Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Supporting information

Extension of the Applicable Range of Fluorescein: A Novel Fluorescein-based Probe for Western Blot Analysis

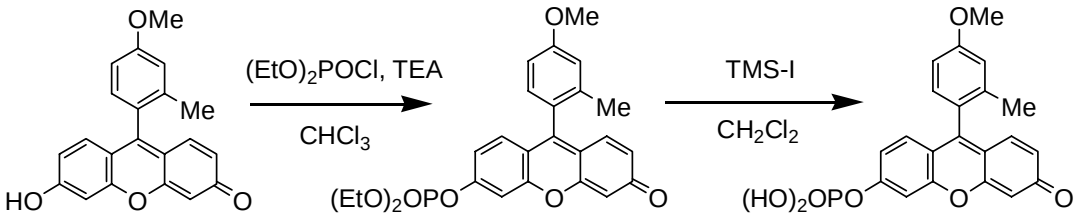
Mako Kamiya[1], Yasuteru Urano[1,2], Nobuyoshi Ebata[3], Masami Yamamoto[3], Jyunichi Kosuge[3] and Tetsuo Nagano[1]

[1]Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan,
[2]PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan
[3]Development Department, Research Products Division, Daiichi Pure Chemicals Co., Ltd., 3-3-1 Koyodai, Ryugasaki-city, Ibaraki 301-0852, Japan.

Materials and General Instrumentation.

General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical or Aldrich Chemical Company, and were used without further purification. Methyl alcohol (MeOH, fluorometric grade) and dimethyl sulfoxide (DMSO, fluorometric grade) used for the spectrometric measurements were purchased from Dojindo. Alkaline phosphatase (molecular weight 160 kDa, EC 3.1.3.1) was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Fluorescein diphosphate (FDP), BODIPY TR-X, succinimidyl ester, DDAO-phosphate, ELF97 phosphate and protein molecular weight standards were purchased from Molecular Probes, Inc. All samples and the kit for Western-blot analysis were purchased from Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan) unless otherwise specified. ^{1}H-NMR spectra were recorded on a JNM-LA300 (JEOL) instrument at 300 MHz. Mass spectra (MS, ESI-TOF) were measured with a JMS-T100LC (JEOL). HPLC purification and analyses were performed on a reverse-phase column (GL Sciences (Tokyo, Japan), Inertsil ODS-3 10mm X 250 mm for purification and Inertsil ODS-3 4.6 mm X 250 mm for analyses) using eluent A and eluent B specified below, fitted on a Jasco PU-1587 system. Absorption spectra were obtained with a 8453 (Agilent) UV/Vis spectrometer and fluorescence spectra were obtained with a Perkin-Elmer LS-50B fluorescence spectrometer. Fluorescence images of the blots were captured with a STORM860 image analyzer (Amersham Biosciences (Tokyo, Japan)) or digital camera through excitation by a trans-illuminator, and acquired images were analyzed with Adobe Photoshop software. All experiments were carried out at 298 K, unless otherwise specified.

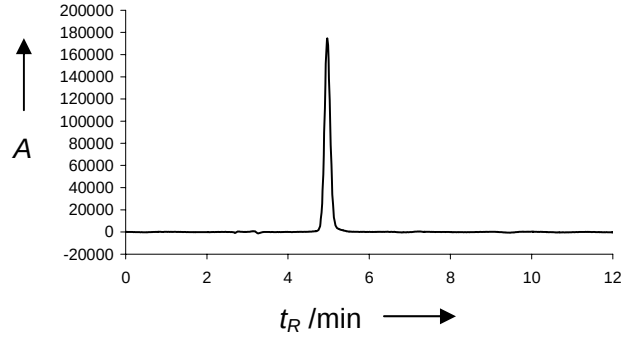
Synthesis and Characterization of TG-Phos.



