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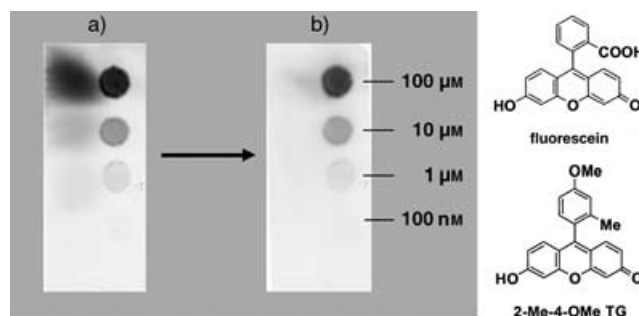
# Extension of the Applicable Range of Fluorescein: A Fluorescein-Based Probe for Western Blot Analysis\*\*

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Western blot immunoanalysis is a powerful method for detecting proteins of interest on a nitrocellulose or polyvinylidene fluoride (PVDF) membrane.<sup>[1–3]</sup> The presence of a target protein is usually detected by using a secondary antibody conjugated with an enzyme such as alkaline phosphatase (ALP), which allows marked signal amplification,<sup>[4]</sup> in combination with a chromogenic, fluorescent, or chemiluminescent probe.<sup>[5–7]</sup> Among these techniques, only fluorescence detection offers high sensitivity and the possibility of multicolor labeling.<sup>[8,9]</sup> Furthermore, fluorescence signals, unlike transient chemiluminescence signals, can be imaged several times and are stable on a dried blot. However, the range of fluorescence probes applicable to Western blot analysis is limited at the present time. In particular, probes based on the fluorescein molecule have rarely been used in this analysis despite their extensive exploitation in other biological techniques.<sup>[10–13]</sup> For example, fluorescein diphosphate (FDP),<sup>[14–17]</sup> a fluorescein-based ALP probe, is seldom used to detect the activity of the widely used secondary detection enzyme ALP in Western blot analysis. Nevertheless, the superior optical properties of fluorescein, such as high molar extinction coefficients and high quantum yields of fluorescence in aqueous media as well as efficient excitation by an argon ion laser (488 nm), make the development of fluorescein-based probes for ALP suitable for Western blot

analysis highly desirable. Herein, we report a new probe for ALP based on a fluorescein derivative that we developed recently<sup>[18]</sup> which works effectively even on Western blots and makes it possible to perform multicolor labeling.

A critical requirement of probes for Western blot analysis is that the hydrolysis product has adequate affinity for the blot. We confirmed that fluorescein generated by ALP-catalyzed hydrolysis of FDP, lacks sufficient affinity for the blocked membrane in Western blotting (Figure 1), and we



**Figure 1.** Difference in affinity for the blot between 2-Me-4-OMe TG and fluorescein. Solutions of various concentrations (100  $\mu$ M, 10  $\mu$ M, 1  $\mu$ M, 100 nM) of 2-Me-4-OMe TG (right lane) and fluorescein (left lane) were dropped onto a blocked nitrocellulose membrane and imaged a) before and b) after washing.

suggest that this low affinity may be attributed to the carboxylic group of the fluorescein molecule which makes the hydrophilicity rather high. We then examined the affinity of 2-Me TG, one of our recently reported fluorescein derivatives (TokyoGreens, TG),<sup>[18]</sup> in which the carboxylic group of the traditional fluorescein molecule is replaced with a methyl group. We found that a spot of a solution of 2-Me TG dropped onto the membrane remained well defined and was not readily washed out (see Supporting Information). This result suggested that TokyoGreens, which do not have a carboxylic group, are more suitable than traditional fluorescein as the fluorophore in probes for Western blot analysis. TokyoGreens also offer the advantage of precise control of the fluorescence emission output. We thus considered 2-Me-4-OMe TG (Figure 1), which shows strong fluorescence (as fluorescein does) and becomes essentially nonfluorescent upon alkylation of its phenolic group, as a suitable core fluorophore for our new probe. Incorporation of a phosphate group into the phenolic group of 2-Me-4-OMe TG gave TG-Phos, a new probe for ALP whose hydrolysis product has a high affinity for Western blots, unlike the hydrolysis product of FDP (Scheme 1).

