Electronic Supplementary Material (ESI) for Chemical Communications.


**Supporting Material**

# The Nitrilimine-Alkene Cycloaddition is an Ultra Rapid Click Reaction

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## 1. Kinetic Analysis

Acrylamide was added to a solution of hydrazonyl chloride **1**(5 μM, 2mL) in 1:1 acetonitrile-50 mM phosphate buffer at different pH and varying concentrations of chloride. The florescence increase at 480 nm due to the formation of pyrazoline product was monitored by PTI QM-40 fluorescent spectrophotometer with 320 nm excitation.

## 2. SfGFP2AcrK expression

Plasmid pET-sfGFPS2TAG was used to express sfGFP incorporated with AcrK at its S2 position.(*1*) *E. coli* BL21(DE3) cells were transformed with pEVOL-pylT-PrKRS and pET-sfGFPS2TAG and plated on LB agar plate containing chloramphenicol (Cam) (34 μg/mL) and ampicillin (Amp) (100 μg/mL). A 5 mL overnight culture was prepared from a single colony. This overnight culture was used to inoculate 500 mL of LB medium supplemented with Cam and Amp. Cells were grown at 37°C in an incubator (250 r.p.m.) and the protein expression was induced with 1 mM IPTG, 0.2% arabinose and 2 mM AcrK when OD600 reached 0.6. After 8h induction, cells were harvested, resuspended in a lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0), and sonicated in an ice/water bath four times (4 min each, 10 min interval to cool the suspension below 10 °C between each pulse). The cell lysate was centrifuged (60 min, 10,000 g, 4 °C). The supernatant was incubated with 3 mL Ni-NTA resin (Qiagen) (2 h, 4 °C). The slurry was then loaded to a column and the protein-bound resin was washed with 30 mL of the wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0). Protein was finally eluted by the elution buffer containing wash buffer plus 250 mM imidazole. Eluted fractions were collected, concentrated and buffer exchanged to 10 mM ammonium bicarbonate using an Amicon Ultra-15 Centrifugal Filter Devices (10k MWCO, Millipore). The protein purity was analyzed by 15% SDS-PAGE.

## 3. pH dependence of the labeling reaction in the presence of chloride

Hydrazonyl chloride **1**(5 mM in acetonitrile, 15 μL) was added to the solution of sfGFP2AcrK (5 μM, 500 μL) in 1:1 acetonitrile-50 mM phosphate buffer that also contained 50 mM sodium chloride (pH 6-10). The solution was incubated at room temperature for 15 min and then quenched by 500 mM acrylamide. The labeled-protein was further purified using the Ni-NTA resin (5 μL). The protein-bound resin was centrifuged (10 min, 13.4k) and washed with water for 4 times. After boiling the resin in 6X protein loading buffer (375 mM Tris-HCl, 10% SDS, 30% Glycerol, 0.03% bromophenol blue, and 600 mM DTT) and filtration to remove the Ni-NTA resin, the labeled protein was subjected to 15% SDS-PAGE analysis. In-gel fluorescence detection was performed using BioRad ChemiDoc XRS+ Imaging system before the gel was stained by Coomassie blue.

## 4. Chloride dependence of the labeling reaction at pH7

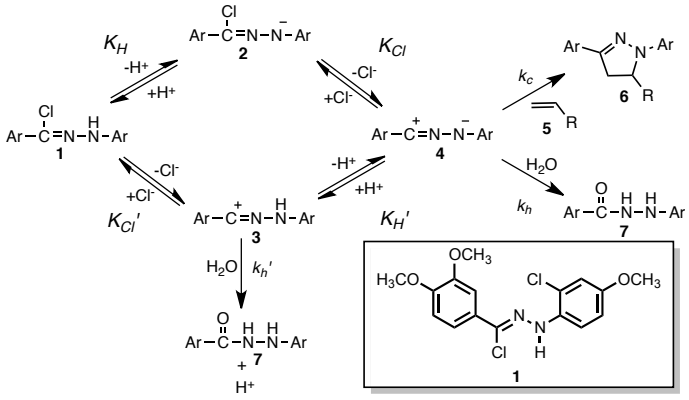
Hydrazonyl chloride **1**(5 mM in acetonitrile, 15 μL) was added to the solution of sfGFP2AcrK (5 μM, 500 μL) in 1:1 acetonitrile-50 mM phosphate buffer (pH 7)

that contained a sodium chloride concentration varying from 0 to 200 mM. The solution was incubated at room temperature for 30 min and then quenched by 500 mM acrylamide. The labeled-protein was further purified using the Ni-NTA resin (5 μL). The protein-bound resin was centrifuged (10 min, 13.4k) and washed with water for 4 times. After boiling the resin in 6X protein loading buffer (375 mM Tris-HCl, 10% SDS, 30% Glycerol, 0.03% bromophenol blue, and 600 mM DTT) and filtration to remove the Ni-NTA resin, the labeled protein was subjected to 15% SDS-PAGE analysis. In-gel fluorescence detection was performed using BioRad ChemiDoc XRS+ Imaging system before the gel was stained by Coomassie blue.

**5. The labeling reaction at pH 10 without chloride**

Hydrazonyl chloride **1**(5 mM in acetonitrile, 15 μL) was added to the solution of sfGFP2AcrK (5 μM, 500 μL) in 1:1 acetonitrile-50 mM phosphate buffer (pH 10). The solution was incubated at room temperature for 1-4 min and then quenched by 500 mM acrylamide. The labeled-protein was further purified using the Ni-NTA resin (5 μL). The protein-bound resin was centrifuged (10 min, 13.4k) and washed with water for 4 times. After boiling the resin in 6X protein loading buffer (375 mM Tris-HCl, 10% SDS, 30% Glycerol, 0.03% bromophenol blue, and 600 mM DTT) and filtration to remove the Ni-NTA resin, the labeled protein was subjected to 15% SDS-PAGE analysis. In-gel fluorescence detection was performed using BioRad ChemiDoc XRS+ Imaging system before the gel was stained by Coomassie blue.

