

SCIENCE IMAGING SYSTEMS

# Application Note

No.19

基礎編：Fluorescent Gel Shift Assay

FLA-2000 / FLA-3000G

## はじめに

今回は、蛍光検出によるゲルシフトアッセイを紹介します。DNA-タンパク質複合体と、遊離DNAとの間に生じる泳動の移動度の差を利用して、DNA結合タンパク質について研究する手法です。この方法はタンパク質とDNAとの相互作用を簡便かつ高感度に検出する方法として広く使用されています。

生命現象の多くはDNA結合タンパク質が関係しており、その働きとして、遺伝子の発現調整や発現誘導などの制御機構に関与していることが知られつつあります。

今回は、Imaging Research社のDr. Peter RammおよびNezar Rghei氏にご執筆頂きました。検出には、FLA-2000を用いています。原文が英語ですので正確な内容をお伝えするために翻訳せず、原文のまま掲載することにしました。

## Contents

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## Summary

- フルオレセイン標識DNAを用いて、蛍光検出をすることができました。
- AP2転写因子を0.18 $\mu$ gまで検出することができました。
- 直線性のある検量線を得ることができました。

FLA

# 1 Introduction

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Specific interactions between proteins and DNA sequences regulate many cellular processes, including gene expression, DNA repair, and DNA packaging. Therefore, techniques to evaluate these interactions are important tools in molecular biology.

Interactions of regulatory proteins with their DNA binding sites are commonly studied by filter binding, DNA footprinting, methylation interference, and gel shift assay (also known as the gel retardation assay)[1]. Of these the gel shift assay is the most widely used. It is based on the observation that DNA molecules bound by protein complexes migrate more slowly than unbound DNA, in a non-denaturing gel matrix [2,3].

In conducting a typical gel shift assay, protein extracts are incubated with labeled oligonucleotides to form specific complexes. The DNA/protein mixture is then resolved on a non-denaturing polyacrylamide gel followed by autoradiographic or fluorescence detection. On the resolved gel, the position of the oligonucleotide - protein complex will be shifted relative to the position of the unbound oligonucleotides.

Advantages of the gel shift method are:

- a) it can be used to identify the presence of a specific interaction even in crude extracts;
- b) the procedure is relatively quick and simple;
- c) it can provide data regarding the sequence specificity and affinity of the DNA-binding interaction to the putative binding site [1].

Most gel shift assays have used isotopic label. Disadvantages of this include issues of disposal, safety, and the extended period of time required for exposure. In contrast non-radioactive detection (commonly with fluorescence labeling or DNA intercalating dye) avoids the problems of radioisotopes, and allows direct and immediate imaging of gels without need for film.

In summary, the gel shift assay is a valid and widely used method for measuring protein-DNA interactions. In this application note, we describe the use of the Fluorescent / radioisotope imaging system FLA-2000 (Fuji Photo Film Co., Ltd., Tokyo) to image a fluorescent gel shift assay

ゲルシフト法のメリット

- a) 簡単なサンプル処理でも特異的な相互作用を検出可能。
- b) 操作が早くて簡単。
- c) シーケンスとDNA-タンパク質結合の親和力の関係を得ることができる。

