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SnoopLigase-mediated peptide-peptide conjugation and purification

Corresponding author:

Mark Howarth

Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK.

mark.howarth@bioch.ox.ac.uk

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SnoopLigase peptide-peptide coupling

SnoopLigase-mediated peptide-peptide conjugation and purification

Can M Buldun[†,1], Irsyad N. A. Khairil Anuar[†,1], and Mark Howarth[†*]

[†]Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK.

[1]Equal contribution

*Correspondence and requests for materials should be addressed to M.H. (email: mark.howarth@bioch.ox.ac.uk).

Summary/Abstract

Covalently linking together different proteins can enhance functionality for a range of applications. We have developed the SnoopLigase peptide-peptide conjugation method to easily and specifically link proteins fused to the peptides SnoopTagJr or DogTag via an isopeptide bond. SnoopLigase conjugation has been applied for enhancing enzyme resilience and for antigen oligomerization to enhance vaccine efficacy. Following conjugation, SnoopLigase and unreacted substrates can be removed by solid-phase immobilization of SnoopLigase, yielding purified protein-protein conjugates. Here we describe procedures for designing tag-fused proteins, SnoopLigase purification, and ligation of SnoopTagJr and DogTag. We further define steps for purification of ligated product and quantification of ligation success.

Key Words

bioconjugation, vaccination, nanobiotechnology, chemical biology, protein engineering, split protein, protein design

1. Introduction

Protein-protein conjugation is a key tool to extend protein function. Synthetic protein assemblies bring major potential for new advances in biotransformation, vaccination, diagnostics and therapeutics ***(1)***. Covalent conjugation of proteins is often preferable to non-covalent assembly, because of the easier analysis of interactions and the enhanced resilience in real-world applications, including inside the body.

Various protein-protein covalent conjugation methods are available to the user, including split inteins ***(2)***, Sortase A ***(3)*** and Butelase 1 ***(4)***. However, there can be challenges for the application of such systems in terms of the maximum conversion, precise requirements for tag location, and the high concentration of reacting species required ***(1)***. Rather than peptide bond formation, we have focused on the opportunities arising from directing isopeptide bond formation ***(5)***. SpyLigase is a tripartite split of the CnaB2 domain from the *Streptococcus pyogenes* FbaB protein ***(6)***. SpyLigase mediates the formation of an isopeptide bond between the lysine in KTag and aspartic acid in SpyTag. SpyLigase was a useful proof of principle for the three-part splitting of a protein for ligation purposes. However, the yield was rarely above 60% and needed inconvenient reaction conditions (i.e. 4 °C with 1.5 M trimethylamine N-oxide for >24 h). Recent work by Wen-Bin Zhang's laboratory re-designed this system to produce SpyTag/BDTag peptide-peptide ligation by SpyStapler, showing activity even inside cells ***(7)***.

We recently introduced an alternative tripartite protein split for conjugating peptide tags called SnoopLigase ***(8)***. SnoopLigase covalently links DogTag and SnoopTagJr via an

Asn-Lys isopeptide bond (Fig. 1A, B). Compared to SpyLigase, SnoopLigase delivers better substrate conversion (more than 95% with a range of different targets) and is active under a broader range of conditions ***(8)***. Because of SnoopLigase's high substrate conversion even down to 2.5 μM partner concentration ***(8)***, we envision that SnoopLigase will find application in many different applications (Fig. 1C) ***(1)***.

We have demonstrated the application of SnoopLigase as a simple way to enhance enzyme resilience. For both β-lactamase and phytase, cyclization using SnoopLigase meant that the enzyme retained solubility and activity following boiling (Fig. 1C) ***(8)***. The resilience from SnoopLigase cyclization surpassed the resilience obtained from our previous SpyRing cyclization approach ***(9)***. SnoopLigase was applied in a different way to accelerate vaccine assembly. Monomeric antigens from pathogens do not lead to effective vaccines, but assembly into nanoparticles can enhance the strength and persistence of protective antibody responses ***(10)***. Antigens from malaria or cancer bearing SnoopTagJr were efficiently multimerized by reaction with the DogTag-linked nanoparticle IMX313 (Fig. 1C) ***(11)***.

Separating products from unreacted starting material and catalyst is often as challenging as reactions themselves. However, solid-phase immobilization of SnoopLigase to streptavidin or HaloLink resin enabled the ligated SnoopTagJr-DogTag product to be eluted with high purity, free from SnoopLigase or unligated substrates. In this chapter we describe the methods and principles to successfully design, express, ligate and purify conjugates of SnoopTagJr and DogTag.

2. Materials

2.1. Equipment

1. Incubators and shakers appropriate for growing bacterial cultures.
2. Centrifuges: floor-standing centrifuge capable of spinning 1 L bacterial cultures at 5,000 *g* and benchtop centrifuge capable of spinning 1.5 mL tubes at 16,900 *g*.
3. Sonicator.
4. PCR Machine.
5. Nanodrop for protein quantification.
6. Electrophoresis apparatus for SDS-PAGE.
7. Thermomixer.
8. Gel Imager.
9. 20 mL Econo-Pac Chromatography Columns (Bio-Rad).
10. 1 mL Polyprep Chromatography Columns (Bio-Rad).
11. Spectra/Por 3 3,500 Da MWCO dialysis membrane (Spectrum Labs).
12. 10 kDa molecular weight cut-off (MWCO) spin filters for concentrating proteins.

2.2. Reagents

1. Expression vectors pET28a-AviTag-SnoopLigase (plasmid # 105626), pET28a-HaloTag7-SnoopLigase (plasmid # 105627) and pET28a-SUMO-DogTag (plasmid # 105629) are available from Addgene (www.addgene.org).
2. Target proteins with SnoopTagJr and DogTag.
3. His-tagged SUMO-protease Ulp1, available commercially (e.g. Thermo Fisher Scientific, 12588018) or from Addgene (plasmid # 64697).

