**A Flexible Single-Step Detection of Blotted Antigen Using a Fusion Protein between Protein A and Green Fluorescent Protein**

Takashi Aoki,1,† Mamiko Miyashita,1 Hiroyoshi Fujino,1,2 and Hiroyuki Watabe1

1Department of Biochemistry, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan
2Katayama Chemical Industries Co., 3-26-22 Higashi-Nanba, Amagasaki 660-0892, Japan

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A green fluorescent protein mutant (S147P GFP) was fused with protein A and expressed in *Escherichia coli*. This fusion protein (PA-GFP147) was used in immunoblotting studies as a new detection system, designated as “flexible single-step detection (FSSD)”. In FSSD, the detection of blotted antigen was done in one step, and the antigen-antibody reaction can be monitored by UV-irradiation in real time. The reaction time, therefore, can be flexibly controlled by monitoring the green fluorescence.

**Key words:** green fluorescent protein; protein A; fusion protein; immunoblotting

Green fluorescent protein (GFP) from *Aequorea victoria* is now widely used in cell biology as a marker for gene expression and a fusion tag in studying protein localization and secretion systems in living cells,1-3 and it is also a useful tool for the studies outside cells.4,5 We previously reported the construction of a fusion protein between GFP and protein A (PA) and its application to Western blotting.4,5 PA-GFP fusion protein expressed in *Escherichia coli* is a metabolically labeled antibody-specific ligand and it was used in a blotting study instead of an enzyme-labeled secondary antibody. Here, we present an improved PA-GFP detection system, designated “flexible single-step detection (FSSD)”, which is more simple, rapid, and sensitive. A blotted antigen is usually detected by the following three steps: primary antibody reaction, enzyme-labeled secondary antibody reaction, and visualization of the enzyme. However, FSSD described here is a real time monitoring of the antigen-antibody reaction, and the detection is done in only one step in which the above three steps are combined.

The wild type GFP or S147P GFP was fused with protein A and expressed in *E. coli*. A modified gfp cDNA was prepared from plasmid TU#659 by standard PCR using the following primers (5'-TTT GAATTTC ATGAGTAAAAGGAAAGAATTTTCT-3’ and 5’-CCCAAGCTTCTATTGTGTATGTTCATCCATGCC-3’, EcoRI and HindIII sites are underlined). The fragment was digested with EcoRI and HindIII, then cloned into the *E. coli* expression vector pKK223-3 (Pharmacia Biotech, Uppsala, Sweden). The resulting plasmid was designated pGFPwt. To increase the fluorescent activity at high temperatures, serine 147 of wild type GFP was changed to proline,10 and this plasmid was designated pGFP147P. The DNA fragment encoding protein A was prepared from pAGFP1274 by PCR using 5’-GGG GAATTTC ATGGAAACAAGCATAACCTGC-3’ and 5’-TTTGAATTCCCAGGATCGTTTCAAAGCT-3’ as primers. The fragment was then cloned into the EcoRI site of pGFPwt and pGFP147P, and the resulting plasmids were designated pAGFPwt and pAGFP147P, respectively (Fig. 1a). The DNA was sequenced using a fluorescence imaging analyzer FMBIO-100 (Takara Shuzo Co. Ltd., Tokyo, Japan).

The strains harboring pAGFPwt or pAGFP147P were grown in 200 ml of LB broth until midlog phase at 37°C. After adding 0.3 mm (final concentration) isopropyl-β-d-thiogalactopyranoside (IPTG; Wako Pure Chemical Industries, Ltd., Osaka, Japan), the cells were grown at 25°C for 18 h and harvested by centrifugation. Cells were suspended in 20 ml of Tris-HCl buffer (pH 8.0) containing 10 mM EDTA and 30 mM NaCl (TEN buffer), and disrupted by sonic oscillation. The supernatant obtained by centrifugation was put on a column (2.5 × 4 cm) containing rabbit IgG immobilized on Sepharose 6B (Pharmacia Biotech) and the bound materials were eluted with TEN buffer containing 9 M urea. Fractions emitting green fluorescence under a UV light at

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1 To whom correspondence should be addressed. Fax: +81-13323-3-1346; E-mail: aoki@hoku-iroy-u.ac.jp

**Abbreviations:** GFP, Green fluorescent protein; PA, protein A; FSSD, flexible single-step detection; IPTG, isopropyl-β-d-thiogalactopyranoside; CBB, Coomassie brilliant blue; AFP, α-fetoprotein; BSA, bovine serum albumin; HRP, horseradish peroxidase; DAB, 3,3’-diaminobenzidine tetrahydrochloride
Fig. 1. Construction of Recombinant Plasmids and Fluorescence Intensity of PA-GFPs.

