Cell-Free Expressed Protein in Fluorescent Gel Shift Assays

ABSTRACT

Cell-free expression is a convenient option for rapid protein production. In vitro-expressed (IVE) proteins can be used for functional analyses such as protein:protein and protein:nucleic acid interactions. Here we used transcription factor c-Jun to demonstrate optimization of a fluorescent gel shift assay using IVE proteins. The parameters optimized include fluorophore, oligo concentration, cell-free expression system selection and binding buffer conditions. Fluorescence and IVE give a rapid, convenient alternative to radioactive detection for studying nucleic acid-binding proteins.

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Introduction

Cell-free expression systems generate protein from a plasmid DNA template in as little as 60 minutes. Advances in extract technology make it possible to produce nanogram to microgram quantities of protein in self-contained scalable reactions. The availability of extracts from a variety of different systems (mammalian, plant, bacterial and insect) make it increasingly simple to complement, and even substitute, traditional cell-based protein expression methods with a cell-free alternative.

The gel shift assay, also known as a gel retardation or electrophoretic mobility shift assay (EMSA), is a common technique used to detect protein:nucleic acid interactions. Labeled nucleic acid is incubated with the protein of interest, then analyzed using electrophoresis and a nondenaturing gel. Protein:nucleic acid complexes migrate more slowly during gel electrophoresis than unbound or free nucleic acid. Thus, a shift in the mobility of a nucleic acid fragment after incubation with a particular protein or cell extract indicates an interaction.

Gel shift assays have numerous applications including:

- Determining whether a protein in a nuclear extract is able to bind a nucleic acid probe sequence
- Identifying the protein or proteins that bind the nucleic acid sequence (e.g., via antibody-mediated "supershift" or by reconstituting shifts with purified protein)
- Mapping the interaction site by site-directed mutagenesis of the nucleic acid or mutagenesis of the protein
- Studying kinetic binding (e.g., determine K_d dissociation constant of the protein:nucleic acid interaction)

Gel shift assays are typically performed with purified protein or crude extract and short radiolabeled DNA fragments. Interaction specificity is determined by competition with excess unlabeled DNA. If the interaction is specific, incubation with the same DNA (specific competitor) will decrease the shift intensity, while incubation with unrelated DNA (nonspecific competitor) will result in no change in the shift intensity. Nonspecific interactions would be affected by adding either competitor.

While gel shift assays are relatively simple to perform, they can require significant optimization(1) . The nucleic acid (DNA or RNA), oligo length and form (single- or double-stranded) are dependent on the protein of interest. The nucleic acid used, both labeled and competitors, must be titrated to find an optimum concentration. The reaction conditions (time, temperature and buffer composition) will determine whether a weak or transient interaction can be detected. In addition, the electrophoresis conditions also may require optimization to ensure the complex is stable during analysis. The assay conditions used in the Gel

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Shift Assay System (Cat.# E3300) support many protein:nucleic acid interactions and are a good starting point for further optimization.

Traditionally, ^{32}P end-labeled DNA fragments are used as probes in gel shift assays. With the decrease in popularity and availability of radioactivity, nonradioactive methods such as fluorescent or chemiluminescent alternatives are being used with greater frequency. Fluorescence, which has the advantage of allowing direct in-gel detection with a fluorescence scanner, is the focus of this study.

While purified protein and cell nuclear extracts are typically used as the protein source for gel shift assays, in vitro-expressed (IVE) protein is increasingly recognized as a convenient alternative. Here, we demonstrate the use of IVE protein in gel shift assays using fluorescently labeled DNA. As a model, we chose c-Jun, a member of the AP1 family of transcription factors, which forms homodimers that bind AP1 sites in vitro.