4. SnoopTagJr peptide (GKLGSIEFIKVNKGY) solid-phase synthesized. Dissolve to 10 mM in DMSO and store at -80 °C. This peptide is longer than the minimal SnoopTagJr sequence and includes a tyrosine to facilitate absorbance measurement.
5. EDTA-free mixed protease inhibitors tablets (Roche) to inhibit bacterial proteases during protein purification.
6. 100 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol: 17.4 mg PMSF in 1 mL isopropanol. Store at −20 °C.
7. 420 mM isopropyl-β-D-thiogalactopyranoside (IPTG) solution: 100 mg IPTG in 1 mL MilliQ water. Sterile-filter and store at −20 °C.
8. 50 mg/mL kanamycin solution: 1 g of kanamycin in 20 mL MilliQ water. Sterile-filter and store at −20 °C.
9. 20% (w/v) glucose solution: 200 g/L D-glucose in MilliQ water. Autoclave and store at 25 °C.
10. Ni-NTA agarose resin (Qiagen) for purification of His-tagged proteins.
11. HiCap Streptavidin agarose (Qiagen) or HaloLink resin (Promega).
12. 10% (v/v) Tween 20.
13. InstantBlue (Expedeon) or Coomassie solution to stain polyacrylamide gels.
14. GST-BirA protein as described ***(12)***. See section 3.3.1.
15. Streptavidin, available commercially (e.g. Thermo Fisher Scientific, 434301).
16. Glutathione HiCap resin for purification of GST-tagged proteins (Qiagen, 30900).
17. 50 mM D-Biotin solution: 12.2 mg D-biotin in 1 mL DMSO. Store at −20 °C.

18. 100 mM ATP solution: 55.1 mg adenosine 5′-triphosphate disodium salt hydrate in 1 mL MilliQ water. Store in aliquots at −80 °C.
19. 1 M magnesium chloride solution: 203 mg magnesium chloride hexahydrate in 1 mL MilliQ water. Store at 25 °C.

2.3. Buffers, Media and Cells

1. *E. coli* expression strain such as BL21 (DE3) RIPL (Agilent) which enables good protein expression with rare codons.
2. Lysogeny broth (LB): 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl. Autoclave and store at 25 °C.
3. Ni-NTA binding buffer: 50 mM Tris base, 300 mM NaCl, pH-adjusted to 7.8 with HCl.
4. 50 mM Tris borate pH 8.0 (50 mM Tris, pH-adjusted with boric acid) and Tris borate pH 7.25.
5. 50% (v/v) glycerol solution: 25 mL glycerol made up to 50 mL with MilliQ water.
6. 6× SDS loading buffer [0.23 M Tris–HCl pH 6.8, 24% (v/v) glycerol, 0.23 M sodium dodecyl sulfate (SDS), 120 μM bromophenol blue].
7. Tris phosphate pH 7.0 (25 mM phosphoric acid, pH-adjusted with Tris base) containing 0.01% (v/v) Tween 20.
8. Tris phosphate pH 7.0 (25 mM phosphoric acid, pH-adjusted with Tris base) containing 300 mM NaCl, 500 mM imidazole pH 7.0 and 0.01% (v/v) Tween 20.
9. Tris phosphate pH 7.0 (25 mM phosphoric acid, pH-adjusted with Tris base) containing 500 mM imidazole pH 7.0.

10. Tris phosphate pH 7.0 (25 mM phosphoric acid, pH-adjusted with Tris base) containing 2 M imidazole pH 7.0.
11. 50 mM glycine pH 3.0 (pH-adjusted with HCl) with 300 mM NaCl and 0.01% (v/v) Tween 20.
12. 50 mM glycine pH 3.0 (pH-adjusted with HCl).
13. 50 mM glycine pH 2.0 (pH-adjusted with HCl).
14. 1 M Tris–HCl pH 9.5.

3. Methods

3.1. Design of SnoopTagJr and DogTag fusion constructs

The amino acid sequences of SnoopTagJr and DogTag are shown in Fig. 2. Both SnoopTagJr and DogTag can be used as either N-terminal or C-terminal fusions. The terminus for fusion should be chosen based on the features of the protein of interest, e.g. distance from a binding site or active site, or prior experience of the protein tolerating other fusions. If an initial construct shows low reaction rate, it is worth testing SnoopTagJr or DogTag at the opposite terminus, to see if reaction rate is increased. When using proteins of interest that form multimeric assemblies, it is important to keep in mind that SnoopTagJr and DogTag need to be surface-accessible even after the multimer has formed. SnoopTag ***(13)*** and SnoopTagJr can be used interchangeably but reaction rate will be higher with SnoopTagJr ***(8)***. When using SnoopTag or SnoopTagJr at the C-terminus, it is recommended to add a C-terminal GS ***(8, 14)***.

The tags should be spaced from the protein of interest with an appropriate linker. We have had good experiences with the sequence GSGESG (e.g. Addgene plasmid # 35050 ***(15)***, # 102831 ***(16)***, # 72323 ***(13)***, # 105628 ***(8)***). G/S-rich linkers show a good balance of

flexibility, solubility and protease resistance ***(17)***. while the E in the spacer increases hydrophilicity. In case of slow or incomplete reaction, it is worth testing a linker with an additional GSG or $(GSG)_2$.

If the protein of interest (POI) is to be cyclized ***(8)***, we suggest testing both orientations, DogTag-POI-SnoopTagJr and SnoopTagJr-POI-DogTag. The linker length should be chosen based on the distance between the N- and C-termini in the POI. Besides terminal fusions, DogTag can be placed into protein loops [SnoopTagJr showed low reactivity in loops ***(18)***]. Which loop is suitable for DogTag insertion needs to be tested empirically for each POI. We suggest first testing surface-exposed and flexible loops as identified from structural data (e.g. crystal structure B-factor) or homology modelling. Permissive stretches for internal protein tagging can also be obtained by sequence-based prediction ***(19)***. A good starting point for linkers is $(GS)_3$ on either side of DogTag ***(8)***.

To maximize expression yield, ideally codon usage should be optimized for the expressing cell type. However, codon usage will be less important when expressing in a strain such as *E. coli* BL21 (DE3) RIPL. In case of low expression, it is worth checking for potential secondary structure in the mRNA near the Shine-Dalgarno site. Adjusting codon usage to get a Ribosome Binding Calculator score of at least 10,000 should favor good expression yield ***(20)***.

3.2. SnoopLigase production

Choose Biotin-SnoopLigase or HaloTag7-SnoopLigase for your application (*see* **Note 1**). The expression vectors pET28a-AviTag-SnoopLigase and pET28a-HaloTag7-SnoopLigase from Addgene contain an N-terminal His-tag for Ni-NTA purification.

