Supplementary Material: An improved variant of TEV protease that does not need reducing agents

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Protocol for Producing TEV Protease

The following protocol provides reasonable yields of TEV protease with minimal effort, and can easily be adapted to whatever combination of equipment and methods are available in your laboratory. This is not an extensively optimized protocol, so you should feel free to experiment with different conditions if those are more convenient for you as they may lead to equally good (or possibly better) yields.

1. Order the plasmid for the TEV Hexa mutant from Addgene (plasmid #222868). TEV Triple is also available from AddGene (plasmid #92414) but has no advantages compared to TEV Hexa. Addgene ships these plasmids as bacterial stabs in BL21-CodonPlus-RIL *E. coli* cells.
   - Once received, streak and isolate bacteria from the stab culture onto an LB agar plate (See https://www.addgene.org/protocols/streak-plate/ for directions).
   - The plate should include carbenicillin (or ampicillin) at 100 μg mL^{-1} to select for bacteria carrying the plasmid with the TEV protease gene. In addition, to maintain the additional plasmid (pRIL) in the BL21-CodonPlus-RIL cells with the genes for tRNAs for rare codons, you should also include chloramphenicol (30 μg mL^{-1}).
   - As the gene for TEV protease is controlled by a tac promoter rather than a T7 promoter, the use of a DE3 *E. coli* strain is not necessary for protein expression.
   - We have also tried using regular BL21 cells rather than CodonPlus-RIL cells; when using regular BL21 cells expression levels of TEV protease drop by roughly one-third.
2. For long-term storage of cells for protein expression, it is advisable to generate glycerol stocks from single colonies from plates of transformants and then to store the glycerol stocks at −80°C (See https://www.addgene.org/protocols/create-glycerol-stock/ for directions).
3. Inoculate a 250 mL culture of ZYM-5052 media [1] supplemented with 100 μg L^{-1} carbenicillin and 30 μg L^{-1} chloramphenicol and grow for ≈60 hours at 25°C in a 2 L baffled flask.
   - A single colony from a plate of transformants, or a scraping from a glycerol stock stored at −80°C, can be used to inoculate the culture.
   - For our incubator, shaking at 125 rpm provides a good amount of aeration for 2 L baffled flasks.

- We find using autoinducing media and a long growth time at a relatively low temperature convenient because we can set up a culture on a Friday afternoon and come back to harvest it Monday morning.
- Ampicillin can be used instead of carbenicillin. However, we prefer to use carbenicillin as it is more stable so it is better suited for maintaining antibiotic selection for long incubation times.

4. Harvest the cultures by centrifugation (≈3000×rcf for ≈20 min) and then resuspend the resulting cell pellets in 25 mL of ice-cold lysis buffer (250 mM NaCl, 20 mM imidazole, 20 mm phosphate (pH 7.4), 2 mM DTT, and protease inhibitor cocktail).

   - Our lysis buffer includes a protease inhibitor cocktail of 1 mM AEBSF, 50 μM bestatin, 15 μM E-64, 15 μM pepstatin, and 8.5 μM phosphoramidon. However, this is not needed if samples are processed quickly and kept on ice whenever possible. Alternatively, other protease inhibitor cocktails can be used (such as Roche cOmplete™ protease inhibitor cocktail), as long as inhibitors that react with cysteine (such as N-ethylmaleimide (NEM) and iodoacetamide) are not included [2]. Interestingly, E-64 (an irreversible inhibitor of cysteine proteases) does not inhibit the activity of TEV protease [2].
   - DTT is included as a reducing agent when we generate TEV Triple to prevent intermolecular disulfide bonds from forming. It should not be needed for TEV Hexa but it does not hurt to include it.
   - Many other procedures include glycerol (typically at 10%) in buffers used with TEV protease to increase its solubility (and therefore reduce the chance of accidental precipitation). We find including glycerol is not necessary for the highly soluble TEV Triple and TEV Hexa mutants.

5. After lysis by ultrasonication, clarify the lysates by high-speed centrifugation (42,000×rcf for 30 min at 4°C).
6. Purify the lysates by immobilized metal affinity chromatography (IMAC).

