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## Site-specific protein modification using immobilized sortase in batch and continuous-flow systems

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### Abstract

Transpeptidation catalyzed by sortase A allows the preparation of proteins that are site-specifically and homogeneously modified with a wide variety of functional groups, such as fluorophores, PEG moieties, lipids, glycans, bioorthogonal reactive groups and affinity handles. This protocol describes immobilization of sortase A on a solid support (sepharose beads). Immobilization of sortase A simplifies downstream purification of a protein of interest after labeling of its N- or C-terminus. Small batch and larger scale continuous flow reactions require only a limited amount of enzyme. The immobilized enzyme can be reused for multiple cycles of protein modification reactions. The described protocol also works with a Ca<sup>2+</sup>-independent variant of sortase A with increased catalytic activity. This heptamutant variant of sortase A (7M) was generated by combining previously published mutations and this immobilized enzyme can be used for the modification of calcium-sensitive substrates or in instances where low temperatures are needed. Preparation of immobilized sortase A takes 1–2 days. Batch reactions take 3–12 hours and flow reactions proceed at 0.5 mL per hour, depending on the geometry of the reactor used.

### Keywords

Protein synthesis; flow reactor; immobilized enzyme; protein modification; continuous flow reaction; batch reaction; sortase; calcium independent sortase A

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### Author contributions

MDW, CPG and HLP conceived of the idea; MDW, TW, CST, CPG and AEMB performed the immobilization reactions and optimized the sortase reactions in batch and in flow; JRI developed the heptamutant sortase and ZL and LK assisted in sortase and protein production; MDW, TW, CST, CPG, JRI and HPL wrote the manuscript; SDG and HLP supervised the project.

### Competing financial interest.

The authors declare no competing financial interest.

## CATEGORIES

Isolation; separation and purification; Protein functionalization; Chemical Modification

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## Introduction

Proteins are attractive therapeutics, diagnostic tools and imaging agents, but their application as such often requires the installation of additional, non-genetically encoded functional groups. Various methods for protein labeling exist, but these often lack specificity and thus may compromise protein function. Attachment of polyethylene glycol (PEGylation), incorporation of dyes and other functional groups, as well as the generation of antibody-drug conjugates usually rely on modification of endogenous cysteine or lysine residues, which can lead to heterogeneous products, unspecific labeling and loss of protein function. For example, PEGylation, while increasing circulatory half-life<sup>1</sup>, may cause a decrease or even loss of biological activity due to the chemical modification(s) imposed<sup>2</sup>. Such results have also been observed when preparing antibody-drug conjugates<sup>3</sup>. Heterogeneity after labeling may confound accurate dosing and complicate drug approval and pharmaceutical production.

The transpeptidation reaction catalyzed by sortase A allows site-specific protein modification with functional groups of choice, such as dyes<sup>4</sup>, biotin<sup>4</sup>, click-handles<sup>5</sup>, nucleic acids<sup>6</sup>, carbohydrates<sup>7</sup>, lipids<sup>8</sup>, and PEG moieties<sup>9</sup>. The modification is installed in a controlled and site-specific fashion, in near-quantitative yields. Engineered variants of Sortase A with increased catalytic activity allow more rapid protein modification at lower temperatures, enabling a wider range of applications<sup>10</sup>.

## Advantages of sortase-mediated reactions using immobilized sortase

A sortase-mediated reaction is comprised of enzyme, substrate and the desired functional group to be installed. This reaction allows functionalization of a (poly)peptide of choice at its N-terminus<sup>11-13</sup>, C-terminus<sup>4,14</sup>, at a specific internal site<sup>15,16</sup>, or at both termini using two orthogonal sortases<sup>11</sup>. Upon completion of the reaction, the labeled product requires further purification. To this end, both sortase and the input substrate can be engineered to contain an affinity tag, most commonly His<sub>6</sub>, at their C-termini. This tag is lost upon successful transpeptidation of substrate, so that His-tagged sortase A and unreacted substrate can be removed in a single step by affinity chromatography on a Ni-NTA matrix. Gel filtration can then be used for further purification of the product by removal of excess free peptide<sup>17</sup>. Affinity chromatography cannot always resolve input material from labeled product, either because both species contain an affinity tag (as applied to N-terminal labeling reactions<sup>11</sup>) or because the intrinsic properties of substrate and/or product cause non-specific binding to the affinity resin<sup>15</sup>. In these situations, product purification by gel filtration and/or ion exchange chromatography are possible alternatives. As described in this report, the use of immobilized sortase accelerates the purification process and obviates the need for removal of sortase.

A concern frequently raised regarding sortase-mediated reactions is the amount of sortase A required, as enzyme and substrate are typically used at near equimolar concentrations. This is of particular worry for large-scale reactions, which therefore require sizable amounts of enzyme, whose presence can complicate downstream purification steps in large-scale applications. Immobilizing sortase A on a solid support can overcome these technical limitations as it allows using sortase A in continuous-flow reactions. The prepared sortase columns can be re-used, affording scale-up of the reactions and minimizing batch-to-batch variation. The amount of sortase A required for large-scale reactions may thus be decreased and downstream processing is simplified. Even for small-scale batch reactions, the use of immobilized sortase facilitates analysis of reactions by simplifying the composition of the mixture of proteins in solution. Sortase A mutants with increased activity can further decrease the amount of sortase needed for large-scale reactions. They can be used at lower concentrations, either in solution or when immobilized. Moreover, these mutant sortases can function at reduced temperatures (4 °C), compared to 20–37 °C used for sortagging<sup>10</sup> with the wild type enzyme facilitating labeling of sensitive proteins. However, both wild type Sortase A and the evolved pentamutant sortase (5M) require Ca<sup>2+</sup> as a cofactor, compromising their utility in applications in the presence of Ca<sup>2+</sup> or in combination with buffers such as phosphate-buffered saline. We recently described a sortase A variant, termed heptamutant sortase A (7M), which combines the pentamutant version with increased catalytic activity with mutations that render sortase calcium independent<sup>18</sup>, resulting in an enzyme with increased activity and no longer requiring Ca<sup>2+</sup> (Fig. 2)<sup>19</sup>. The immobilized variant of this enzyme facilitated straightforward modification of aerolysin and greatly simplified the purification of the modified product.