1. Transform an *E. coli* expression strain, e.g. BL21 (DE3) RIPL, with the pET28a-AviTag-SnoopLigase or pET28a-HaloTag7-SnoopLigase plasmid.
2. Grow a 10 mL overnight culture from a single colony in LB with 10 µL 50 mg/mL kanamycin and 200 µL 20% (w/v) glucose at 37 °C.
3. Transfer the overnight culture to 1 L LB with 1 mL 50 mg/mL kanamycin and 37.5 mL 20% (w/v) glucose in a 2.5 L Ultra Yield flask (Thomson Instrument Company).
4. Grow the culture at 37 °C, 200 rpm to an OD_{600} of 0.5.
5. To induce protein expression, add 1 mL of 420 mM IPTG solution.
6. Grow the culture at 30 °C, 200 rpm for 4 h.
7. Harvest cells by centrifugation at 4,000 *g* for 20 min at 4 °C. Discard the supernatant. Store the pellets at -80 °C or continue directly with protein purification.
8. Resuspend the pellet in 10 mL ice-cold Ni-NTA binding buffer (50 mM Tris base, 300 mM NaCl, pH 7.8) containing 10 mM imidazole pH 7.8, 10 mM PMSF and EDTA-free mixed protease inhibitors. Perform all subsequent steps on ice or at 4 °C.
9. Sonicate on ice six times for 30 s, with 1 min breaks between sonication cycles.
10. Centrifuge the lysate for 30 min at 16,900 *g*, 4 °C.
11. Incubate the supernatant with 0.5 mL (packed volume) Ni-NTA agarose for 30 min at 4 °C with end-over-end rotation.
12. Collect the sample in a 20 mL Econo-Pac Chromatography Column (Bio-Rad). Discard the flow-through.
13. Wash the resin five times with 5 mL Ni-NTA binding buffer containing 10 mM imidazole.

14. Wash the resin once with 1 mL of Ni-NTA binding buffer containing 30 mM imidazole.
15. For elution, cap the column and incubate the resin with 0.5 mL of Ni-NTA buffer containing 200 mM imidazole for 5 min. Collect the flow-through.
16. Repeat the elution twice more, but without incubation time.
17. Dialyze the purified protein three times against 1.5 L 50 mM Tris-borate pH 8.0 (*see* **Note 2**) at 4 °C using a Spectra/Por 3 3,500 Da MWCO membrane (Spectrum Labs).
18. AviTag-SnoopLigase only: perform enzymatic biotinylation ***(12)***. See section 3.3 for further details.
19. If required, concentrate the protein using a Vivaspin centrifugal concentrator with 10 kDa MWCO (GE Healthcare). AviTag-SnoopLigase can be concentrated to at least 500 µM. HaloTag7-SnoopLigase can be concentrated to at least 250 µM. Store aliquots at -80 °C. Our experience was that SnoopLigase tolerated freeze-thaw cycles, but this would be preferably avoided. Avoid storing SnoopLigase samples for more than 1 week at 4 °C.

3.3. AviTag-SnoopLigase biotinylation

3.3.1. GST-BirA expression and purification

GST-BirA expression and purification was described in detail previously ***(12)***. This section will briefly outline the production of GST-BirA.

1. Express GST-BirA in *E. coli* BL21 (DE3) RIPL from pGEX-GST-BirA ***(21)*** as described above for AviTag-SnoopLigase and HaloTag7-SnoopLigase.

2. Once the cells are harvested, resuspend the pellet in 15 mL ice-cold PBS (1.44 g/L di-sodium hydrogen phosphate, 0.24 g/L potassium di-hydrogen phosphate, 0.2 g/L KCl, 8 g/L NaCl, pH 7.4) with 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mg/mL lysozyme, 1% (v/v) Triton X-100, 10 mM PMSF and EDTA-free mixed protease inhibitors. Incubate on ice for 30 min. Perform all subsequent steps at 4 °C.
3. Sonicate on ice six times for 30 s, with 1 min breaks between sonication cycles.
4. Centrifuge the lysate for 30 min at 16,900 *g*, 4 °C.
5. Incubate the supernatant with 1 mL glutathione HiCap matrix for 30 min at 4 °C with end-over-end rotation.
6. Collect the sample in a 20 mL Econo-Pac Chromatography Column (Bio-Rad). Discard the flow-through.
7. Wash the resin five times with 10 mL of PBS, 1 mM DTT, 1 mM EDTA and 0.05% (v/v) Triton X-100.
8. Cap the column and incubate the resin with 1 mL elution buffer (50 mM Tris–HCl pH 8.0, 400 mM NaCl, 50 mM reduced glutathione, 1 mM DTT; made fresh each time) for 5 min. Collect the flow-through.
9. Repeat the elution twice more, but without incubation.
10. Measure the protein concentration. Dilute the protein with PBS so that the concentration is less than 50 μM, to reduce the risk of aggregation.
11. Dialyze the purified protein three times against 3 L of PBS at 4 °C using a Spectra/Por 3 3,500 Da MWCO membrane (Spectrum Labs). Store aliquots at -80 °C.

3.3.2. AviTag-SnoopLigase biotinylation with GST-BirA

Biotinylation of AviTag-SnoopLigase has been described in detail ***(12)*** and is summarized here.

1. To 100 μM AviTag-SnoopLigase in 50 mM Tris borate pH 8.0, add 5 μL 1 M magnesium chloride, 20 μL 100 mM ATP, 20 μL 50 μM GST-BirA, and 3 μL 50 mM D-Biotin.
2. Incubate the sample for 1 h at 30 °C.
3. Add a further 20 μL 50 μM GST-BirA and 3 μL 50 mM D-Biotin. Incubate for 1 h at 30 °C.
4. To remove GST-BirA, incubate the sample with 0.1 mL 50% slurry of buffer-washed glutathione HiCap resin for 30 min at 25 °C. Centrifuge the sample and collect the supernatant.
5. To remove free biotin, dialyze the purified protein three times against 1.5 L 50 mM Tris borate pH 8.0 at 4 °C using a Spectra/Por 3 3,500 Da MWCO membrane (Spectrum Labs).
6. If required, concentrate the protein using a Vivaspin centrifugal concentrator with 10 kDa MWCO (GE Healthcare). Biotin-SnoopLigase can be concentrated to at least 500 μM. Store aliquots at -80 °C. From experience, multiple freeze-thaw cycles do not influence enzyme reactivity. Avoid storing GST-BirA for more than 1 week at 4 °C. Biotin-SnoopLigase can also be lyophilized and stored at 25 °C ***(8)***.
7. The extent of biotinylation is tested by streptavidin shift assay. Add 2 μL of 6× SDS-PAGE buffer to 10 μL of 10 μM biotinylated AviTag-SnoopLigase.
8. Incubate at 95 °C for 5 min. Allow the sample to cool to 25 °C.

9. Add 5 µL of PBS containing a 2× molar excess of streptavidin to the samples. Incubate at 25 °C for 5 min.

10. Run samples containing streptavidin alone, biotinylated SnoopLigase alone, and the streptavidin/biotin-SnoopLigase mix on SDS-PAGE.

11. Stain the gel using InstantBlue or Coomassie. In the lane containing biotinylated SnoopLigase and streptavidin, the band corresponding to biotinylated SnoopLigase should have shifted up, indicating interaction with streptavidin. Note that in some cases it will be important to use SnoopLigase that is 100% biotinylated (*see* **Note 3**).