2-Me-4-OMe TG (1) **2** **TG-Phos (3)**

Preparation of Compound 2; A mixture of 2-Me-4-OMe TG (**1**) 15.7 mg (47 μmol), triethylamine 16.4 μl (118 μmol), and chlorophosphoric acid diethyl ester 6.8 μl (47 μmol) in dry chloroform (4 ml) was stirred at room temperature under argon overnight. The reaction mixture was concentrated under reduced pressure. The residue was chromatographed on silica gel with CH_2Cl_2-MeOH (100:3) as the eluent to give compound **2** 26.2 mg (quant) as an orange powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.39 (m, 6H), 2.05 (s, 3H), 3.89 (s, 3H), 4.27 (m, 4H), 6.45 (d, *J* = 2.0 Hz), 6.58 (dd, *J* = 9.7 Hz, 2.0 Hz), 6.86-7.35 ppm (m, 7H). HRMS (ESI^+): calcd for ($[M+Na]^+$), 491.12356; found, 491.11955.

Preparation of TG-Phos (3); To a solution of compound **2** 30.0 mg (64 μmol) in dry dichloromethane (0.5ml), trimethylsilyl iodide 19.7 μl (140 μmol) was added dropwise. The reaction mixture was stirred at room temperature under argon for 3 hours. After evaporation of the chloroform, the residue was purified by semi-preparative HPLC using eluent A (10 mM triethylammonium acetate buffer, pH 6.5) and eluent B (acetonitrile) (A/B = 80/20) to give TG-Phos (**3**) 7 mg (26.5%) as an orange powder. ^{1}H NMR (300 MHz, CD_3OD): δ = 2.02 (s, 3H), 3.90 (s, 3H), 6.48 (d, *J* = 2.0 Hz, 1H), 6.60 (dd, *J* = 9.6 Hz, 2.0 Hz, 1H), 6.96-7.71 ppm (m, 7H). ^{13}C-NMR (75 MHz, CD_3OD); 187.34, 162.35, 161.65, 156.06, 155.69, 139.03, 132.98, 131.55, 130.84, 129.46, 125.56, 119.92, 119.83, 119.20, 116.67, 117.06, 112.81, 108.19, 105.40, 55.90, 23.26. HRMS (ESI^-): calcd for $[M-1]^-$, 411.06336; found, 411.05935. UV/Vis (100 mM sodium phosphate buffer, pH 7.4): λ_{max} = 454 nm, Φ_{fl} = 0.011. HPLC analysis with detection at 454 nm using eluent A (100 mM triethylammonium acetate buffer, pH 6.5) and eluent B (acetonitrile) (A/B = 80/20)



Measurements of Absorption Spectra and Fluorescence Spectra, and *in vitro* alkaline phosphatase (ALP) assay.

TG-Phos and FDP were dissolved in deionized water to obtain 10 mM stock solutions, then diluted with the assay buffer described below to a final concentration of 1 μM. All fluorescence measurements and enzymatic reactions with ALP were performed at 37°C in 3 mL total volume of 100 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM $MgCl_2$ in a 1-cm cuvette. For Figure 2 in the manuscript, various units of ALP (0.081 units for Figure 2A, 0.81, 0.405, 0.081, 0.0405, 0.0081 units for Figure 2B) were added at the 3-minute time point. The excitation wavelength was 491 nm, and the emission wavelength was 510 nm. For determination of the quantum efficiency of fluorescence (Φ_{fl}), fluorescein in 100 mM aq. NaOH (Φ_{fl} 0.85) was used as a fluorescence standard (C. A. Paeker, W. T. Rees *Analyst* **1960**, *85*, 587-600).

Electrophoresis and electroblotting.

Immediately prior to SDS-polyacrylamide gel electrophoresis, protein molecular weight standards and CYP3A2 were diluted with SDS-gel loading buffer (63 mM Tris-HCl buffer, pH 6.8, containing 2 % SDS, 5 % βME, 0.005 % BPB and 15 % glycerol). Protein molecular weight standard solution was prepared to contain 50 ng of each marker protein per 1 μl, then heated at 95 °C for 4 minutes, and 5 μl (250 ng) was loaded per lane (lanes 1-6, 8-13). CYP3A2 solutions were prepared to contain decreasing amounts of CYP3A2 (250 ng, 125 ng, 62.5 ng, 31.2 ng, 15.6 ng, 7.8 ng per 5 μl) and 5 μl was loaded per lane (250 ng for lane 1 and 8 / 125 ng for lane 2 and lane 9/ 62.5 ng for lane 3 and lane 10 / 31.2 ng for lane 4 and lane 11 / 15.6 ng for lane 5 and lane 12 / 7.8 ng for lane 6 and lane 13). After SDS-polyacrylamide gel electrophoresis according to the manufacturer's instrumentations, the proteins were electroblotted onto PVDF membranes using a semidry blotter. The obtained blots were cut into two portions for further staining procedures.