This protocol describes immobilization of *S. aureus* sortase A and its variants on Sepharose beads for modification of either the N- or C-terminus of a substrate in small batch or in continuous flow mode for larger-scale reactions (see Fig. 1 for overall schematic). The continuous flow mode is most effectively applied to C-terminal labeling reactions, where substrate and modifying nucleophile are provided in solution, but may also be applied to N-terminal reactions by pre-reacting the resin with an LPXTG-containing probe to boost yields. Prior to performing conjugation reactions using the continuous flow system, suitability of the designated protein as a substrate for sortase A should be evaluated in small batch reactions. Detailed protocols for production of sortases, sortase substrate design, peptide synthesis, and sortase reactions in solution are provided in previous protocols<sup>20,21</sup>. Sortase A immobilization has been previously described using a different strategy than detailed in this protocol<sup>22</sup>. The protocol outlined here offers a more rapid, covalent immobilization procedure, as well as the use of the calcium-independent heptamutant sortase A to increase the utility of the reaction. In immobilized form, the heptamutant sortase enables a reduction in the amount of enzyme needed, while accelerating the reaction in a wide array of buffers.

### Limitations of the continuous-flow method

Purification and immobilization of sortase, as well as packing a column or cartridge with the sortase-conjugated beads for use in a continuous-flow reaction takes time. However, the ability to reuse immobilized sortase saves both time and resources. Flow reactions for N-

terminal labeling require slightly longer reaction times due to the pre-equilibration of the column with an LPXTG-containing probe. Non-specific binding of the substrate of interest or of the label itself may interfere with the immobilized sortase labeling reaction if blocking procedures are not carefully followed. In our experience, non-specific binding is negligible (Fig. 3 and 4). Nonetheless, the occurrence and extent of non-specific binding must be determined empirically for every reaction. A higher concentration of immobilized sortase may be required to achieve a similar degree of labeling when compared to soluble sortase, as not all sortase active sites may be equally accessible as a consequence of immobilization.

## Experimental design and general considerations

Sortase A or derivatives with increased activity and/or  $\text{Ca}^{2+}$  independence, including the heptamutant sortase A, are expressed and purified as described<sup>20</sup>, followed by immobilization on commercially available cyanogen bromide-activated Sepharose. Free amines of sortase A react with the activated resin, forming an iso-urea or an imidocarbonate derivate (Fig. 1A). For small batch reactions, the sortase-containing beads are mixed with substrate and labeled in a microcentrifuge tube and reacted for 3–12 hrs at 37 °C. The heptamutant sortase A described here can be used at reduced temperatures, as low as 4 °C. Small scale test reactions are performed to optimize the labeling time, labeling temperature and probe-to-substrate ratio. These small batch reactions are also more rapid for small scale labeling reactions. For larger scale preparative reactions we pack a column with immobilized sortase A (10 mg of enzyme in a 5 x 70 mm column), and equilibrate it with sortase reaction buffer at 37 °C. The optimal labeling temperature and probe-to-substrate ratio as determined in the batch reaction provide good starting conditions for the optimization of the flow reaction. We vary the flow-rate and determine the conversion of the substrate by taking aliquots and analyzing them by SDS-PAGE.

For C-terminal labeling of a protein on a larger scale, we load the column with the desired substrate protein bearing a sortase-recognition motif LPXTG at its C-terminus (where X can be any amino acid, although typically glutamic acid is used) (Fig. 5) together with an oligoglycine probe harboring the functional group to be conjugated onto the protein.

For N-terminal labeling, we use a similar approach (Fig. 6). Here, the column is preloaded with a LPETGG-containing peptide, decorated with the substituent of choice at its N-terminus. This allows the acyl intermediate to form on-resin, prior to incubation with the Gly<sub>n</sub>-modified substrate protein to be labeled. This preincubation step is essential to increase the yield of labeled product.

## MATERIALS

### REAGENTS

**Overall Caution**—For all items marked with a **! Caution** marker, please use proper personal protection equipment (PPE; gloves, eye protection, and proper attire). Use of these chemicals should be carried out in a fume hood when possible. For more information, please refer to each item's Material Safety Data Sheet (MSDS)

- Cyanogen bromide-activated-Sepharose 4B (e.g. Sigma-Aldrich, cat. no. C-9142)

- Hydrochloric acid (HCl; EMD Millipore, cat. no. HX0603) ! **CAUTION** Toxic/corrosive
- Sortase A or the heptamutant sortase A (expressed and purified as described<sup>20</sup>. Plasmids are available from Addgene, cat. no. 51138 and 51141)
- Sodium Chloride (American Bioanalytical, cat. no. AB01915)
- Sodium Bicarbonate (American Bioanalytical, cat. no. AB01929)
- Glycine (Sigma, cat. no. 67126)
- Tris Base (American Bioanalytical, cat. no. AB02000)
- Tris-HCl (American Bioanalytical, cat. no. AB02005)
- Common reagents for SDS-PAGE analysis
- Brilliant blue R (Sigma-Aldrich, cat. no. B7920)
- Methanol (MeOH; EMD, cat. no. MX0488) ! **CAUTION** Toxic/flammable
- Acetic acid (AcOH; VWR, cat. no. BDH3094) ! **CAUTION** Corrosive/flammable
- CaCl<sub>2</sub> (EMD, cat. no 208290)
- For C-terminal labeling: Purified target protein (LPETGGHHHHHH at the C-terminus at a 220 μM concentration in Tris-buffered saline (TBS)
- For C-terminal labeling: N-terminal oligoglycine peptide probe: 20 mM in water (synthesis described elsewhere<sup>20</sup>)
- For N-terminal labeling: Purified protein to be used as nucleophile (GGG at the N-terminus)
- For N-terminal labeling: LPXTGG peptide probe: 20 mM stock solution in water, 1 mM solution for reactions (synthesis described in elsewhere<sup>21</sup>).
- Triglycine (Sigma, cat. no. G1377)
- Ethanol (Pharmco-AAPER, cat. no. 111000190) ! **CAUTION** Flammable
- Nickel-nitrilotriacetic acid agarose suspension (Ni-NTA agarose; Qiagen, cat. no. 30230)
- Imidazole (Alfa Aesar, cat. no. A10221) ! **CAUTION** Harmful
- Sample loading LDS-buffer, 4× (Invitrogen, cat. no. NP0008)