3.4. Ligation reaction

The standard reaction condition is 10 µM Biotin-SnoopLigase or HaloTag7-SnoopLigase, 10 µM SnoopTagJr-substrate and 10 µM DogTag-substrate in 50 mM Tris borate pH 7.25 with 15% (v/v) glycerol at 4 °C for 24 h. However, the parameters can be adjusted as required:

- Component concentration: SnoopLigase reaction rate depends on the concentration of all three components. Therefore, it is recommended to use the highest substrate concentration possible, and at least equimolar amount of SnoopLigase. If a protein substrate is supposed to be cyclized by SnoopLigase, note that high concentrations increase the probability of intermolecular conjugation ***(22)***. The balance between cyclization and intermolecular conjugation depends on the substrate construct, e.g. distance between N- and C-termini and linker length. Distant N- and C-termini may require a longer linker to minimise intermolecular conjugation. If initial reaction conditions show a high level of intermolecular conjugation by SDS-PAGE, choose lower substrate concentrations.

- Stoichiometry: The reaction works at 1:1:1 stoichiometry. However, to maximize the conjugation efficiency of one substrate, double the concentration of SnoopLigase and the other substrate.
- pH: The reaction works best at pH 6.5 – 8.5 (Fig. 3A, B).
- Temperature: The reaction works well at 4 – 25 °C (Fig. 3C).
- Glycerol: glycerol is not essential for the reaction, but slightly enhances reaction rate (Fig. 3D).
- NaCl: avoid NaCl if possible. NaCl concentrations above 10 mM reduce SnoopLigase reaction rate (Fig. 3E, F). Preferably dialyze your SnoopTagJr-fusion and DogTag-fusion into a buffer without NaCl before reaction.
- Other additives: Reducing agents do not affect SnoopLigase reaction, so you can use whatever is optimal for your protein of interest. Tween 20 and Triton X-100 are tolerated up to at least 2% (v/v).

Reaction time: Choose reaction time according to substrate concentration. At 10 µM concentration of components, we usually use 24 – 48 h. Cyclization reactions typically occur faster: 0.5 – 24 h ***(8)***.

3.5. Determining efficiency of ligation

1. Samples to be analyzed by SDS-PAGE: (a) SnoopLigase reaction mix and (b) substrate mix, i.e. the same as (b) but no SnoopLigase added. Add 10 µL of sample to a PCR tube.
2. Add 2 µL 6× SDS loading buffer.
3. Heat samples for 5 min at 95 °C in a PCR block with heated lid.

4. Let samples cool down to 25 °C and briefly centrifuge.
5. Run samples on SDS-PAGE.
6. Stain the gel using InstantBlue or Coomassie.
7. Acquire an image of the gel, e.g. using a ChemiDoc XRS+ Imager (Bio-Rad).
8. Quantify the intensity of protein bands (densitometry) using suitable software, e.g. Image Lab Software (Bio-Rad).
9. Calculate reaction efficiency using band intensities (*see* **Note 4**):

- If equimolar substrate concentrations were used, calculate reaction efficiency based only on the bands in the reaction sample:

$$\%\ Product\ formed = \frac{[Product]}{[Product] + [Substrates]} \times 100\%$$

- If one substrate has been used in excess, calculate reaction efficiency by loss of the less concentrated substrate:

$$\%\ Substrate\ reacted = \left(1 - \frac{[Substrate\ after\ reaction]}{[Substrate\ before\ reaction]}\right) \times 100\%$$

3.6. Purification of conjugated product

3.6.1. SnoopLigase capture

1. To 0.5 mL SnoopLigase reaction mix containing 10 µM Biotin-SnoopLigase or HaloTag7-SnoopLigase, add 0.5 µL 10% (v/v) Tween 20. The Tween 20 will decrease the resin sticking to plastic surfaces.
2. Add 25 µL HiCap Streptavidin agarose to reactions containing Biotin-SnoopLigase, or 50 µL HaloLink resin to reactions containing HaloTag7-SnoopLigase. If SnoopLigase concentrations other than 10 µM were used, adjust the resin volumes accordingly.
3. Incubate for 30 min at 25 °C with end-over-end rotation. Incubation time may need to be prolonged, depending on the conjugated proteins.
4. Equilibrate a 1 mL polyprep column (Bio-Rad) with 0.5 mL 50 mM Tris borate pH 8.0 with 0.01% (v/v) Tween 20.
5. Transfer the sample containing the resin into the column. Place the column into a 1.5 mL tube and centrifuge for 1 min at 300 g.
6. Test the capture efficiency by analyzing using SDS-PAGE and Coomassie staining the amount of SnoopLigase and product in the reaction mix before addition of resin, and the amount in the flow-through after SnoopLigase capture.

3.6.2. Product elution

We have established three different methods for product elution. The elution method should be chosen based on the tolerance of the substrate molecules. Imidazole and low pH elution are the more convenient methods**,** while elution by peptide competition is more laborious and expensive but gentler. The procedures described below are for monomeric conjugates, i.e. one SnoopTagJr and one DogTag per product molecule. For multimeric conjugates, the procedure may need to be adjusted (*see* **Note 3**).

3.6.2.1. Product elution by imidazole

Perform all steps at room temperature (~25 °C).

1. To the polyprep column containing the resin from section 3.6.1., add five resin volumes of Tris phosphate pH 7.0 containing 300 mM NaCl, 500 mM imidazole pH 7.0 and 0.01% (v/v) Tween 20. Centrifuge for 1 min at 300 *g*. Repeat the wash once more.
2. Add five resin volumes of Tris phosphate pH 7.0 containing 500 mM imidazole pH 7.0. Centrifuge for 1 min at 300 *g*. Repeat the wash twice more.
3. Centrifuge for 1 min at 300 *g* to remove the remaining buffer.
4. Cap the column. To elute the conjugated product, add one resin volume of Tris phosphate pH 7.0 containing 2 M imidazole pH 7.0. Incubate for 5 min on a Thermomixer at 500 rpm. Centrifuge for 1 min at 300 *g*. The flow-through contains the eluted product.
5. Repeat the elution step twice more.
6. Dialyze the eluted protein three times against 1,000 volumes of a suitable buffer, e.g. PBS, at 4 °C using a 3,500 Da MWCO membrane.

3.6.2.2. Product elution by low pH

Perform all steps at 4 °C or on ice.

1. To the polyprep column containing the resin from section 3.6.1., add five resin volumes of ice-cold 50 mM glycine pH 3.0 containing 300 mM NaCl and 0.01% (v/v) Tween 20. Centrifuge for 1 min at 300 *g*, 4 °C. Repeat the wash once more.

