Preface

Since the discovery of p53 as a tumor suppressor, numerous methods have evolved to reveal the unique structural features and biochemical functions of this protein. Several unique properties of p53 posed a challenge to understanding its normal function in the initial phase of its research. The low levels of p53 in normal cells, its stabilization under situations of genotoxic stress, induction of growth arrest, and apoptosis with stabilization of the protein, obstructed the visibility of its normal, unmutated function. The property of p53 that can sense a promoter and transactivate or inhibit is still not well understood. It is still not known whether it is the absence of the protein that causes tumorigenesis, or if its mutants have a dominant role in inducing cancer.

p53 Protocols comprises eighteen chapters for the study of the diverse properties of p53 and related proteins. The methods included are invaluable for delineating the function of other proteins that may function as tumor suppressors or growth suppressors. The chapters are not presented in any schematic order, for the importance and diversity of the functions of p53 make it impossible to organize them suitably.

We have made a sincere effort to collect the methods most useful to those investigators working on tumor suppressors or growth suppressors. The purpose of *p53 Protocols* is not only to provide investigators with methods to analyze similar biochemical functions, but also to familiarize them with the associated problems that arose during the course of investigations. In keeping with the other volumes of the series, we have provided detailed protocols with troubleshooting notes. Special effort has been made to discuss problems and their solutions to ease the learning curve for standardizing a new method. We did not attempt to cover all of the methods that are currently available. Rather, the emphasis was to present protocols from authors who have used these methods successfully.

We thank Professor John Walker for giving us the opportunity and encouragement to structure and edit this book. We also thank the staff of Humana Press for their work in its behalf. We sincerely thank all contributing authors for sharing the extensive benchtop experience they have developed over the years and also for generating high quality manuscripts. We would like to extend special thanks to our graduate students: Rebecca Frum, for enthusiastically helping us with grammatical editing, communication with

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Purification of Recombinant p53 from Sf9 Insect Cells

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Summary

We describe a method for purifying recombinant p53 from baculovirus infected cells in one step by anion exchange chromatography. The p53 is full-length with no flanking sequences and its expression is driven by the baculovirus polyhedron promoter. We also describe how to concentrate the p53 up to 0.9 mg/mL. By gel filtration analysis, we demonstrate that 20% of the p53 forms a tetramer, and 80% forms a monomer. In a DNA binding assay known as the electromobility shift assay, the purified p53/DNA complex forms a single band the gel. This simple procedure should be useful for investigations into the biochemistry of the p53 protein.

Key Words

anion exchange chromatography, gel filtration chromatography, virus titer

1. Introduction

Human p53 is a protein with a theoretical molecular weight of 43,653 Da, based on its amino acid sequence. p53 is conserved within a wide variety of eukaryotes ***(1)***. The term p53 was coined because it has an apparent molecular weight of 53,000 Da when compared to proteins of known size upon analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After cellular DNA damage, p53 up-regulates the expression of genes that repair DNA, prevent cell cycle progression, and initiate apoptosis. These activities are essential for human tumor suppression and, hence, mutations in the *p53* gene are prevalent in a wide variety of tumors ***(2,3)***. The full-length polypeptide of the gene product can form a homotetramer that has an unusual shape. The tetramer has an increased Stokes' radius when compared to globular proteins of known molecular weight ***(4)***. The homotetramer binds to p53 responsive sequences in the promoters of several genes. Perturbation of the C terminus by acetylation, phosphorylation, or noncovalent interaction with other macromol-

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ecules often increases the affinity of p53 for its responsive element. This communication describes the method we use to routinely generate high levels of human p53 from *Spodoptera frugiperda* 9 (Sf9) insect cells infected with recombinant baculovirus. This method is a modification of the one originally used by Delphin and his colleagues ***(5)***. We also describe an analysis of the purified p53 by gel filtration chromatography and electromobility shift assay.

