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Protein–Protein Interactions

Methods and Applications

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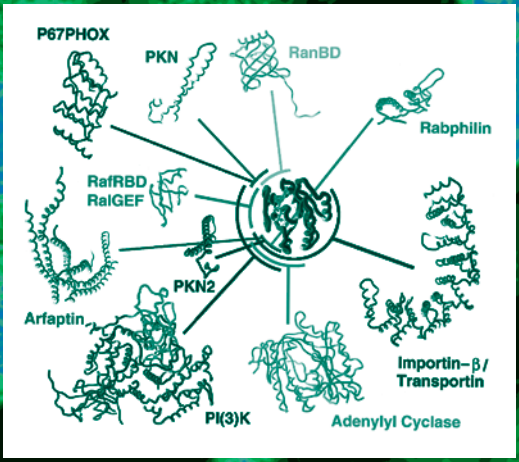

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Fluorescence Gel Retardation Assay to Detect Protein–Protein Interactions

Sang-Hyun Park and Ronald T. Raines

Abstract

A gel mobility retardation assay can be used to detect a protein–protein interaction. The assay is based on the electrophoretic mobility of a protein–protein complex being less than that of either protein alone. Electrophoretic mobility is detected by the fluorescence of a green fluorescent protein variant that is fused to one of the protein partners. The assay is demonstrated by using the interaction of the S-protein and S-peptide fragments of ribonuclease A as a case study.

Key Words

Electrophoresis; fusion protein; gel mobility shift; gel retardation; green fluorescent protein; protein–protein interaction.

1. Introduction

Gel mobility retardation is a useful tool for both qualitative and quantitative analyses of protein–nucleic acid interactions ***(1)***. Here, a gel mobility retardation assay is described that can be used to detect and identify specific protein–protein interactions ***(2,3)***. The assay is based on the electrophoretic mobility of a protein–protein complex being less than that of either protein alone. Electrophoretic mobility is detected by the fluorescence of a green fluorescent protein (GFP) variant that is fused to one of the protein partners.

GFP from the jellyfish *Aequorea victoria* has exceptional physical and chemical properties besides spontaneous fluorescence. These properties include high thermal stability and resistance to detergents, organic solvents, and proteases ***(4–6)***. These properties endow GFP with enormous potential for biotechnical applications ***(7,8)***. Since the cDNA of GFP was cloned ***(9)***, a variety of GFP

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variants have been generated that broaden the spectrum of its application ***(10–15)***. Among those variants, S65T GFP is unique in having increased fluorescence intensity, faster fluorophore formation, and altered excitation and emission spectra than that of the wild-type protein ***(13,16)***.

The fluorescence gel retardation assay described below uses S65T GFP to probe protein–protein interactions in vitro ***(2)***. This method requires fusing S65T GFP to a target protein (X) to create a GFP chimera (GFP–X). The interaction of this fusion protein with another protein (Y) is then analyzed by native gel electrophoresis ***(17)*** followed by the detection of the fluorescence of free (GFP–X) and bound chimera (GFP – X·Y). The fluorescence gel retardation assay is a rapid method to demonstrate the existence of a protein–protein interaction and to estimate the equilibrium dissociation constant (K_d) of the resulting complex.

2. Materials

1. 10 m*M* Tris-HCl (pH 7.5) containing glycerol (5% v/v).
2. Solution of aqueous glycerol (10% v/v).
3. Solution of aqueous acrylamide (30% w/v) and bisacrylamide (8% w/v), filtered through a 0.45-μm filter and stored at 4 °C in the dark.
4. 0.5 *M* Tris-HCl (pH 8.8), filtered through a 0.45-μm filter and stored at 4°C.
5. Electrophoresis buffer (5X stock solution): Tris base (125 m*M*) and glycine (0.96 *M*), which is diluted to a 1X working solution as needed.
6. Solution of aqueous ammonium persulfate (10% w/v).
7. *N′,N′,N′,N′*-tetramethylethylenediamine (TEMED).
8. Native acrylamide (6% w/v) gels (8 × 8 cm, 0.75 mm thick) prepared free of detergents or reducing agents (*see* **Note 1**).
9. Mini-gel electrophoresis apparatus and power supply.
10. Purified protein GFP–X and purified protein Y.
11. Fluorimager system (Molecular Dynamics; Sunnyvale, CA).
12. UV/Vis spectrophotometer.

3. Methods

3.1. Preparation of Polyacrylamide Gel

1. In a 50 mL plastic tube, mix 1 mL of acrylamide/bisacrylamide stock solution, 1.25 mL of 0.5 *M* Tris-HCl (pH 8.8), and 2.75 mL of deionized water.
2. Add 20 μL of ammonium persulfate (10% w/v) and 5 μL of TEMED. Vortex the solution briefly to mix.
3. Using a 5-mL pipet, slowly apply the solution to a mini-gel cast (8 cm × 8 cm, 0.75 mm thick). Insert a 0.75-mm comb and allow the gel to polymerize for 30 min at room temperature.
4. Carefully remove the comb without disrupting the edges of the polymerized wells.

