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# Identification of Proteins Interacting with Single Nucleotide Polymorphisms (SNPs) by DNA Pull-Down Assay

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## Abstract

Single nucleotide polymorphisms (SNPs) are the most abundantly found common form of DNA variation in the human genome. Many genetic association studies have proved that some of these SNPs are involved in regulating several cellular/physiological processes ranging from gene regulation to disease development. Analysis of the protein complex that binds to these SNPs is a crucial step in studying the mechanisms by which gene expressions are regulated in *cis*- or *trans*-acting manner. Commonly used techniques to determine DNA-protein interaction, such as electrophoretic mobility shift assay (EMSA), have limited value for simultaneously analyzing a large number of proteins in the complex. Furthermore, this assay is tedious and time-consuming and often requires radiolabeled probe as well as extensive optimization. Here, we describe a pull-down assay before performing the EMSA, which helps in the detection of differentially-bound protein(s) in an allele-specific manner. The assay is easy to perform and does not require radiolabeling of DNA probes. Biotinylated DNA probe bound to streptavidin beads can be complexed with protein(s) from cell nuclear lysate, nonspecific proteins were washed out, and only protein(s) having high affinity to SNP-specific DNA were detected on SDS-PAGE and identified by mass spectrometry.

### Keywords

SNP; EMSA; Pull-down assay; SDS-PAGE; GWAS; Mass spectrometry

## 1. Introduction

The central goal of human genetics is to identify specific DNA variants that contribute significantly to population variation in each trait or phenotype. The Encyclopedia of DNA Elements (ENCODE) project consortium data demonstrated that ~80% of the human genome is biochemically active [1]. SNPs (single nucleotide polymorphisms) are the most commonly occurring genetic variations in the human genome and account for more than 80% of all variations in the human genome [2]. A SNP is a DNA point variation at a single base pair position with frequency of more than 1% in a population making it different from

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mutation which is rare with frequency of less than 1% [3]. It has been estimated that the human DNA sequence carries a SNP in every 500–1000 nucleotides (The International SNP Map Working Group, 2001) [4]. Over the years, SNPs are shown to have potential to modulate the transcriptional regulation and hence change in gene expression. They have also proved to be extremely informative in disease gene mapping and tracing evolutionary histories of populations [5]. After the completion of sequencing of human genome project, many follow-up association studies have aimed to pinpoint the predisposing SNPs and explore the functional role of these SNPs.

SNPs are distributed throughout the human genome; however, the associated variants identified by the recent genome-wide association studies (GWAS) demonstrated that about 88% of these variants fall outside the protein coding regions, especially the intronic or intergenic regions [6, 7]. Some of those SNPs present in the regulatory (i.e., promoter/enhancer) regions and noncoding RNAs have been shown to produce insightful effects on the transcription of its target genes, and the expression of a substantial proportion of genes is influenced by polymorphism in regulatory elements [5, 8]. The physical locations of these SNPs emphasize the regulatory role of these SNPs, and the challenge is to detect the actual causal variants and define the functional consequence of these regulatory SNPs.

Transcriptional regulation of gene expression is controlled through the binding of sequence-specific DNA binding proteins (i.e., transcription factors) to the regulatory regions of genes. The presence of these proteins/factors is dependent upon the cell type being examined and the stimulus to which the cell are exposed. Knowledge of the transcription factors present during any given time can be important in generating a more thorough understanding of how a cell or tissue responds to its environment. Therefore, identification of these transcription factors or regulatory protein(s) required for the expression of a specific gene can provide a better understanding of the molecular mechanisms involved and might suggest new therapies that specifically target an individual gene or set of genes.