The methods described below outlines: (*i*) infection of cells for virus production; (*ii*) the virus plaque assay; (*iii*) the titer calculation; (*iv*) the optimization of protein expression; (*v*) the infection of Sf9 cells for p53 production; (*vi*) the harvesting of cells and preparation of soluble nuclear lysate; (*vii*) the anion exchange column purification; (*viii*) the concentration of p53; (*ix*) the analysis by gel filtration chromatography; and (*x*) the electromobility shift assay.

2. Materials

2.1. Commercial Products

1. Sf9 cells (Invitrogen, Carlsbad, CA, USA; cat. no. B825-01).
2. Complete insect media: 90% Grace's insect medium, supplemented (Invitrogen; cat. no. 11605-094), 10% heat-inactivated fetal bovine serum (Cellgro-Mediatech, Herndon, VA, USA; cat. no. 35-010-CV) (*see* **Note 1**), 10 µg/mL gentamycin (Invitrogen; cat. no. 15710-064), 0.25 µg/mL Fungizone (also known as amphotericin B; Invitrogen; cat. no. 15290-018), 100 U/mL penicillin/100 µg/mL streptomycin (Bio Whittaker, Walkersville, MD, USA; cat. no. 17-602E).
3. Recombinant full-length human p53 baculovirus (with no flanking coding sequences). Our recombinant virus was originally created by the Prives laboratory ***(6)***.
4. 4% Baculovirus agar (Invitrogen; cat. no. 18300-012).
5. 2X Complete Grace's insect media: 90% 2X Grace's insect media (Invitrogen; cat. no. 11667-037), 20% heat-inactivated fetal bovine serum, 20 µg/mL gentamycin, 0.50 µg/mL Fungizone.
6. Agarose–media overlay: a 1:3 mixture of 4% agarose gel to 2X complete Grace's insect medium. Prepare just prior to use. To prepare, heat agarose to 70°C in a water bath to melt. Cool to 37°C (*see* **Note 2**). Heat 2X complete Grace's insect media to 37°C. Then mix at the appropriate ratio.
7. Q Sepharose® Fast Flow (Amersham Pharmacia Biotech, Piscataway, NJ, USA; cat. no. 17-0510-01).
8. Open column (1.5 × 20 cm) (Bio-Rad, Hercules, CA, USA; cat. no. 737-1521).
9. γ-^{32}P-ATP (6000 Ci/mmol at 150 mCi/mL) (Perkin Elmer Life Science, Gaithersburg, MD, USA; cat. no. NEG-035C).
10. *mdm2* promoter oligonucleotide sequence: 5'-AAAGGAGTTAAGTCCTGAC TTGTCTCCAGCT-3' and its complement (Integrated DNA Technologies, Coralville, IA, USA).