3.2. Gel Electrophoresis

1. Estimate the concentration of protein GFP–X by using the extinction coefficient [ε = 39.2 mM^{-1}cm^{-1} at 490 nm ***(13)***] of S65T GFP.
2. To begin the gel retardation assay, mix protein GFP-X (1.0 μM) with varying amounts of protein Y in 10 μL of 10 m*M* Tris-HCl buffer (pH 7.5) containing glycerol (5% v/v) (*see* **Note 2**).
3. Incubate the mixtures at 20°C for 20 min. Using gel-loading pipet tips, apply the mixtures onto the polyacrylamide gel. Perform the electrophoresis at 4°C at 10 V/cm for 30 min using 1X electrophoresis buffer (*see* **Note 3**).
4. Immediately after electrophoresis, scan the gel with a Fluorimager SI System at 700 V using the built-in filter set (490 nm for excitation; ≥515 nm for emission) (*see* **Notes 4** and **5**).

3.3. Data Analysis

1. Quantify the fluorescence intensities of bound and free GFP–X in the gel scan by using the program ImageQuaNT (Molecular Dynamics; Sunnyvale, CA).
2. Determine the value of **R** (= fluorescence intensity of bound GFP–X/total fluorescence intensity) for each gel lane from the measured fluorescence intensities.
3. Calculate values of K_d for each lane with the equation:

$$K_d = \frac{1-\mathbf{R}}{\mathbf{R}} \times \left([\mathrm{Y}]_{\mathrm{total}} - \mathbf{R} \times [\mathrm{GFP-X}]_{\mathrm{total}} \right) \quad (1)$$

3.4. Case Study

The well-characterized interaction of the S-15 and S-protein fragments of ribonuclease A has been used to demonstrate the potential of the fluorescence gel retardation assay to detect protein–protein interactions ***(2)***. The assay has also been used to detect the interaction of CREB and importins ***(18)***, and that of cyclophilin and the capsid protein p24 of HIV-1 ***(19)***. In the first example, a GFP chimera, S15-GFP(S65T)-His6, is produced by standard recombinant DNA techniques and affinity-purified from bacteria. A fixed quantity of S15–GFP(S65T)–His_6 is incubated with a varying quantity of S-protein prior to electrophoresis in a native polyacrylamide gel. After electrophoresis, the gel is scanned with a fluorimager and the fluorescence intensities of bound and free S15–GFP(S65T)–His_6 are quantified (**Fig. 1**). The value of K_d for the complex formed in the presence of different S-protein concentrations is calculated from the values of **R** and the total concentrations of S-protein and S15–GFP(S65T)–His_6 by using eq. (1). The average (± SE) value of K_d is (6 ± 3) ×10^{-8} *M*.

4. Notes

1. Polymerized, unused gels can be store at 4°C for up to 1 mo. To prevent the gels from getting dry, they can be wrapped in wet paper towels and sealed in a plastic bag.

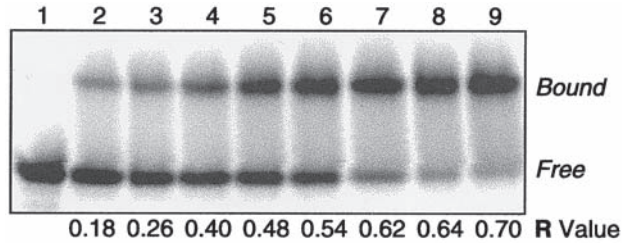

Fig. 1. Gel retardation assay of a protein–protein interaction. Gel retardation assay of the interaction of S15–GFP(S65T)–His_6 with varying amounts of S-protein. Lanes 1–9, 1 μ*M* S15–GFP(S65T)–His_6 and 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 μ*M* S-protein, respectively. The relative mobilities of free and bound S15–GFP(S65T)–His_6 in an acrylamide (6% w/v) gel are 0.72 and 0.47, respectively. The value of **R** is obtained for each lane, and values of K_d are calculated by using eq. (1), with the average being $K_d = (6 \pm 3) \times 10^{-8}$ *M*.

2. Pilot experiments may need to be conducted to determine the proper concentration range of GFP–X and Y. A good starting point is to adjust the concentration of GFP–X to the K_d value of complex, if known, and to prepare serial dilutions of Y so that its concentrations spans from $K_d \times 10$ to $K_d/10$. For example, if K_d = 1.0 μ*M*, mix 1.0 μ*M* GFP–X with 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 μ*M* Y.
3. The gel, electrophoresis apparatus, and electrophoresis buffer should be pre-equilibrated at 4°C before electrophoresis.
4. Although it is desirable to scan the gel immediately after electrophoresis, gels can be stored at 4°C for a few hours before scanning without compromising the resolution and fluorescence sensitivity.
5. It is not necessary to remove the gel from the glass plates before scanning. Most glassware used for casting gels are not fluorescent. The surface of the glass to be scanned should be rinsed with deionized water to remove any residual buffer and acrylamide, and wiped dry.

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