The electrophoretic mobility shift assay (EMSA), also known as gel retardation or gel shift assay, is widely used for detection of sequence-specific DNA binding proteins [9, 10]. This assay can be used to determine, in both a qualitative and quantitative manner (densitometry), if a particular transcription factor is present within the nuclei of the cells and tissue of interest or to identify an unknown DNA binding protein which may control the expression of a gene of interest. The assay is based upon the ability of a transcription factor to bind in a sequence-specific manner to a radiolabeled oligonucleotide probe and retard its migration through a polyacrylamide gel. Nevertheless, this technique has its limitations; it requires a radiolabeled probe and results in nonspecific protein bands in SDS-PAGE, which makes it difficult to quantitate sequence-specific interacting protein. To overcome this difficulty, we herewith describe the DNA pull-down assay which helps to wash out nonspecific proteins, thereby allowing the proteins possessing strong binding affinity to DNA, which can be detected by the EMSA. These proteins can later be identified by mass spectrometry. The assay is relatively simple and does not require radiolabeled probes. Further, this technique can detect the allele-specific interaction of protein(s) in wild type versus mutated genotype of SNP. Furthermore, this technique also showed enrichment of low abundant targets as well as isolation of intact complex.

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## 2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to reagents.

### 2.1. Nuclear Extract Isolation

1. Phosphate-buffered saline (PBS), pH 7.4.
2. 0.5 M sodium fluoride (NaF), store at 4 °C.
3. 100 mM phenylmethylsulfonyl fluoride (PMSF) solution in isopropanol, store at −20 °C.
4. Dithiothreitol (DTT), store at −20 °C.
5. Leupeptin, store at −20 °C.
6. 1.25 M β-Glycerophosphate disodium salt, store at 4 °C.
7. 1 M sodium vanadate, store at −20 °C.
8. 1 M potassium chloride (KC1), store at room temperature (RT).
9. 1 M HEPES, stored at 4 °C.
10. 1 M magnesium chloride hexahydrate ($MgCl_2$), stored at RT.
11. 1 M sucrose, stored at RT.
12. 10% Igepal CA-630 (NP-40), stored at RT.
13. 1 M sodium chloride (NaCl), stored at RT.
14. 0.5 M EDTA, stored at RT.
15. PBS buffer containing inhibitors (PBSI): 0.5 mM PMSF, 25 mM β-glycerophosphate, 10 mM NaF, stored at 4 °C.
16. Buffer A: 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM sucrose, 0.5% NP-40, stored at 4 °C.
17. Buffer B: 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 2.5% glycerol, stored at 4 °C.
18. Buffer D: 20 mM HEPES, pH 7.9, 100 mM KC1, 0.2 mM EDTA, 8% glycerol, stored at 4 °C.

### 2.2. Protein Assay

1. BCA Protein Assay reagent kit: Reagent A containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1 M sodium hydroxide, and Reagent B containing 4% cupric acid.

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2. Albumin standard 2 mg/mL.
3. 96-well plate.
4. Microplate spectrophotometer, benchmark plus.
5. Microplate manager version 5.2.

### 2.3. Pull-Down Procedure

1. Dynabeads M-280 streptavidin-coated beads.
2. Magnetic separation tube rack.
3. 2× B/W buffer [10 mM Tris-HCl (ph 7.5), 1 mM EDTA, 2 M NaCl].
4. 5× EMSA buffer (100 mM HEPES (pH 7.2), 160 mM KCl, 0.5 mM EDTA (pH 8) 50% glycerol, 350 ng/μL BSA, 40 ng/μL poly dI-dC, 12.5 mM DDT).
5. Phosphate-buffered saline (PBS), pH 7.4.
6. Rocking platform.
7. Microcentrifuge.
8. Wash buffer (1× EMSA buffer plus 500 mM NaCl).
9. Biotinylated (5′-biotin TEG) labeled sense strand DNA (wild type and mutated). Non-labeled oligo for antisense DNA (wild type and mutated) (Integrated DNA Technologies, Coralville, IA) (*see* Note 1).