2.2. Buffers

1. Isotonic buffer: 10 m*M* Na_2HPO_4, pH 7.2, 130 m*M* NaCl, 1 m*M* diethylenetriaminepentaacetic acid dianhydride (DTPA) (Sigma, St. Louis, MO, USA; cat. no. D 6518).
2. Cell lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 150 m*M* NaCl, 0.5% (v/v) Nonidet® P-40.
3. Phosphate-buffered saline (PBS): 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 137 m*M* NaCl, 2.7 m*M* KCl.
4. Buffer A: 20 m*M* Tris-HCl, pH 8.0, 12% sucrose, 2 m*M* ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2 m*M* phenylmethylsulfonylfluoride (PMSF) (Fisher Scientific, Tustin, CA; cat. no. A 270184G005) (*see* **Note 3**), 5 m*M* dithiothreitol (DTT), 1 m*M* DTPA.
5. Buffer B: 20 m*M* Tris-HCl, pH 8.0, 2 m*M* EGTA, 2 m*M* PMSF, 10 m*M* DTT, 50 µg/mL leupeptin (Roche Molecular Biochemicals, Indianapolis, IN, USA; cat. no. 1017128), 10 µg/mL pepstatin (Roche Molecular Biochemicals; cat. no. 1524488), 10 µg/mL E-64 (Roche Molecular Biochemicals; cat. no. 1585673), 1 m*M* DTPA.
6. Buffer C: 40 m*M* Tris-HCl, pH 8.0, 10 m*M* DTT, 1 m*M* DTPA.
7. Laemmli protein denaturation buffer: 1.25 *M* Tris-HCl, pH 6.8, 20% glycerol (v/v), 0.02% bromphenol blue (w/v), 5% SDS (w/v), 10% β-mercaptoethanol (v/v).
8. 5X DNA binding buffer: 50 m*M* Tris-HCl, pH 7.5, 250 m*M* NaCl, 5 m*M* $MgCl_2$, 2.5 m*M* EDTA, 2.5 m*M* DTT, 20% (v/v) glycerol, 0.1 mg/mL poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech; cat. no. 27-7880-02)
9. 10X Electrophoretic mobility shift assay (EMSA) gel loading buffer: 250 m*M* Tris-HCl, pH 7.5, 40% (v/v) glycerol, 0.2% (w/v) bromphenol blue, 0.2% (w/v) xylene cyanol.
10. TE: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA.

3. Methods

3.1. Infection for Virus Production

1. Seed each of two T-25 flasks with 2×10^6 Sf9 cells (in the log-phase of growth) in 5 mL of complete Grace's insect media and incubate at 27°C in a nonhumidifying incubator with no added CO_2. Wait 2 to 3 h for the cells to attach to the bottom of the flask. Cells must be at least 50% confluent prior to the next step.
2. Transfer 20 µL of P1 (passage 1 virus) from virus stock stored at 4°C to each flask. Briefly rock flasks to ensure even distribution of virus.
3. At 8 d postinfection, remove the media containing the P2 virus to a 15-mL conical plastic tube and centrifuge (2500*g* for 5 min) to remove cell debris.
4. Transfer 2 mL of the supernatant (P2 virus) to a new tube and store at –80°C for long-term storage. Store the remaining portion of the P2 virus at 4°C.
5. To generate P3 virus, seed 2×10^7 Sf9 cells in each of four T-175 flasks with 25 mL of complete Grace's insect media.

6. At 12 h postseeding, add 500 µL of P2 virus from 4°C stock to each flask to infect cells.
7. At 9 d postinfection, harvest the P3 virus as described above.
8. Store the majority of the P3 virus at 4°C for use within 2 yr, and store a small aliquot at –80°C for long-term storage. The P3 virus will be used to determine the titer.

3.2. Plaque Assay

1. Prewet the 10-cm tissue culture dishes (Falcon 353003) with complete Grace's insect media and seed with 5×10^6 Sf9 cells that are in the log phase of growth. Prepare two dishes for each virus dilution you plan to use (we usually use six dilutions), plus an extra pair for mock infection.
2. Rock dishes for 10 min at room temperature (8 side-to-side motions/min) on a platform rocker.
3. Stop rocker and ensure the platform is level to produce an evenly distributed monolayer of cells. After 10 min, use a microscope to inspect a few dishes to ensure that the cells are evenly distributed.
4. Remove dishes to a level area, and allow cells to attach to bottom surface (approx 30 min). Cells should be approx 50% confluent on the dish bottom.
5. Prepare 10-fold serial dilutions of virus inoculum in 1-mL total vol of complete Grace's insect media. The dilutions should range from 100-fold to 10^7-fold.
6. Aspirate all but 2 mL of the medium in pre-seeded dishes.
7. Carefully add 1 mL of the diluted virus dropwise over the course of 90 s, taking special care not to disturb the monolayer of cells.
8. Incubate plates at room temperature on a rocking platform (approx 2 side-to-side motions/min) for 1 h.
9. Aspirate remaining media and overlay the infected cells with 10 mL of agarose–media overlay.
10. Incubate cells at 27°C in the incubator until plaques are formed (approx 5 to 6 d).
11. View cells at 30× magnification with a dissecting microscope and count plaques as distinct opaque white dots.