Choosing the Right Fluorophore

Fluorescence scanners vary in the fluorophores they optimally detect. Therefore, you may need to empirically determine which fluorophore can be detected with the greatest sensitivity and lowest background by a scanner. We tested the sensitivity of the Typhoon™ Trio+ imager (GE Healthcare) for Cy®5 and Cy®3 fluorescently labeled oligos. Four oligos containing consensus AP1 DNA binding sites were generated (Integrated DNA Technologies):

- AP1-Fwd Cy5 (5´-Cy®5-CGC TTG ATG AGT CAG CCG GAA-3´)
- AP1-Fwd Cy3 (5´-Cy®3-CGC TTG ATG AGT CAG CCG GAA-3´)
- AP1-Rev (5’-TTC CGG CTG ACT CAT CAA GCG-3’)
- AP1-Rev Cy5 (5’-Cy®5-TTC CGG CTG ACT CAT CAA GCG-3’)

The oligos were then annealed in three combinations:

- Cy5 AP1: AP1-Fwd Cy5 + AP1-Rev
- 2Cy5 AP1: AP1-Fwd Cy5 + AP1-Rev Cy5
- Cy3 AP1: AP1-Fwd Cy3 + AP1-Rev

Annealed oligos as well as a titration of single-stranded oligos (5 picomoles to 5 femtomoles) were analyzed on a 5% nondenaturing polyacrylamide TBE gel. Figure 1 shows the annealing reactions worked well; all double-stranded oligos show the predominant species as a higher molecular weight than the single-stranded oligos. The sensitivity of detection of the Cy®5-labeled oligo was 5 fmol, while the sensitivity of the Cy®3-labeled oligo was 50 fmol. Subsequent studies were performed using 2Cy5 AP1 in order to maximize sensitivity. Because bromophenol blue (BPB) was detected as a band slightly above the 2Cy5 AP1 double-stranded oligo in the Cy®5 channel, we omitted it from gel shift samples and used it only in the oligo control as a means to track electrophoresis.

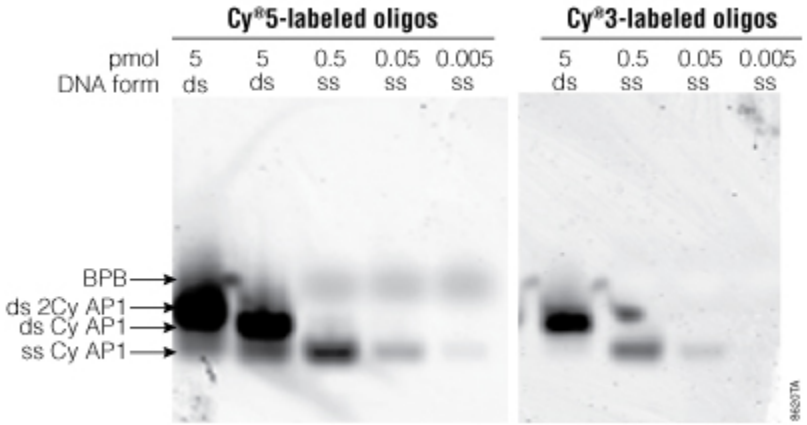

Figure 1. Detection sensitivity of Cy®5- and Cy®3-labeled DNA oligos. Double-stranded (ds) Cy5 AP1, 2Cy5 AP1 or Cy3 AP1 and single-stranded (ss) AP1-Fwd Cy5 or AP1-Fwd Cy3 were analyzed. Oligos were titrated from 5pmol to 0.005pmol (5fmol) and resolved on a 5% nondenaturing polyacrylamide TBE gel run at 4°C using 100V for 1 hour, then scanned with the Typhoon™ Trio+ Imager (GE Healthcare). Bromophenol blue (BPB) was detectable in the Cy®5 channel.

Optimizing Fluorescent Oligo Concentration

The amount of labeled oligo used for the gel shift will affect the ability to specifically detect nucleic acid:protein interactions and to perform kinetic analyses. Excess labeled oligo can saturate detection and make it difficult to compete with unlabeled oligo. Also, if kinetic studies are planned, at least a 50% shift of all oligo is required to determine the dissociation constant for the interaction. Ideally, the minimum amount of oligo that can still easily be detected during a shift should be used.

To optimize fluorescent oligo concentration, we tested gel shifting and oligo competition using HeLa nuclear extracts and purified c-Jun [Recombinant Human AP1 (rhAP1)]. HeLa nuclear extract was used under standard conditions provided in the *Gel Shift Assay System Technical Bulletin* #TB110. Purified c-Jun was used with a modified 5X gel shift buffer(2) . Figure 2, Panel A, shows the results of the gel shift assay using 500 fmol of 2Cy5 AP1. For the HeLa Extract, a shift was observed, and adding the specific competitor reduced the shift intensity, while no change in intensity was observed with the nonspecific competitor. When the purified c-Jun was used, competitor effect was not obvious.