## EQUIPMENT

- Amicon ultra concentrators 3 or 10 kDa MW cutoff (Millipore, cat. no. UFC900324 and UFC901024). The cut off range is dependent on the size of the protein being labeled.
- Dialysis cassettes (Thermo Scientific, cat. no. 66330 or 66110)

- 25 mL polypropylene gravity columns with fritted bottom (Bio-Rad, cat. no. 732–1010)
- Rotating mixer/shaker
- Tubes or vials for immobilized sortase storage
- GE Healthcare, Tricorn 5/100 column
- Micropipettes (5–1000 L)
- Centrifuge that supports centrifugation at 2080g
- 37°C incubator
- 1.5 mL centrifuge tubes
- Corning Costar spin-X centrifuge tube filters (SIGMA, cat. no. CLS8161)
- Superdex 75 (10/300 GL)
- Heating block
- Centrifuge that supports centrifugation at 2500g
- GENIE Plus syringe pump (Kent scientific)

#### REAGENT SETUP

- **Sepharose swelling buffer:** 1 mM HCl in water, store at 4 °C
- **NaHCO<sub>3</sub>/NaCl coupling buffer:** 0.1 M NaHCO<sub>3</sub> pH 8.3, 0.5 M NaCl
- **AcOH/NaCl buffer:** 0.1 M AcOH pH 4.0, 0.5 M NaCl
- **Blocking solution:** 0.2 M glycine in water
- **TBS buffer:** 50 mM Tris-HCl pH 7.4, 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.
- **Sortase reaction buffer:** 500 mM Tris-HCl, pH 7.4, 1.5 M NaCl, 100 mM CaCl<sub>2</sub>. (10× stock).
- **Purification buffer:** 50 mM Tris-HCl, pH 7.5, 150 mM NaCl. Store at 4 °C.
- **Column washing solution:** 1 mM triglycine, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM CaCl<sub>2</sub>

## PROCEDURE

### Part 1 - IMMOBILIZATION OF SORTASE TIMING 8–9 hours

- 1| Take 30 mg of stock sortase A or heptamutant sortase A and exchange the buffer to NaHCO<sub>3</sub>/NaCl coupling buffer through dialysis or with spin concentrators. Dilute the sortase to a total volume of 7 mL of coupling buffer.
- 2| Split 1 g of dry cyanogen-bromide activated Sepharose into two 25 mL polypropylene gravity flow columns.
- 3| Add 20 mL of cold 1 mM HCl swelling solution to each column and cap each end. Incubate for 7 min at 4 °C rotating in an end-over-end mixer. After uncapping, gently vacuum the liquid through the bottom frit. Repeat this step 5 times.

*Note:* Good swelling of the Sepharose beads is required to obtain good immobilization efficiencies.

**CRITICAL STEP:** Do not let the beads dry out.

- 4| Combine the beads into one column and flow 200 mL of cold distilled water through the column with vacuum at 4 °C to wash the beads.
- 5| Flow through with vacuum 10 mL of NaHCO<sub>3</sub>/NaCl coupling buffer at 4 °C.
- 6| Immediately after washing add the 7 mL of sortase A solution from step 1. Remove a small amount (~50 µL) of the supernatant for coupling efficiency analysis (step 15). Rotate in an end-over-end mixer at RT (room temperature, 20–25 °C), for 2 h.

*PAUSE POINT:* The coupling can be done at 4 °C overnight.

**CRITICAL STEP:** Avoid amine-containing buffers, such as TRIS, or other nucleophilic buffers, as they will react with cyanogen bromide-activated Sepharose.

- 7| Remove the supernatant by gravity flow and carefully collect it to keep as a control (step 15).
- 8| Flow through 20 mL NaHCO<sub>3</sub>/NaCl coupling buffer using vacuum to wash the resin.
- 9| Modify any remaining unreacted cyanate ester groups with 15 mL blocking solution for 2 hours at RT, rotating in an end-over-end shaker.  
*PAUSE POINT:* The blocking step may be carried out for 16 h at 4 °C.
- 10| Flow through 15 mL ice-cold NaHCO<sub>3</sub>/NaCl coupling buffer using vacuum to wash the resin.
- 11| Flow through with vacuum 15 mL 0.1 M AcOH/NaCl buffer at pH 4 to wash the resin.
- 12| Repeat steps 10 and 11 five times.



- 13| Flow through 15 mL of ice-cold TBS buffer using vacuum to wash the resin, Repeat this step three times.
- 14| Resuspend the beads with an equivalent volume of TBS buffer to obtain a 50% slurry and transfer to a tube or vial for long term storage.

*PAUSE POINT:* Immobilized sortase from *S. aureus* can be stored at 4 °C for 3 months or until degradation or loss of efficiency is observed. The heptamutant sortase has a shorter lifetime of 3–4 weeks. Longer storage at 4 °C in the absence of a bacteriostatic or bactericidal is not recommended, as the protein may degrade or contamination may occur. The resin can be frozen in 20% glycerol at –20 °C or lyophilized in the presence of sucrose and stored at –20°C for even longer periods. For freeze-drying, we recommend gradually freezing the slurry at –20 °C before lyophilizing to minimize damage to the beads. After reconstitution, wash the resin with TBS buffer.

- 15| Analyze aliquots of the supernatant taken before and after immobilization (from steps 6 and 7) by SDS-PAGE to estimate sortase A coupling efficiency and the concentration of enzyme that is immobilized on the beads. It will be beneficial to create a serial dilution of the initial sample to achieve a better coupling efficiency estimate. The coupling efficiency should be 90–100%.
- 16| You can now either use the beads for small-scale batch labeling reaction to test their function and determine optimal reaction conditions (perform the steps in Part 2); or, if you are already satisfied that the beads will perform their function, flow reactions for large scale protein production (perform the steps in Part 3).

## Part 2 - BATCH LABELING USING IMMOBILIZED SORTASE A: Optimization of the batch reaction

### TIMING 1.5 days

- 17| Add 20–150 μM of immobilized sortase A (~7.5 mg mL<sup>-1</sup> resin, 300 μM) or 5–25 μM of immobilized heptamutant sortase A (~7.5 mg mL<sup>-1</sup> resin, 440 μM) to a solution containing 10–50 μM target protein and 1–2 mM peptide probe in 1× sortase buffer (final concentrations). The final reaction volume should be at least 100 μL. The controls to be included are: (a) resin only, (b) target protein only, (c) probe only, (d) target protein combined with the resin, (e) probe combined with the resin and (f) probe combined with the target protein (in the absence of resin).