   - We filter our clarified lysates using Phenomenex glass fiber/cellulose acetate syringe filters prior to IMAC to increase the longevity of our IMAC columns.
   - We keep our samples on ice or at 4°C when possible during purification. However, our chromatography instruments and the buffers used with them are at room temperature. As long as samples are processed quickly, it is not critical to keep TEV protease samples cold at every step of the purification protocol.
   - For IMAC, we use a Cytiva (Marlborough MA) Äktaprime plus liquid chromatography system with a 5 mL Cytiva HisTrap column (which theoretically is able to bind in excess of 200 mg of His-tagged protein). Before applying the sample, the column is equilibrated with "binding buffer" (500 mM NaCl, 50 mM phosphate (pH 7.4), 20 mM imidazole). After applying the sample, we wash the column with more binding buffer and then elute His-tagged proteins using a steadily increasing gradient of "elution buffer" (500 mM NaCl, 50 mM phosphate (pH 7.4), 500 mM imidazole).
   - If using a "Superloop", it is best to clean out the loop after purifying TEV protease before purifying regular His-tagged proteins. The residual amount of material that can persist in the Superloop can result in unwanted on-column cleavage. Likewise, it is a good idea to reserve an IMAC column for TEV protease purifications as residual TEV protease bound to the IMAC column may cause problems with on-column cleavage.
   - We have also successfully used Cytiva His GraviTrap columns (which theoretically are able to bind in excess of 40 mg of His-tagged protein) for purification. For purification with a GraviTrap column, we recommend using a wash buffer with an intermediate concentration of imidazole (50-75 mM) after applying the sample to the column to remove weakly-binding contaminates before releasing the His-tagged protein from the column with elution buffer containing 500 mM imidazole.

7. Combine elution fractions containing protein as determined by monitoring the A_{280}.
    - We sometimes see two peaks in our chromatogram from IMAC. The first peak (eluting at a lower imidazole concentration) corresponds to metal-binding bacterial proteins, whereas the second peak (eluting at a higher concentration of imidazole) corresponds to 7×His-tagged TEV protease. We find that 7×His-tagged TEV protease elutes when the imidazole concentration in the gradient elution exceeds ≈300 mM.
    - If purifying TEV Triple, it is a good idea to add DTT to a final concentration of 2 mM to the pooled IMAC fractions so that disulfide bonds do not begin to form.
    - We sometimes will skip the additional purification detailed in the next three steps and use TEV protease directly after IMAC purification. Although there will be some impurities in the TEV protease sample at this point, TEV protease is usually added in a 1:50 or 1:100 molar ratio when it is used to cleave substrate. Consequently, whatever impurities there are in the TEV protease sample will be further diluted in the cleavage reaction sample (and should be removed by whatever additional post-cleavage purification steps are carried out to separate fusion tags from target proteins).
8. If carrying out size-exclusion chromatography (SEC), concentrate the combined IMAC elution fractions to a volume appropriate for your loading loop.
    - Our SEC system has a 5 mL loading loop, so we typically concentrate our samples to a bit less than half of that (≈2 mL) using 10 kDa Amicon Ultra centrifugal filters.
    - We filter our concentrated TEV protease using 0.2 μm syringe filters prior to SEC to increase the longevity of our SEC columns.
    - When concentrating the TEV protease prior to SEC, we typically reach concentrations of around 300 μM (≈10 mg mL^{-1}). This is approximately half of the maximum solubility of TEV Triple and TEV Hexa and we have had no problems with precipitation at this step. That said, if you are generating TEV protease on a larger scale, it may be necessary to add 10% glycerol at this point to increase TEV protease solubility, to use a larger sample loading loop, and/or to carry out multiple SEC runs, to avoid over-concentrating/precipitating your protein.
9. Carry out SEC to further purify TEV protease.
    - For SEC we use an Äktaprime plus liquid chromatography system with a Cytiva HiPrep 16/60 Sephacryl S100 column and a 150 mM NaCl, 50 mM phosphate (pH 7.4) running buffer ("SEC buffer"). With this column, we find that fractions corresponding to 58–73 mL into the SEC run contain TEV protease.
10. Pool SEC fractions containing protein and quantify the concentration of TEV protease by measuring the A_{280}.
    - The TEV Triple and TEV Hexa constructs both have molar absorptivities at 280 nm of 31,970 L mol^{-1} cm^{-1}.
    - If needed, we further concentrate the SEC-purified TEV protease using 10 kDa Amicon Ultra centrifugal filters.
    - If purifying TEV Triple, it is a good idea to add DTT to a final concentration of 2 mM at the pooled SEC fractions so that disulfide bonds do not begin to form.
11. TEV Triple and Hexa can be stored in SEC buffer at 4°C for a few days if they will be promptly used. For long-term storage, they should be diluted with glycerol to achieve a 50% glycerol concentration and then stored at −80°C.
    - We usually aim for a final TEV protease concentration of 25 μM (0.87 mg mL^{-1}) for our frozen stocks of TEV protease. Although TEV Triple and TEV Hexa can be stored at much higher concentrations, we find this concentration convenient for our use.

12. Our typical yield of TEV Hexa from this protocol using a 250 mL culture is around 14 mg (corresponding to ≈56 mg L^{-1} of culture). This protocol can easily be scaled up if more protease is needed.

References

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