# Expression and Purification of His-Tagged TEV Protease Triple Mutant (L56V, S135G, S219V) in BL21(DE3) RIL Cells

The expression vector pH6TevMut produces TEV protease with an N-terminal hexahistidine tag. This TEV protease construct carries three mutations relative to WT. The S219V mutant has been shown to be more stable and more efficient as a catalyst than the wild-type protease [1]. The addition of L56V and S135G mutations drastically improves solubility [2].

We found that auto-induction with BL21(DE3)RIL cells (Stratagene) is much better than IPTG induction. Note: For optimal growth 500mL media should be placed in a 2L baffled flask to give better aeration. Recipes for media are included at end of document.

**Expression BL21(DE3)RIL cells:**

1. Transform pH6TevMut into BL21(DE3)RIL cells and plate on LB/Kan/Cap plates.
2. Pick 3-4 colonies from freshly transformed BL21(DE3)RIL cells and inoculate 5ml of LB containing 30 ug/ml of Kanamycin and 34 ug/ml of Chloramphenicol. Incubate overnight at 37°C with 225 rpm shaking.
3. The next morning, inoculate 0.5L of ZYP-5052 rich media for auto-induction containing 100 μg/ml Kanamycin and 25 μg/ml of Chloramphenicol with 0.5ml overnight culture.
4. Incubate at 25°C with 290 rpm shaking for ~24 hours (overnight).
5. Monitor growth the next day at $OD_{600}$ until the reading plateaus. This usually takes ~1-2 hours. Induction happens very close to saturation, so it is critical that you don't harvest too early.
6. Harvest by centrifugation at 6,500 rpm for 10 minutes at 4°C. Weigh the cell pellet and store at -80°C. Typical yield is ~11g wet cell pellet per 500mL induction.
7. Check expression by SDS-PAGE. You should see a single band for Tev protease at 28.5kDa.

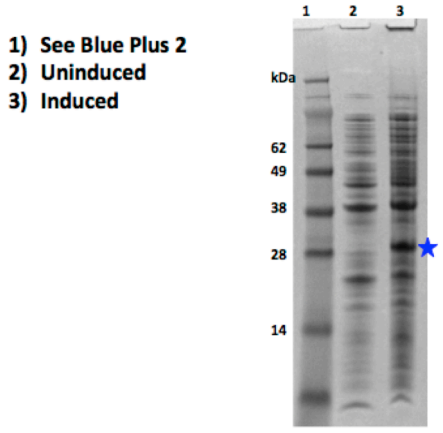


**Figure 1: Expression of TevMut via auto-induction.**

**H6-TEV protease (L56V, S135G, S219V) Amino Acid Sequence:**

M G S S H H H H H H G G G E S L F K G P R D Y N P I S S T I C H L T N E S D G H T T
S L Y G I G F G P F I I T N K H L F R R N N G T L V V Q S L H G V F K V K N T T T L Q
Q H L I D G R D M I I I R M P K D F P P F P Q K L K F R E P Q R E E R I C L V T T N F
Q T K S M S S M V S D T S C T F P S G D G I F W K H W I Q T K D G Q C G S P L V S T
R D G F I V G I H S A S N F T N T N N Y F T S V P K N F M E L L T N Q E A Q Q W V S
G W R L N A D S V L W G G H K V F M V K P E E P F Q P V K E A T Q L M N E L V Y S
Q

Number of amino acids: 254
Molecular weight: 28794.6
Theoretical pI: 8.66

**Purification largely follows protocol detailed in ref #2.**

| Lysis Buffer | Wash Buffer | Elution Buffer | Dialysis Buffer (4L) |
|---|---|---|---|
| 50 mM $NaH_2PO_4$<br>0.3 M NaCl<br>pH 7.5 | 50 mM $NaH_2PO_4$<br>0.3 M NaCl<br>25 mM Imidazole<br>10% Glycerol<br>* 5mM BME<br>pH 7.5 | 50mM $NaH_2PO_4$<br>0.3M NaCl<br>300mM Imidazole<br>10% Glycerol<br>* 5mM BME<br>pH 7.5 | 25mM $NaH_2PO_4$<br>0.2M NaCl<br>* 5mM BME<br>10% Glycerol<br>pH 8.0 |

* Add BME fresh on the day of purification

***Note: Tev protease tends to precipitate when over-handled, so it's important for the IMAC elutions to be as concentrated as possible so that you avoid having to concentrate the protein.*** **All steps are carried out at 4°C:**

1. Equilibrate Qiagen Ni-NTA Resin in 50mL conical tubes using lysis buffer (use 2mL packed resin per ~11g wet cell paste).
2. Thaw cell pellet on ice and resuspend in lysis buffer (6mL per gram weight pellet).
3. Add 200 μg/mL of lysozyme.
4. Sonicate on ice for 10 minutes, 5sec. on/10sec. off. (Allow for appropriate cooling periods in between bursts to avoid overheating, which will cause aggregation of protein).
5. Centrifuge the lysate at 22k rpm for 20 minutes at 4°C to pellet cellular debris. Save supernatant.
6. Add supernatant to pre-equilibrated resin and rock on a rotary shaker for 45min to 1 hour at 4°C.
7. Spin down at 3,500 rpm for 10 minutes at 4°C in a benchtop centrifuge to pellet resin. Note: At this point a brown color should be detected on the resin indicating lots of bound protein.