Green/red dual detection on the blots.

BODIPY TR-X, succinimidyl ester was dissolved in anhydrous DMSO to obtain 10 mM stock solution and diluted with 0.1 N sodium bicarbonate buffer, pH 8.3, to a final concentration of 5 μM dye. To stain total proteins on PVDF membranes, the blots were first equilibrated in two changes of 0.1 N sodium bicarbonate buffer, pH 8.3, for 10 minutes each, and then incubated with BODIPY TR-X, succinimidyl ester for 30 minutes with agitation. The blots were washed in three changes of 100% methanol for 10 minutes each to remove dye bound non-specifically to the membrane. After having been equilibrated three times with Tris-buffered saline, pH 7.6, containing 0.1% Tween20 (T-TBS) for 10 minutes each, the blots were blocked for 60 minutes in T-TBS containing 5 % skim

milk. A mouse monoclonal anti-CYP3A2 antibody, the primary antibody, was diluted 1:1000 in T-TBS containing 0.5% skim milk and the blots were incubated for 1 hour in this antibody solution. The blots were rinsed in T-TBS three times for 10 minutes each, then further incubated for 1 hour in a 1:1000 dilution of an anti-mouse ALP-conjugated secondary antibody, followed by washing with T-TBS three times for 10 minutes each. Finally, in order to detect the presence of CYP3A2, TG-Phos or FDP was diluted with Tris-based assay buffer (Bio-Rad Laboratories, AP Conjugate Substrate Kit) to a final concentration of 10 μM. The blots were incubated in this substrate solution without agitation for 2 minutes at room temperature, then immediately rinsed with distilled water and imaged with a STORM860 image analyzer or trans-illuminator. Images a) and b) in Figure 3 and Figure S5 were captured separately by a STORM860 image analyzer, and image c) in Figure 3 and Figure S5 was obtained through simultaneous excitation with a trans-illuminator.

Figure S1. Difference in affinity for the blot between 2-Me TG and fluorescein. Solutions of various concentrations (100 μM, 10 μM, 1 μM, 100 nM) of 2-Me TG (right lane) or fluorescein (left lane) were dropped on blocked nitrocellulose membrane, and imaged with a STORM860 image analyzer a) before and b) after washing.

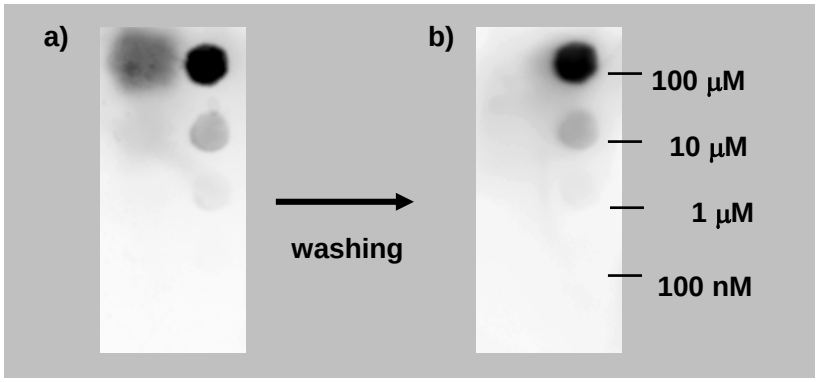


Figure S2. Absorption and fluorescence spectra of 1 μM TG-Phos (a) and FDP (b) solution before (dashed line) and after (solid line) reaction with ALP. Assay buffer was 0.1 M Tris-HCl buffer, pH 7.4, containing 0.5 mM $MgCl_2$. Excitaion wavelength was 491 nm.