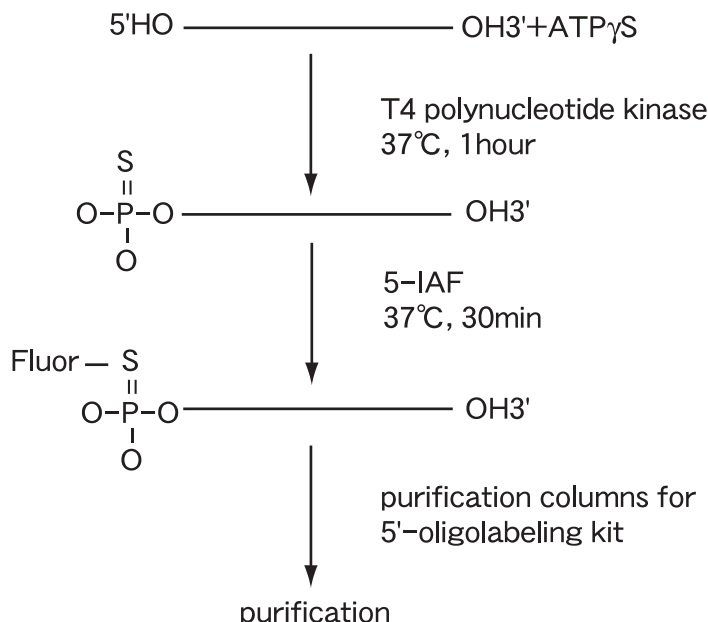
## 2 Materials and Methods

### Labeling and Annealing of Consensus Oligonucleotide

The AP2 oligonucleotide (5'-GATCGAACTGACCGCCCCGCGCCCCGT-3') was end labeled with fluorescein. AP2 oligonucleotide (14 pmol) was combined with 50  $\mu$ M ATP $\gamma$ S, 1X T4 polynucleotide kinase reaction buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, and 5 mM dithiothreitol) and 1  $\mu$ l T4 polynucleotide kinase in a total reaction volume of 50  $\mu$ l. The reaction mixture was incubated at 37°C for 1 hour.

The T4 polynucleotide kinase was inactivated by heat for 10 min at 70°C. Then, the reaction mixture was placed on ice and 2  $\mu$ l of 25mM 5-Iodoacetamidofluorescein (5-IAF) was added. The reaction mixture was incubated for 30 min at 37°C. The end result of these reactions is the attachment of a single fluorescein molecule to the 5'-phosphorothioated oligonucleotide. The unreacted 5-IAF and byproducts were removed using purification columns supplied with the Oligolabeling Kit, according to the manufacturer's recommendations.

The oligonucleotide was annealed to itself by incubation at 70°C for 2 min and was then allowed to cool for a period of 30 min (to approx. 30°C).



**Fig.2-1 End Labeling the Oligonucleotide**

#### Fig.2-1 オリゴヌクレオチドの末端標識

a) AP2 oligonucleotideに ATP $\gamma$ S, 1X T4 polynucleotide kinase reaction buffer, T4 polynucleotide kinaseを加え、37°C で1時間インキュベートする。

b) 70°C で10分間加熱した後、5-Iodoacetamidofluorescein (5-IAF)を加え、37°C で30分間インキュベートする。

c) Oligolabeling Kitに含まれるカラムで精製した後、70°C で2分間加熱し30分間放冷する。

**Fig.2-1**

## ■ DNA Binding Reactions

DNA binding reactions using AP2 extract (Promega, Madison, WI) were assembled by combining AP2 extract, 5X gel shift binding buffer (5 mM MgCl<sub>2</sub>, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl pH 7.5, and 0.25 µg/µl poly dIdC in 20% glycerol), and oligonucleotides (Table 2-1).

**Table 2-1**

	Positive Reaction (Labeled AP2 Only)	Specific Competitor (Cold AP2)	Non-Competitor (Cold SP1)
Nuclease-free water	3µl	2µl	2µl
5X gel shift binding buffer	2µl	2µl	2µl
AP2 extract (1.4 mg/ml)	1µl	1µl	1µl
Cold AP2 oligo (1.75 pmol/ml)	-	1µl	-
Cold SP1 oligo (1.75 pmol/ml)	-	-	1µl

**Table 2-1 Assembly of DNA binding reactions.**

The reaction mixtures were incubated for 10 min at room temperature. Then, 4 µl of (0.2 pmol/µl) 5'-end-fluorescein-labeled AP2 oligonucleotide were added to each reaction. The reaction mixtures were incubated for an additional 20 min at room temperature.

Gel shift assay was performed on a two-fold dilution series of AP2 extract to shift the 5'-end-fluorescein-labeled AP2 consensus oligonucleotide. Five reactions were set up by combining 2 µl 5X gel shift binding buffer, 2 µl nuclease-free water, and 2 µl of AP2 extract (2.8, 1.4, 0.7, 0.35, and 0.18 µg, respectively). In addition, a negative control reaction was set up in which nuclease-free water was substituted for the AP2 extract. The reaction mixtures were incubated for 10 min at room temperature. Subsequently, 4 µl of (0.2 pmol/µl) 5'-end-fluorescein-labeled AP2 oligonucleotide were added to each reaction. The reaction mixtures were incubated for 20 min at room temperature.

**Table 2-1 DNA結合反応の溶液組成**

## ■ Electrophoresis of DNA-protein complexes

A 4% nondenaturing polyacrylamide gel (500 µl 2% bisacrylamide, 2 ml 40% acrylamide, 1 ml 10X TBE, 625 µl 80% glycerol, 15.9 ml H<sub>2</sub>O, 10 µl TEMED, and 150 µl 10% ammonium persulfate) was cast in a Mini-PROTEAN II gel apparatus (BioRad, Hercules, CA) and was set to polymerize overnight. All electrophoresis was performed using 0.5X TBE buffer. After running the gel for 30 min at 100V, the samples were loaded and resolved for 45 minutes.