3.3. Titer Calculation

The titer is the number of plaque-forming units (pfu)/mL of virus. Use the following formula to calculate titer:

pfu/mL = (1/dilution) × number of plaques produced by the inoculum on dish.

The pfu/mL for the p53 virus is typically 10^8. To determine the amount of inoculum needed for p53 protein production, we use the following formula:

$$\text{mL of inoculum} = \frac{(\text{multiplicity of infection [MOI]} \times \text{total number of cells to be infected})}{\text{titer of virus (pfu/mL)}}$$

We typically use an MOI of 6 for large-scale p53 production.

3.4. Optimization of Protein Expression

The optimal time for p53 expression post-virus infection is obtained from a simple timecourse experiment followed by SDS-PAGE and Coomassie® staining.

1. Seed seven T-25 flasks with 3×10^6 Sf9 cells in 5 mL of complete Grace's insect media.
2. Infect six of the seven flasks with 125 µL (approx 4 MOI) of virus inoculum.
3. Collect cells on day 2, 3, 4, 5, 6 and 10 postinfection.
4. Collect the uninfected cells on day 2.
5. Remove cells by scraping the surface of the flasks with disposable cell scrapers (Falcon®; cat. no. 353086).
6. Pour the cell–medium mixture into 15-mL plastic tubes with screwtops. Centrifuge the cells (3200*g* for 5 min) at 4°C in a swinging bucket rotor. In all subsequent steps, maintain cells at 0°C by keeping the tubes on wet ice.
7. Remove supernatant and add 5 mL of ice-cold PBS. Gently resuspend cells by flicking the bottom of the tube with a finger, then centrifuge, and remove supernatant.
8. Add 300 µL of cell lysis buffer and resuspend cells by vortex mixing.
9. Sonicate cells in a bath sonicator (Fisher Scientific 550 Sonic Dismembrator, setting 5) in continuous mode for 10 s with 1 min rest intervals. Sonicate five times, and centrifuge the lysed cells (3200*g* for 5 min) to remove particulate material.
10. Add 10 µL of supernatant to 10 µL of Laemmli protein denaturation buffer. Vortex mix, boil for 5 min, and load onto a 10% Laemmli gel. After electrophoresis, stain gel with Coomassie Blue dye.

One typically finds that 3 d postinfection is optimal for p53 expression (**Fig. 1**). The p53 protein can be detected by eye as a protein with an apparent molecular weight of 53 kDa (*see* **Note 4**).

3.5. Infection of Sf9 Cells for p53 Purification

1. Seed 5×10^6 Sf9 cells in the log phase of growth into a T-75 flask containing 15 mL of complete Grace's insect media. Incubate overnight at 27°C.
2. Mix 2 mL of complete Grace's insect media with 300 µL of P3 p53 virus stock (6 MOI) to create inoculum.
3. Remove old media from cells and replace with 2.3 mL inoculum.
4. Rock flasks at a rate of 4 side-by-side motions/min for 1 h at room temperature.
5. Add 13 mL of complete Grace's insect media and incubate at 27°C in nonhumidified incubator, 0% CO_2 for 72 h.

In a typical preparation, p53 is purified from 14 T-75 flasks of infected cells.

