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Site-specific N-terminal labeling of proteins using sortase-mediated reactions

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ABSTRACT
For many proteins, the N- or the C-terminus make essential contributions to substrate binding, for protein-protein interactions, or for anchoring the proteins to a membrane. In other circumstances, at least one of the termini is buried within the protein, rendering it inaccessible to labeling. The possibility of selective modification of one of the protein’s termini may present unique opportunities for biochemical and biological applications. We describe sortase-mediated reactions to selectively label the N-terminus of a protein with a variety of functional groups. If sortase, the protein of interest, and a suitably functionalized label are available, the reactions usually require less than 3 hours.

INTRODUCTION
Modification of proteins with fluorophores or other compounds of interest enables creation of novel biological tools to study cellular pathways and molecular mechanisms \textsuperscript{1,2}. Conjugation of a toxic moiety or antigen to a targeting antibody expands the use of these proteins for cellular delivery purposes, while reducing toxic side effects \textsuperscript{3-5}. The key to tagging the protein of interest
without disrupting its structure or function is selective site-specific labeling. Specific labeling at the N-terminus of a protein is often the only option available, either because of the constraints imposed by the protein’s topology \(^6-^8\), or because the native C-terminus is essential for function (e.g., ubiquitin) \(^9,^{10}\) and/or cellular membrane anchoring \(^11,^{12}\), which renders cytosolic portions of proteins inaccessible to added sortase if labeling is to be conducted on intact cells.

Maleimide and NHS-ester derived probes are commonly used to modify proteins, as they are reactive with thiol and amino-groups of cysteine and lysine, respectively \(^13-^{15}\). While cysteines and lysines can be introduced at the N-terminus of a protein, the use of side chain-reactive probes lacks selectivity and may also compromise the active site of the protein being labeled. Genetic engineering approaches allow site-specific modification, but may interfere with protein structure \(^16,^{17}\). While the sortase-mediated labeling method described here overcomes many of these challenges, the possibility of unintended alterations that interfere with protein function should always be considered in design and interpretation.

Sortases are expressed by Gram-positive bacteria. They are essential in cell wall biosynthesis \(^18-^{21}\) and covalent attachment of proteins to the peptidoglycan cell wall. Additional background on sortase enzymes and a detailed protocol for C-terminal labeling using sortases can be found in (this issue of) Nature Protocols [ref]. In the specific instance of N-terminal labeling described here, the protein to be labeled is engineered with an exposed stretch of glycines or alanines at its N-terminus when using sortase A from *Staphylococcus aureus* or *Streptococcus pyogenes*, respectively. A peptide decorated with a functional group of choice (fluorophores, biotin, lipids, nucleic acids, carbohydrates, etc) and comprising a sortase-recognition motif LPXTG/A sequence (X being any amino acid \(^22-^{29}\)) at its C-terminus is then added to the reaction together with sortase. Sortase A cleaves between the Thr and Gly/Ala residues, forming a thioester intermediate with the peptide probe. Nucleophilic attack by the N-terminally modified protein of interest resolves the intermediate, resulting in the formation of a covalent bond between the peptide probe and the N-terminus of the protein (Fig.1).

Alternatively, depsi-peptides can be used for N-terminal labeling \(^29,^{30}\). Depsi-peptides feature an ester linkage between the threonine and glycine, instead of an amide peptide bond to yield a more effective leaving group. By using depsi-peptides, the probe concentration in the reaction can be lowered while maintaining yields.
ENGINEERING THE PROTEIN OF INTEREST

Proteins to be labeled at the N-terminus must display glycine (SrtA, *S. aureus*) or alanine (SrtA, *S. pyogenes*) residues at their N-terminus. Using standard molecular cloning methods, a stretch of one to five glycines or two to five alanines is introduced, immediately following the initial methionine, which is often removed by methionylaminopeptidase. The requisite number of glycines/alanines should be determined empirically, as it depends on the exposure of the N-terminus, although usually three residues suffice. A linker may be interposed between the N-terminal glycines/alanines and the remainder of the protein to improve accessibility.

In those cases where the initial methionine is not (completely) removed after protein synthesis, we use an alternative strategy to expose glycine residues at the N-terminus: a thrombin cleavage site (Leu-Val-Pro-Arg-Gly) is inserted to precede the glycine stretch. Thrombin cleaves between the Arg and Gly residues, thus ensuring that upon cleavage these glycines are exposed on the protein molecule to be labeled. **Critical Step:** Although thrombin is somewhat specific, we recommend that before choosing this option, the user confirm that the protein of interest is not itself directly susceptible to thrombin cleavage.