## ■ Detection using the FLA-2000

The FLA-2000 was set in Fluorescence mode, using 473 nm excitation, and a 520 nm emission filter. Instrument sensitivity was set at 100, and a 100 µm resolution was selected. The images produced by the FLA-2000 were analyzed using Analytical Imaging Station (AIS™) software (Imaging Research Inc., St. Catharines, ON, Canada).

### 3 Results and Discussion

#### ■ Calibration of Fluorescence Intensity

An independent calibration of observed fluorescence to fluor concentration was performed to determine linearity of FLA-2000 response within the concentration range of interest. To create the calibration image, a twofold serial dilution of 5'-fluorescein labeled M13 primer was made (Fig.3-1-a) ranging from 1000 to 62.5 femtomoles (fmol) in a black, 1536 well plate (Corning Costar Co., Cambridge, MA).

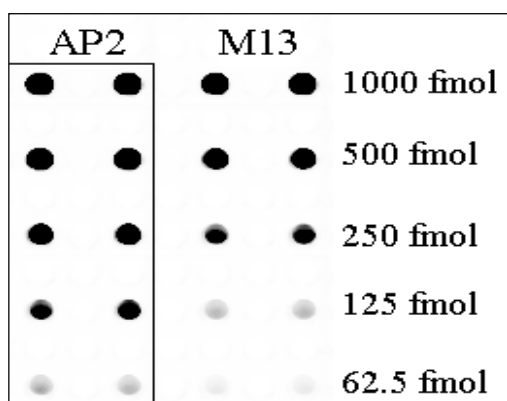


Fig.3-1-a

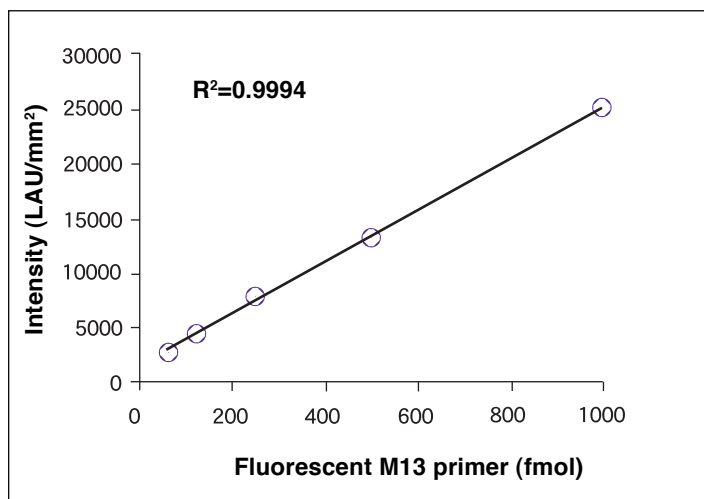


Fig.3-1-b

Fluorescence intensity (LAU/mm<sup>2</sup>) was calibrated to concentration (fmol), using calibration functions provided within the AIS software. Instrumental response was highly linear (Fig.3-1-b). In that the AP2 and M13 molecules were labeled in the same way, the M13 fluorescence could be used as a direct and linear reflection of the AP2 concentration.

Following calibration, the intensity of shifted bands in the gel was read in raw intensity units of the FLA-2000 (LAU/mm<sup>2</sup>) and was reported in units of fmol M13 primer (reflecting AP2).

**Fig.3-1 Calibration of FLA-2000 to units of concentration using known amounts of fluorescent M13 primer.**

a: Image of a twofold dilution series of M13 primer and AP2 oligonucleotides in a 1536 well plate. Concentrations refer to the M13 primer.

b: Calibration function derived from the above image. This function describes the relation between fluorescence intensity (Linear Arbitrary Units, LAU / mm<sup>2</sup>) as seen by the FLA-2000, and the concentration of M13 primer.

**Fig.3-1 FLA-2000の検量線**  
既知量の蛍光標識M13プライマーを使用。

a: M13プライマーとAP2 オリゴヌクレオチドを2倍ずつ段階希釈。1536ウェルプレートを使用。図中の濃度はM13プライマーに対応。

b: aの画像から作成した検量線。この関数は、蛍光強度とM13プライマーの濃度の関係を示している。

## Gel Shift Assay

A gel shift assay was performed using the fluorescein labeled AP2 oligonucleotide and a nuclear extract containing AP2 protein. The complex of the oligonucleotide and protein migrated as a single band (Fig.3-2). The formation of the band was inhibited completely by adding unlabeled AP2 oligonucleotide, a specific competitor, to the DNA binding reaction. The formation of the complex was inhibited only slightly when SP1 oligonucleotide, a noncompetitor, was added. These results suggest that the DNA/protein complex is the result of a specific DNA-protein interaction.