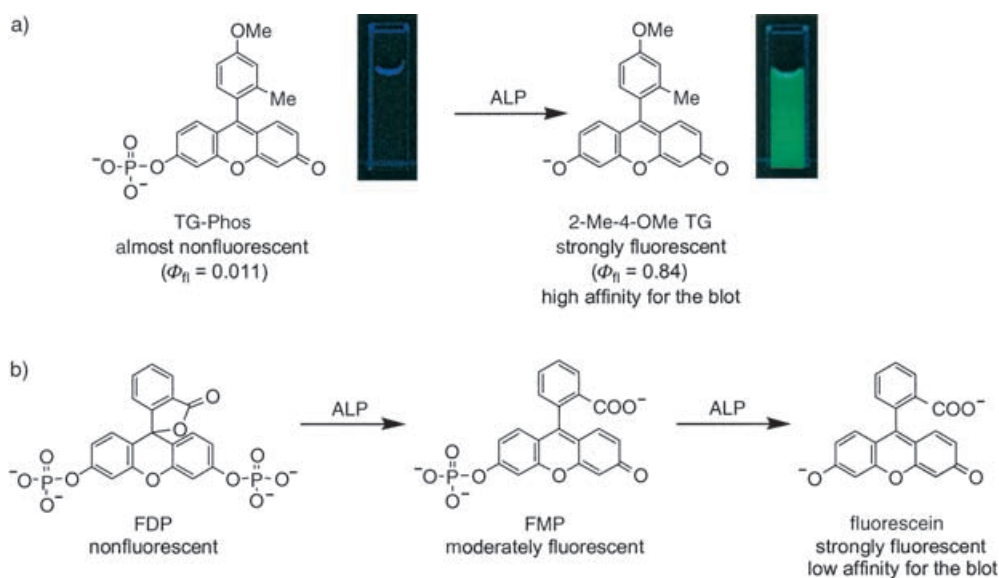
TG-Phos is stable and almost nonfluorescent, but is converted into strongly fluorescent 2-Me-4-OMe TG upon one-step hydrolysis by ALP (see Scheme 1 a, Figure 2 a, and Supporting Information). We observed a linear relationship between ALP activity and the initial rate of increase of fluorescence intensity (Figure 2 b). In the case of FDP, which is hydrolyzed first to moderately fluorescent fluorescein monophosphate (FMP) and then to strongly fluorescent fluorescein, the rate of increase of fluorescence is slow (Scheme 1 b, Figure 2 a). Additionally, there is poor linearity

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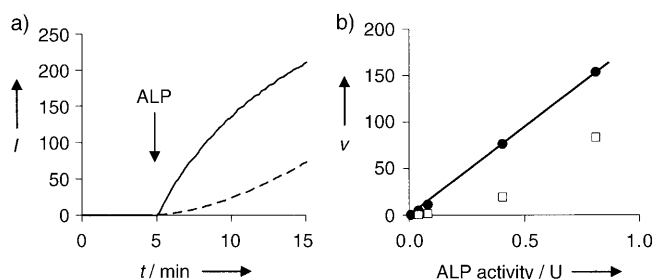
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**Scheme 1.** Reaction scheme of a) TG-Phos and b) FDP with ALP to yield fluorescent hydrolyzed products.