We then asked whether decreasing the labeled oligo to 50 fmol (thus changing the molar excess of competitor from 10- to 100-fold) would clarify competition. As expected, lowering the 2Cy5 AP1 input to 50 fmol improved the shift competition for both the HeLa extract and purified c-Jun (Figure 2, Panel B). In addition, purified c-Jun shifted all labeled oligo, making it possible to perform future kinetic analyses.

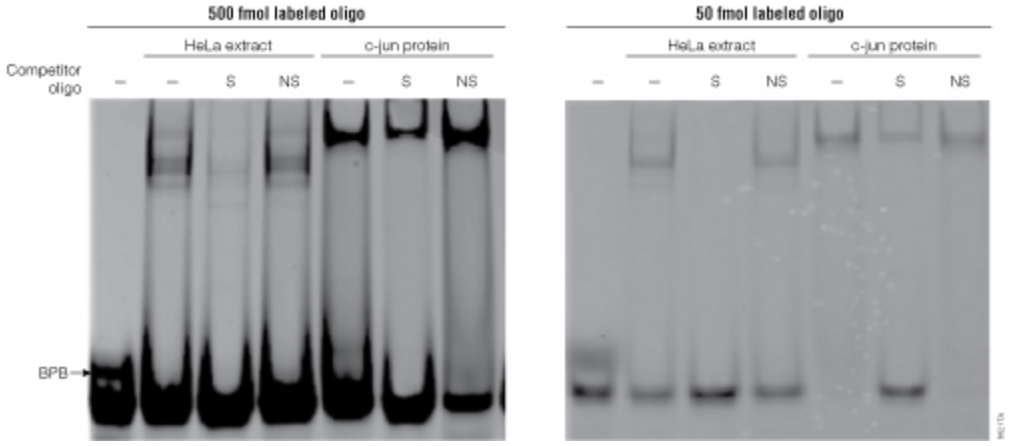

Figure 2. Optimization of fluorescent oligo concentration for gel shift competition assay with HeLa nuclear extract and purified c-Jun. Two amounts of double-stranded 2Cy5 AP1 oligo were tested (500 fmol and 50 fmol). Competitions were performed with 5 pmol specific (S) or nonspecific (NS) competitor. Protein was preincubated with competitor 10 minutes at room temperature before subsequent 20-minute incubation with 2Cy5 AP1. The total EMSA reactions (10µl) were analyzed on a 5% nondenaturing polyacrylamide TBE gel run at 4°C using 70V for 1 hour, then scanned with the Typhoon™ Trio+ Imager (GE Healthcare). Bromophenol blue (BPB) was included in the oligo-only control to track electrophoresis.

Expressing the c-Jun Protein in vitro

c-Jun was expressed from the pFN19K:c-Jun construct using the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170). This system uses rabbit reticulocyte lysate to express protein in a 90-minute reaction at 30°C. The pFN19K:c-Jun plasmid contains T7 and SP6 promoters with the c-Jun coding sequence downstream of the HaloTag® fusion tag (pFN19K HaloTag® T7 SP6 Flexi® Vector, Cat.# G1841). The HaloTag® fusion tag covalently reacts with a chloroalkane ligand allowing specific labeling or capture(3) . After expression, HaloTag® fusions were covalently labeled with the HaloTag® TMR Ligand (Cat.# G8251), allowing fluorescent detection even under denaturing conditions. Expression of c-Jun in the reticulocyte lysate system resulted in a single predominant product (Figure 3).