2. Add five resin volumes of ice-cold 50 mM glycine pH 3.0. Centrifuge for 1 min at 300 *g*, 4 °C. Repeat the wash twice more.

3. Centrifuge for 1 min at 300 *g* to remove remaining buffer.

4. Cap the column. To elute the conjugated product, add one resin volume of ice-cold 50 mM glycine pH 2.0 and incubate for 1 min. Centrifuge for 1 min at 300 *g*, 4 °C into a 1.5 mL tube containing 0.3 resin volumes 1 M Tris–HCl pH 9.5.

5. Repeat the elution step twice with centrifugation into the same 1.5 mL tube and without addition of fresh Tris–HCl.

6. Dialyze the eluted protein three times against 1,000-fold excess volume of a suitable buffer, e.g. PBS, at 4 °C using a 3,500 Da MWCO membrane.

3.6.2.3. Product elution by peptide competition

3.6.2.3.1. Peptide competitor generation

The DogTag:SnoopTagJr competitor peptide is generated from His-tagged SUMO-DogTag and SnoopTagJr peptide. Here we describe the liquid phase production of competitor peptide. Alternatively, a solid-phase approach could be used, as described previously ***(8)***.

1. Express and purify SUMO-DogTag in *E. coli* from pET28a-SUMO-DogTag, as described above for AviTag-SnoopLigase and HaloTag7-SnoopLigase.

2. Perform a conjugation reaction using 75 µM Biotin-SnoopLigase or HaloTag7-SnoopLigase, 75 µM SUMO-DogTag and 150 µM SnoopTagJr peptide in 50 mM Tris borate pH 7.25 with 15% (v/v) glycerol at 4 °C for 4 h.

3. Capture the ligase and elute the reaction product using the imidazole elution method described above. Dialyze the eluted product three times against 1,000 volumes of 50 mM Tris borate pH 7.5 at 4 °C using a 3,500 Da MWCO membrane.

4. Concentrate the SUMO-DogTag:SnoopTagJr conjugate to ≥ 100 µM using a 10 kDa MWCO spin filter.
5. To the concentrated SUMO-DogTag:SnoopTagJr conjugate, add His-tagged SUMO-protease Ulp1 at 1:50 molar ratio.
6. Incubate for 45 min at 25 °C.
7. Add Tween 20 to a final concentration of 0.01% (v/v).
8. To deplete the His-tagged proteins (SUMO and Ulp1), add Ni-NTA agarose at 1:4 reaction volume. Incubate for 1 h at 25 °C, rotating end-over-end.
9. Centrifuge the sample for 1 min at 16,900 *g*. Collect the supernatant containing the DogTag:SnoopTagJr competitor peptide. The competitor peptide contains 3 tyrosine residues and therefore allows concentration determination by OD_{280}. Measure the OD_{280} and calculate the molar concentration using the molar extinction coefficient of 4,470 M^{-1} cm^{-1}.

3.6.2.3.2. Product elution by peptide competition

1. To the polyprep column containing the resin from section 3.6.1., add five resin volumes of Tris phosphate pH 7.0 containing 0.01% (v/v) Tween 20. Centrifuge for 1 min at 300 *g*. Repeat the wash four more times.
2. Cap the column. To elute the conjugated product add two resin volumes of 100 µM DogTag:SnoopTagJr competitor solution generated above.
3. Incubate for 4 h at 37 °C on a Thermomixer at 500 rpm.
4. Centrifuge for 1 min at 300 *g* and collect the supernatant.

5. Dialyze the eluted protein three times against 1,000 volumes of a suitable buffer, e.g. PBS, at 4 °C using a 3,500 Da MWCO membrane.

3.6.3. Resin regeneration and re-use

Having eluted the reaction product from immobilized Biotin SnoopLigase or HaloTag7-SnoopLigase, the remaining SnoopLigase resin can be regenerated, stored for at least 2 weeks at 4 °C, and used for new conjugations at least eight times ***(8)***. Perform all steps at 4 °C or on ice to minimize protein hydrolysis.

1. To the polyprep column containing SnoopLigase-linked resin (from section 3.6.2.), add five resin volumes of ice-cold 50 mM glycine pH 2.0. Centrifuge for 1 min at 300 *g*, 4 °C. Repeat the wash once more.
2. Add five resin volumes of 50 mM Tris borate pH 8.0. Centrifuge for 1 min at 300 *g*, 4 °C. Repeat the wash once more.
3. Optional: To store the resin, cap the column and add two resin volumes of 50 mM Tris borate pH 8.0 containing 0.05% (w/v) sodium azide (Caution: sodium azide is toxic). Store at 4 °C. No obvious loss in SnoopLigase activity was detected for storage up to 2 weeks. Longer storage times have not been tested.
4. To re-use the SnoopLigase resin, centrifuge the column for 1 min at 300 *g*, 4 °C. Add five resin volumes of 50 mM Tris borate pH 8.0 with 0.01% (v/v) Tween 20. Centrifuge for 1 min at 300 *g*, 4 °C. Repeat the wash twice more.
5. To start a new ligation reaction, add 10-20 resin volumes of 10 µM of each SnoopTagJr substrate and DogTag-substrate in 50 mM Tris borate pH 7.25 with 15% (v/v) glycerol. Incubate for 24 h at 4 °C on a Thermomixer at 800 rpm. Reaction conditions can be adjusted as described in section 3.4.

6. Elute the product as described in section 3.6.2.

7. If required, start the regeneration process again.

4. Notes

1. Biotin-SnoopLigase requires enzymatic biotinylation of AviTag-SnoopLigase, to enable subsequent solid-phase capture. HaloTag7-SnoopLigase does not require further processing, but the size of HaloTag7 might cause steric hindrance with some substrates, causing inefficient conjugation. HaloTag7 contains an intramolecular disulfide bond. During expression and purification, a fraction of the proteins might form undesirable intermolecular disulfide bonds. If homodimerization of HaloTag7 is problematic for the anticipated application, e.g. when purifying multimeric SnoopTagJr:DogTag conjugates, we suggest isolating the monomeric version by size exclusion chromatography or using the C61S C262S mutant HaloTag7SS instead ***(8, 23)***.

2. AviTag-SnoopLigase and HaloTag7-SnoopLigase are soluble in a variety of buffers. However, SnoopLigase reaction works most efficiently in the absence of NaCl, so we recommend dialyzing the proteins into buffer without NaCl.