SYNTHESIS OF LPXTG/A-CONTAINING PEPTIDES

Many core facilities devoted to peptide synthesis can deliver modified peptides for use in N-terminal sortase-catalyzed labeling. Alternatively, commercial providers are a readily accessible source of these materials. However, manual synthesis is cost effective, expands the range of modifications possible for the individual user, and for those reasons it is included in this protocol. The following section provides a protocol for the synthesis of 5(6)-TAMRA, biotin labeled, and NHS ester linked probes for sortase A-mediated reactions. Attachment of fluorophores allows microscopy of internalized labeled proteins, while biotin attachment is especially useful for tagging proteins for pull-down experiments with streptavidin beads. For N-terminal reactions, the probe is linked to the N-terminus of a LPETGG peptide for *S. aureus* and a LPETAA peptide for *S. pyogenes* sortase A. Although any amino acid can be placed between the proline and threonine, we prefer glutamic acid or other polar amino acids to aid in precipitation of crude peptide after cleavage from the solid phase resin. To reduce the time required for synthesis and purification, Fmoc-Lys(biotin)-OH, Fmoc-Lys(5-TAMRA)-OH, and other pre-conjugated building blocks can be obtained commercially. These building blocks should be coupled to the leucine residue of the sortase recognition sequence.
MATERIALS
REAGENTS

- N,N-Dimethylformamide (DMF; Applied Biosystems, cat. no. GEN002007) *Caution* Flammable/toxic
- Acetonitrile (ACN; JT Baker Analytical, cat. no. 9017-03) *Caution* Flammable/toxic
- N-methyl-2-pyrrolidone (NMP; Sigma Aldrich, cat. no. 328634-2L) *Caution* Flammable/irritant/toxic
- Diisopropylethylamine (DIPEA; Fisher BioReagent, cat. no. BP592500) *Caution* Highly flammable/corrosive
- Dichloromethane (DCM; VWR, cat. no. JT9305-3) *Caution* Carcinogen
- Dimethylsulfoxide (DMSO; EMD Chemicals Inc, cat. no. MX1458-6) *Caution* Irritant/flamable
- Piperidine (Sigma Aldrich, cat. no. 104094) *Caution* Flammable/corrosive
- Pyridine (Sigma Aldrich, cat. no. 270407) *Caution* Highly flammable/toxic
- Diethyl ether (EMD Chemicals Inc, cat. no. EX0185-8) *Caution* Highly flammable/harmful
- Trifluoroacetic acid (TFA; Sigma Aldrich, cat. no. T6508) *Caution* Strongly corrosive/toxic
- Triisopropylsilane (TIS; Sigma Aldrich, cat. no. 233781) *Caution* Flammable.
- 5(6)-carboxy-tetramethylrhodamine (5(6)-TAMRA; Novabiochem, cat. no, 815030)
- Biotin (Sigma Aldrich, cat. no. B4501)
- Fmoc-Ala-OH (Novabiochem, cat. no. 852003)
- Fmoc-Lysine(Mtt)-OH (EMD biosciences, cat. no. 04-12-1137)
- Fmoc-Cys(Trt)-OH (Novabiochem, cat. no. 852008)
- Fmoc-Gly-OH (Novabiochem, cat. no. 852001)
- Fmoc-Thr(tBu)-OH (Novabiochem, cat. no. 852000)
- Fmoc-Glu(OtBu)-OH(Novabiochem, cat. no. 852009)
- Fmoc-Pro-OH (Novabiochem, cat. no. 852017)
- Fmoc-Leu-OH (Novabiochem, cat. no. 852011)
- Fmoc-ε-caproic acid (Novabiochem, cat. no. 852053)
2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; Novabiochem, cat. no. 851006) Caution Irritant/harmful

Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; Novabiochem, cat. no. 851009) Caution Irritant/harmful

Ninhydrin (Eastman, cat. no. 2495) Caution Harmful

Potassium cyanide (Sigma-Aldrich, cat. no. 31252) Caution Highly toxic/hazardous to the environment

Phenol (J.T. Baker, cat. no. 2858-04) Caution Toxic/corrosive

Rink amide resin SS, 100-200 mesh, 1% DVB (Advanced Chemtech, cat. no. SA5030)