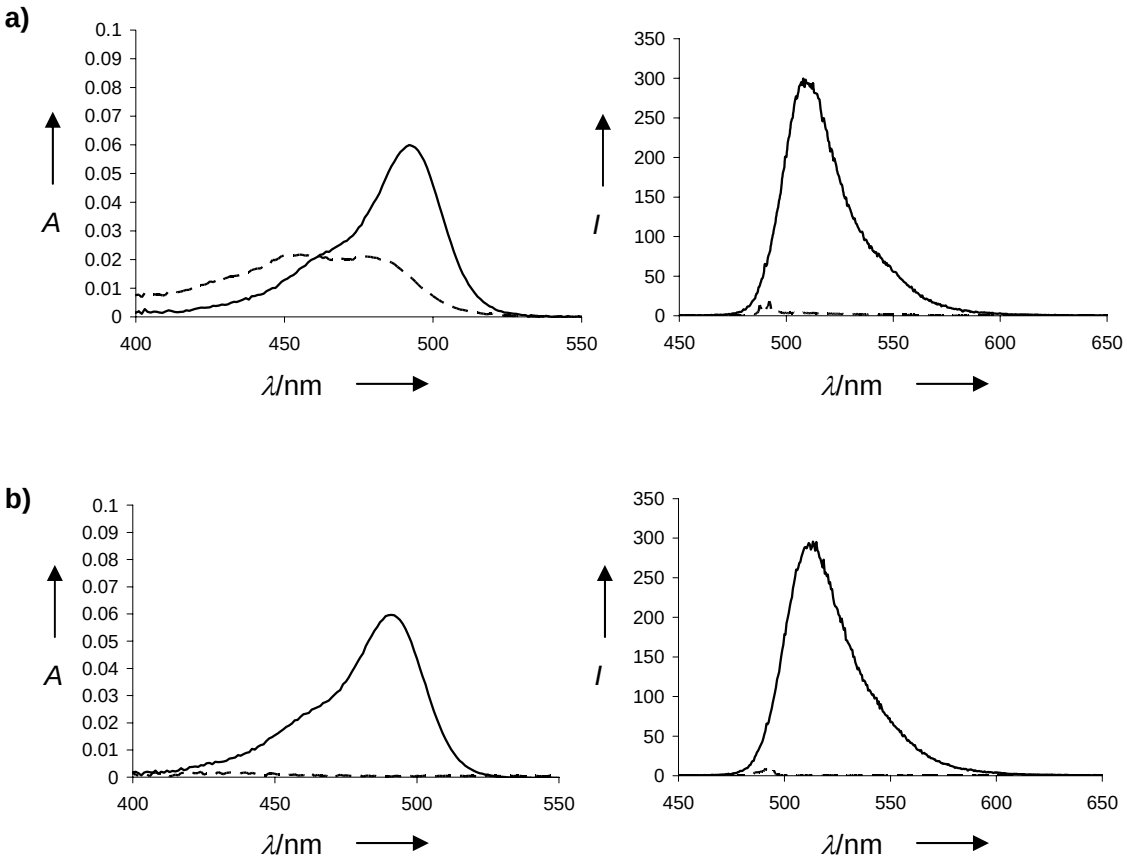


Figure S3. Tracing the conversion of TG-Phos to 2-Me-4-OMe TG by ALP, using HPLC with eluent A (10 mM sodium phosphate buffer, pH 7.4) and eluent B (acetonitrile) (A/B = 75/ 25). Absorbance at 254 nm was monitored. Peaks at 3.9 minutes and 28.8 minutes correspond to TG-Phos and 2-Me-4-OMe TG, respectively.

