avoid warming the annealed oligonucleotide before adding the Klenow fragment.

Problem: Most of the bands on the EMSA gel show only a black smear or show no protein binding at all.

[Step 27]

Solution: The EMSA reactions contain either too much or too little nonspecific competitor. Make a titration series with three to five random probes to determine the ideal amount of competitor DNA.

DISCUSSION

Various methods exist to identify differential transcription factor activity in an unbiased way. Genomic screening methods like DNA microarray analysis or serial analysis of gene expression are powerful techniques to detect differences in steady-state mRNA levels (Velculescu et al. 1995; De Haro and Panda 2006). However, many transcription factors are regulated extensively at the post-transcriptional and/or post-translational levels. Proteomic methods can, in principle, be used to screen for transcription factor regulation at the protein level. However, these methods are generally ineffective at detecting transcription factors because of the low levels at which these proteins are expressed (Reddy et al. 2006). DDDP complements these methods by using an activity assay to select specifically for transcriptionally competent DNA-binding proteins.

A key feature of DDDP is the use of a random DNA library, which enables the screening of a maximum number of potential DNA-binding sites within a minimum length of DNA sequence. The question arises: What is the chance of finding functional binding sites in synthetic DNA sequences that have not been evolutionarily tuned for efficient transcription factor binding? Transcription factors have, in general, a very high affinity for their binding sites. We have shown previously that a single mismatch in a transcription factor recognition site reduces its binding affinity by, at most, one order of magnitude (Fonjallaz et al. 1996). Therefore, most factors should also bind to such imperfect binding sites. Consequently, this reduces greatly the number of binding sites that have to be present in the library. As outlined in greater detail (Reinke et al. 2008), if one allows for at most one mismatch in the binding site, a binding site for a typical eukaryotic transcription factor can still be found (with a probability of more than 99%) in as few as 11,000 bps of random DNA sequence (Fig. 5).
Like any other screening method, DDDP has its limitations. Successful use of DDDP only identifies a binding site, not the transcription factor itself. Nevertheless, after narrowing down the binding site, bioinformatic methods can greatly facilitate the identification of the corresponding transcription factor. Alternatively, the DNA probe obtained by the screen can be used to track the protein as it is purified biochemically, or it can be used directly in an affinity purification procedure (Kadonaga and Tjian 1986). Unfortunately, DDDP cannot be performed in saturating conditions. Also, different transcription factors can have different requirements (e.g., salt concentration, temperature, cofactors, etc.) for DNA binding. Nevertheless, DDDP can be used in any biological system that allows for the preparation of concentrated protein extracts. It is also a relatively inexpensive genome-wide screening method that can be performed easily in any normally equipped molecular biology laboratory.

REFERENCES


**Caution**

[$\alpha^{32}\text{P}]d\text{CTP}$

[$\alpha^{32}\text{P}]d\text{CTP}$ is a radioactive substance. When planning an experiment that involves the use of radioactivity, consider the physico-chemical properties of the isotope (half-life, emission type, and energy), the chemical form of the radioactivity, its radioactive concentration (specific activity), total amount, and its chemical concentration. Order and use only as much as needed. Always wear appropriate gloves, lab coat, and safety goggles when handling radioactive material. X rays and gamma rays are electromagnetic waves of very short wavelengths either generated by technical devices or emitted by radioactive materials. They might be emitted isotropically from the source or may be focused into a beam. Their potential dangers depend on the time period of exposure, the intensity experienced, and the wavelengths used. Be aware that appropriate shielding is usually made of lead or other similar material. The thickness of the shielding is determined by the energy(s) of the X rays or gamma rays. Consult the local safety office for further guidance in the appropriate use and disposal of radioactive materials. Always monitor thoroughly after using radioisotopes. A convenient calculator to perform routine radioactivity calculations can be found at [http://www.graphpad.com/calculators/radcalc.cfm](http://www.graphpad.com/calculators/radcalc.cfm).

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**Recipe**

**EMSA binding buffer (5X)**

- 1 mM DTT (dithiothreitol)
- 0.1 mM EDTA
- 25 mM HEPES-KOH, pH 7.6
- 150 mM NaCl
- 50 ng/µL poly(dI-dC)
- 200 ng/µL salmon sperm DNA (sheared)

**Recipe**

**EMSA loading buffer**

- 15% (w/v) Ficoll
- 0.4% (w/v) Orange G
Recipe

**NaCl (Sodium chloride)**

To prepare a 5 M solution: Dissolve 292 g of NaCl in 800 mL of H₂O. Adjust the volume to 1 L with H₂O. Dispense into aliquots and sterilize by autoclaving. Store the NaCl solution at room temperature.