EQUIPMENT

3 mL syringe equipped with fritted glass filter (New England Peptide, AC0-003)
Glass column with a fritted glass filter bottom
Wrist Action shaker (St. John Associate Inc.)
Swinging bucket centrifuge (Beckman)
HPLC system (Agilent 1100 series)
Reverse phase C18 column (Waters Delta Pak 15 µm, 100 Å, 7.8 x 300 mm)
Liquid chromatography/mass spectrometry LC/MS
Nuclear Magnetic Resonance (NMR) spectrometer
Vacuum line
Lyophilizer
Microfuge tubes
Test tube racks
Syringes
Graduated cylinders
Heating block
Erlenmeyer flasks
50 mL polypropylene conical tubes (Corning)

REAGENT SETUP

Kaiser test solution A dissolve 500 mg of ninhydrin in 10 mL ethanol.
• **Kaiser test solution B** dissolve 80 g of phenol in 20 mL of ethanol.
• **Kaiser test solution C** dissolve 1.3 mg potassium cyanide (20 μmol) in 20 mL of water. Add 2 mL of the potassium cyanide solution to 100 mL of pyridine.
• **20% piperidine in NMP** mix 20 mL piperidine and 80 mL NMP.
• **Cleavage cocktail** (95% TFA, 2.5% H₂O, and 2.5% TIS) mix 4.75 mL TFA, 125 μL H₂O and 125 μL TIS
• **Buffer A HPLC** 0.1% TFA in H₂O
• **Buffer B HPLC** 0.1% TFA in ACN
• **Buffer A LC/MS** 0.1% formic acid in H₂O
• **Buffer B LC/MS** 0.1% formic acid in ACN

**Kaiser Test TIMING 5 min**
Monitor peptide couplings by performing a Kaiser test 34.

1. Mix 2 μL of solution A, 2 μL of solution B and 4 μL of solution C in a microcentrifuge tube.
2. Add 5-10 dried beads of the rink amide resin to the mixture at room temperature.
3. Heat the tube to 95˚C for 3 min. A dark blue color indicates incomplete coupling.

*Note:* This test works on primary amines and does not work for testing the attachment of an amino acid to a Pro residue. Alternative methods such as the acetaldehyde/p-chloroanil test or microcleavage can be used to monitor these reactions 35,36.

**Microcleavage Test TIMING 45 min**

1. Prepare a 30 μL solution of 95% TFA and 5% H₂O.
2. Add 5-10 beads to the solution and cleave for 20 min.
3. Take 5 μL of the supernatant and dilute in 30 μL of H₂O and analyze by mass spectrometry.

**A) TAMRA-LPETGG Probe**

*Note:* Use Fmoc-Ala-OH in place of Fmoc-Gly-OH to make probes for S. pyogenes sortase A

**Resin Preparation TIMING 15 min**
Add 100 µmol of Rink amide resin (167 mg) into a capped glass column with a fritted glass filter bottom, solvate the resin in dichloromethane (DCM) (7 mL) by shaking for 15 min in a wrist-action shaker and remove the DCM by vacuum filtration.

**Deprotection** TIMING 30 min

Add 20% piperidine solution in N-methyl-2-pyrrolidone (NMP) (7 mL) and shake for 15 min to remove the resin’s Fmoc protecting groups.

Remove the piperidine solution by vacuum filtration and wash the resin three times with NMP (7 mL), three times with DCM (7 mL) and an additional time with NMP.

**Coupling Reaction** TIMING 2-3 h until pause point, 3.5 h per coupling cycle

Dissolve Fmoc-Gly-OH (89 mg, 300 µmol), HBTU (114 mg, 300 µmol), and DIPEA (104 µL, 600 µmol) in NMP (7 mL) and add to the resin. Shake the suspension for 2 h at room temperature.

Remove the reaction solution by vacuum filtration and wash the resin three times with NMP (7 mL) and three times with DCM (7 mL). Confirm the coupling reaction by performing a Kaiser test.

*Note:* If the reaction is incomplete repeat steps 4-5 with half the amount of reagents used for a standard coupling and shake for 1 h.

**PAUSE POINT:** The resin can be stored at 4 °C after drying under vacuum. **CRITICAL STEP:** At this stage, store peptides in their Fmoc-protected form.

Repeat steps 1-5 with Fmoc-Gly-OH (89 mg, 300 µmol), Fmoc-Thr(OtBu)-OH (119 mg, 300 µmol), Fmoc-Glu(OtBu)-OH (127 mg, 300 µmol), Fmoc-Pro-OH (101 mg, 300 µmol), Fmoc-Leu-OH (106 mg, 300 µmol), Fmoc-ε-aminocaproic acid (85 mg, 300 µmol).

