TEV protease production and cleavage

Risk assessment

▷ Wear gloves, safety glasses, lab coat



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This is a bench card. Full protocol available online.

›› *Preparative expression*

(1.) Inoculate 50 mL LB containing carbenicillin at 100 mg/L with a single fresh colony of *E. coli* BL21(DE3) carrying the TEV expression plasmid. Incubate overnight at 37 °C, 180 rpm.

(2.) Retain the equivalent of 0.5 OD mL of cells from the pre-culture in 70 µL 1 × SDS-PAGE sample buffer 🗎 SOP 0045 as the uninduced reference; the autoinduction medium has no uninduced phase.

(3.) Inoculate 1 L TB autoinduction medium with 10 mL of the overnight pre-culture in a baffled Erlenmeyer flask. Incubate at room temperature (20–25 °C), 180 rpm, for 18–24 h, typically overnight.

(4.) Retain a second sample of the same cell equivalent at harvest density.

(5.) Harvest the cells at 4 000 × *g* for 20 min at 4 °C. Discard the supernatant and weigh the wet pellet.

›› *Lysis*

(1.) Resuspend the pellet in 5 mL cold TEV lysis buffer per 1 g wet mass. Supplement AEBSF to 0.5 mM, $MgCl_2$ to 2 mM, and DNase I to 40 U/mL final. Keep the lysate on ice.

(2.) Lyse the cells by sonication on ice (0.125 in horn; 6 cycles of 30 s on, 60 s off, at 30–40% amplitude) or by two passes through a high-pressure homogenizer.

(3.) Clear the lysate by centrifugation at 35 000–50 000 × *g* for 20 min at 4 °C.

(4.) Retain 20 µL of the cleared lysate, and resuspend a comparable aliquot of the pellet in 1 × SDS-PAGE sample buffer as the insoluble fraction.

›› *Affinity chromatography (HisTrap FF on ÄKTA)*

(1.) Filter the cleared lysate through a 0.45 µm filter immediately before loading.

> ***Critical:*** An unfiltered lysate clogs the HisTrap FF crude inlet within minutes at the loading flow rate; do not skip filtration.

(2.) Run the FPLC variant, route *Alternative C* of 🗎 SOP 0045, on the 5 mL HisTrap FF crude column, with TEV lysis buffer as the binding buffer and TEV elution buffer as the eluent in place of the phosphate-based pair given there. The load is the 50–75 mL of cleared, filtered lysate from 1 L of culture. Where no FPLC is available, run route *Alternative A* by gravity instead.

(3.) Analyze the peak fractions by SDS-PAGE 🗎 SOP 0007 and inspect the FPLC chromatogram. Pool the fractions carrying a single sharp band at 70 kDa (His6-MBP-TEV) or 28 kDa (His7-TEV-Arg5), taking the peak proper and leaving a shoulder on either side of it out rather than pooling to baseline.

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> ***Critical:*** The 3C linker in pOPIN is not a TEV substrate, so the fusion protein stays intact. pRK793 self-cleaves instead: the freed MBP (43 kDa) carries no His tag and washes through, but fusion that failed to cleave (72 kDa) is tagged and does bind.

›› *Dialysis and concentration*

(1.) Add TCEP to 0.5 mM to the pooled fractions. Pre-rinse the dialysis tubing and transfer the material into it.

(2.) Dialyze overnight at 4 °C against 1 × dialysis buffer at a sample-to-buffer ratio of $1:25$ or higher.

> ***Critical:*** 1.5 L dialysis buffer brings a sample of up to 60 mL below 20 mM imidazole. Split larger pools across two baths.

(3.) Recover the dialysate. The in-bag TCEP has dialysed out by now, so add TCEP again to 0.5 mM final. Clear the dialysate at 20 000 × *g* for 15 min at 4 °C to remove any precipitate.

(4.) Determine the concentration spectrophotometrically at 280 nm. Bring to 2 g/L with 1 × dialysis buffer. If below 2 g/L, concentrate over an ultrafiltration device (SOP 0045).

> ***Critical:*** Do not concentrate past 2.0 g/L. The protease precipitates against the membrane.

> ***Quality assurance:*** A typical His-TEV preparation yields on the order of 30 mg per 1 L of culture.

(5.) Add 1.0 vol glycerol diluent. Mix until homogeneous, do not shake.

›› *Aliquot, storage, and lot record*

(1.) Aliquot the protease into 50–100 µL portions and store at −20 °C.

(2.) Record the following lot metadata in the protease lot log:

- Lot number as `MM/YYYY`, following the labelling convention in SOP 0030.
- Date of preparation and contributor.
- Final concentration (mg/mL) and total yield (mg).
- Number and size of aliquots.
- Specific activity (mg protease required to cleave 1 mg of 70 kDa substrate at 4 °C overnight).
- Storage buffer composition and storage location.

›› *Standard TEV activity assay*

(1.) Dilute the TEV-cleavable test substrate to 0.5 g/L with TEV assay buffer. Any purified His-MBP fusion carrying an `ENLYFQ/G` site will work. Activity is normalized to a nominal substrate mass of 70 kDa.

> ***Critical:*** One lot test consumes about 220 µL of the diluted substrate. The substrate must be purified before the test is started.