3. When purifying multimeric conjugates (e.g. ligating an antigen onto a heptameric scaffold) ***(11)***, it is important to use 100% biotinylated AviTag-SnoopLigase (streptavidin shift assay, section 3.3.) or well purified monomeric HaloTag7-SnoopLigase, to prevent SnoopLigase contamination in the eluted product. Furthermore, the SnoopLigase capture and elution protocols need to be adjusted for multimeric conjugates. Use double the amount of resin to capture SnoopLigase and increase the incubation period on the resin to 12 h. For imidazole elution, use 1 M

492 instead of 0.5 M imidazole in the wash buffer, and 3.5 M instead of 2 M imidazole in
493 the elution buffer.

494 4. SDS-PAGE densitometry as an analytical method has a limited dynamic range. If
495 substrate concentrations higher than 10 µM have been used in the conjugation
496 reaction, dilute the samples to be analyzed on SDS-PAGE to 10 µM substrate
497 concentration before loading on the gel.

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Figure legends:

Fig. 1. Schematic of SnoopLigase reaction. (A) SnoopLigase directs the formation of an isopeptide bond between DogTag and SnoopTagJr, allowing covalent conjugation of two proteins of interest, X and Y. The isopeptide bond is represented in red. Figure adapted from ***(8)***. **(B)** Chemical representation of isopeptide bond formation. Glu803 in SnoopLigase promotes isopeptide bond formation between Asn854 of DogTag and Lys742 of SnoopTagJr. Numbering is based on Protein Data Bank (PDB) 2WW8. **(C)** Potential applications of SnoopLigase-based peptide-peptide conjugation.

Figure 2. Amino acid sequence alignment of SnoopLigase, SnoopTagJr and DogTag. Key residues for reaction are underlined. Numbering is based on PDB 2WW8.

Figure 3. SnoopLigase reaction in a range of conditions. (A) pH-dependence. SnoopTagJr-AffiHER2 and SUMO-DogTag were ligated using SnoopLigase (10 μM each) for 2 h in Tris phosphate citrate (25 mM phosphoric acid and 25 mM citric acid, pH-adjusted with Tris) with indicated pH. **(B)** pH-dependence. SnoopTagJr-AffiHER2 and SUMO-DogTag were ligated using SnoopLigase (10 μM each) for 1.5 h at 4 °C in Tris borate with 15% (v/v) glycerol with the indicated pH. **(C)** Temperature-dependence. As in (B) at pH 7.25 at 4 to 37 °C. **(D)** Glycerol-dependence. As in (B) at pH 7.25 with 0−40% glycerol. **(E)** NaCl-dependence. As in (B) at pH 7.25 with 0-512 mM NaCl. **(F)** Effect of NaCl on time-course. As in (B) at pH 7.25 with or without 137 mM NaCl. Data are mean of triplicate ± 1 SD. Some error bars are too small to be visible. Data based on ***(8, 18)***.

Figure 4. Purification of SnoopLigase reaction product. (A) Cartoon of solid-phase SnoopLigase purification. SnoopTagJr- and DogTag-linked proteins are covalently

583 conjugated using biotin-SnoopLigase. Then streptavidin-agarose (SA-agarose) binds biotin-
584 SnoopLigase, unreacted proteins are washed away, and ligated proteins are eluted. Red
585 represents the isopeptide bond. **(B)** Cartoon of peptide competitor production. SUMO-
586 DogTag:SnoopTagJr covalent conjugate is produced by SnoopLigase and imidazole
587 conjugate elution. SUMO is then cleaved from this conjugate by SUMO-protease Ulp1
588 (gray). Incubation with Ni-NTA resin depletes the His-tagged SUMO and Ulp1, yielding
589 purified DogTag:SnoopTagJr peptide. **(C)** Analysis of product from SnoopLigase
590 purification using three different elution methods. SnoopTagJr-AffiHER2 and SUMO-
591 DogTag were ligated using biotin-SnoopLigase (10 μM each) for 16 h at 4 °C. Biotin-
592 SnoopLigase was captured with streptavidin-agarose, followed by glycine pH 2.0 or
593 imidazole, or peptide elution. Analysis by SDS-PAGE with Coomassie staining. Data
594 adapted from ***(8)***.
595
596