*Note:* The Kaiser test does not work for verifying the extent of the Leu coupling, since the N-terminus of Pro is a secondary amine. To test this coupling reaction, one can use the chloroanil test or microcleavage. Note that the orthogonal protecting groups may not be fully removed during this abbreviated cleavage.

After removing the Fmoc on the ε-aminocaproic acid residue, add a solution of 5(6)-TAMRA (52 mg, 120 µmol), PyBOP (63 mg, 120 µmol), and DIPEA (42 µL, 240 µmol) and shake overnight at room temperature. To prevent quenching of the fluorophore, wrap the column in aluminum foil.

Repeat step 5 and perform the Kaiser test to check the TAMRA coupling.
**Cleavage from Resin** **TIMING 3 h**

9 Suspend the resin in cleavage solution consisting of 95% TFA, 2.5% H2O, and 2.5% TIS (5 mL) for 2 h at room temperature.

10 Elute the cleavage solution into 90 mL of ice cold (-20 °C) diethyl ether and rinse the resin with an additional 3 mL of the cleavage solution into the ether.

11 Store the ether solution at -20 °C for 20 min to precipitate the peptide. Centrifuge the suspension at 1,900 g for 15 min at 4 °C, decant the supernatant and gently evaporate the remaining ether under reduced pressure.

**Pause point:** The crude peptide can be stored as a solid at -20 °C.

**Critical step:** Verify the identity and purity by LC/MS analysis (linear gradient 5→45% LC/MS buffer B over 10 min). If LC/MS shows that the crude peptide is of sufficient purity, the next steps (12-14) may be omitted and the peptide may be used directly in sortase reactions.

**HPLC purification**

12 Dissolve the dried peptide in H2O (2 mL) and centrifuge at 14,000 rpm for 10 min in a tabletop centrifuge to remove particulate matter.

*Note:* Up to 50% of tert-butanol may be added to peptides that do not dissolve in pure H2O. Also spin filters or syringe filters may be used to remove particulate matter.

13 Purify the centrifuged supernatant by reverse-phase HPLC on a C18 column using a 10-75% buffer B gradient.

14 Analyze the fractions for product by LC/MS and lyophilize the desired fractions to dryness.

*Note:* TAMRA containing probes consist of a mixture of regio-isomers that will likely result in two product peaks during reverse phase HPLC purification. The different isomers have no effect on labeling.

**Critical step:** Verify the identity and purity by LC/MS analysis (linear gradient 5→45% LC/MS buffer B over 10 min) and NMR spectroscopy.

**Pause point:** The lyophilized peptide can be stored at -20 °C indefinitely.

**B) Biotin-LPETGG Probe**

1 Use the same reaction conditions as for synthesis of the TAMRA-LPETGG probe through the Fmoc deprotection step of the Leu residue. At this point, add a solution of biotin (74 mg, 300
µmol), HBTU (114 mg, 300 µmol) and DIPEA (104 µL, 600 µmol) in NMP (7 mL); shake for 2 h.

2 Remove the reaction solution by vacuum filtration, wash the resin, and check the success of biotin coupling with a Kaiser test (remaining free amines).

3 Cleave the product from the resin as indicated in steps 9-11 for the TAMRA probe.

4 Purify by reverse phase HPLC as indicated in steps 12-14 of the TAMRA probe

**Critical step:** Verify the identity and purity by LC/MS analysis (linear gradient 5→45% B in 10 min) and NMR spectroscopy.

**Pause point:** The lyophilized peptide can be stored at -20 °C indefinitely.

C) Other LPETGG Probes

For the addition of other functional groups or acid-labile substituents, we recommend using probes conjugated via NHS ester couplings.

1 Synthesize LPETGG as described above.

2 Remove the Fmoc on the Leu according step 2 of the general method.

3 Cleave the peptide from the resin as described in steps 9-11.

4 Purify the peptide by HPLC, steps 12-14 or if of sufficient purity as indicated by LC/MS, use the crude peptide directly after precipitation from the cleavage solution into diethyl ether.

5 Dissolve the lyophilized peptide in DMSO.

6 Add three equivalents of the LPETGG peptide in DMSO to the NHS ester probe and add 5 equivalents of DIPEA.

7 Incubate the reaction for 12 h at RT.

8 Dilute the reaction to 25% DMSO with H2O and purify by HPLC as indicated above.

*Note:* For fluorescent probes, protect the reaction mixture from light by covering it in aluminum foil.