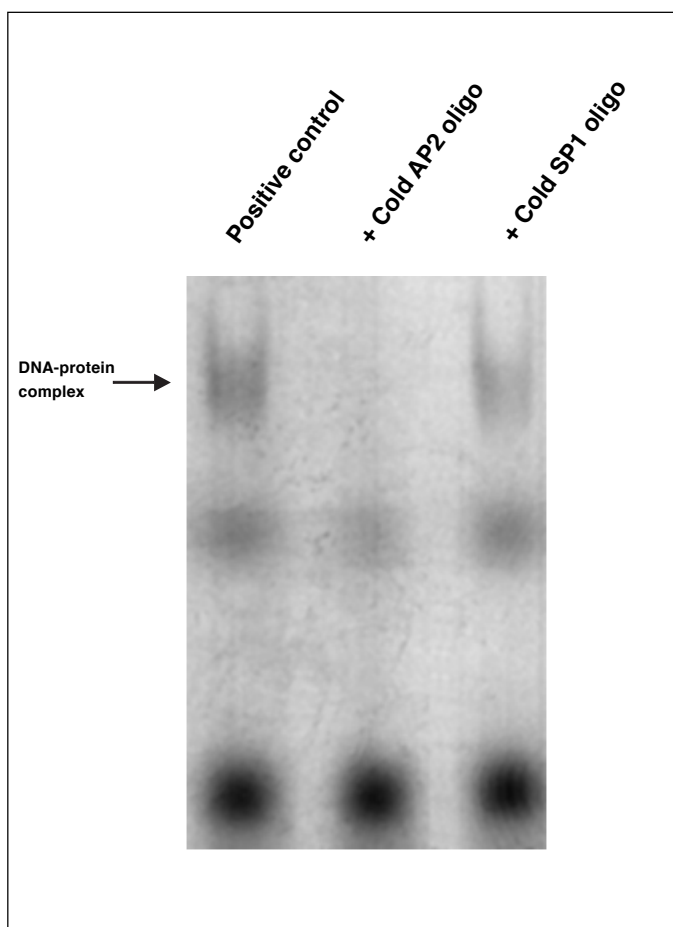


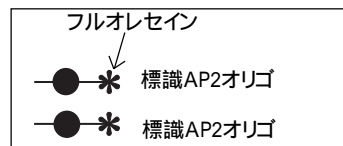
Fig.3-2

**Fig.3-2 Gel shift assay using AP2 extract.**

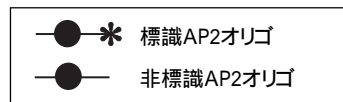
Lane 1: 5'-End-fluorescein-labeled AP2 consensus oligonucleotide incubated with 1.4 mg/ml AP2 extract.  
 Lane 2: Labeled AP2 oligonucleotide plus unlabeled AP2 oligonucleotide (competitor) incubated with AP2 extract.  
 Lane 3: Labeled AP2 oligonucleotide plus unlabeled SP1 oligonucleotide (noncompetitor).  
 The shifted band (arrow) corresponds to the DNA-protein complex.

**Fig.3-2 AP2抽出物を使ったゲルシフトアッセイ**

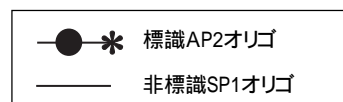
Lane 1 : Positive control  
 5'末端フルオレセイン標識したAP2結合オリゴヌクレオチドとAP2抽出物をインキュベートしたもの。



Lane 2 : +Cold AP2 oligo  
 標識したAP2結合オリゴヌクレオチドと標識していないAP2結合オリゴヌクレオチドを混合したものと、AP2抽出物をインキュベートしたもの。



Lane 3 : +Cold SP1 oligo  
 標識したAP2結合オリゴヌクレオチドと標識していないSP1オリゴヌクレオチドを混合したものと、AP2抽出物をインキュベートしたもの。



Formation of detectable amounts of the complex required only small quantities of AP2 extract. The shifted band was detectable using as little as 0.18  $\mu\text{g}$  of AP2 extract (Fig.3-3). The relation between measured complex formation and AP2 extract concentration (Fig.3-4) was quite linear ( $R^2 = 0.98$ ).