### 2.4. SDS-PAGE

1. 2× Laemmli sample buffer: 950 μL of Laemmli sample buffer mixed with 50 μL of 2-mercaptoethanol.
2. 4–15% gradient polyacrylamide gel in Tris–HCl.
3. Running buffer (10×): 250 mM Tris, 1.92 M glycine, 1% SDS. Prepare 1× running buffer by diluting 100 mL of 10× buffer with 900 mL deionized water.

## 3. Methods

### 3.1. Nuclear Extract Isolation

1. All procedures are done on ice or in a controlled temperature room at 4 °C.
2. Remove medium from cultured cells and wash them once with cold PBS. Add PBSI buffer (1 mL/10-cm dish), and harvest cells with a rubber scraper. Cells are collected into a 50-mL conical tube, which is centrifuged at 550× *g* for 5 min (*see* Note 2).

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1. We design 30–40-base-long (can be up to 500 bp) oligonucleotides keeping the SNP position in center. The sequence is sent to Integrated DNA Technologies (IDT). 5’-biotinylated sense and antisense singlestranded oligonucleotides are synthesized, and the sequence is verified by IDT.

2. A 50-mL conical tube is used to ensure a maximal yield of cells.

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3. Remove supernatant. Transfer the pellet to a 1.5-mL microcentrifuge tube, and centrifuge at 1500× *g* for 30 s.
4. To buffers A, B, and D, add the following inhibitors: 0.5 mM PMSF, 1 mM $Na_3VO_4$, 0.5 mM DTT, 1 μg/mL leupeptin, 25 mM β-glycerophosphate, and 10 mM NaF.
5. Remove supernatant and resuspend the pellet in two package cell volume of buffer A with inhibitors. Keep on ice for 10 min. Vortex briefly, and centrifuge at 2600× *g* for 30 s.
6. Remove supernatant, and resuspend the pellet in 2/3 package cell volume of buffer B with inhibitors.
7. Sonicate the mixture for 5 s. Centrifuge at 10,400× *g* for 5 min (*see* Note 3).
8. Dilute the supernatant isovolumetrically with buffer D with inhibitors. Aliquot and store at −80 °C (*see* Note 4).

### 3.2. Protein Assay

1. Prepare standard albumin concentrations at 50, 100, 250, 500, 750, 1000, 1500, and 2000 μg/mL.
2. Pipet 10 μL of standards and samples each in duplicate onto a 96-well plate.
3. Mix 50 parts of buffer A with 1 part of buffer B.
4. Add 200 μL of mixed buffer A and B to standards and samples.
5. Incubate plate at 37 °C for 30 min.
6. Read the protein content at 562 nm on a microplate spectrophotometer.

### 3.3. Pull-Down Procedure

1. Dissolve single-stranded DNA to make duplex. Resuspend the oligonucleotide to 100 μM in water, and mix both single strands in a 1:1 ratio. For preparation of duplex, use 62 μL sense oligonucleotide and 62 pL antisense oligonucleotide, and add 376 μL water. Anneal this mixture by heating to 95 °C and then cooling slowly to room temp. Store this at –20 °C (*see* Note 5).
2. Resuspend the Dynabeads™ in the vial (i.e., vortex for >30 s or tilt and rotate for 5 min). Use 40 μL of beads for each reaction. Wash the beads with buffer on magnetic rack. Remember to include a beads only control (*see* Note 6).
3. Resuspend the beads according to the direction in 2× B/W buffer in twice the original volume.

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[3]. In our experience, a 5-s sonication is sufficient to break the cells. Sonication for a longer period may disrupt the nucleus.
[4]. Nuclear extracts so prepared do not have detectable cytosolic proteins.
[5]. For DNA 4 μg of biotinylated double-stranded oligonucleotides are used for each assay; we mix 4 μg of sense with 4 μg of antisense oligonucleotides to generate 4 μg of 5′-biotinylated double-stranded oligonucleotide in each experiment.
[6]. Add an equal volume of washing buffer, or at least 1 mL, and mix (vortex for 5 s, or keep on a roller for at least 5 min). Place the tube on a magnet for 1 min and discard the supernatant. Remove the tube from the magnet, and resuspend the washed Dynabeads™ in the same volume of washing buffer as the initial volume of Dynabeads™ taken from the vial.