8. Decant supernatant. Resuspend resin in lysis buffer (~5-10mL) and pour into column. Drain. Repeat to get all the resin transferred into the column. Drain. Save flow through.
9. Add 5 column volumes (CV) wash buffer, shake gently to resuspend beads. Drain. Add 5CV wash buffer. Drain. Repeat until you've washed with a total of 50 CV of wash buffer.
10. Elute the protein with 8CV of elution buffer. Make first elution 0.5CV and last elution 4CV to ensure all the protein is off of the beads.
11. Analyze fractions by SDS-PAGE.

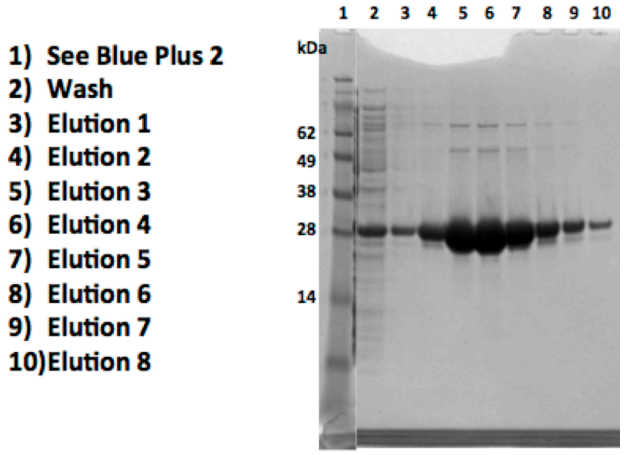


**Figure 2: IMAC purification of H6TevMut.**

12. Pool only the most concentrated fractions and dialyze (10K MWCO dialysis tubing). Place in 2L of cold dialysis buffer for ~2 hours then swap into 2L fresh dialysis buffer and let it dialyze overnight. The point of the dialysis swap is to remove as much of the imidazole as possible.
13. Overnight dialysis usually results in little to no precipitation of protein, but if any is present, it is removed by centrifugation.
14. Measure the protein concentration by absorbance at 280 nm (Extinction Coefficient = 33710 $M^{-1}cm^{-1}$). A concentration of 100 -150 µM is usually sufficient. Note: concentrating routinely causes precipitation, so this should be a last-case scenario.
15. Reference #2 stores the enzyme in this buffer at -80°C, but we've noticed some loss of activity over time under these conditions. Therefore, if possible, store the protein in 50% glycerol rather than 10%. Typical yields from are ~15 mg per 0.5L induction.

**Activity Test of H6TEVmut**

Buffer Conditions: 50 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 7.5
Test substrate: HisMBP-RPA70AB, 71.7kDa; Cleavage products: 44.5kDa & 27.2kDa

The molar ratio of substrate:enzyme was 50:1 and after mixing, samples were removed at various time points. Gel loading buffer was added and the samples were boiled to stop the reaction. Samples were analyzed by SDS-PAGE. As seen in Figure 3, cleavage is much faster at room temperature.

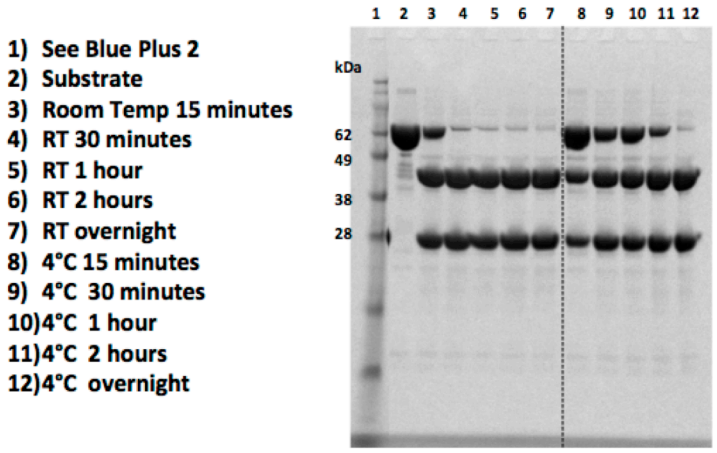


**Figure 3: Activity test of H6TevMut. Room temperature vs. 4°C cleavage.**

**ZYP-5052 rich medium for auto-induction [3]:**
**This medium allows growth to high densities. It requires good aeration, so use 500mL medium in 2L baffled flasks and shake at high speeds. Studier has found that high concentrations of kanamycin are required for selection.**