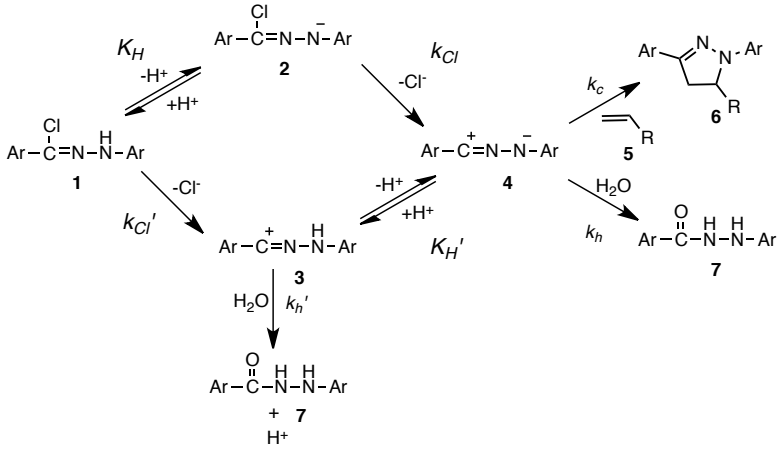
**6. Equation derivation**



**Scheme 1**

As indicated by **Scheme 1**, when [H$^+$]>>$K_H$ and [Cl$^-$]>>$K_{Cl}$, the formation rates of **6** and **7** and the consumption rate of **1** follow a relation defined by $\frac{d\mathbf{1}}{dt} = \frac{d\mathbf{6}}{dt} + \frac{d\mathbf{7}}{dt}$, in which $\frac{d\mathbf{6}}{dt} = k_c \times \mathbf{5} \times \mathbf{4}$, $\frac{d\mathbf{7}}{dt} = k_h \times \mathbf{4} + k_{h'} \times \mathbf{3}$, $\mathbf{3} = \mathbf{1} \times \frac{K_{Cl'}}{[Cl^-]}$, and $\mathbf{4} = \mathbf{1} \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]}$. Therefore the consumption rate of **1** can be defined as $\frac{d\mathbf{1}}{dt} = (k_c \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]} \times$

$[\mathbf{5}] + k_h \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]} + k_h' \cdot \frac{K_{Cl'}}{[Cl^-]}) \times \mathbf{1}$ that can be integrated to give $\mathbf{1} = \mathbf{1_0} \times e^{-\left(k_c \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]} \times \mathbf{5} + k_h \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]} + k_{h'} \times \frac{K_{Cl'}}{[Cl^-]}\right)t}$. At any time, $\mathbf{6} + \mathbf{7} = \mathbf{1_0} - \mathbf{1}$ and $\frac{\mathbf{7}}{\mathbf{6}} = \frac{k_h \times \mathbf{4} + k_{h'} \times \mathbf{3}}{k_c \times \mathbf{5} \times \mathbf{4}} = \frac{k_h \times \mathbf{4} + k_{h'} \times \frac{[H]^+}{K_H} \times \mathbf{4}}{k_c \times \mathbf{5} \times \mathbf{4}} = \frac{k_h + k_{h'} \times \frac{[H]^+}{K_H}}{k_c \times \mathbf{5}}$. Therefore, $\mathbf{6} + \frac{k_h + k_{h'} \times \frac{[H]^+}{K_H}}{k_c \times 5} \times \mathbf{6} = \mathbf{1_0} - \mathbf{1_0} \times e^{-\left(k_c \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]} \times \mathbf{5} + k_h \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]} + k_{h'} \times \frac{K_{Cl'}}{[Cl^-]}\right)t}$ that can be simplified to give $\mathbf{6} = \frac{\boldsymbol{k_c} \times [\mathbf{5}]}{\boldsymbol{k_c} \times [\mathbf{5}] + \boldsymbol{k_h} + \boldsymbol{k_{h'}} \times \frac{[H^+]}{K_{H'}}} \times \mathbf{1_0} \times (1 - e^{-\left(k_c \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]} \times \mathbf{5} + k_h \times \frac{K_H \times K_{Cl}}{[H^+] \times [Cl^-]} + k_{h'} \times \frac{K_{Cl'}}{[Cl^-]}\right)t})$ as eq. 1. in the main manuscript.