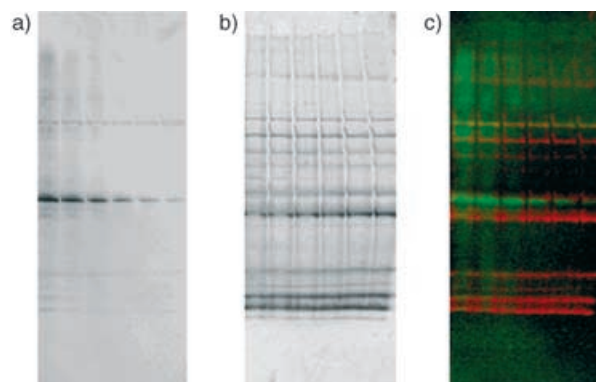
**Figure 2.** In vitro reaction of TG-Phos or FDP with ALP. a) Increase in fluorescence of 1  $\mu$ M solutions of TG-Phos (—) and FDP (----) upon reaction with ALP. b) Correlation between ALP activity and the initial rate of increase of fluorescence using TG-Phos (●) or FDP (□).  $I$  = increase in fluorescence (a.u.);  $\nu$  = initial rate of increase of fluorescence (a.u. min<sup>-1</sup>)

between ALP activity and the initial rate of increase of fluorescence owing to the involvement of the two-step hydrolysis (Figure 2b). The results of a dot-blot assay indicated that TG-Phos is greatly superior to ELF97 phosphate and shows about two-times higher sensitivity than DDAO phosphate, both of which have been used as fluorescence probes for Western blots (see Supporting Information). These results indicate that TG-Phos is a superior probe for ALP and allows the quantitative detection of ALP activity with high sensitivity.

We then examined if a protein of interest could be visualized on the blot by using TG-Phos after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting. CYP3A2 was employed as the target protein, and detection with an ALP-labeled secondary antibody and TG-Phos yielded sharp, CYP3A2-specific green-fluorescent bands whose intensity was proportional to the amount of protein. The development of the fluorescence signal was very rapid, occurring in seconds to minutes, and CYP3A2 could be detected at the nanogram level. Furthermore, the fluores-

cence signal was not easily washed out and was stable on dried blots. In contrast, the green-fluorescent bands derived from FDP were poorly defined and could be easily washed out, making it difficult to detect CYP3A2 on the blot (see Supporting Information).

We next examined simultaneous green/red dual detection of CYP3A2 and total proteins on the same blot. The blot was stained with an amine-reactive BODIPY dye (BODIPY TR-X, succinimidyl ester) to detect total proteins, followed by TG-Phos in combination with an ALP-conjugated secondary antibody to visualize CYP3A2. We succeeded in visualizing all the proteins in the profile as red-fluorescent signals, together with CYP3A2 as a green-fluorescent signal at the same time on the same blot (Figure 3). Such simultaneous two-color detection is a powerful method because it becomes



**Figure 3.** Green/red dual detection of CYP3A2 and total proteins on the same blot after SDS-PAGE and electroblotting onto a PVDF membrane. a) Specific staining of CYP3A2 using ALP-conjugated antibody and TG-Phos. b) Detection of total protein using BODIPY TR-X, succinimidyl ester. c) Simultaneous dual detection of CYP3A2 as green-fluorescent bands and total proteins as red-fluorescent bands. The six lanes contain protein molecular-weight standards (250 ng) and decreasing amounts of CYP3A2 (250 ng, 125 ng, 62.5 ng, 31.2 ng, 15.6 ng, 7.8 ng).

possible to detect two kinds of proteins on a single blot and obviates the need to make a replica for visualizing total proteins, as is usually required for chemiluminescent or colorimetric detection.

In conclusion, we have developed a fluorescence probe for alkaline phosphatase based on the TokyoGreen scaffold which is suitable for Western blot analysis. The new probe, TG-Phos, yields the strongly fluorescent hydrolysis product, 2-Me-4-OMe TG, which has a high affinity for the blot, in a one-step reaction with ALP. Moreover, TG-Phos provides high sensitivity and a good linear response to ALP and is suitable for multicolor labeling. The design strategy presented here is not limited to ALP but could be extended to other detection enzymes and may provide a general method for simple and rapid detection of proteins on Western blots.

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