(a) The wild type or S147P gfp cDNAs were cloned into pKK223-3, then the DNA fragment encoding protein A was placed into the EcoRI site. The resulting plasmids were designated as pPAGFPwt and pPAGFP147P, respectively. (b) Purified PA-GFPwt and PA-GFP147 were blotted onto a nitrocellulose membrane as 4 mm spots and the fluorescence signal excited by the UV light at 365 nm was captured on a diskette using a gel imaging system (Fotodyne Inc., Hartland, WI, USA) with a green filter. (c) A series of purified rabbit IgG samples (100–1600 ng/spot) were blotted onto nitrocellulose membrane. The membrane was then blocked with 10% skimmed milk for 30 min at room temperature. The membrane was rinsed with TBS containing 0.05% Tween 20 (TWEEN-TBS) for 2 min and incubated in Tween-TBS containing 1% BSA and 3 μg/ml PA-GFPwt or PA-GFP147 for 30 min. After the rinse with Tween-TBS for 2 min, the fluorescence signal excited by the UV light at 365 nm was recorded.

365 nm (CSL-6A, Cosmo Bio Co., Ltd., Tokyo, Japan) were pooled and dialyzed against 10 mM Naphosphate buffer (pH 7.0). The fusion proteins obtained from pPAGFPwt and pPAGFP147P were designated as PA-GFPwt and PA-GFP147, respectively. From 200 μl cultures, 4.2 mg of PA-GFPwt and 6.5 mg of PA-GFP147 were obtained, and their purity (>95%) was confirmed by SDS-PAGE.

The absorption and emission spectra of purified PA-GFPs (0.1 mg/ml solution) were compared using a spectrophotometer. As expected, they both absorbed the 395 nm light and emitted green fluorescence (peak emission at 505 nm). PA-GFP147 was 1.6- and 2.2-fold higher than PA-GFPwt in absorbance at 395 nm and fluorescence intensity at 505 nm, respectively. A difference of fluorescence intensity was also observed on the membrane. PA-GFPs were spotted directly (Fig. 1b), or the binding ability to the spotted rabbit IgG was examined (Fig. 1c). In both cases, the fluorescence intensity of PA-GFP147 was superior to that of PA-GFPwt. From these results, PA-GFP147 was used for the subsequent experiments.

To prepare a stable complex between IgG and PA-GFP147, the optimal binding ratio was examined. First, 1.8 μg of PA-GFP147 was incubated with 5.4–12.6 μg (1:3–1:7) of purified rabbit IgG for 5 min and analyzed by agarose gel electrophoresis (Fig. 2a). Two fluorescence bands, one of free PA-GFP147 and the other of PA-GFP147-IgG complex, were clearly visible. Even when the reaction was continued overnight, there was essentially no change in the electrophoretic pattern.

Next, PA-GFP147 was mixed with rabbit whole serum which was an antiserum against human α-fetoprotein (AFP) (Fig. 2a). If serum was added excessively, a portion of the PA-GFP147-IgG complex immediately precipitated (lanes 4–6). Also in the serum, the electrophoretic pattern and complex/free ratio after overnight incubation were basically identical to those of 5-min incubation. After overnight incubation, the reaction mixture was centrifuged and the fluorescence intensity of supernatant was measured. When 18 μg of PA-GFP147 was incubated with the excessive serum (6 μl or over), the fluorescence of supernatant decreased markedly. In contrast, the
PA-GFP147-IgG complex prepared under excessive PA-GFP147 existed stably as a soluble form, and therefore, it is possible to use this complex as a PA-GFP147 labeled antibody for immunoblotting studies.

