

NCL: small test ligations at $\text{pH} \approx 6.5$

TEV cleavage: 550 μg of GST-ENLYFOC-Pax(38-557)-FLAG in 1.4 mL of FLAG elution buffer / glycerol was concentrated to 900 μL .

550 μg in 900 μL

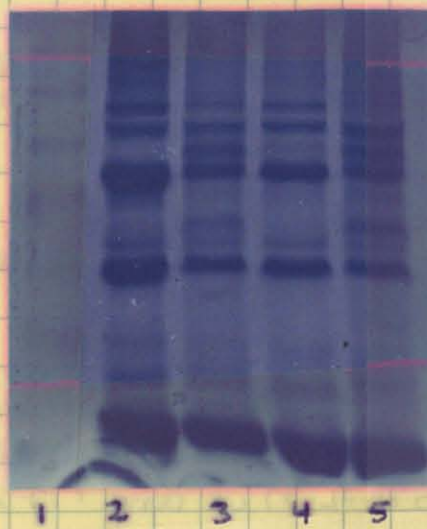
100 μL 10 \times mTEVp buffer

22 μL mTEVp

} inc. at 37 $^{\circ}\text{C}$ for 4.5 h.
(366 μL cut protein)

To the 366 μg of cut protein in 1 mL of 50 mM Tris (and EDTA and DTT) was added 1 mL water to dilute the Tris buffering to 25 mM at pH 8.1. 1.5 mL (243 μg) of the total 2 mL soln. was concentrated to 40 μL in a 50 MWCO centrifugal filter (Millipore).

10 μL was removed (tube A) and combined with 10 μL of 25 mM Tris-HCl, 150 mM NaCl, pH 8.1. The remaining 30 μL was added to 250 μL of lyophilized pY-SBn. 30 μL of 100 mM MES, 150 mM NaCl, pH 6.1 was added (resulting in a pH \approx 6.1 to 6.5 by pH paper.) The solution appeared mostly dissolved, but was a bit cloudy. For rxn b and c, 20 μL was transferred to a new eppendorf. For d 20 remained in the original tube. Thiols were added as listed below and the rxns were incubated at rt for 24 h. To each tube was added 20 μL of SDS reducing buffer and the samples were boiled 5 min. 20 μL were loaded / lane for Coomassie and Western blot.

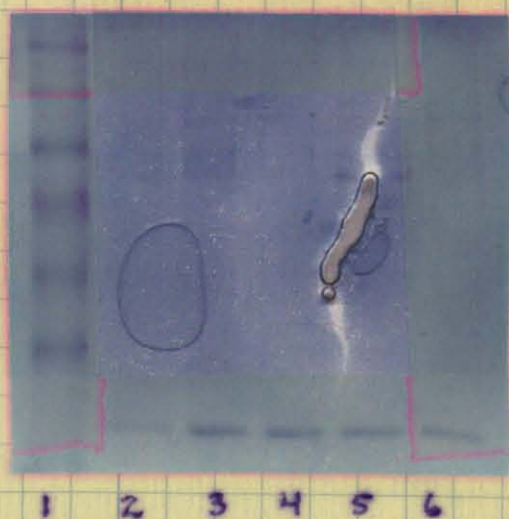


↑↑↑ uncut GST-Pax-FL
↑↑↑ ligation product
↑ Cys-Pax-FLAG
← truncation
↑ mTEVp

- In 1 benchmark protein ladder
 2 rxn A: (control) Cys-Pax-FLAG at pH 8.1 (no peptide thioester or thiols)
 3 rxn B: Cys-Pax-Fl w/ thioester and (after 1/2 dilution) 50 mM MES, pH = 6.1 a bit cloudy (v. slightly, but mostly dissolved) 20 μL
 4 rxn C: 20 μL prot/thioester w/ 100 mM MESNA (2 μL from 1M MESNA in the above MES buffer) and 0.2% v/v thiophenol, clear at t=24
 5 rxn d: (In original thioester container) w/ 0.2% v/v thiophenol and 20 μL prot/pep. (0.8 μL of a 1/20 dilution of thiophenol) a bit cloudy at t=24 h.

NCL - small test ligations (48 h. rxn time in various conditions)

- TEV cleavage to give 240 μg of Cys-Pax(38-557)-FLAG. The protein was concentrated to 40 μL (6 $\mu\text{g}/\mu\text{L}$) at pH 8.1 using the Millipore 50 kDa MWCO centrifugal filters.
- The 40 μL of concentrated protein soln. were added to 250 μg of lyophilized Ac-HHHHHH-Pax(2-367)-SBN in a 1.5-mL eppendorf. The material appeared to be fully dissolved.
- 10 μL of the Cys-Pax-Fl/thioester was removed and combined w/ 10 μL of 25 mM Tris, 150 mM NaCl, pH 8.1 (rxn A). To this was added 2% v/v thiophenol (0.8 μL of a 1/20 dilution.)
- To the remaining 30 μL of protein/thioester was added 100 mM MES, 150 mM NaCl, pH 6.1. 20 μL was removed (rxn B) and added to an eppendorf w/ 2% v/v thiophenol. Rxn C was set up similar to B, but also had the addition of a 100 mM final concentration of MESNA.
- To the 20 μL remaining in the original thioester eppendorf was added 1.0 ~~0.8~~ 2% v/v of thiophenol (4 μL of a 1/20 dilution.) After 24 and 48 hours, rxn A (!?) and C were completely clear, while B and D had some precipitation.



- 1 benchmark protein ladder
- 2 before concentration
- 3 rxn A, t=48 h
- 4 rxn B, t=48 h (slightly cloudy)
- 5 rxn C, t=48 h
- 6 rxn D, t=48 h (slightly cloudy)

Grr! There is no protein here (perhaps explaining the absence of precipitation in rxn A.)