Figure 1

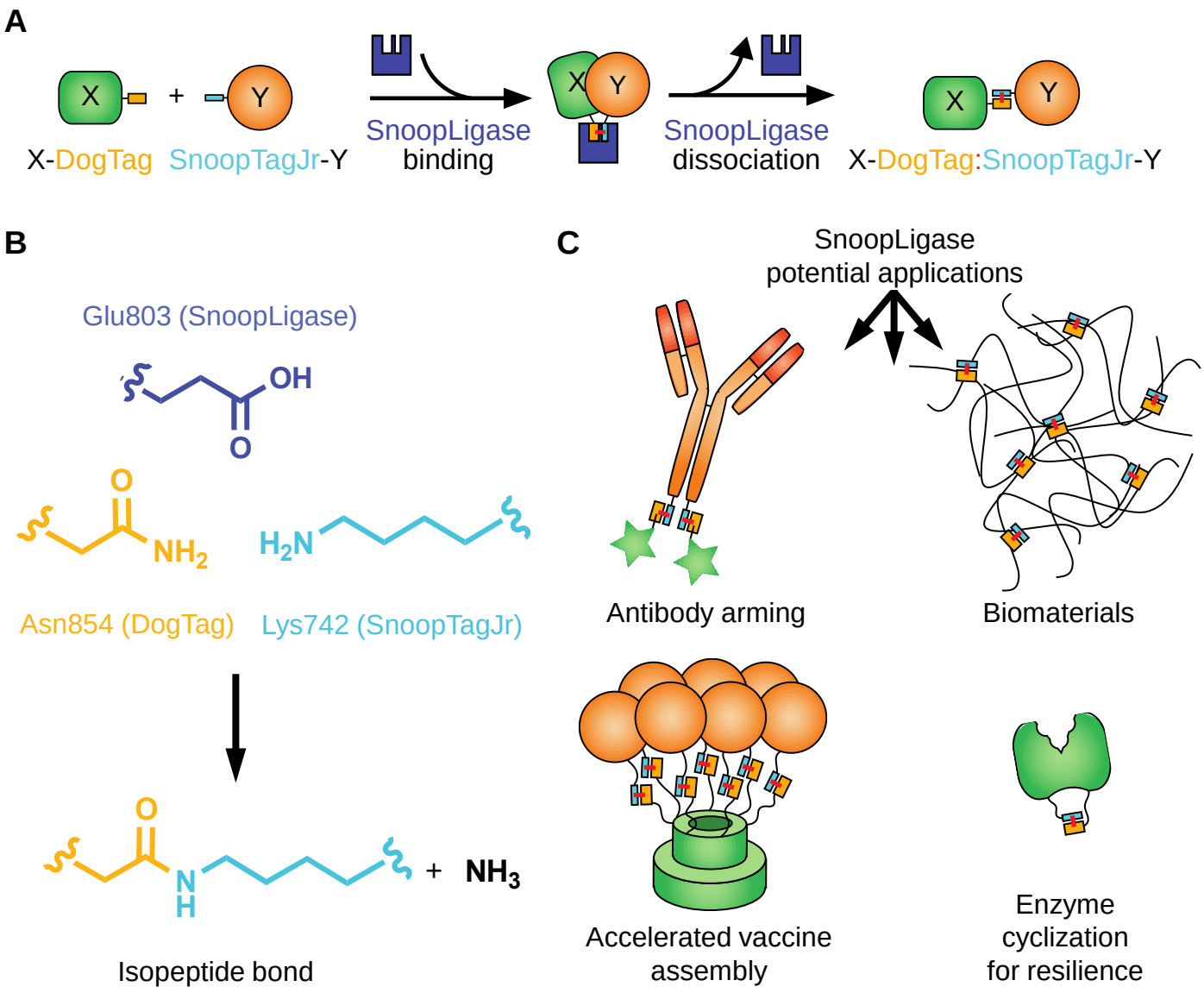

A
X
+
Y
X-DogTag SnoopTagJr-Y
SnoopLigase
binding
SnoopLigase
dissociation
X-DogTag:SnoopTagJr-Y
B
Glu803 (SnoopLigase)
OH
O
NH2
H2N
Asn854 (DogTag)
Lys742 (SnoopTagJr)
N
H
+ NH3
Isopeptide bond
C
SnoopLigase
potential applications
Antibody arming
Biomaterials
Accelerated vaccine
assembly
Enzyme
cyclization
for resilience

Figure 2

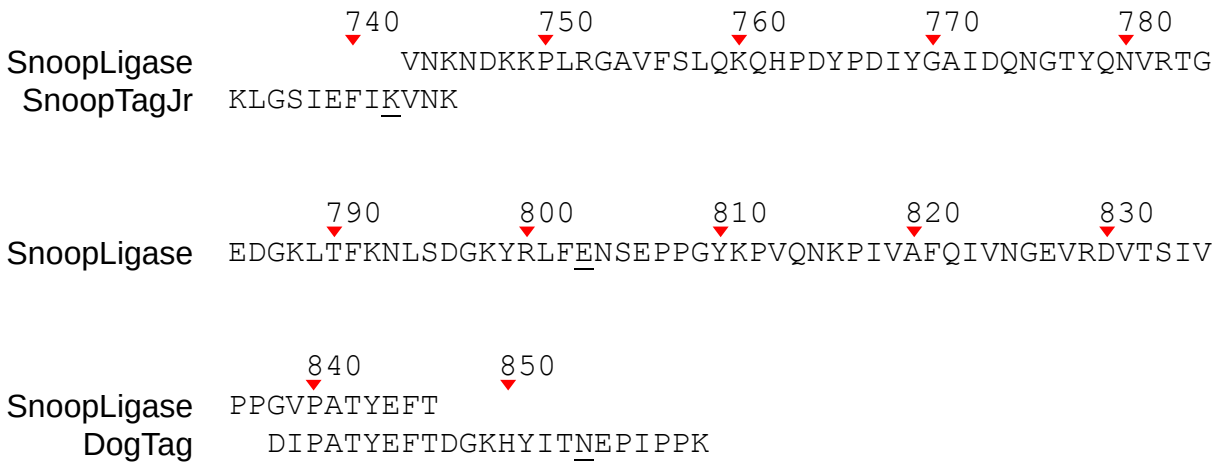

740
750
760
770
780
SnoopLigase VNKNDKKPLRGAVFSLQKQHPDYPDIYGAIDQNGTYQNVRTG
SnoopTagJr KLGSIEFIKVNK
790
800
810
820
830
SnoopLigase EDGKLTFKNLSDGKYRLFENSEPPGYKPVQNKPIVAFQIVNGEVRDVTSIV
840
850
SnoopLigase PPGVPATYEFT
DogTag DIPATYEFTDGKHYITNEPIPPK