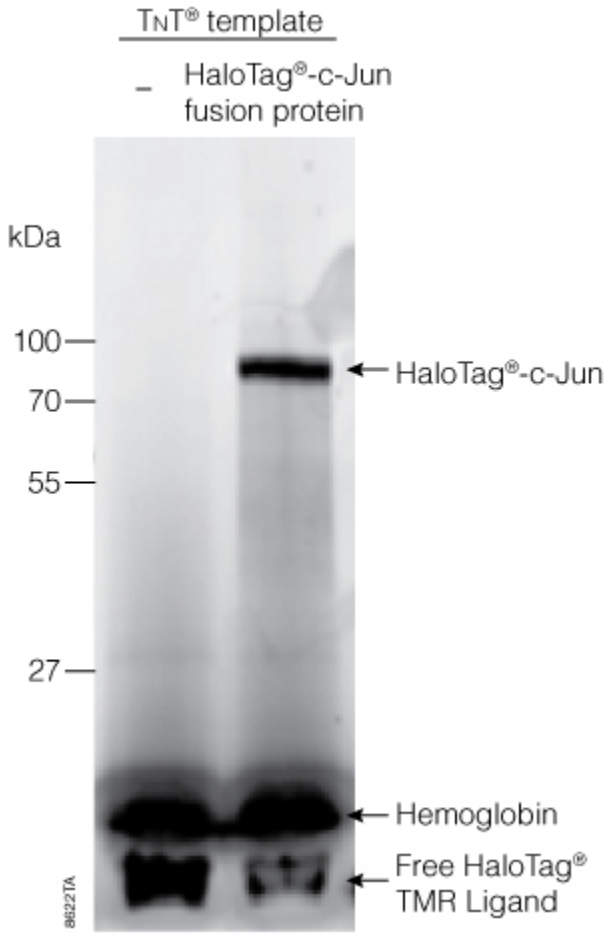

Figure 3. Expression of c-Jun in the TNT® T7 Quick Coupled Transcription/Translation System. TNT® reticulocyte lysate reactions were incubated without DNA (–) or with the pFN19K Flexi®:c-Jun plasmid (c-Jun) for 90 minutes at 30°C. Lysates were then incubated with the HaloTag® TMR Ligand (1µM final) for 30 minutes on ice. A 2.5µl aliquot of the reaction was analyzed on a 4–20% SDS polyacrylamide gel run at room temperature using 80V for 1 hour. TMR-labeled HaloTag® fusion was detected using the Typhoon™ Trio+ Imager (GE Healthcare).

Optimizing the Gel Shift Assay with IVE c-Jun

We tested in vitro-expressed c-Jun without purification in a gel shift assay. Gel shifts were performed with 2µl of TNT® extract +/– c-Jun expression under the same conditions used for purified c-Jun [Figure 2, modified buffer containing 0.01mg/ml poly(dI-dC)•poly(dI-dC)]. The results are shown in Figure 4. For the TNT® reticulocyte lysate-based system, clear shifts were seen with the lysate alone as well as lysate expressing c-Jun. Competition assays showed the shift in the TNT® lysate alone is not specific to AP1. In contrast, TNT® reticulocyte lysates expressing c-Jun gave a specific shift.

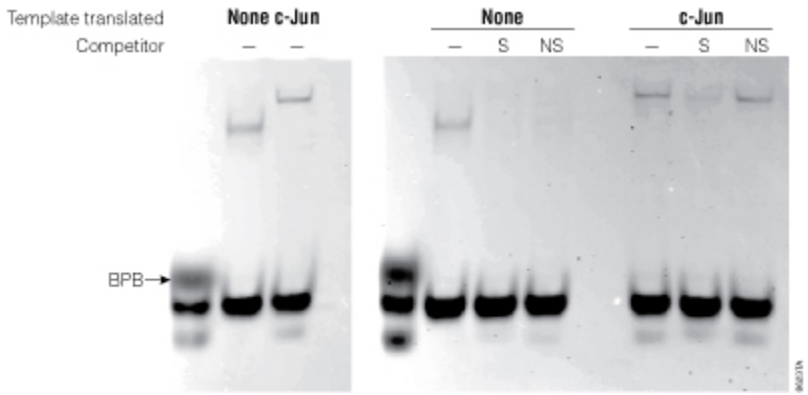

Figure 4. Gel shift assay with c-Jun expressed in the TNT® T7 Quick Coupled Transcription/Translation System. TNT® reticulocyte lysates alone or lysates expressing c-Jun from the pFN19K:c-Jun construct were tested for their ability to shift the 2Cy5 AP1 oligo. Competitions with specific (S) and nonspecific (NS) competitor were performed. Protein was preincubated with competitor 10 minutes at room temperature before subsequent 20-minute incubation with 2Cy5 AP1. The total EMSA reactions (10µl) were analyzed on a 5% nondenaturing polyacrylamide TBE gel run at 4°C using 70V for 1 hour, then scanned with the Typhoon™ Trio+ Imager (GE Healthcare). Bromophenol blue (BPB) was included in the oligo-only control to track electrophoresis. Bromophenol blue (BPB) was included in the oligo-only control to track electrophoresis.