N-TERMINAL SORTAGGING REACTIONS PERFORMED IN SOLUTION

**MATERIALS**

The protocol for expression and purification of the various sortase A is found (elsewhere) in (this issue of) Nature Protocols.

**REAGENTS**
- Purified target oligoglycine/alanine protein in buffer (no phosphate-based buffer if a Ca\textsuperscript{2+}-dependent sortase A is used)
- Purified sortase A in buffer (no phosphate-based buffer if a Ca\textsuperscript{2+}-dependent sortase A is used)
- LPETGG- or LPETAA-based peptide probes (if using \textit{S.aureus} or \textit{S.pyogenes} sortase A, respectively) stock solution: 5 mM in DMSO or water (10\times stock). If a polypeptide is used, then dissolve it in buffer (no phosphate-based buffer if a Ca\textsuperscript{2+}-dependent sortase A is used)
- 4x loading LDS-buffer (Invitrogen, cat. no. LC5800)
- Common reagents for SDS-PAGE analysis (10 or 12\% acrylamide gels)
- Tris Hydrochloride, Tris-HCl (American Bioanalytical, cat. no. AB02005-05000)
- Sodium Chloride, NaCl (American Bioanalytical, cat. no. AB01915-10000)
- Calcium Chloride Dyhydrate, CaCl\textsubscript{2}•H\textsubscript{2}O (Mallinckrodt Chemical, cat. no. 4160)
- Brilliant blue R (Sigma-Aldrich, cat. no. B7920)
- Methanol, MeOH (EMD, cat. no. MX0488-1)
- Acetic acid, AcOH (VWR, cat. no. BDH3094)
- Ethanol, EtOH (Pharmco-AAPER, cat. no. 111000190)

**EQUIPMENT**
- Micropipettes
- 1.5 mL centrifuge tubes
- Centrifuge for 1.5 mL centrifuge tubes
- 37 °C water bath or thermocycler
- Equipment for SDS-PAGE, western-blot, Coomassie staining
- Fast protein liquid chromatography (FPLC) system with size exclusion and ion exchange columns
- Liquid chromatography/mass spectrometry (LC/MS)
- Amicon ultra spin concentrators 10 kDa NMWL (Millipore)

**REAGENT SETUP**
- **Sortase buffer:** 500 mM Tris-HCl pH 7.5, 1.5 M NaCl, 100 mM CaCl\textsubscript{2} (not required if using a Ca\textsuperscript{2+}-independent sortase) (10\times stock).
- **Size exclusion chromatography buffer:** 50 mM Tris-HCl pH 7.5, 150 mM NaCl.
**Coomassie blue staining**: Dissolve 1.25 g Brilliant Blue R in a mixture of methanol (200 mL), water (250 mL) and acetic acid (50 mL). Store in a dark container at RT.

**Destaining solution**: Mix water, ethanol and acetic acid in a ratio of 6:3:1. Store at RT.

PROCEDURE

A) **Setting up the reaction conditions** TIMING 8 h

(i) Mix 0.5-1 mM LPETG/A-containing probe, 10-50 μM target protein, 20-150 μM sortase A in 1× sortase buffer (final concentrations). The controls to be included are: target protein only, sortase only, oligoglycine probe only, target protein and sortase, target protein and oligoglycine probe, sortase and oligoglycine probe.

(ii) Incubate the reactions at RT or at 37 °C. Take 1 μl aliquots at 15’, 1, 3, 5 h. Add 1× SDS-gel loading buffer to the aliquots to stop the reaction and boil 2 min.

**PAUSE POINT**: The 1 μl aliquots can be frozen at -20 °C until further analysis.

(iii) Analyze the 1 μl aliquots by SDS-PAGE (10% or 12% acrylamide for substrates in the 12-80 kDa range) followed by Coomassie staining.

**Note**: Some products and starting material proteins have similar molecular weights. A gradient gel of appropriate size and porosity may achieve better separation.

**Anticipated result**: A successful sortase reaction results in the formation of the acyl-enzyme intermediate. Because the concentration of the protein to be labeled is never sufficiently high to resolve all covalent sortase-peptide intermediates, the sortase-peptide intermediate will be detected in all the reactions. Also, the acyl-enzyme intermediate is rather resistant to reducing and denaturing conditions. Thus, one can detect sortase-peptide adducts by fluorescent scanning or western-blot if the LPETG/A peptide contains a tag (e.g., dye or biotin). A sortase reaction often yields a reaction product with a distinct mobility from that of the input substrate and the hydrolysis product. The ability to distinguish the various intermediates critically depends on the MW of the anticipated products and on the gel systems used to analyze them. In cases where the MW difference is too small to be detected by gel, LC/MS analysis can be used to monitor the reaction and provide an estimated yield.