Figure 3

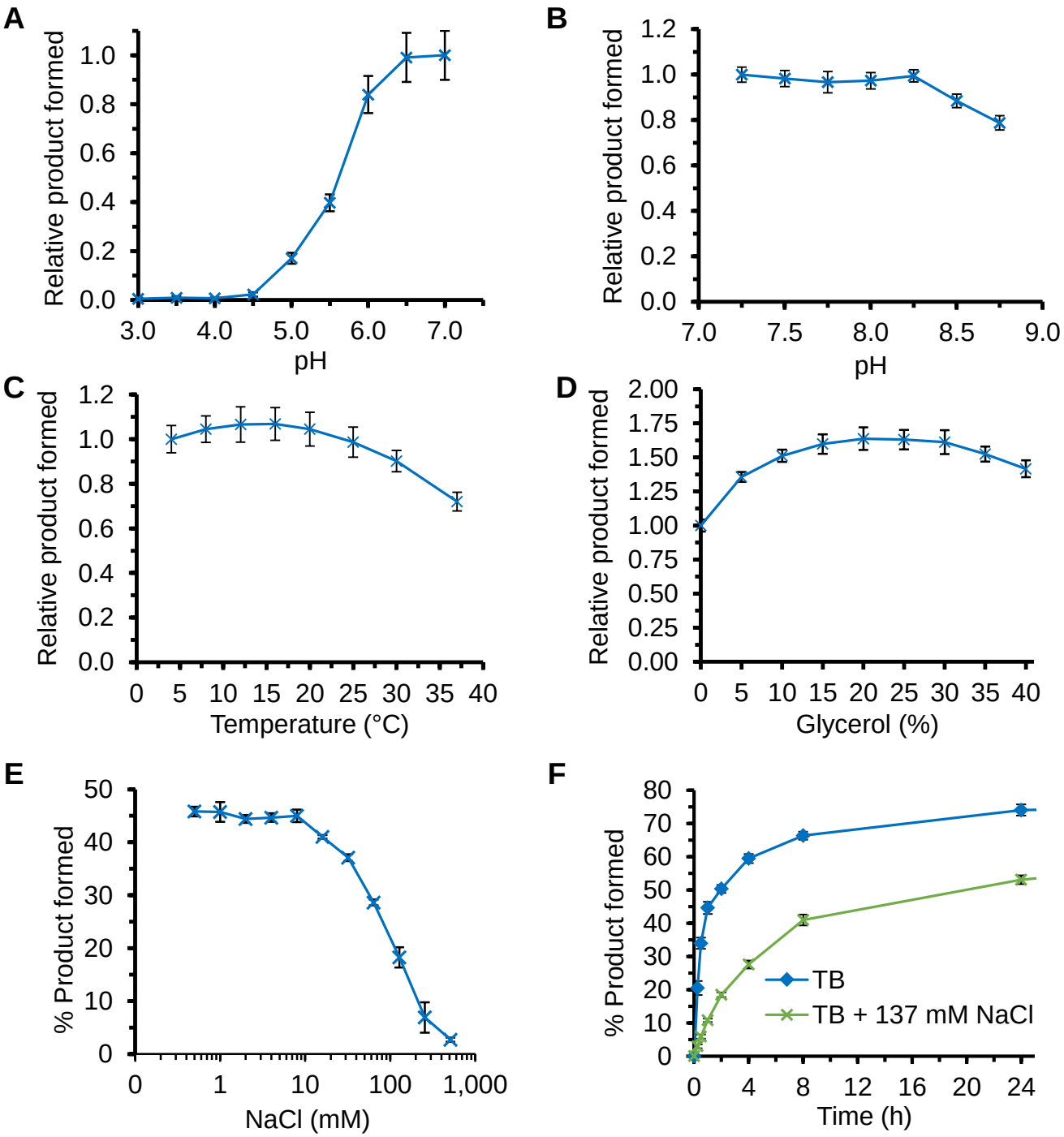
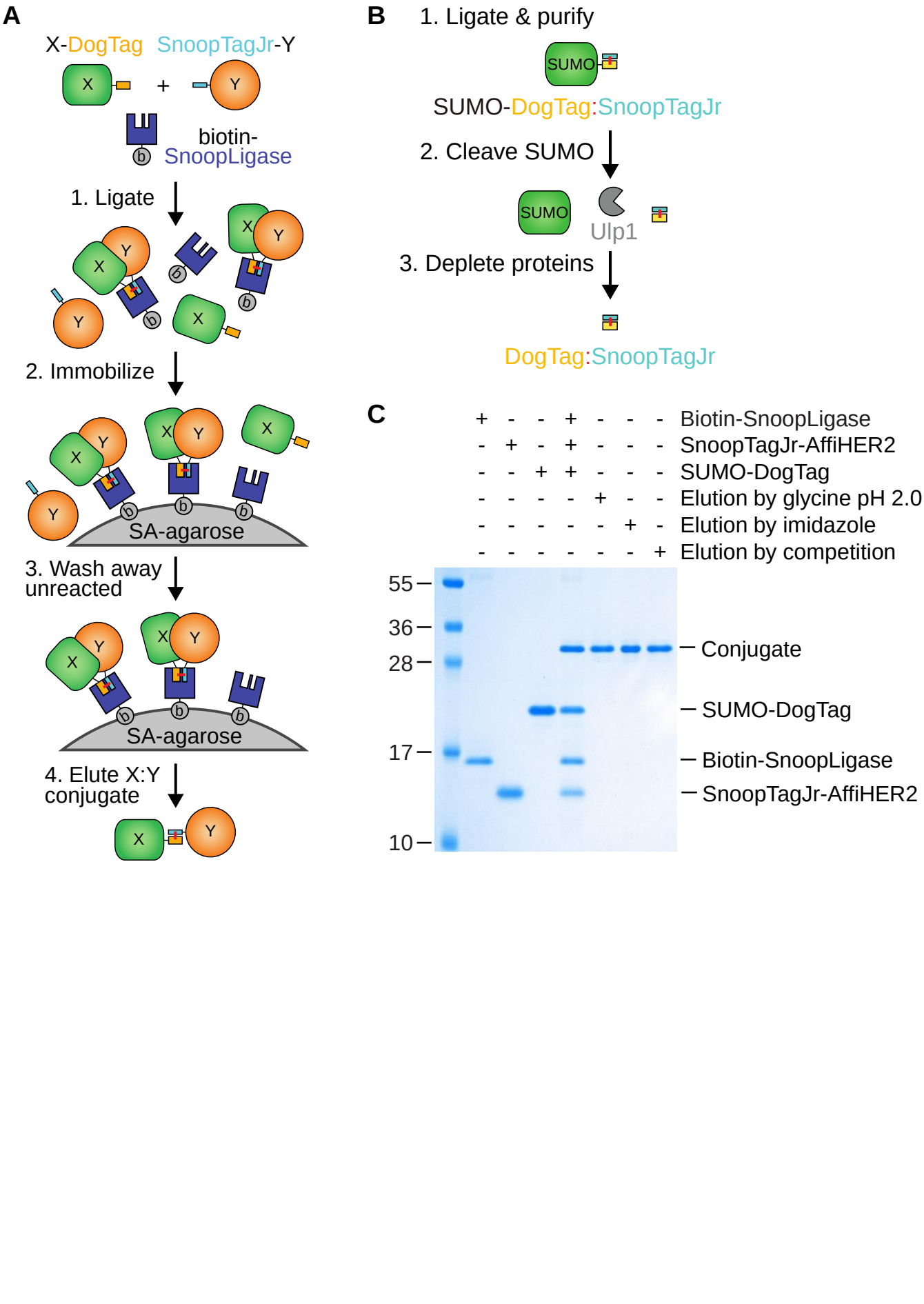

A
Relative product formed
1.0
0.8
0.6
0.4
0.2
0.0
3.0
4.0
5.0
6.0
7.0
pH
B
Relative product formed
1.2
1.0
0.8
0.6
0.4
0.2
0.0
7.0
7.5
8.0
8.5
9.0
pH
C
Relative product formed
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
Temperature (°C)
D
Relative product formed
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
30
35
40
Glycerol (%)
E
% Product formed
50
40
30
20
10
0
0
1
10
100
1,000
NaCl (mM)
F
% Product formed
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
Time (h)
TB
TB + 137 mM NaCl

Figure 4


A
X-DogTag SnoopTagJr-Y
X
+
Y
biotin-
SnoopLigase
1. Ligate
2. Immobilize
SA-agarose
3. Wash away
unreacted
SA-agarose
4. Elute X:Y
conjugate
B
1. Ligate & purify
SUMO
SUMO-DogTag:SnoopTagJr
2. Cleave SUMO
SUMO
Ulp1
3. Deplete proteins
DogTag:SnoopTagJr
C
+ - - + - - - Biotin-SnoopLigase
- + - + - - - SnoopTagJr-AffiHER2
- - + + - - - SUMO-DogTag
- - - - + - - Elution by glycine pH 2.0
- - - - - + - Elution by imidazole
- - - - - - + Elution by competition
55
36
28
17
10
Conjugate
SUMO-DogTag
Biotin-SnoopLigase
SnoopTagJr-AffiHER2