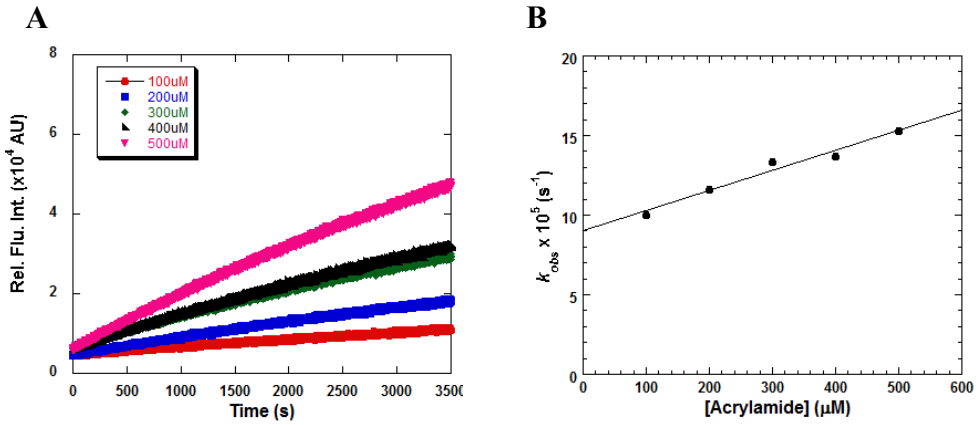

**Scheme 2**

In **Scheme 2**, the two dechlorination steps become rate-limiting. Given that $[H^+] >> K_H$, the consumption rate of **1** is defined as $\frac{d\mathbf{1}}{dt} = k_{Cl'} + \frac{k_{Cl} \times K_H}{[H]^+}$. Therefore $\mathbf{1} = \mathbf{1_0} \times (\mathbf{1} - \boldsymbol{e}^{-\left(\boldsymbol{k_{Cl}} \times \frac{\boldsymbol{K_H}}{[\boldsymbol{H^+}]} + \boldsymbol{k_{Cl'}}\right)\boldsymbol{t}})$. Since $\mathbf{6} + \mathbf{7} = \mathbf{1_0} - \mathbf{1}$ and $\frac{\mathbf{7}}{\mathbf{6}} = \frac{k_h \times \mathbf{4} + k_{h'} \times \mathbf{3}}{k_c \times \mathbf{5} \times \mathbf{4}} = \frac{k_h \times \mathbf{4} + k_{h'} \times \frac{[H]^+}{K_H} \times \mathbf{4}}{k_c \times \mathbf{5} \times \mathbf{4}} = \frac{k_h + k_{h'} \times \frac{[H]^+}{K_H}}{k_c \times \mathbf{5}}$ still stand, the formation of **6** can be described as $\mathbf{6} = \frac{\boldsymbol{k_c} \times [\mathbf{5}]}{\boldsymbol{k_c} \times [\mathbf{5}] + \boldsymbol{k_h} + \boldsymbol{k_{h'}} \times \frac{[H^+]}{K_{H'}}} \times \mathbf{1_0} \times (\mathbf{1} - \boldsymbol{e}^{-\left(\boldsymbol{k_{Cl}} \times \frac{\boldsymbol{K_H}}{[\boldsymbol{H^+}]} + \boldsymbol{k_{Cl'}}\right)\boldsymbol{t}})$ that is eq. 6 presented in the main manuscript.

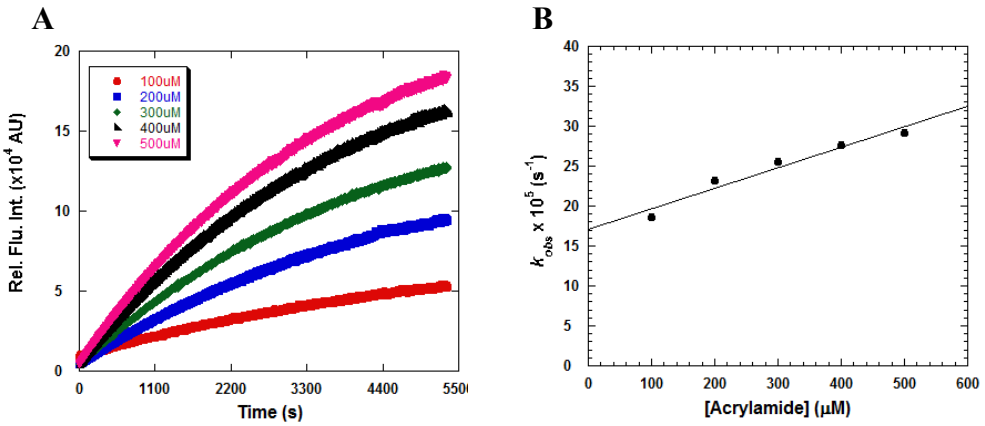
## 7. References


[1] Lee, Y.-J.; Wu, B.; Raymond, J. E., Zeng, Y.; Fang, X.; Wooley, K. L., Liu, W. R. A Genetically Encoded Acrylamide Functionality, *ACS Chem. Biol.* **2013,** 8, 1664-1670.

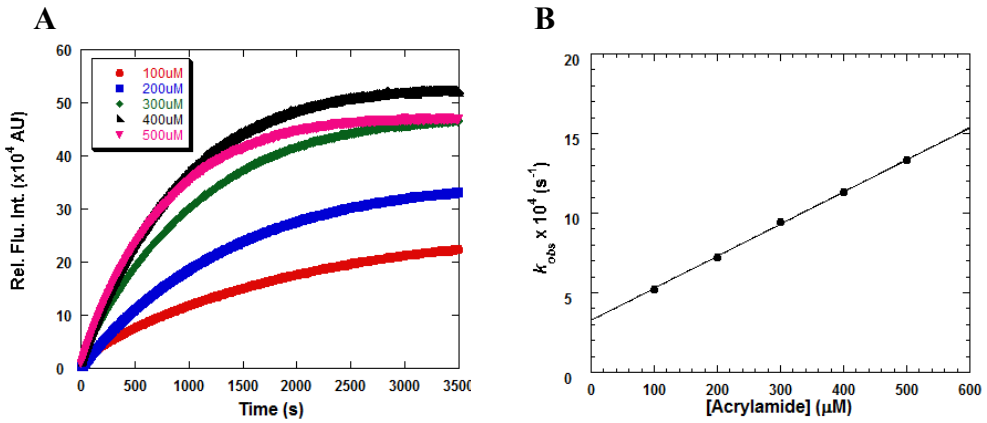
## 8. Supplementary Figures



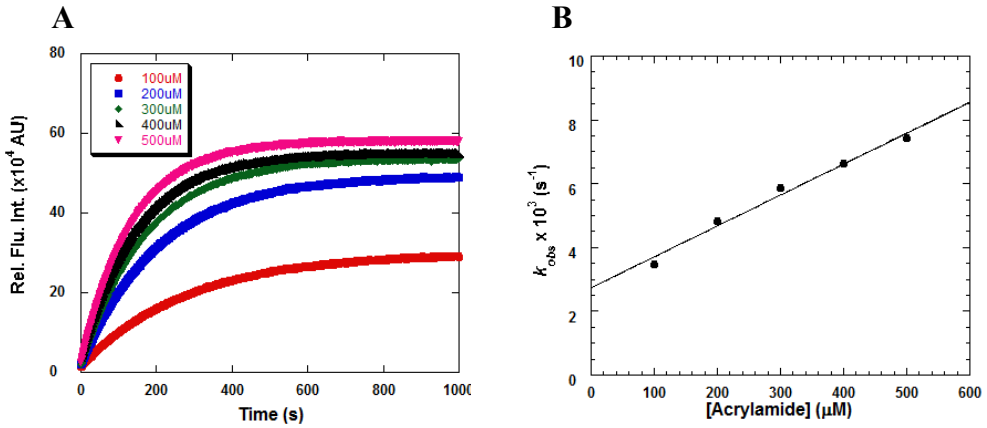

**Supplementary Figure 1** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 6 with 50mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