(B) **Purification**
1 Load the sortase reaction into a spin concentrator with an appropriate MW cutoff and centrifuge to remove unreacted (low MW) probe.

2 As needed, purify the protein of interest by size exclusion chromatography using Superdex 75 or 200 resin, depending on its Stokes’ radius. Analyze fractions by SDS-PAGE and/or LC/MS. Note: If the protein is sufficiently pure at this point, it can be concentrated and is ready for use.

3 If further purification is needed, concentrate the product-containing FPLC fractions with a spin concentrator. Then purify the product by ion-exchange chromatography (Mono Q or Mono S resin depending on the charge distribution of the molecule of interest) with a 0 - 1 M gradient of NaCl. Analyze fractions by SDS-PAGE and/or LC/MS and concentrate the product-containing fractions for use or storage. Note: In those rare instances where sortase has properties similar to the product of interest, an initial Ni-NTA affinity chromatography step can be added since the sortase has a His$_6$ tag and will bind to the resin. If the protein of interest has a His$_6$ tag, this added purification will not work and a different tag should be used.
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<th>Possible cause</th>
<th>Solution</th>
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<td>No or low protein labeling</td>
<td>Not enough LPXTG/LPXTA probe added to the reaction</td>
<td>Reaction conditions have to be determined ad-hoc. Increase the amount of probe to 10 mM and/or decrease the amount of protein nucleophile</td>
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<td>pH of the reaction buffer not compatible with sortase activity</td>
<td>Ensure that the pH of the reaction buffer is appropriate. Check the pH of the stock solutions. Especially the pH of the probe solutions can be low, due to residual traces of TFA. Lyophilize the probe solution multiple times with water to remove TFA. Neutralize the probe solution with aq NaHCO₃ or by adding additional buffer if the pH remains too low</td>
</tr>
<tr>
<td>Proteolysis of sortase</td>
<td>Verify the integrity of sortase upon reaction by Coomassie staining and/or anti-His blot. The amount of sortase before and after reaction should be equal. If not, consider the presence of a contaminating protease, which most probably co-purified with the protein of interest. We recommend further purification of the protein nucleophile.</td>
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<tr>
<td>N-terminal is not adequately exposed</td>
<td>Increase the length of the poly glycine/alanine nucleophile. If the initial Met is not removed during protein</td>
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Wrong strain of sortase is used

Sortase is inactive

Not enough sortase added to the reaction

Detection of a white fluffy precipitate during the sortase-labeling reaction

Protein precipitates during the labeling reaction

Ca\(^{2+}\) precipitate

High concentration of protein, especially when attempting protein-protein fusions

The protein of interest

expression a thrombin cleavage site should be added prior to the nucleophilic Gly/Ala residue.

Ensure that you use sortase A from S. pyogenes for alanine-based nucleophiles and sortase from S. aureus for glycine modified proteins.

Test the preparation of sortase using N-terminal modified GFP as the nucleophile. Too high concentration of DMSO in reaction mixture, originating from peptide stock solution.

Sortase concentration has to be titrated for each substrate to be labeled.

Increase the substrate concentration and/or decrease the amount of protein to be labeled.

Do not use phosphate buffers if working with a Ca\(^{2+}\)-dependent sortase.

Optimize reaction temperature and incubation time. Less protein may be sufficient to achieve the same reaction yield without precipitation.

Perform the labeling reaction at RT and
Product and starting material comigrate on gel. MW of product and starting material are similar. Precipitates at high temperatures. Extend the reaction time. 10% glycerol may also prevent precipitation. Analyze by LC/MS or use a gradient gel.
Figure 1. **N-terminal labeling of proteins.** A peptide probe containing the LPXTG sortase recognition motif and a functional moiety of choice is incubated with *S. aureus* Sortase A. Sortase cleaves the Thr-Gly bond in the motif and via its active site Cys residue forms an acyl intermediate with Thr in the peptide. A protein N-terminally engineered with a series of glycine residues then resolves the intermediate, thus regenerating the active site cysteine on sortase and conjugating the peptide probe to the N-terminus of the protein.
REFERENCES