Reactions may be performed in PBS (instead of sortase buffer) if using the heptamutant sortase A.

*Note:* Removal of aliquots from the supernatant to monitor the reaction (see step 18) will increase the concentration of the immobilized sortase A. Use a reaction volume of 100 μL to minimize concentration increase and to allow direct comparison with sortase A reactions in solution.

- 18| Swirl the beads using an end-over-end shaker or thermomixer (800 rpm) and take aliquots (2 μL) from the supernatant after 1, 2, 4, 8 and 16h.

*Note:* Different temperatures have to be screened to arrive at the optimal reaction conditions. For the regular sortase A, the optimal reaction temperature is between RT and 37°C. The heptamutant sortase is active at reduced temperatures. Most commonly, heptamutant reactions are performed between 15 °C and RT although temperatures as low as 4 °C can be used for particularly sensitive experiments.

- 19| Remove the beads by centrifuging through spin filters at 280g, 5 min, 4 °C. Recover the supernatant and immediately denature the proteins by adding 1× LDS sample buffer and boiling for 2 min. The beads may be washed several times with TBS buffer and saved for further use.
- 20| Analyze the samples by SDS-PAGE followed by fluorescence scanning, western blot or Coomassie staining, depending on the probe being used.
- 21| **Purification of the batch reaction.** Repeat the batch reaction using the optimized reaction time and temperature. Use a reaction volume of 100 µL minimum.
- 22| Transfer the reaction to a centrifuge tube filter, centrifuge at 280g, 5 min, 4 °C and collect the fluid phase.
- 23| Wash the resin beads with 1× sortase buffer, centrifuge again, and collect the fluid phase.

*Note:* The beads may be stored at 4 °C in TBS buffer and may be reused until degradation or loss of activity is observed (as evidenced by decreased conversion of the substrate on Coomassie staining, fluorescence staining or western blot).

- 24| Combine the yield from steps 22 and 23.
- 25| Load the protein onto a previously washed and equilibrated size exclusion column Superdex 75 (10/300 GL). Elute the protein with 25 mL purification buffer at a flow rate of 0.5 mL h<sup>-1</sup>.
- 26| Collect 1.3 mL fractions and combine the positive fractions for protein (based on the UV spectra). Concentrate using an Amicon concentrator at 2080g at 4°C.

PAUSE POINT: Final storage depends on the stability of the protein being labeled.

Usually 4 °C for short-term, while -20°C or -80°C with 10% glycerol is optimal for longer durations

### Part 3 - Protein labeling by flow reactions TIMING 7–16 h

- 27| To pack a column of immobilized sortase A, carefully resuspend the immobilized sortase A slurry to obtain a homogenous solution. Then pack the suspension of immobilized sortase A into a 5 × 100 mm column following the manufacturer's protocol. Final loading volume is 5 × 70 mm immobilized sortase A (bead bed) containing 10 mg of protein.

- 28| Flow through 3 mL of TBS buffer to wash the column.
- PAUSE POINT: The column can be stored at 4 °C, for up to 3 months for sortase A. Columns containing the immobilized heptamutant variant can be stored for 3 weeks. The column can be reused many times until loss of activity or degradation is observed.
- 29| For large-scale protein labeling experiments follow the steps in option A if you want to do C-terminal labeling, or option B if you want to do N-terminal labeling.

### A C-TERMINAL LABELING (LPXTG-CONTAINING PROTEIN) TIMING

- i. Wash the column with 3 mL of 1× sortase buffer.
- ii. Incubate the column at 37 °C and wait 2 h before proceeding to the next step. If using the heptamutant sortase the temperature may be reduced.
- iii. Mix the protein substrate (345 µL of a 220 µM stock), the oligoglycine probe (150 µL of a 20 mM stock), 10× sortase reaction buffer (300 µL), and water (2205 µL) in a centrifuge tube.
- iv. Inject the reaction mixture in the sortase A-immobilized column at a flow rate of 0.5 mL h<sup>-1</sup> using a syringe pump at 37 °C. Collect the eluate.
- v. Inject 1.5 mL of 1 mM of the oligoglycine peptide in 1× sortase reaction buffer after the final amount of protein substrate has flowed through the column. Collect the eluate.
- vi. Combine the eluate from steps iv and v. vii. Purify the protein by gel filtration (see **Steps 30–31**) and regenerate the column as described in **Steps 32–33**.

### B N-terminal protein labeling (GGG-containing protein) TIMING

- i. Prepare solution A by mixing 300 µL of LPETG-containing probe (1 mM), 150 µL 10× sortase buffer, and 1050 µL deionized water (total volume 1500 µL).
- ii. Prepare solution B by mixing 450 µL of the GGG N-terminal protein (55 µM), 600 µL of LPETG containing probe (1 mM), 300 µL of 10× sortase buffer, and 1650 µL of deionized water (total volume 3000 µL).
- iii. Inject solution A into the immobilized sortase A column. Store the column at 37 °C for 0.5 h. *Note:* This step allows the LPETG containing probe to fully react with the sortase to pre-form the LPET-sortase covalent intermediate. If using the heptamutant sortase the temperature may be reduced.
- iv. Pass mixture B through the column at a flow rate of 0.5 mLh<sup>-1</sup>. Collect 0.5 mL fractions from the column.
- v. Reinject the void volume (0.9 mL) back through the column at 0.5 mLh<sup>-1</sup> to elute any remaining free protein.

- vi. Combine the fractions from the column and concentrate using an Amicon concentrator at 2080g at RT.
- vii. Purify the protein by gel filtration (see **Steps 30–31**) and regenerate the column as described in **Steps 32–33**.
- 30| Purification of the labeled protein.** Load the concentrated protein onto a previously washed and equilibrated size exclusion column Superdex 75 (10/300 GL) and elute the proteins with 25 mL eluting solution at a flow rate of 0.5 mL h<sup>-1</sup>.
- 31|** Collect 1.3 mL fractions and combine the positive fractions for protein (based on the UV spectra). Concentrate using a centrifugal concentrator at 2500g at 4°C.
- PAUSE POINT: Final storage depends on the stability of the protein being labeled.
- Usually 4 °C for short-term (3 days), while –20°C or –80°C with 10% glycerol is optimal for longer durations.
- 32| Regeneration of the immobilized sortase column.** Wash the immobilized sortase column with 3 mL of column washing solution at 30 mLh<sup>-1</sup>
- 33|** Wash the column with 3 mL of TBS buffer by hand injection.
- PAUSE POINT: The column can be stored at 4 °C until the next sortase reaction.