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4. Divide the beads into different tubes as required.
5. Add annealed oligonucleotide to the beads. 25 μL of annealed oligonucleotide for a total of 8 μg of DNA. Add water to bring the volume up to dilute the 2× B/w buffer to 1×.(for 80 μL of beads in 2× B/W buffer, add 25 μL of annealed DNA and 55 μL of water for a total of 160 μL).
6. Incubate the mixture at RT, rotating, for 15 min.
7. Wash the beads 3× using the magnetic stand to separate the beads each time. Add 8 μL of 1 μg/μL poly dI-dC per 1 mL of this wash buffer to make the first wash after the binding reaction.
8. After the final wash, resuspend the beads in 1× B/W buffer without NaCl, and transfer the beads with bound DNA into a fresh tube.
9. Separate again and add your binding reaction cocktail.
10. Make up a binding reaction cocktail, and keep on ice. 1× binding reaction (500 μL total): 100 μL 5× EMSA buffer and 100 μg of nuclear lysate water to 500 μL.
11. After the final wash with 1× B/W buffer (no salt) from above, add 500 μL of binding cocktail to the beads.
12. Incubate at RT, rotating, for 20 min.
13. Pull down the beads and wash 3× with 300 μL of wash buffer, using buffer spiked with dI-dC for the first wash.
14. With the final wash, transfer the beads to a new tube (this prevents carrying over proteins stuck to the plastic) (*see* Note 7).
15. Separate the beads, remove the wash buffer, and add 25 μL of 1× Laemmli SDS loading buffer, with B-ME.
16. Boil the beads for 5 min, spin down, separate, and load supernatant onto a gel (*see* Note 8).

### 3.4. SDS-PAGE

1. Remove 4–15% gradient “ready to use” gel from plastic, remove the comb, and cut the tape along the black line across the entire gel. Pull out the gel (*see* Note 9).
2. Set up the electrophoresis unit.
3. Place the gel onto the mini-gel holder cassette of the electrophoresis unit.
4. Load the 10-well gel with 5 μL of standards or 25 μL of the supernatant protein samples.
5. Add 1× running buffer to the electrophoresis cell.

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[7]. The incubation is important to centrifuge the complex at a low speed. A higher speed of centrifugation may disrupt the complex.
[8]. The step causes dissociation of transcription factors from the complex.
[9]. We have found the Bio-Rad mini-gel to be convenient. However, mini-gels from other manufacturers may be used.

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6. Cover the electrophoresis cell with the cell lid.
7. Connect the electrophoresis unit to a power supply. Set the voltage at 150 V.
8. Run the gel at 150 V until the marker dye has run off the edge of the gel.
9. Remove gel and rinse with 1× transfer buffer precooled in ice (4 °C).
10. Set up the electrophoretic transfer cell.
11. Soak fiber pad, filter paper, and nitrocellulose membrane in 1× transfer buffer before placing them in the gel cassette.
12. Place nitrocellulose membrane between gel and anode. Inspect to make sure that no air bubble is trapped between gel and membrane.
13. Connect the electrophoretic transfer unit to a power supply.
14. Place the unit on ice or at 4 °C.
15. Run the gel at 90 V for 1 h.
16. Stain the gel with Coomassie brilliant blue R-250 (*see* Note 10).
17. Protein bands which show significant binding difference between wild-type and mutated DNA used for mass spectrometry analysis.

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[10]. Please *do not* stain the gels using boiling or microwave to speed up the staining or destaining process. Please *never* use overhead projector foils for gel scanning as this prevents the gel from being used for MS experiments.

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