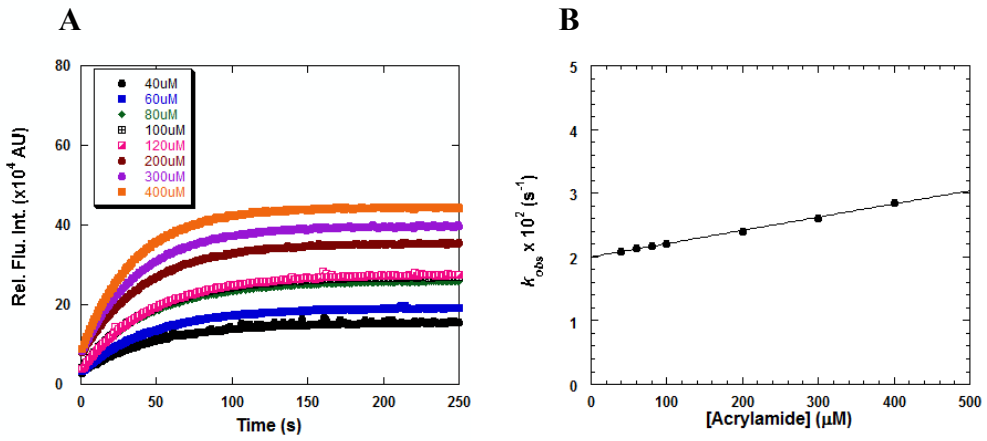

**Supplementary Figure 2** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 7 with 50 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



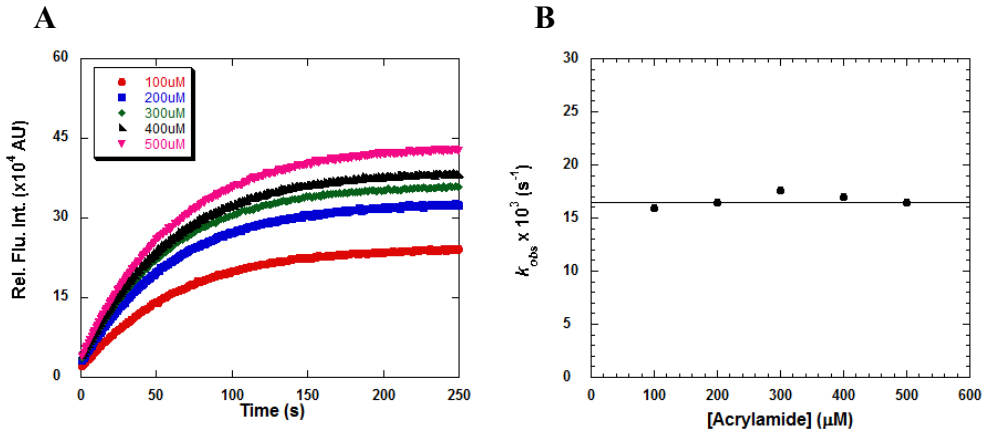

**Supplementary Figure 3** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 8 with 50 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



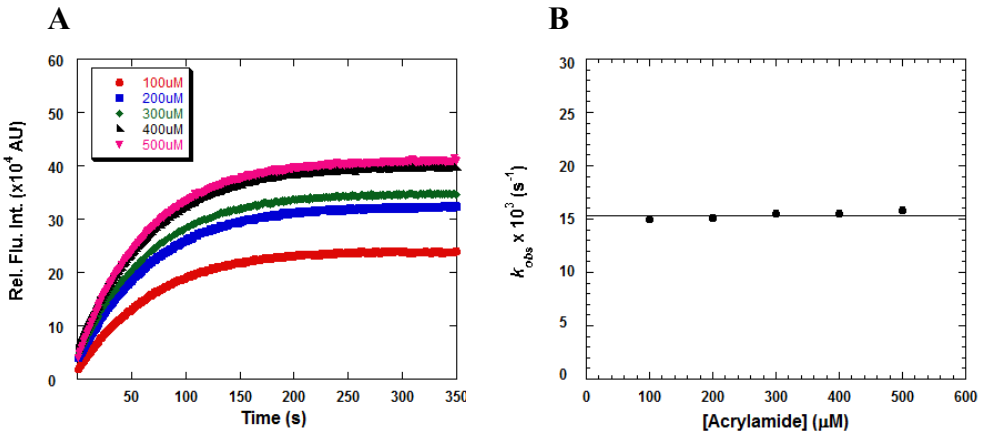

**Supplementary Figure 4** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 50 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



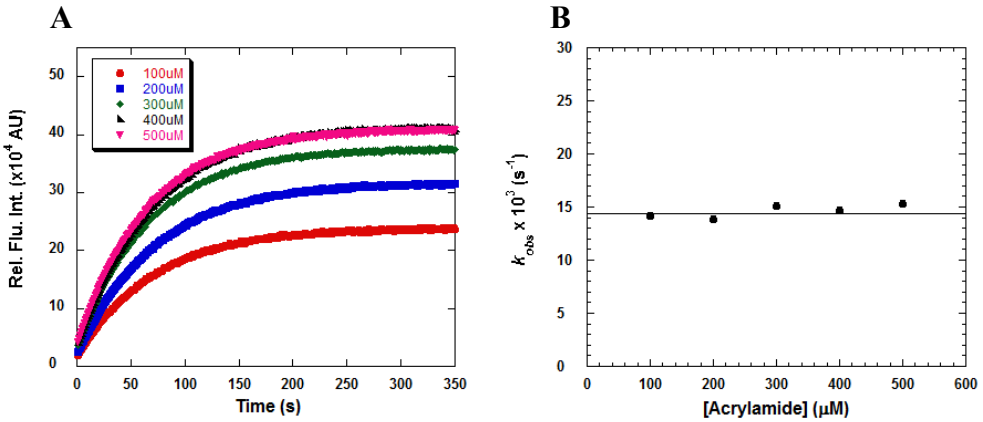

**Supplementary Figure 5** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 10 with 50 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