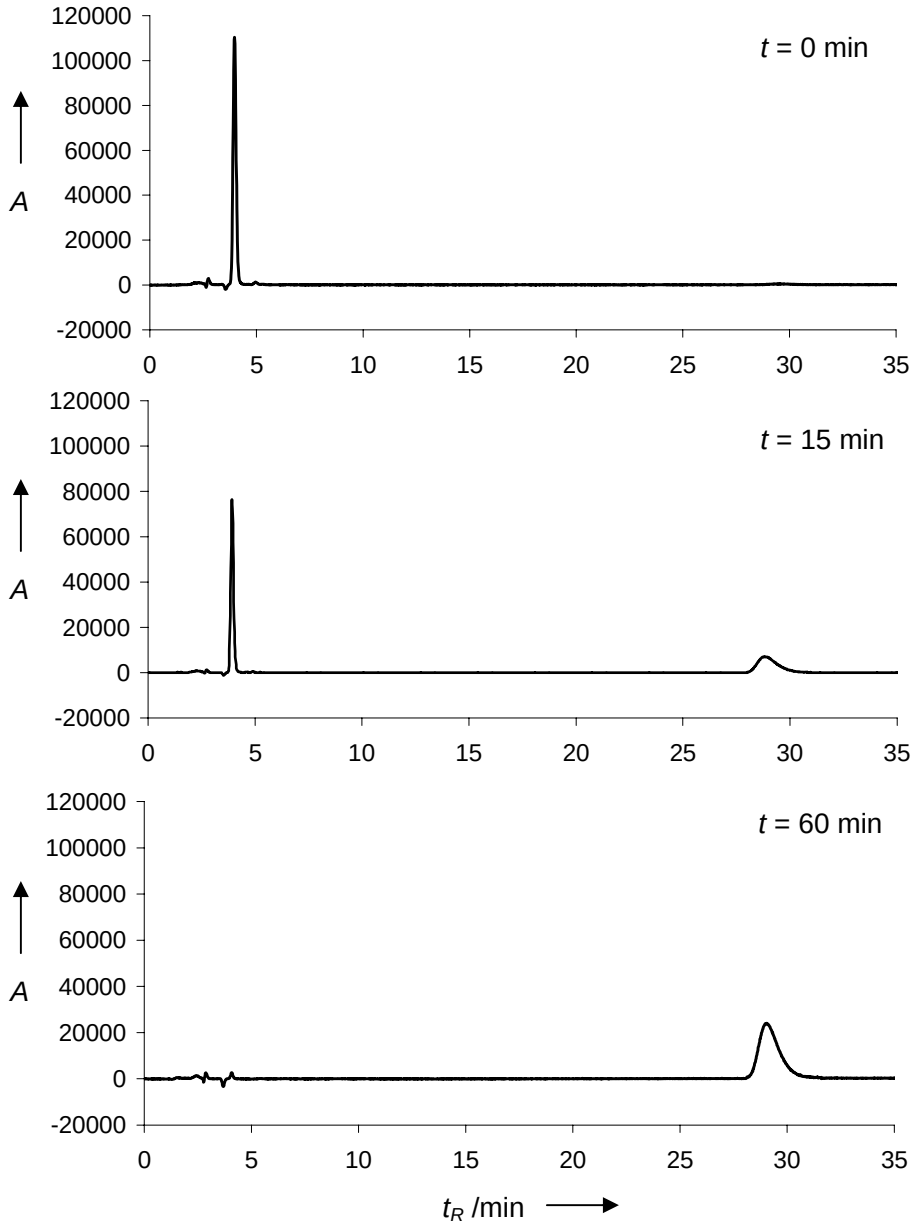


Figure S4. Fluorescence excitation and emission spectra of 2-Me-4-OMe TG (solid line) and BODIPY TR-X, SE (dashed line). a) The excitation spectra collected by using the respective emission maxima (511 nm, 617 nm). Both fluorophores can be simultaneously excited by UV illumination (trans-illuminator) or separately excited by blue light and red light (STORM860 image analyzer). b) The emission spectra collected by using the respective excitation maxima (491 nm, 589 nm). The fluorophores have their emission maxima at sufficiently separated wavelengths. Spectra of 2-Me-4-OMe TG were measured in 0.1 N sodium phosphate buffer, pH 7.4, while those of BODIPY TR-X, SE were measured in MeOH.