To reduce background nonspecific DNA-binding activity from the lysates, we increased the concentration of poly(dI:dC) in the binding buffer to 0.05mg/ml. In addition, to improve competition, we increased the molar excess of competitor by reducing 2Cy5 AP1 and increasing the competitor input. Figure 5 shows the results of this optimization. Extracts alone no longer showed a shift, and 4µl of TNT® lysate expressing c-Jun clearly showed a specific shift. Titration of TNT® reticulocyte lysates from 2µl to 6µl (maximum volume that could be added to the reaction) gave a dose-dependent shift.

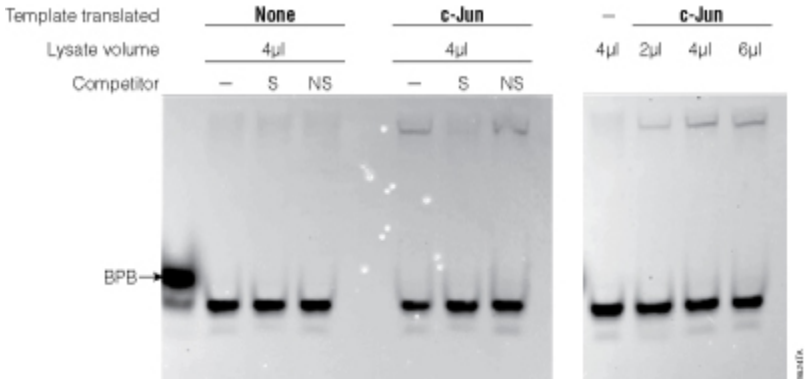

Figure 5. Use of in vitro-expressed c-Jun for fluorescent gel shift. The TNT® T7 Quick Coupled Transcription/Translation System was used to express c-Jun from the pFN19K:c-jun construct. Control lysates (–) or lysates expressing c-Jun were tested for their ability to shift the 2Cy5 AP1 oligo under modified reaction conditions [25 fmol labeled oligo, 7 pmol specific (S) or nonspecific (NS) competitor, 0.5mg/ml poly(dI:dC)]. Protein was preincubated with competitor 10 minutes at room temperature before subsequent 20-minute incubation with 2Cy5 AP1. The total EMSA reactions (10µl) were analyzed on a 5% nondenaturing polyacrylamide TBE gel run at 4°C using 70V for 1 hour, then scanned with the Typhoon™ Trio+ Imager (GE Healthcare). Bromophenol blue (BPB) was included in the oligo-only control to track electrophoresis. Bromophenol blue (BPB) was included in the oligo-only control to track electrophoresis.

Discussion and Conclusion

Here we demonstrated the use of in vitro-expressed c-Jun protein in a fluorescent gel shift assay. We show the importance of optimization of experimental parameters specific to cell free extracts and fluorescent labels. First and foremost, select an optimal extract for protein expression. Rabbit reticulocyte lysates, wheat germ extracts, insect cell extracts and *E. coli* S30 extracts are all common systems that can be used for cell-free protein expression(4) . Extract selection should be based on efficiency of full-length protein expression and minimal shifting of the nucleic acid probe. As demonstrated here, if extracts shift the probe in the absence of specific protein expression, binding conditions may be optimized. In addition, fluorophore type and detection sensitivity should be tested prior to performing any gel shift assay to ensure that optimal conditions are used for detection.

The primary advantages for use of cell-free expression and fluorescently labeled oligos in gel shift assays are the considerable time savings and eliminating the need for radioactivity. With cell-free expression, no protein purification may be needed. Typical in vitro expression reaction times range from 1 to 2 hours, allowing the user to go from expression plasmid to gel shift within an afternoon. Creating a HaloTag® fusion protein has the additional advantage of allowing easy determination of expression levels and subsequent purification, if desired. The quick turnaround time in protein expression allows for rapid screening of proteins and protein mutants for mapping specific regions involved in nucleic acid binding activity.

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