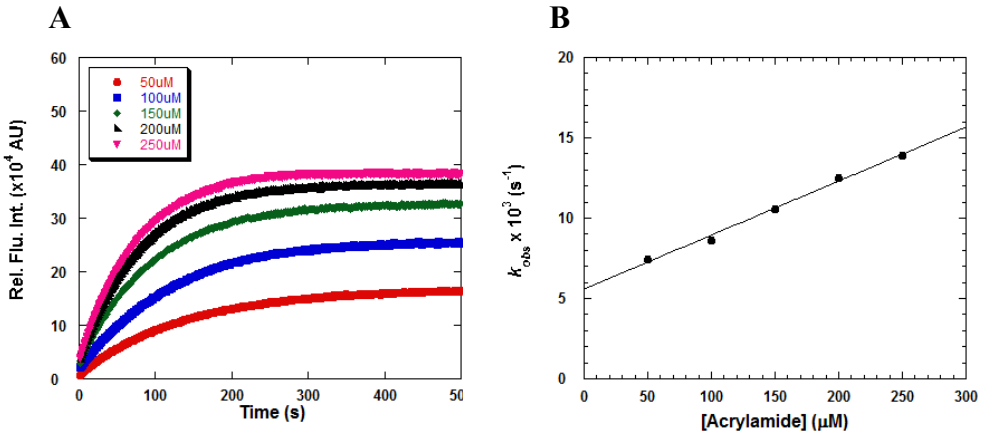

**Supplementary Figure 6** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 without chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



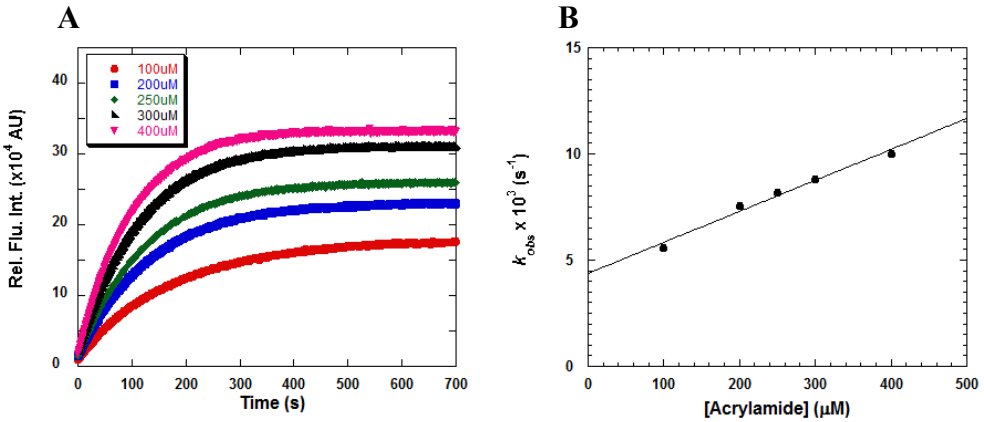

**Supplementary Figure 7** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 0.1 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



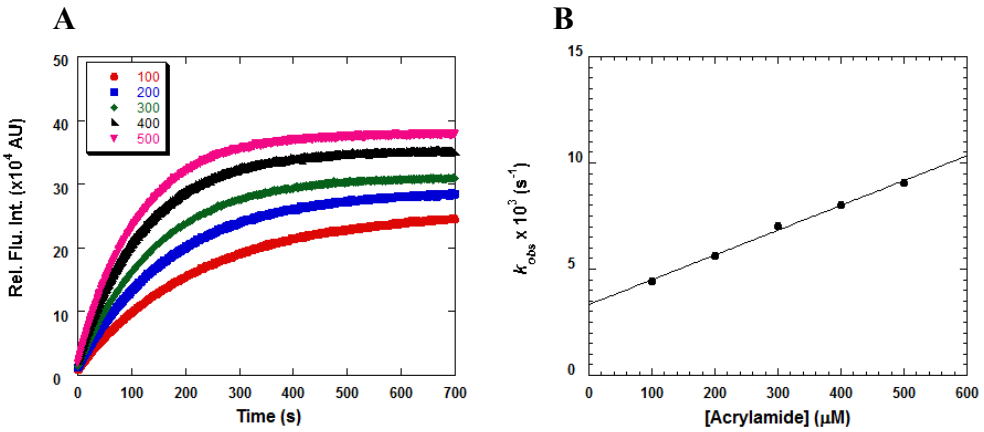

**Supplementary Figure 8** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 1 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



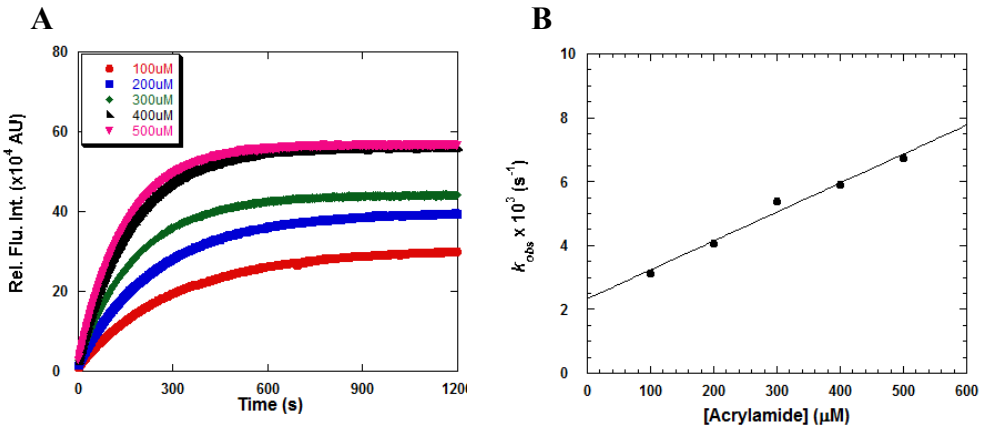

**Supplementary Figure 9** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 10 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