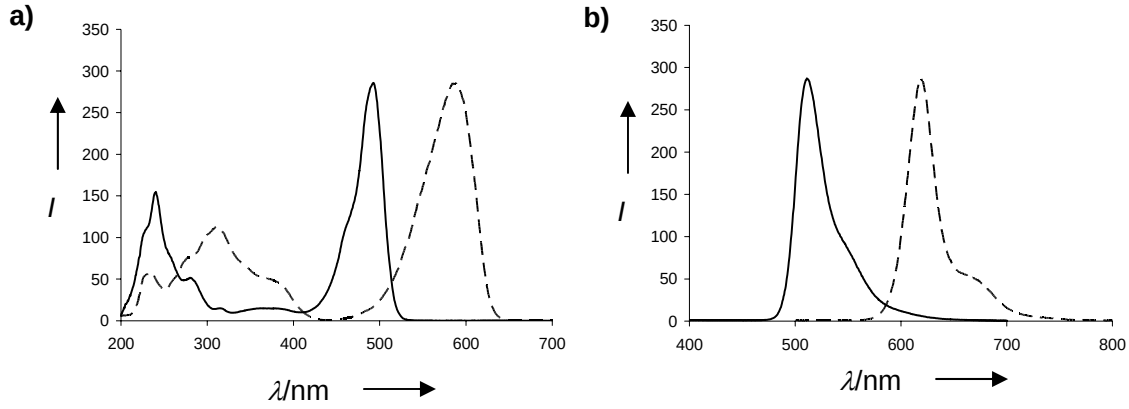


Figure S5. Green/red dual detection of CYP3A2 and total proteins by using FDP. a) Specific staining of CYP3A2 using ALP-conjugated antibody and FDP. b) Detection of total protein using BODIPY TR-X, succinimidyl ester. c) Simultaneous dual detection of CYP3A2 as green fluorescent bands and total proteins 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).

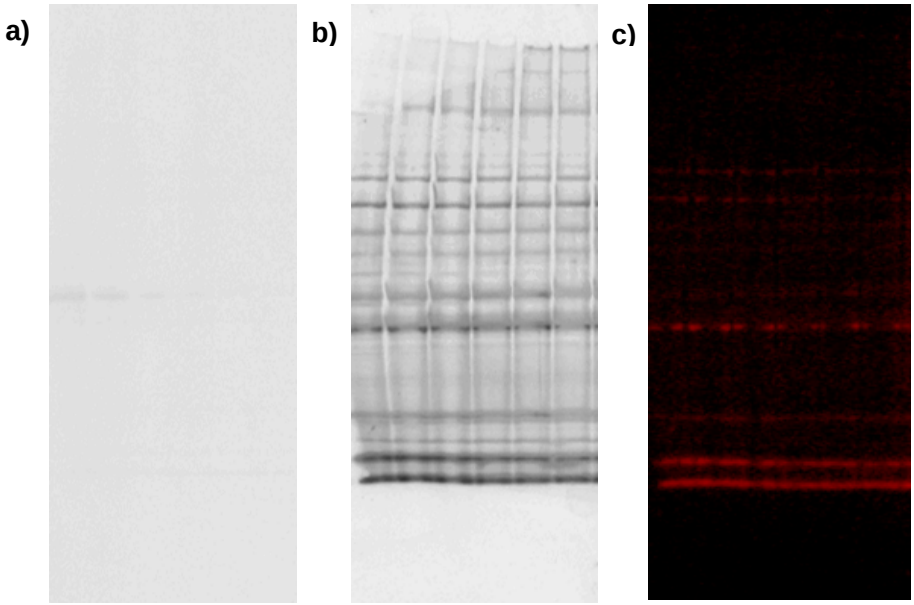


Figure S6. Dot-blot assay for the comparison of sensitivity between TG-Phos and two fluorescence probes currently used for Western blot analysis (ELF97 phosphate and DDAO-phosphate). Various amounts of ALP (3.9 μg, 1.3 μg, 0.39 μg, 0.13 μg) were directly dropped onto nitrocellulose membrane, and the membranes were air-dried for 30 minutes, blocked for 60 minutes in T-TBS containing 5 % skim milk, and washed with T-TBS three times for 5 minutes each. The membranes were then incubated in a 10 μM solution of TG-Phos, ELF97 phosphate or DDAO-phosphate in Tris-based assay buffer (Bio-Rad Laboratories, AP conjugate Substrate Kit) without agitation for 5 minutes at room temperature, then immediately rinsed with distilled water and imaged. a) Fluorescence detection using ELF97 phosphate (left) and TG-Phos (right). Images generated by excitation with UV lamps (365 nm), were captured with a digital camera. b), c) Fluorescence detection using DDAO-phosphate (left) and TG-Phos (right). Images were separately captured and quantified by means of a STORM860 image analyzer. R_{fl} stands for the fluorescence ratio of each spot to the spot ⑤.