The human AFP blotted onto the membrane was made visible by the PA-GFP147-IgG complex, designated as “FSSD”, or the conventional color detection method using horseradish peroxidase (HRP) and 3,3′-diaminobenzidine tetrahydrochloride (DAB). AFP was purified from human placenta and its antiserum was prepared from young male rabbits by a standard procedure. The procedure of FSSD was as follows: 20 μl of the antiserum against AFP and 300 μl of purified PA-GFP147 (0.6 mg/ml) were mixed and incubated for 5 min at room temperature. Then, this solution was added to 4.68 ml of TBS (50 mM Tris-HCl, 200 mM NaCl, pH 7.4) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20, and used as “anti-AFP working solution”. The working solution prepared was stable for the repeated freezing and thawing. The precipitation did not completely occur, and a decrease in fluorescence activity was also not observed by repeated use and storing at −20°C at least 2 months. Blocking of the membrane was done in TBS containing 10% skimmed milk (Snow Brand Milk Products, Co., Tokyo, Japan) for 30 min at room temperature. The membrane was rinsed with TBS containing 0.05% Tween 20 (Tween-TBS) for 2 min and incubated in the anti-AFP working solution with gentle shaking. The incubation was continued for 2 h at room temperature and then at 4°C for 18 h. The reaction was monitored by UV-irradiation, and the greenish fluorescent bands on the mem-
brane were recorded after rinsing the membrane with Tween-TBS for 2 min. The procedure for FSSD is summarized and compared with the standard HRP/DAB method (Fig. 2b).

Western blots are shown in Fig. 3. In FSSD, a 6.25 ng band were detected by the first 2-h reaction and the fluorescence intensity of each band increased with the reaction time. Although the fluorescence intensity and sensitivity will vary with some parameters, e.g., antibody titer and dilution factor, these parameters can be regulated by the flexible reaction time. It is clear that the sensitivity of FSSD is lower than that of the HRP/DAB detection and recent chemiluminescence systems. However, the sensitivity and specificity of FSSD may be sufficient for rapid screening, e.g., fraction monitoring in protein purification and measuring the expression of recombinant proteins. As shown in Fig. 3b, non-specific extra bands were rarely detected in FSSD, which may be owing to the simple procedure. Moreover, in FSSD, since it is easy to stop the reaction by UV-monitoring before the extra bands appear, the resulting background is clear.

This work is an improvement of the previous detection technique of blotted antigen using PA-GFP. The points of improvement were that (i) S147P GFP was used instead of wild type GFP; and (ii) the detection procedure was simplified and designated as FSSD. Although the sensitivity is not sufficient to analyze picogram level samples, FSSD has unique and useful features for immunoblotting studies. We hope that FSSD is applied to many studies and various results are accumulated by many researchers. These results will help the further improvement of this system.

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**Fig. 3. Detection of Blotted Human AFP by FSSD.**

*(a)* A series of human AFP samples was put on two polyacrylamide gels (lanes 2-8; 400, 200, 100, 50, 25, 12.5, and 6.25 ng, respectively). SDS-PAGE was done as described by Laemmli using a broad range molecular weight standards kit (Bio-Rad). The proteins were separated on 0.1% SDS-12% polyacrylamide gels (10 x 8 x 0.15 cm) and stained with 0.25% Coomassie brilliant blue (CBB) R-250 or electroblotted onto a nitrocellulose membrane. Electrophoresis was done at a constant voltage of 20 V for 90 min using a semi-dry transblotter. Molecular weight standards were put on lane 1. The blotted AFP bands were made visible by FSSD. In FSSD, the incubation time with the anti-AFP working solution was indicated in parenthesis. (b) Human AFP (200 ng) was mixed with the *E. coli* cell extracts which were prepared from the strain harboring pRK223-3 and put on lane 2. The sample was diluted 1:2, 4, 8, 16, 32, and 64 and put on lanes 3, 4, 5, 6, 7, and 8, respectively. Molecular weight standards are in lane 1. The samples were put on three gels, and the upper panel is the CBB-staining and the lower two panels are the blotting results. AFP bands were made visible by FSSD (2 h-incubation) or HRP/DAB detection. HRP/DAB detection was done as follows: The blocked membrane was incubated with the antiserum against AFP (diluted 1:1000 with TBS containing 1% BSA and 0.05% Tween 20) for 1 h at room temperature. The membrane was then incubated in TBS containing 2.7 µg/ml secondary antibody (HRP-labeled anti-rabbit IgG) and 1% BSA for 1 h. The HRP was made visible with TBS containing 0.02% DAB and 0.01% H2O2 for 10 min.
References