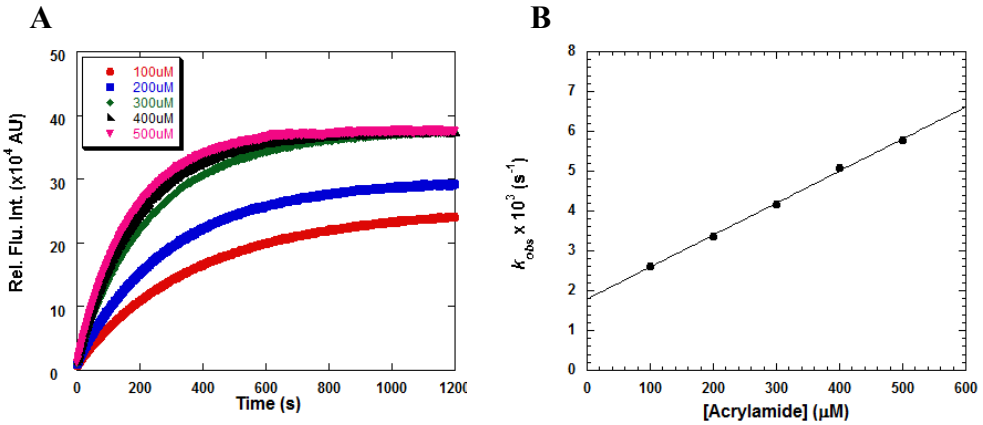

**Supplementary Figure 10** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 20 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



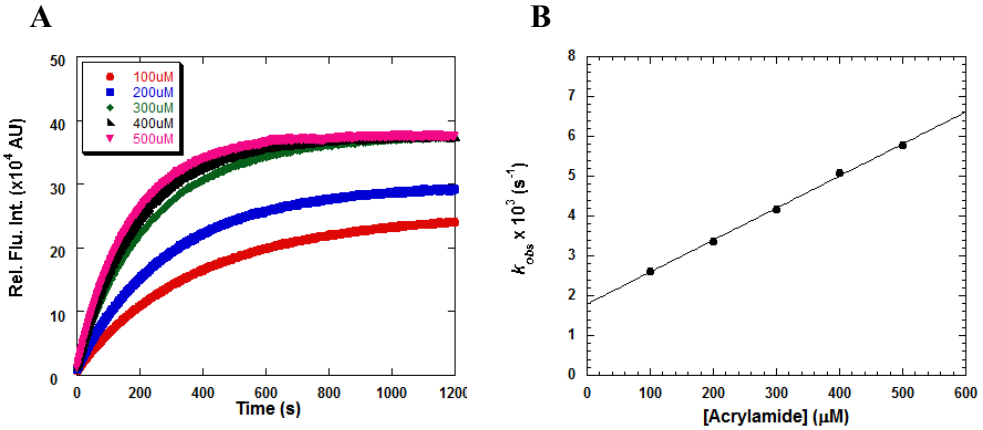

**Supplementary Figure 11** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 30 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



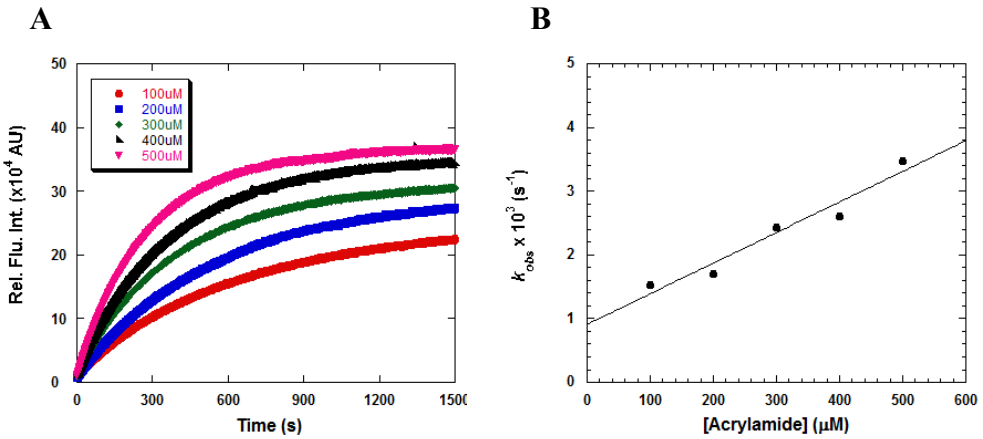

**Supplementary Figure 12** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 40 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



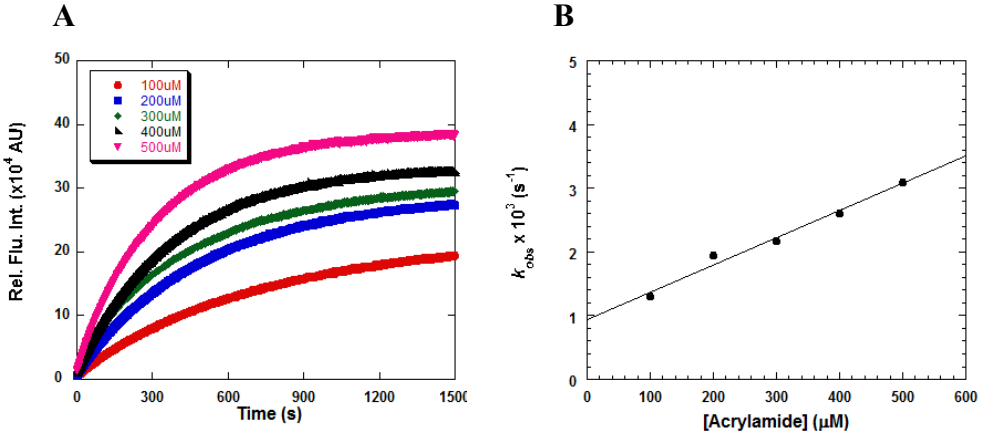

**Supplementary Figure 13** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 50 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