(2.) Thaw two separate TEV protease aliquots of the lot. From each, prepare 50 µL at 0.4 g/L in assay buffer. For a lot at 1 g/L this is 20 µL lot plus 30 µL buffer.

> ***Critical:*** The two aliquots carry the replication of the assay. Splitting one aliquot into two series replicates the pipetting but not the aliquot.

(3.) Carry each replicate down a five-point two-fold series in reaction tubes SOP 0031. Pre-fill tubes 2 to 5 with 25 µL assay buffer, transfer 25 µL from tube 1 into tube 2, mix, and continue with a fresh tip:

Tube	TEV concentration	TEV mass per assay	TEV per 1 mg substrate
1	0.400 g/L	2.000 µg	0.200 mg
2	0.200 g/L	1.000 µg	0.100 mg
3	0.100 g/L	0.500 µg	0.050 mg
4	0.050 g/L	0.250 µg	0.025 mg
5	0.025 g/L	0.125 µg	0.013 mg

(4.) Transfer 5 µL of each dilution of both series into fresh tubes. Add 20 µL of the 0.5 g/L substrate dilution (10 µg substrate; 25 µL total). Mix gently, spin down briefly, and incubate overnight at 4 °C.

(5.) Stop each reaction with 8 µL 4 × SDS-PAGE sample buffer SOP 0007 and heat to 95 °C for 5 min. 18 h
Prepare substrate-only (20 µL substrate dilution plus 5 µL assay buffer) and protease-only (5 µL of tube 1 of either series plus 20 µL assay buffer) controls.

> ***Critical:*** A His6-MBP-TEV lot and the uncleaved substrate differ by under 2 kDa and co-migrate on this gel; a His7-TEV-Arg5 lot at 28 kDa does not. Use the protease-only lane to place the protease, and read cleavage from the product bands rather than from the substrate position.

(6.) Resolve both series and the two controls on one 12% SDS-PAGE gel SOP 0007, loading 10 µL per lane. Stain with Coomassie SOP 0010. Look for the substrate band at 68.8 kDa to disappear in favor of the His-MBP (44 kDa) and Rab7 (23.7 kDa) product bands.

> ***Quality assurance:*** The twelve samples fit a 12-well comb; cast a 15-well gel to carry a molecular-weight marker alongside them. Keeping both series on one gel means the two endpoints are compared under identical staining.

Optional: *Using TEV protease for tag cleavage*

(1.) Verify that the fusion places `ENLYFQ/G` between the tag and the target, with at least three residues of flexible linker between the cleavage site and the folded domain of the target. Cleavage leaves the Gly on the target N-terminus.

(2.) Match the cleavage mixture to the following reaction conditions:

- Buffering system: Tris, HEPES, phosphate, or MES buffer (pH 6.0 to pH 9.0).
- Temperature: 4–37 °C; activity peaks near 34 °C, so 37 °C costs little. The default, 4 °C overnight, is chosen for the target rather than for the protease.
- Reductant: 1 mM TCEP, or 1–5 mM DTT.
- Monovalent salt: fully active at low salt; half as active at 500 mM NaCl; keep the digest near 150 mM where the target allows.
- Imidazole: dilute or exchange an IMAC eluate to 20 mM or below before a long digest.
- Tolerated protease inhibitors: PMSF and AEBSF (1 mM), TLCK (1 mM), pepstatin A (1 mM), bestatin (1 g/L), E-64 (3 g/L), Roche "complete" cocktail (1 ×).

(3.) Avoid conditions incompatible with TEV protease activity:

- Zn^{2+} at 5 mM or above, Cu^{2+} at 1 mM or above, Co^{2+} at 10 mM or above. Add EDTA up to 1 mM to quench divalent metals.
- Glycerol or sorbitol exceeding 40% (w/v).

- Iodoacetamide and NEM are potent TEV protease inhibitors.
- For detergents, verify per detergent before scaling up; some are inhibitory.

(4.) Add TEV to the pooled chromatographic fractions at the ratio indicated by the current lot's activity assay (typically 0.05 mg TEV per 1 mg target). Take a zero-time sample for SDS-PAGE, mix with sample buffer, and freeze immediately. 📖

(5.) Pre-rinse the dialysis tubing or cassette and transfer the cleavage mixture into it. Add TCEP to 0.5 mM directly into the dialysis vessel before sealing. Dialyze against the target's storage or next-step buffer at a sample-to-buffer ratio of at least $1:100$ for 16 h at 4 °C. ⏲ 16 h 📖

(6.) Remove the His6-MBP-TEV protease and any released His-tagged portion of the substrate by reverse IMAC 📄 SOP 0045. The cleaved, tag-free target appears in the flow-through.

> ***Critical:*** Remove EDTA and strong reductants such as DTT before reverse IMAC; EDTA strips Ni^{2+} from the resin and DTT reduces it. The preceding dialysis does this provided the dialysis buffer contains neither. TCEP at 1 mM, as used throughout this protocol, is tolerated on immobilized nickel and does not have to be removed. ⬅

(7.) Confirm complete cleavage by SDS-PAGE 📄 SOP 0007 against the zero-time sample. Expect loss of the tagged precursor band and gain of two product bands. 🐞

🖉 Recipe (available online) 🐞 Troubleshooting (available online) 📖 Notes (available online)

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