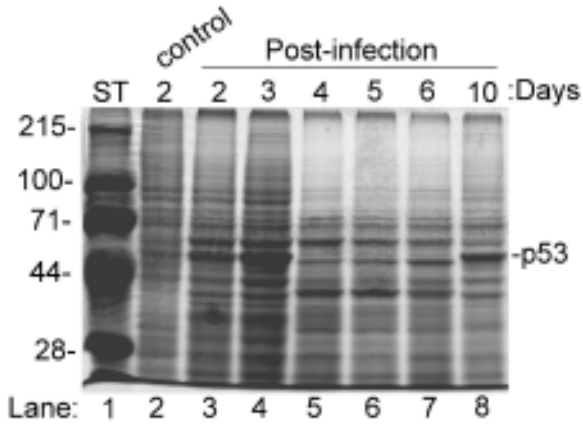

Fig. 1. Equal vol of soluble cell lysate was loaded onto a 10% polyacrylamide gel. Lane 1, molecular weight standards; lane 2, lysate from uninfected cells; lanes 3–8, lysate from infected cells obtained at indicated days postinfection.

3.6. Harvesting Cells and Preparation of Soluble Nuclear Lysate

1. Scrape cells from flasks, without draining the media, using cell scrapers. Pour cell–media mixture into 50-mL plastic tubes with screwtops.
2. Centrifuge at 4°C (3200*g* for 5 min), remove media, and wash cell pellet 2× with ice-cold isotonic buffer.
3. To lyse cells, resuspend the cell pellet in 100 mL of Buffer A plus 0.2% (v/v) Triton® X-100 by gentle inversion. Transfer cell lysate to appropriate centrifuge tubes (*see* **Note 5**). Centrifuge the nuclei at 5600*g* for 8 min and discard the supernatant.
4. Resuspend the pellet in 100 mL of Buffer A plus 0.1% (v/v) Triton X-100 by gentle inversion. Centrifuge the nuclei at 5600*g* for 8 min and discard the supernatant.
5. Lyse nuclei by adding 30 mL of Buffer B plus 0.5 *M* NaCl and vortex vigorously. Allow the mixture to remain on ice for 20 min.
6. Transfer the lysate to centrifuge tubes (Ultra-Clear™, 1 × 3.5 in.; Beckman-Coulter, Fullerton, CA, USA) and add more Buffer B plus 0.5 *M* NaCl to top off if necessary. Centrifuge mixture at 100,000*g* for 60 min at 4°C in a swinging bucket rotor.
7. Remove supernatant and dilute five-fold with Buffer B plus 0.1 *M* NaCl. Gently mix, and centrifuge the diluted mixture at 20,000*g* for 30 min at 4°C.
8. Remove soluble nuclear lysate (supernatant) and aliquot to new tubes. These may be stored at –80°C indefinitely at this point (*see* **Note 6**).

3.7. Anion Exchange Column Purification of p53

1. Rinse Q Sepharose Fast Flow beads with water.
2. Equilibrate Q Sepharose Fast Flow beads with Buffer C plus 0.1 *M* NaCl, according to the manufacturer's instructions.
3. Pour equilibrated beads into an open 1.5 × 20 cm column up to a final column vol of 15 mL.
4. Wash the column with 50 mL of Buffer C plus 0.1 *M* NaCl.
5. Thaw soluble lysate on ice and add 75 mL to column. The flow rate of the column should be approx 0.625 mL/min. Allow lysate to completely pass through column.
6. Wash column with 50 mL Buffer C plus 0.1 *M* NaCl.
7. Wash column with 50 mL of Buffer C plus 0.2 *M* NaCl.
8. Elute p53 with Buffer C plus 0.4 *M* NaCl in three successive 15-mL fractions. The second fraction contains the highest level of p53.

3.8. Concentrating p53

p53 tends to bind irreversibly to ultrafiltration membranes during concentration. We have found that pretreating the membranes with bovine serum albumin (BSA) helps to prevent some of the p53 loss during ultrafiltration.