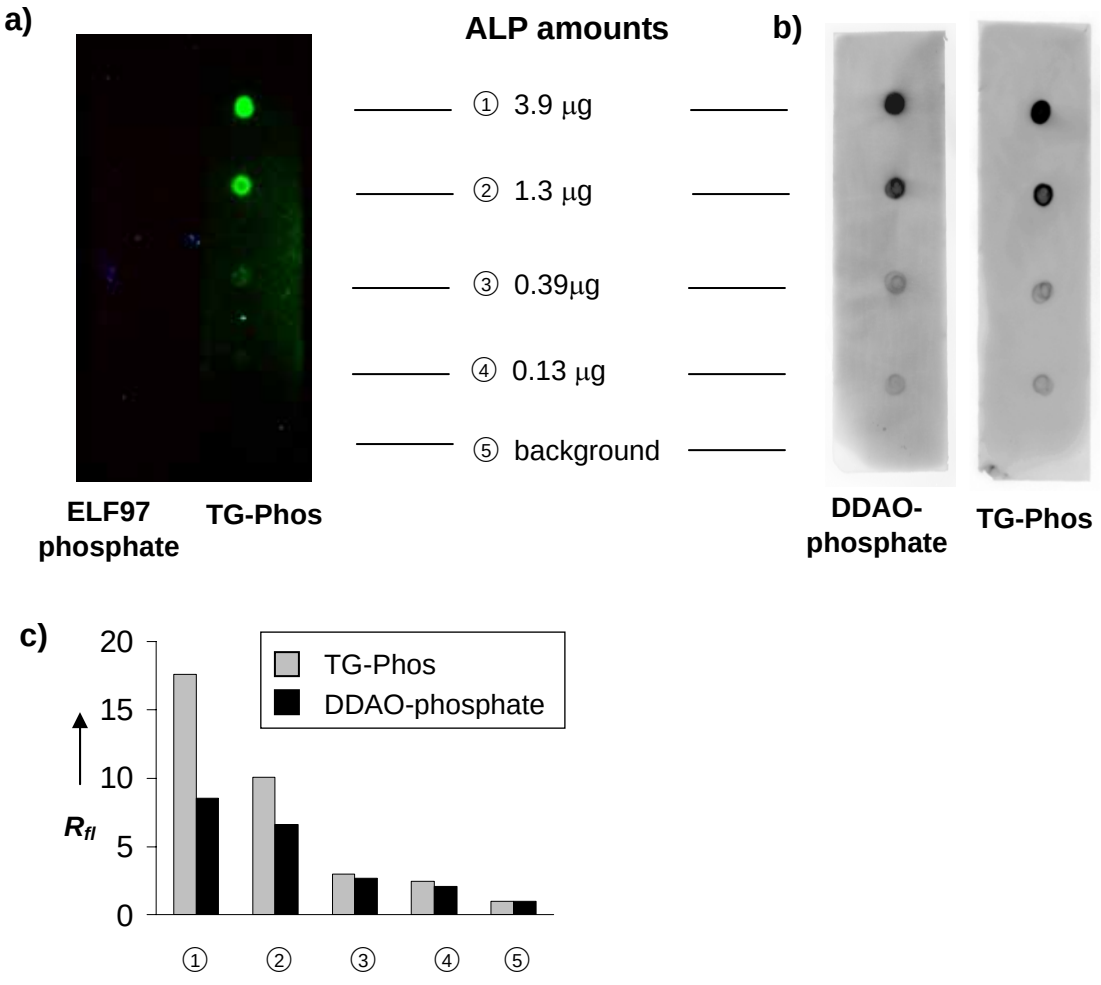


Figure S7. Stability of TG-Phos and its ALP-catalyzed hydrolysis product in Tris-based assay buffer, pH 9.5 (Bio-Rad Laboratories, AP Conjugate Substrate Kit), which was used for fluorescence detection on the blot. The fluorescence increase of a 1 μM solution of TG-Phos was observed with or without addition of ALP (8 units) at 5 minutes. Fluorescence signals derived from TG-Phos and its ALP-catalyzed hydrolysis product were unchanged for at least 1 hour.