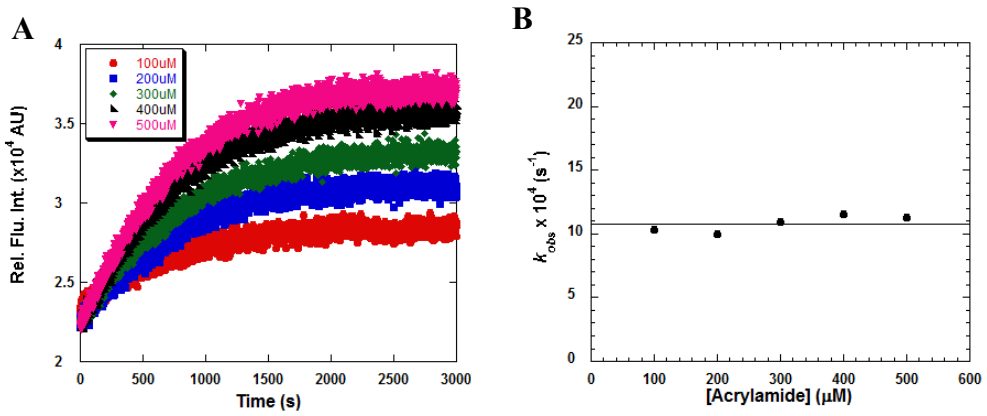

**Supplementary Figure 14** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 60 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



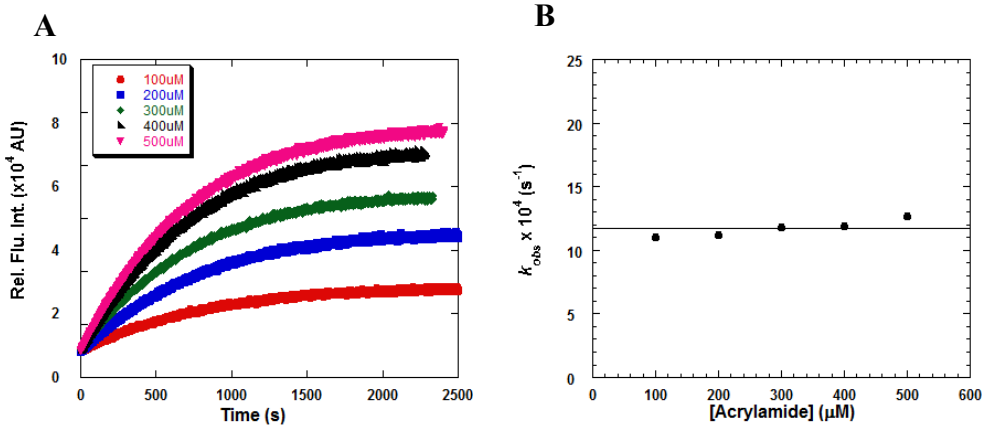

**Supplementary Figure 15** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 80 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



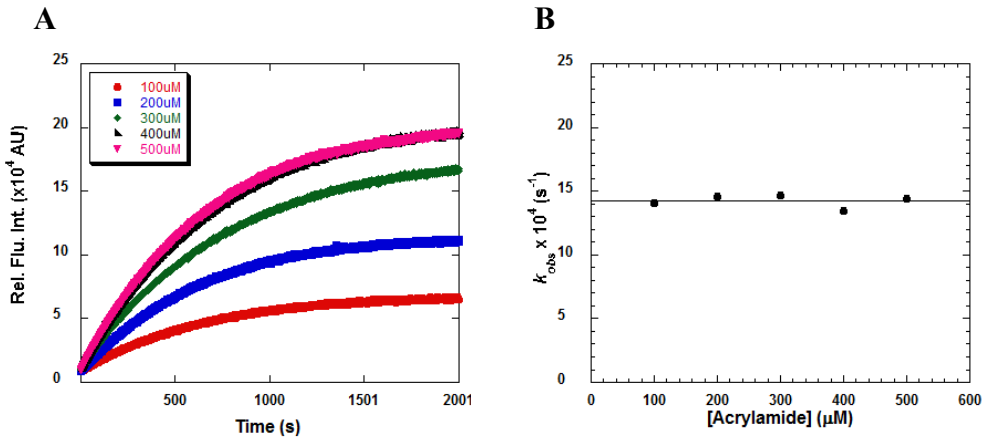

**Supplementary Figure 16** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 9 with 100 mM chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



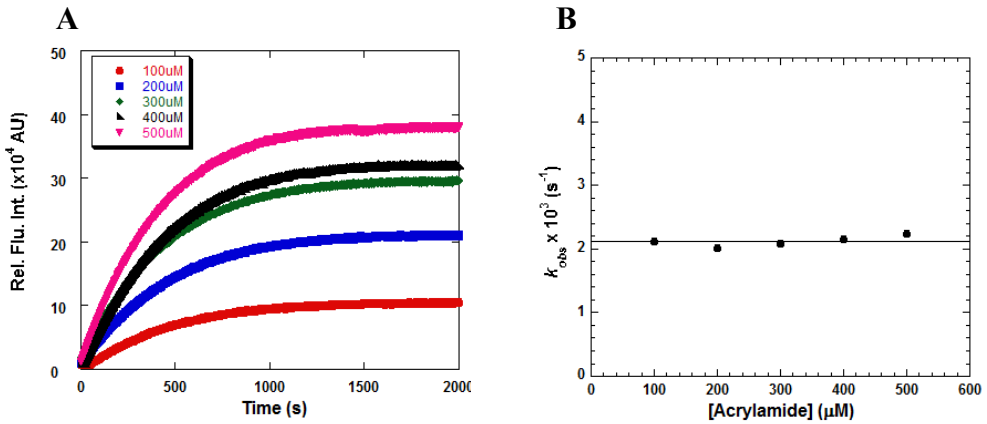

**Supplementary Figure 17** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 5 without chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