1. Pretreat YM30 Centricon® (Millipore, Bedford, MA, USA) with 2 mL of 0.01 mg/mL (w/v) BSA (Sigma; cat. no. A-7906) dissolved in water.
2. Centrifuge for 10 min at 5000*g* and remove collected filtrate.
3. Rinse the Centricon 12X with water.
4. Centrifuge 2 mL of water through the Centricon for 10 min at 5000*g*.
5. Place purified p53 into pretreated Centricon and centrifuge at 5000*g* per manufacturer's instructions.
6. Determine the final p53 protein concentration by Bradford assay ***(7)*** or by comparison of p53 protein band intensity to the intensity of known amounts of BSA separated by 10% SDS-PAGE (we usually perform both tests).

Figure 2 shows the unconcentrated and concentrated p53 compared to BSA standards on a gel stained with Coomassie Blue dye. In the second fraction from the column, we obtain an initial concentration of 0.2 mg/mL of p53. After the Centricon step, the p53 concentration is between 0.6–0.9 mg/mL. However, approx 50% of the p53 is lost due to its nonspecific binding to the Centricon during the concentration step.

3.9. Analysis by Gel Filtration Chromatography

p53 can form a tetramer through an oligomerization domain in its C terminus. One method to demonstrate that p53 forms a tetramer is gel filtration analy-

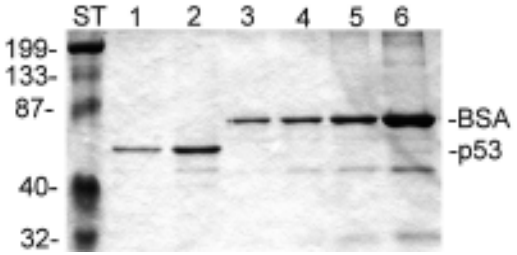

Fig. 2. Purification of p53 through a Q Sepharose Fast Flow column. ST, MW standards; lane 1, unconcentrated p53; lane 2, p53 after ultrafiltration-mediated concentration; lane 3, BSA protein standard (1 μg); lane 4, BSA protein standard (2 μg); lane 5, BSA protein standard (4 μg); lane 6, BSA protein standard (10 μg).

sis. The p53 that we purify contains approx 20% tetramer and 80% monomer by comparison to protein standards of known MW.

Concentrated p53 (94 μL, 0.5 mg/mL) was injected onto a 300 × 7.8 mm Bio-Sil SEC 250-5 high-performance liquid chromatography (HPLC) column, 5-μm particle size (Bio-Rad) equilibrated with 100 m*M* $NaHPO_4$, pH 7.06, 1 m*M* EDTA. The HPLC components were a Varian 9050 UV/*VIS* detector, a Varian 9012 pump, and a Varian 9100 autosampler. The flow rate was 1 mL/min, and the p53 elution profile was monitored at a wavelength of 280 nm. **Figure 3** shows a chromatograph of p53 protein. Comparison to molecular weight (MW) standards indicates that the monomer has a MW of 69.3 kDa, and the tetramer has a MW of 328 kDa. The ratio of monomer to tetramer was 4:1.

3.10. Electromobility Gel Shift Assay

We use a standard DNA binding assay called the EMSA to determine whether p53 is functional. Briefly, p53 is incubated with radiolabeled synthetic double-stranded DNA containing a p53-responsive element. The p53/DNA complex is separated from the nonbound DNA by electrophoresis. The gel is dried and exposed to X-ray film to visualize the DNA bound to p53. To ensure specificity of the binding, we usually run a number of controls. Negative controls include nonaddition of p53 to the reaction mixture, excess nonradiolabeled DNA (competitive inhibitor), and excess poly(dI-dC)·poly(dI-dC) (nonspecific inhibitor).

3.10.1. Annealing Complementary Strands

1. Dissolve complementary single-stranded oligonucleotides containing consensus sequence in TE at a concentration of 1 μg/μL. We used the second p53 response element within the P2 promoter of human *mdm2*.