## TIMING

Steps **1–5**, 1.5 h

Steps **6–8**, 2.5 h

Steps **9–15**, 3–4 h

Steps **16–20**, 1 d

Steps **21–26**, 4 h

Steps **27–28**, 1h

Step **29 A**: Steps **i–ii** 1–2 h, Steps **iii–x** 7–8 h, Steps **xi–xii** 3–4 h

Step **29B**: Steps **i–v** 1.5 h, Steps **v–viii** 2.5 h, Steps **ix–xv** 3–4 h

Steps **30–31**, 1.5–4 h

Steps **32–33**, 30 min

## TROUBLESHOOTING

See Table X for troubleshooting guidelines.

## ANTICIPATED RESULTS

The anticipated loading of the resin using the above described conditions is approximately 7.5 mg of sortase A per milliliter of packed resin, and this resin can be stored for several weeks to months at 4 °C depending on the used sortase variant.

A successful sortase reaction will result in the formation of product, which, depending on the probe used, can be visualized by fluorescence scanning (if using a fluorescent dye), western-blot (if using biotin or an affinity tag) or Coomassie staining (if substrate and product differ in apparent MW sufficiently to be visualized by SDS-PAGE). Figures 3 and 4 present a successful batch C-terminal and N-terminal labeling reaction, using immobilized heptamutant sortase A and sortase A respectively. Sortase A should not be visible in any of the samples, as it is immobilized and removed by filtration/centrifugation in the case of batch reactions. Since an acyl intermediate may form, a decrease in protein signal is expected in the control samples containing just the target protein and immobilized sortase when performing C-terminal labeling. On some occasions proteins will non-specifically bind to the resin. This can be inferred by comparing the amount of protein obtained upon reaction with the amount of input material.

For large scale flow reactions, we typically use a flow rate of 0.5 mL/h with a residence time of the protein on the column of 2 h. Under these conditions we obtain a yield in the range of 65–90% of purified labeled protein. Reaction rates and flow rates are protein-dependent and need to be determined empirically prior to scaling up the reaction.

### Troubleshooting

Troubleshooting guidelines can be found in Table 1.

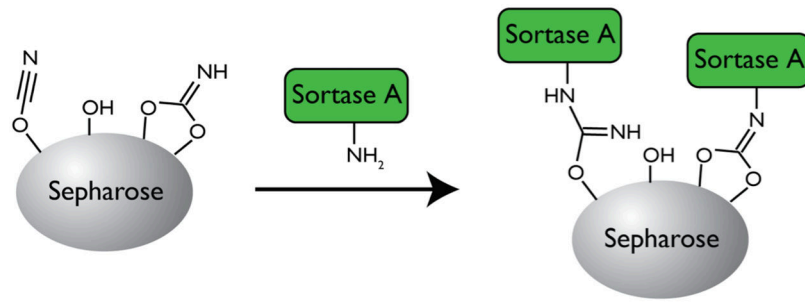
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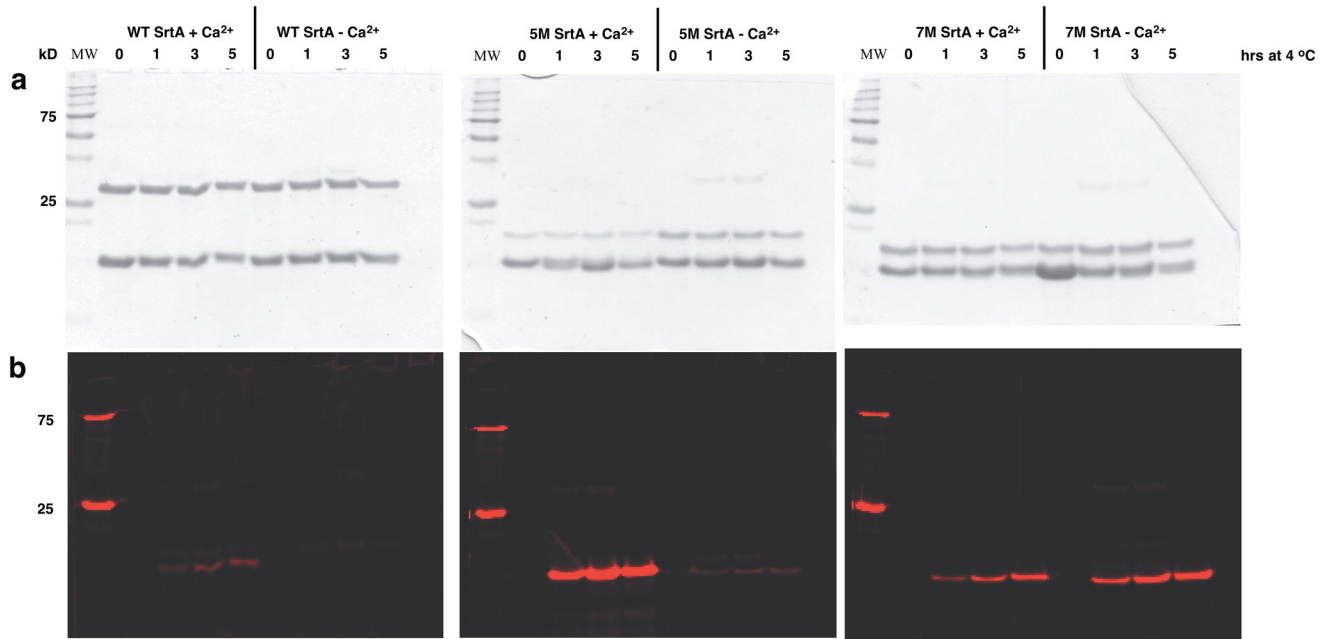
**SUMMARY**

Sortase A can be used for the site-specific modification of proteins. This protocol describes its immobilization on sepharose beads allowing for larger scale flow reactions or easy separation from the enzyme in batch reactions.