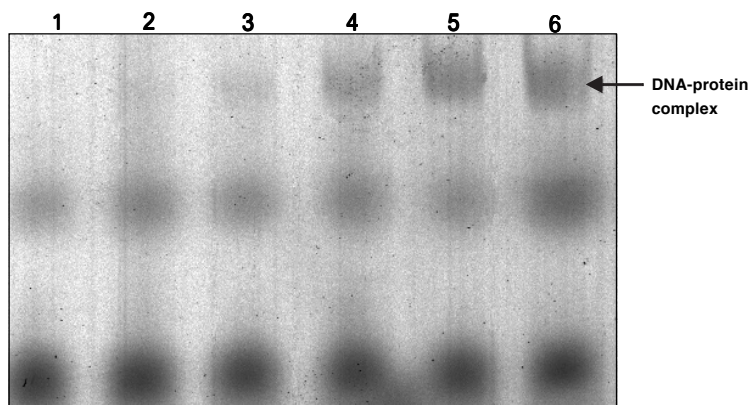


Fig.3-3

Fig.3-3 Fluorescent gel shift with varying concentrations of AP2 extract.

Fig.3-3 種々のAP2抽出物濃度における蛍光ゲルシフト画像

Lane 1 : Negative control  
 Lane 2 : 0.18 $\mu\text{g}$   
 Lane 3 : 0.35 $\mu\text{g}$   
 Lane 4 : 0.70 $\mu\text{g}$   
 Lane 5 : 1.4 $\mu\text{g}$   
 Lane 6 : 2.8 $\mu\text{g}$

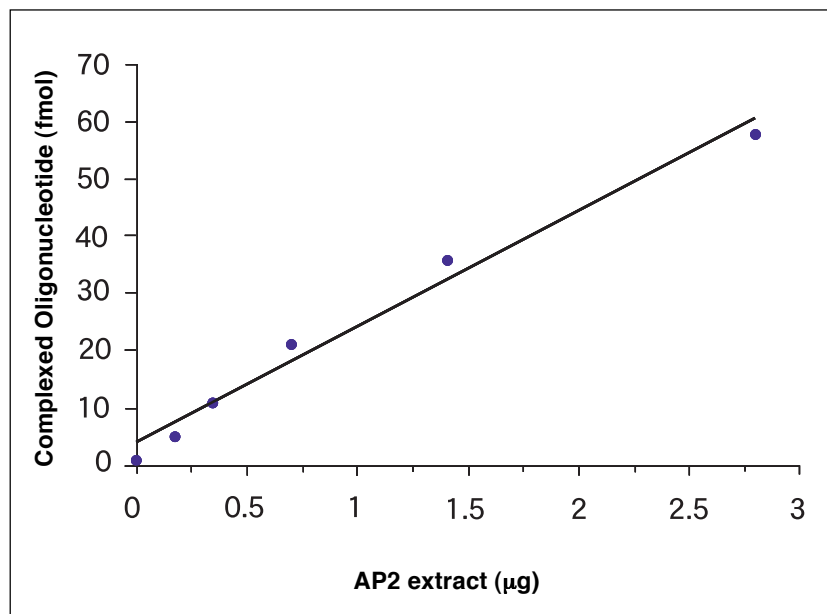


Fig.3-4

Fig.3-4 Correlation between measured amount of complexed oligonucleotides detected in the gel, and the amount of AP2 extract present in the reaction mixture.

Fig.3-4 AP2抽出物とAP2結合オリゴヌクレオチドとの関係

To summarize, the fluorescent gel shift assay offers a safe, rapid, and sensitive alternative to radioactivity based methods for studying DNA-protein interaction. The FLA-2000 provides immediate and sensitive detection of this assay.

## 4 References

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- 1) Carey, J., Gel retardation, *Methods in Enzymology*, 208, 103-117 (1991).
- 2) Fried, M., Crothers, D.M., Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis, *Nucl. Acids Res.*, 9, 6505-6525 (1981).
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著者

Nezar Rghei, M.Sc.  
Peter Ramm, Ph.D.  
( Imaging Research Inc. )

編集

三浦 研二  
長島 真喜子  
大岡 留里子  
石井 幸  
( 富士写真フイルム )

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 **FUJIFILM**

富士写真フイルム株式会社

●本書についてのお問い合わせは

東京本社 ■ イメージング&インフォメーション事業本部 ライフサイエンスグループ

〒106-8620 東京都港区西麻布2-26-30 TEL (03)3406-2201  
FAX (03)3406-2158

E-mail : [sginfo@tokyo.fujifilm.co.jp](mailto:sginfo@tokyo.fujifilm.co.jp)  
ホームページ : <http://fujifilm.co.jp/bio>

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