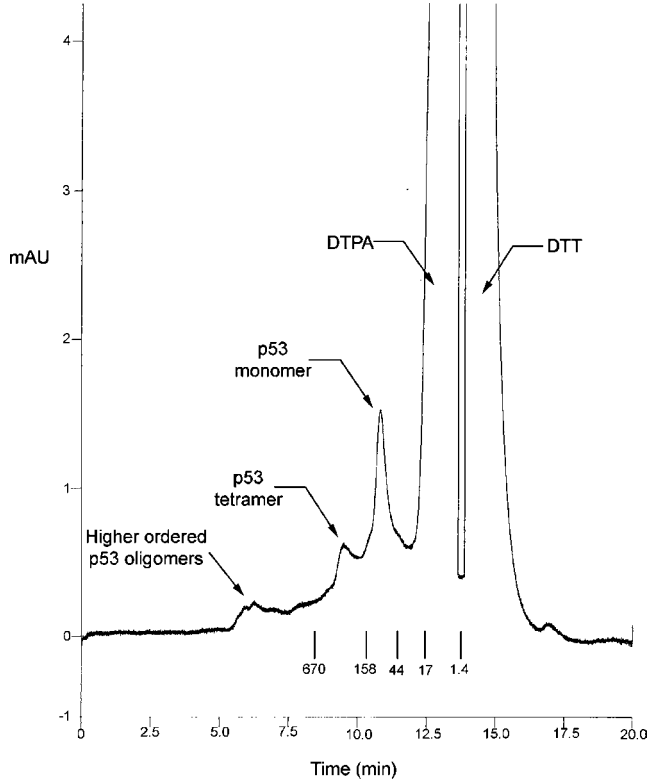

Fig. 3. Gel filtration analysis of purified p53. Chromatogram of concentrated p53 eluted from a Bio-Sil SEC 250-5 column monitored at 280 nm. Elution positions of protein standards are depicted within the chromatogram and their MWs (in kDa) are shown.

2. Add 20 µL of each oligonucleotide to a polylethylene tube and heat to 85°C for 5 min.
3. Remove from heat source and allow sample to cool down to room temperature on benchtop for 3 h.

3.10.2. End-Labeling Double-Stranded Oligonucleotide with γ-^{32}P-ATP

1. Add the following reagents together in the following order: (*i*) 5 µL T4 polynucleotide kinase 10X buffer (Roche Molecular Biochemicals); (*ii*) 2 µL T4 polynucleotide kinase (Roche Molecular Biochemicals); (*iii*) 1 µL DNA probe (1 µg/µL); (*iv*) 3 µL γ-^{32}P-ATP (6000 Ci/mmol at 150 mCi/mL); and (*v*) 39 µL water for a total of 50 µL.
2. Mix and incubate at 37°C for 10 min. Stop the reaction by adding 1 µL of 0.5 *M* EDTA, pH 8.0.

3.10.3. Removal of Unreacted γ-^{32}P-ATP from Radiolabeled DNA

Gel filtration is used to remove unreacted γ-^{32}P-ATP from DNA. The radiolabeled DNA will be excluded from the column beads, and the γ-^{32}P-ATP will be trapped within the column beads.

1. Centrifuge G-50 spin column (Roche Molecular Biochemicals; cat. no. 1 273 965) with its collection tube at 1100*g* for 2 min at room temperature.
2. Discard the collection tube and eluted buffer. Keep the column in the upright position and add 50 µL of the quenched reaction mixture to the center of the column bed (it is essential that the mixture not contact the sides of the column during pipeting).
3. Place the column into a new collection tube, while maintaining it in an upright position. Centrifuge on a swinging bucket rotor at 1100*g* for 4 min at room temperature (*see* **Note 7**).
4. Recover the labeled DNA in the collection tube, and remove 1 µL for scintillation cocktail counting. We typically achieve a specific radioactivity in the range of 0.7–3 × 10^7 counts per minute $\left(\frac{\text{cpm}}{\mu\text{g}}\right)$.
5. Dilute radiolabeled DNA with TE to a final concentration of 6000 cpm/µL (*see* **Note 8**).