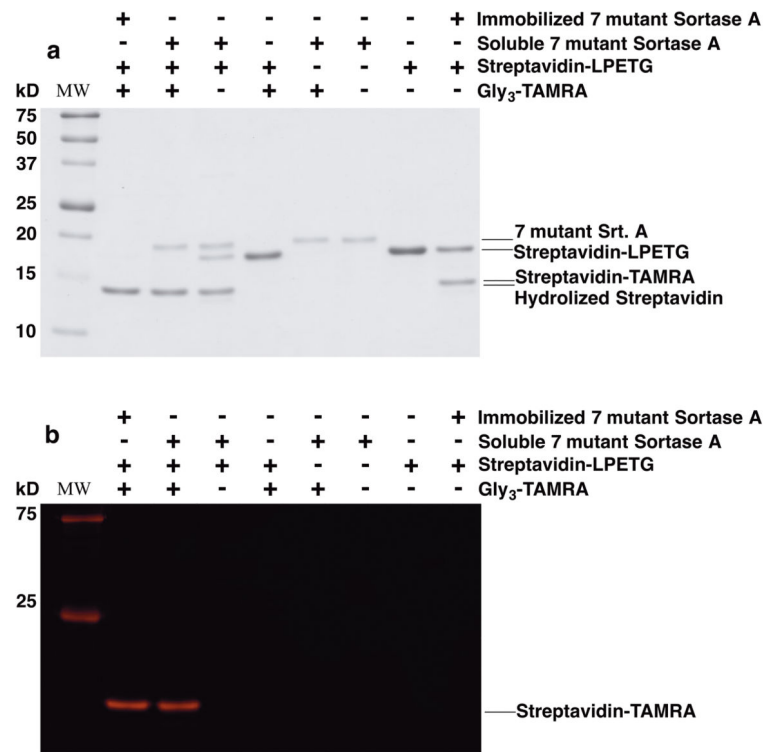
**Figure 1. Schematic representation of the sortase immobilization on Sepharose beads**  
Free amines of sortase A react with the activated ester, forming an iso urea or an imidocarbonate derivate.





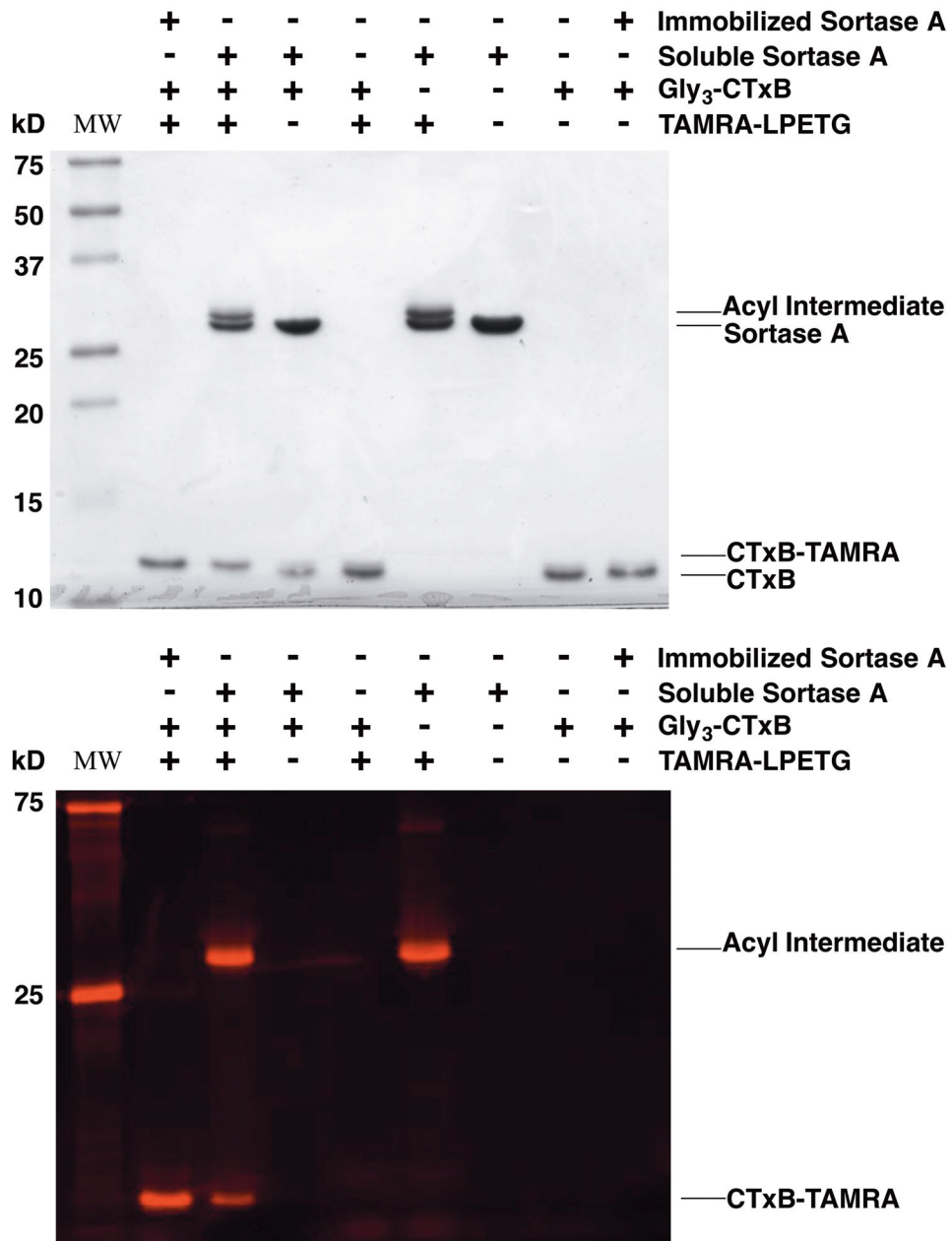
**Figure 2. Comparison of wildtype (WT), pentamutant (5M) and heptamutant (7M) Srt A activity in the presence or absence of  $\text{Ca}^{2+}$**

In this experiment, 30  $\mu\text{M}$  VHHF4-LPETG-6xHis, 0.5 mM GGG-TAMRA and 5  $\mu\text{M}$  of the appropriate sortase were incubated at 4  $^{\circ}\text{C}$  for the indicated time in either (+  $\text{Ca}^{2+}$ ) 50 mM Tris, pH 7.5, 150 mM NaCl supplemented with 10 mM  $\text{CaCl}_2$  or (–  $\text{Ca}^{2+}$ ) PBS supplemented with 10 mM EGTA. Equal amounts of the reaction mixtures were loaded on a 15% SDS-page gel. The mixtures were analyzed using in gel fluorescence scanning with emission filter 580 nm (b) and staining with coomassie brilliant blue (a). The band at 15 kD in the fluorescence image corresponds to TAMRA labeled VHHF4. No fluorescent signal is observed for WT Srt A.