ZYP-5052 rich medium for auto-induction
Add 1 M $MgSO_4$ and 1000x metals mix before adding 20xNPS to avoid precipitate

| Large scale | 200 ml total | 500 ml total | 1 liter tot | Final conc |
|---|---|---|---|---|
| ZY | ~186 ml | ~464 ml | ~928 ml | |
| 1 M $MgSO_4$ | 0.2 ml | 0.5 ml | 1 ml | 1 mM |
| 1000x metals mix* | 0.2 ml | 0.5 ml | 1 ml | 1x |
| 50x5052 | 4 ml | 10 ml | 20 ml | 1x |
| 20xNPS | 10 ml | 25 ml | 50 ml | 1x |
| Antibiotics, as needed: | | | | |
| Kanamycin (25 mg/ml) | 0.8 ml | 2 ml | 4 ml | 100 µg/ml |
| Chloramphenicol (25 mg/ml) | 0.2 ml | 0.5 ml | 1 ml | 25 µg/ml |

20xNPS: (NPS = 100 mM $PO_4$, 25 mM $SO_4$, 50 mM $NH_4$, 100 mM Na, 50 mM K)

| To make 100 ml: | To make 1 liter: | |
|---|---|---|
| 90 ml water | 900 ml water | |
| 6.6 g $(NH_4)_2SO_4$ | 66 g $(NH_4)_2SO_4$ | = 0.5 M |
| 13.6 g $KH_2PO_4$ | 136 g $KH_2PO_4$ | = 1 M |
| 14.2 g $Na_2HPO_4$ | 142 g $Na_2HPO_4$ | = 1 M |

add in sequence in beaker, stir until all dissolved
pH of 20-fold dilution in water should be ~6.75
Autoclave

50x5052: (5052 = 0.5 % glycerol, 0.05% glucose, 0.2% alpha-lactose)

| To make 100 ml: | To make 1 liter: |
|---|---|
| 25 g glycerol (weigh in beaker) | 250 g glycerol (weigh in beaker) |
| 73 ml water | 730 ml water |
| 2.5 g glucose | 25 g glucose |
| 10 g α-lactose | 100 g α-lactose |

Add in sequence in beaker, stir until all dissolved.
Lactose is slow to dissolve -- may take two hours or more at room temperature. Can speed up by heating in microwave oven.
Autoclave

1 M $MgSO_4$
24.65 g $MgSO_4$-$7H_2O$
Water to make 100 ml
Autoclave.

The 1x trace metals mixture is an attempt to saturate almost any metal-containing target protein, even at high levels of expression. The 1x concentrations are below toxic levels, as tested by growth in different concentrations of the metals individually. Target proteins produced at 100 mg/liter would have a concentration of 2 μM for a protein of 50,000 Da or 10 μM for a protein of 10,000 Da. If the metal content of an expressed protein is known, a saturating amount of that metal can be added rather than 1x metals mix.

1000x trace metals mixture (100 ml in ~50 mM HCl)

| Add to 36 ml sterile water: | | MW | 1x conc |
|---|---|---|---|
| 50 ml | 0.1 M $FeCl_3$-$6H_2O$ (dissolved in ~0.1 M HCl = 100-fold dil of conc HCl) DO NOT AUTOCLAVE $FeCl_3$ STOCK!!!! | 270.30 | 50 μM Fe |
| 2 ml | 1 M $CaCl_2$ | 110.99 | 20 μM Ca |
| 1 ml | 1 M $MnCl_2$-$4H_2O$ | 197.91 | 10 μM Mn |
| 1 ml | 1 M $ZnSO_4$-$7H_2O$ | 287.56 | 10 μM Zn |
| 1 ml | 0.2 M $CoCl_2$-$6H_2O$ | 237.95 | 2 μM Co |
| 2 ml | 0.1 M $CuCl_2$-$2H_2O$ | 170.486 | 2 μM Cu |
| 1 ml | 0.2 M $NiCl_2$-$6H_2O$ | 237.72 | 2 μM Ni |
| 2 ml | 0.1 M $Na_2MoO_4$-$5H_2O$ | 241.98 | 2 μM Mo |
| 2 ml | 0.1 M $Na_2SeO_3$-$5H_2O$ | 263.03 | 2 μM Se |
| 2 ml | 0.1 M $H_3BO_3$ | 61.83 | 2 μM $H_3BO_3$ |

Autoclave the stock solutions of the individual metals, except 0.1 M $FeCl_3$ in 1/100 volume conc HCl.
A brief precipitate appeared upon addition of $Na_2SeO_3$, which redissolved rapidly

Store at room temperature

## References

1. Kapust, R.B., et al., *Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency.* Protein Eng, 2001. 14(12): p. 993-1000.
2. Cabrita, L.D., et al., *Enhancing the stability and solubility of TEV protease using in silico design.* Protein Sci, 2007. 16(11): p. 2360-7.
3. Studier, F.W. *Protein production by auto-induction in high-density shaking cultures* Protein Expression and Purification 41 (2005) 207–234.