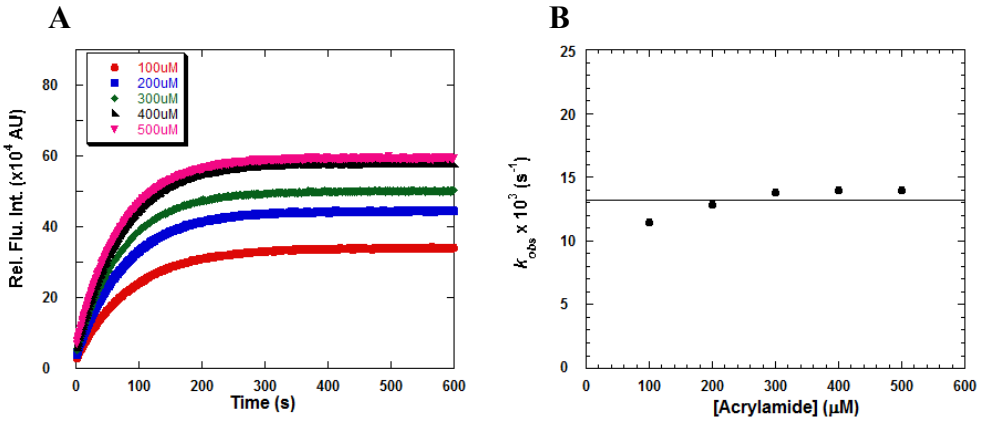

**Supplementary Figure 18** (**A**)The fluorescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 6 without chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



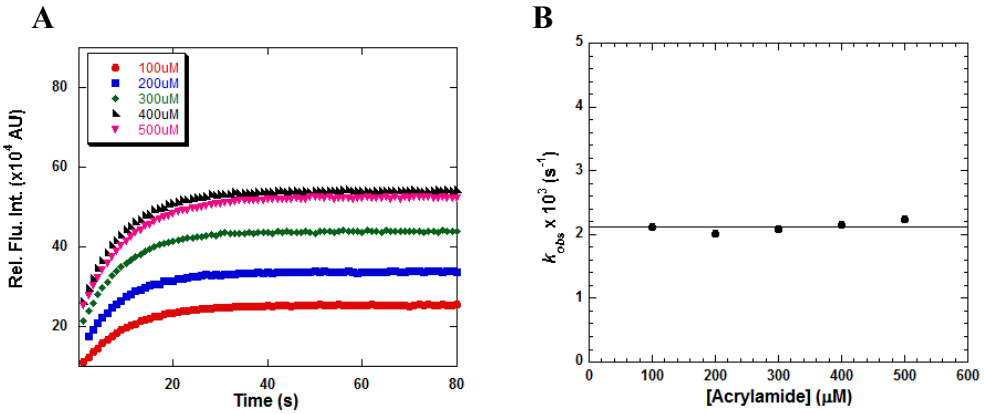

**Supplementary Figure 19** (**A**)The florescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 7 without chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.



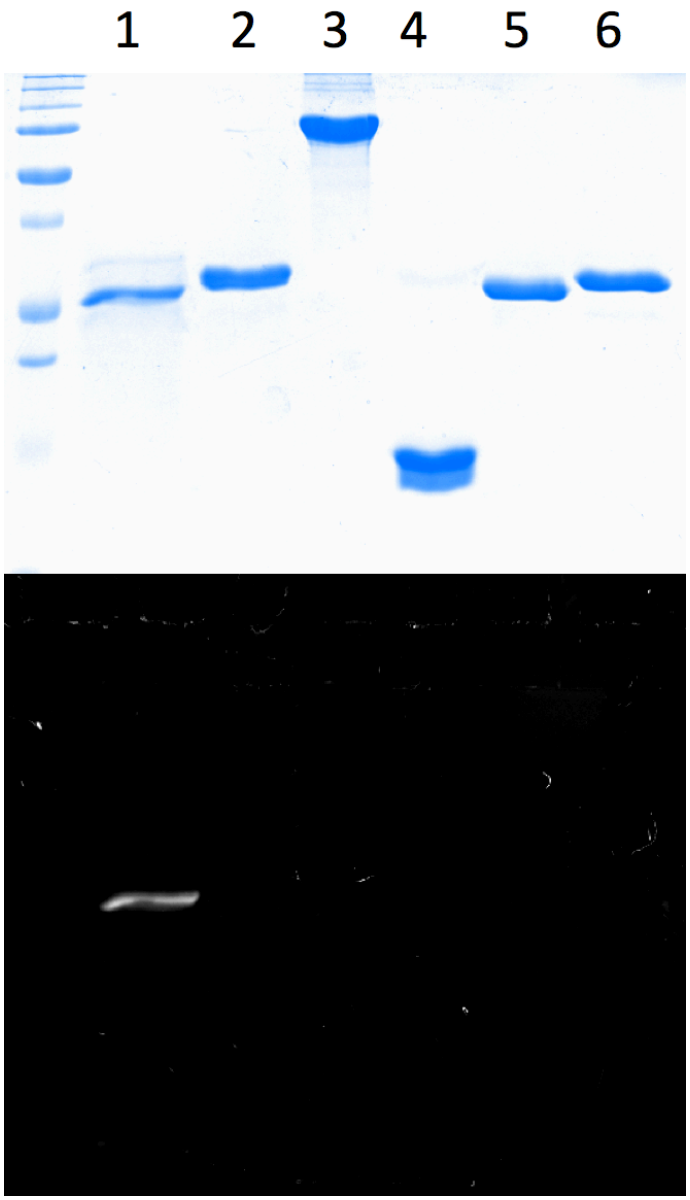

**Supplementary Figure 20** (**A**)The florescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 8 without chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.




**Supplementary Figure 21** (**A**)The florescence increase of the formation of pyrazoline between Hydrazonyl chloride **1** and acrylamide at pH 9 without chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.




**Supplementary Figure 22** (**A**)The florescence increase of the formation of pyrazoline between hydrazonyl chloride **1** and acrylamide at pH 10 without chloride. (**B**) The liner dependence of $k_{obs}$ of the reaction between hydrazonyl chloride **1** and acrylamide on acrylamide concentration.




**Supplementary Figure 23**: The selective labeling of sfGFP2AcrK with diarylnitrilimine. Proteins (**1**: sfGFP2AcrK; 2: sfGFP-p53 peptide fusion; 3: BSA; 4: lysozyme; 5: sfGFP; 6: sfGFP incorporated with a meta-trifloromethyl-phenylalanine at its S2 site) were incubated with 5 mM hydrazonyl chloride **1** for 20 min before they were analyzed by SDS-PAGE. The top panel shows the Coomassie-blue staining of the gel and the bottom panel shows the fluorescent imaging of the gel before it was Coomassie-blue stained. The data clearly demonstrated the specific reaction between acrylamide and nitrilimine.