**Figure 3. Comparison of C-terminal specific labeling of a LPETG containing streptavidin protein with a Gly<sub>3</sub>-TAMRA fluorophore using immobilized heptamutant sortase A (batch) or heptamutant sortase A in solution**

The LPETG containing streptavidin and Gly<sub>3</sub>-TAMRA were prepared as described in the protocol, as well as the expression and immobilization of sortase A. In this experiment, 50  $\mu$ M LPETG containing streptavidin and 2 mM Gly<sub>3</sub>-TAMRA were incubated overnight with either 25  $\mu$ M immobilized sortase A (lane 1) or 10  $\mu$ M sortase A in solution (lane 2). The equal amounts of the reaction mixtures were loaded on a 15% SDS-page gel and stained with coomassie brilliant blue (a). In gel fluorescence scanning with emission filter 580 nm yielded image b. TAMRA labeled product was only found in the lanes containing sortase, protein, and probe. This gel confirms that immobilization of sortase A did not compromise its activity. The coomassie-stained image A illustrates the absence of sortase A in solution, when using immobilized sortase A for the reaction.



**Figure 4. Comparison of N-terminal specific labeling of a Gly<sub>3</sub> containing Cholera toxin B (CtxB) with a LPETG containing TAMRA fluorophore using immobilized sortase A (batch) or sortase A in solution**

The LPETG containing TAMRA and Gly<sub>3</sub>-CtxB were prepared as described in the protocol, as well as the expression and immobilization of sortase A. In this experiment, 50  $\mu$ M LPETG containing TAMRA and 30  $\mu$ M Gly<sub>3</sub>-CtxB were incubated overnight with either 50  $\mu$ M immobilized sortase A (lane 1) or 50  $\mu$ M sortase A in solution (lane 2). The equal amounts of the reaction mixtures were loaded on a 15% SDS-page gel. The mixtures were analyzed after in gel fluorescence scanning with emission filter 580 nm (**b**) and staining with coomassie brilliant blue (**a**). The two fluorescent bands around 10 kD (lanes 1–2) in the

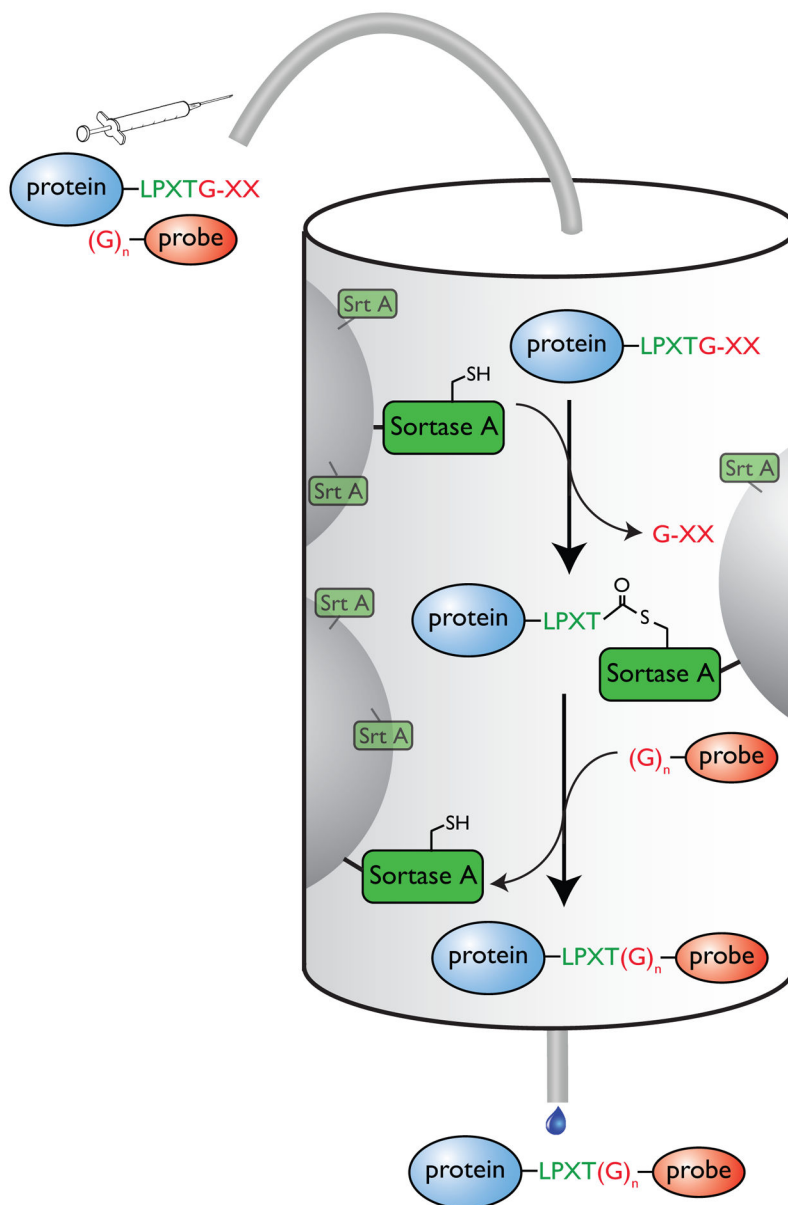
fluorescence image indicate near complete conversion to the labeled product. The coomassie-stained image A illustrates the absence of sortase A in solution, when using immobilized sortase A for the reaction.