3.10.4. DNA Binding Reaction

1. Add the following in a polyethylene tube:
   (*i*) 3.5 µL 5X DNA binding buffer; (*ii*) 1 µL poly(dI-dC)·poly(dI-dC) (1 µg/µL); (*iii*) 6 µL water; and (*iv*) 3 µL p53 protein (0.2–0.5 µg/µL) for a total of 13.5 µL.
2. Mix and leave at room temperature for 10 min.
3. Add 2 µL of radiolabeled DNA (6000 cpm/µL) and incubate at room temperature for 20 min.
4. Add 2 µL of 10X EMSA gel loading buffer to quench the DNA binding reaction. Mix.
5. Load quenched samples onto 6% nondenaturing polyacrylamide gel and electrophorese with 0.5X TBE running buffer. **Figure 4** shows an autoradiogram of a p53 EMSA.

4. Notes

1. To heat inactivate fetal bovine serum, first thaw at 4°C. Then incubate in water bath at 56°C for 30 min.
2. Use extra caution to ensure that temperature is no higher than 37°C and no lower than 35°C. A higher temperature will kill the insect cells, and a lower temperature will cause the agarose–media overlay to form lumps.
3. Create PMSF stock solution by preparing a 0.1 *M* solution in 100% ethanol. Be careful not to inhale the PMSF powder during handling.
4. Sometimes it is difficult to distinguish between p53 and virus proteins in the Coomassie-stained gel. To ensure that p53 is expressed, perform an immunoblot

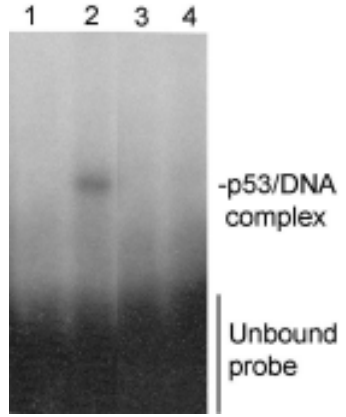

Fig. 4. EMSA assay to detect p53 binding to DNA. An autoradiograph of a gel demonstrating p53 binding to DNA. *mdm2* p53 responsive element-2 was incubated with p53 under various conditions to demonstrate specificity of p53 binding activity. Lane 1, absence of p53 from the reaction mixture; lane 2, complete reaction mixture; lane 3, complete reaction mixture plus excess unlabeled *mdm2* p53 responsive element-2; lane 4 complete reaction mixture plus excess poly(dI-dC)·poly(dI-dC).

analysis with a commercially available antibody. After the immunoblot image is recorded, stain the membrane with Coomassie Blue dye for 15 min and air dry. Within 1 h, many abundant proteins can be detected on the membrane. Comparison of the immunoblot image with the Coomassie-stained membrane will indicate which stained protein is p53.

5. We use 50 mL Oak Ridge polycarbonate centrifuge tubes (Nalge, Rochester, NY, USA) for centrifugation at *g* forces between 4000 and 20,000*g*.
6. Delphin et al. reported that p53 undergoes oxidation when p53 is stored in the absence of DTT ***(5)***. It has been our experience that p53 is extremely sensitive to oxidation as well. Ensure that DTT is fresh whenever buffers are made, and remake the buffers every 3 mo. We purge our solutions with N_2 gas for 5–10 min to remove dissolved O_2.
7. We use the Centra CL2 IEC centrifuge equipped with a 52212 IEC rotor. One must use some caution in choosing the correct centrifuge–rotor combination. We found that at 1100*g*, some heavy swinging buckets do not achieve the vertical position in a timely manner, the result of which forces the sample to the side of the column prior to its migration to the collection tube. The recovery of purified DNA from these preparations is often very low.
8. Only dilute a portion of the radiolabeled DNA. If an experiment must be repeated later, then use a more concentrated solution of radiolabeled DNA to compensate for the radioactivity decay.

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