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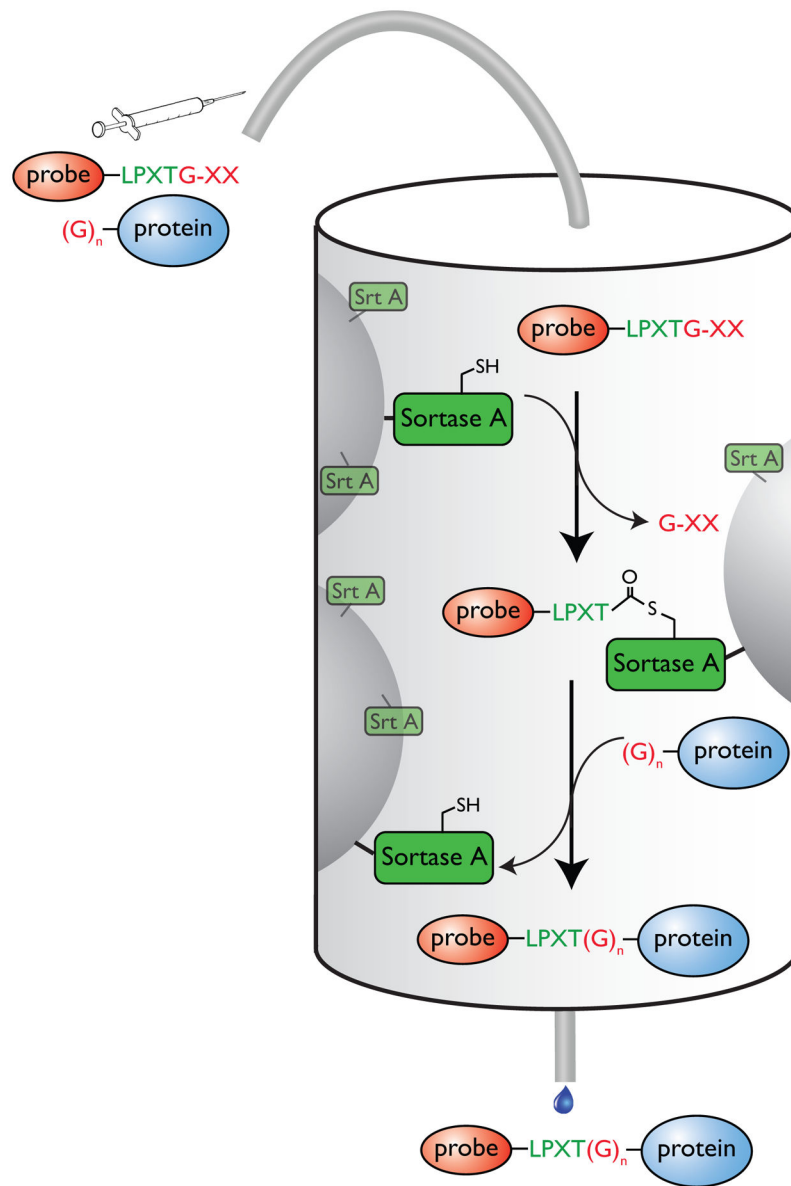
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**Figure 5. C-terminal labeling of proteins using immobilized sortase**

A protein C-terminally modified with the LPXTG motif and a peptide probe containing a series of N-terminal glycine residues are injected onto a column with *S. aureus* sortase A immobilized on Sepharose beads. Sortase forms an acyl-enzyme intermediate with Thr on the protein's recognition motif. Nucleophilic attack of the peptide probe resolves the intermediate, thus ligating the peptide probe to the C-terminus of the protein and regenerating the active site Cys on sortase. Labeled protein is eluted from the column.



**Figure 6. N-terminal labeling of proteins using immobilized sortase**

A protein N-terminally modified with a series of glycine residues and a peptide probe containing C-terminal LPXTG *S. aureus* motif are injected onto a column with *S. aureus* sortase A immobilized on Sepharose beads. Sortase forms an acyl-enzyme intermediate with Thr residue within the peptide's recognition motif. Nucleophilic attack of the protein resolves the intermediate, thus ligating the peptide probe to the N-terminus of the protein and regenerating the active site Cys on sortase. Labeled protein is eluted from the column.

Table 1

## Troubleshooting

Problem	Possible reason	Solution
Poor immobilization	<p>The cyanogen bromide-activated Sepharose is no longer reactive</p> <p>The protein is diluted in a buffer incompatible for immobilization</p>	<p>Check the expiration date of the Sepharose</p> <p>Avoid the use of amine- containing buffers</p>
Incomplete conversion for C- terminal protein modification	<p>Wrong buffer</p> <p>The ratio between the C-terminal protein-LPXTGG and GGG-label is not optimal.</p> <p>Sortase is degraded</p> <p>C-terminus is not well exposed</p> <p>The protein to be labeled is not a suitable substrate for sortase</p> <p>Hydrolysis of the C-terminal LPXTGG protein</p>	<p>The buffer pH should be ~ pH 7.5. If <i>S. aureus</i> Sortase A is used the buffer should contain 1mM CaCl<sub>2</sub> and phosphate-based buffers should be avoided.</p> <p>Increase the amount of nucleophile. The conditions need to be determined empirically.</p> <p>Prepare a fresh batch of immobilized sortase.</p> <p>Extend the C-terminus of the protein introducing a linker (Gly<sub>4</sub>Ser)<sub>2</sub> immediately upstream of the sortase motif.</p> <p>Reaction conditions should be tested in batch using soluble and immobilized enzyme, before scaling up to the continuous-flow system.</p> <p>Although uncommon, this may occur more frequently with the heptamutant sortase. Lower the temperature and/or reaction time.</p>
Incomplete conversion for N- terminal protein modification	<p>No acyl-intermediate was formed</p> <p>The ratio between the N-terminal protein-LPXTGG and GGG-protein is not optimal.</p> <p>The flow rate may be too high or too low.</p> <p>Sortase is degraded</p> <p>N-terminus is not exposed</p> <p>The protein to be labeled is not a suitable substrate for sortase</p> <p>Hydrolysis of the LPXTGG probe</p>	<p>Load the LPXTGG-containing (poly)peptide on the immobilized sortase column and let incubate at 37°C for 1 – 2 h.</p> <p>Decrease the amount of injected protein or increase the amount of LPXTGG probe for the incubation.</p> <p>Use SDS-PAGE to check the conversion rate and adjust the flow rate.</p> <p>Prepare a fresh batch of immobilized sortase.</p> <p>Increase the length of the Gly<sub>n</sub> N- terminus</p> <p>Reaction conditions should be tested in batch using soluble and immobilized enzyme, before scaling up to the continuous-flow system.</p> <p>This may occur more frequently with the heptamutant sortase. Reduce the temperature and/or reaction time.</p>