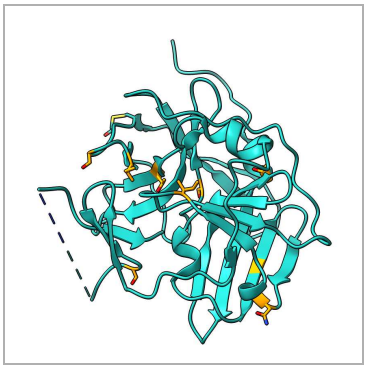

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We use this protocol and it's working

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Purification of 10xHis-SuperTEV

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ABSTRACT

SuperTEV is a mutated version of TEV (Tobacco Etch Protease) a cysteine protease widely used in labs as it is highly specific to a cleavage sequence that can be genetically encoded. Depending on the lab, it has been reported that the protease is unstable or purifies with low yields. Efforts have been made for many years (Tropea, 2009, Methods Mol Biol) to improve its solubility. Here we report our platform's efforts in generating a new TEV variant, called SuperTEV, that incorporates 9 mutations identified by different groups in recent years applied together at once. We find that the protein is easily produced and purified in large amounts and is functional.

The list of mutations on the canonical TEV protease are the following (with references that also identified the same mutations):

T17S (directed evolution, increased solubility and production) van den Berg et al., 2006 and Wei et al., 2012
L56V (rational design, improved solubility) Cabrita et al., 2007 and Wei et al., 2012
N68D (directed evolution, increased solubility and production) van den Berg et al., 2006 and Wei et al., 2012
I77V (directed evolution, increased solubility and production) van den Berg et al., 2006 and Wei et al., 2012
S135G (rational design, increased solubility) Cabrita et al., 2007 and Wei et al., 2012
I138T (increased catalytic activity; TEV3) Sanchez and Ting, 2019
S153N (increased catalytic activity; TEV3) Sanchez and Ting, 2019
T180A (increased catalytic activity; TEV3) Sanchez and Ting, 2019
S219V (inhibits autoproteolysis) Kapust, 2001

References:

Wei et al., 2012. Protein Expression and Purification
Sanchez and Ting, 2019. Nature Methods
van den Berg et al., 2006. Journal of Biotechnology
Cabrita et al., 2007. Protein Science
Kapust et al., 2002. Biochemical and Biophysical Research Communications

GUIDELINES

This protocol is written with the expectation of standard bacterial culture and basic purification knowledge

MATERIALS

Plasmid Addgene #193833 (https://www.addgene.org/193833)
BL21 (DE3) cells (Lucigen)
AutoTB + trace elements (Formedium) or TB + trace elements (Formedium)
Glycerol (Applichem)
AktaGO (Cytiva)
HiFliq Ni-NTA columns (ProteinArk)
12-14 kDa dialysis tubing (SpectraPor)
Dialysis clips (SpectraPor)
Emulsiflex
5 M NaCl
1 M HEPES, pH 7.5
2.5 M imidazole pH 7.5 (We recommend, Sigma #56749-1KG for low background absorbance)
1 M DTT
1 M IPTG
4X LDS loading dye (Genscript)
NuPage 4-20% SDS-PAGE Gels (Thermofisher)
8M Urea

BEFORE START INSTRUCTIONS

Recommended to have ready before starting alongside Materials.

-Autoclaved flasks
-Autoclaved LB media
-Autoclaved AutoTB media
-Buffers
-Liquid nitrogen

Growing bacterial cultures (6 L)

4d

1 Transform the bacterial plasmid expressing the 10xHis-SuperTEV into BL21 (DE3) cells. Plate on to LB-Agar plates + Kanamycin. Grow overnight at 37 °C or over the weekend at 25 °C. 1d

2 In an afternoon, pick a streak of cells and innoculate into 200 mL of LB + Kanamycin media. Note: for every 1 L of expression culture, you will require 10-20 mL of pre-culture. Grow 1d

overnight at 37 °C

3 The next morning, innoculate 20-40 mL of preculture into 3 flasks containing 2 L of Autoinduction TB media (2 L in a 5 L flask is appropriate). Shake in an incubator at 37 °C for 3-4 hours until OD600 ~0.8-1. Take a 1 mL sample of the culture. Immediately change the temperature to 18 °C and continue shaking overnight (approximately 20:00:00) 20h

> **Note**
>
> Regular LB or TB media can also be used. Induction will be at the same point as the temperature change with 0.5 mM IPTG

4 Take a 1 mL sample of the culture and measure the OD600. Harvest the culture by centrifuging at 5000 x g, 10°C, 00:30:00 . Transfer the pellets to 50 mL tubes directly or resuspend in minimal amounts wash buffer buffer transferring. Pellets can be used immediately or store at -20 °C indefinitely. 30m

5 Confirm expression by running an SDS-PAGE gel to observe the appearance of a band around 25 kDa that would represent the production of the 10xHis-SuperTEV. 40m

Recommended recipe to prepare SDS-PAGE samples. Mix 1:4, 4X LDS loading dye and 8 M Urea, to make a Urea loading dye.

For samples, calculate the amount of sample needed to prepare. We use the following formula:

1/OD600 x 100 uL = **volume in uL to centrifuge down** 14000 rpm, 00:01:00 .

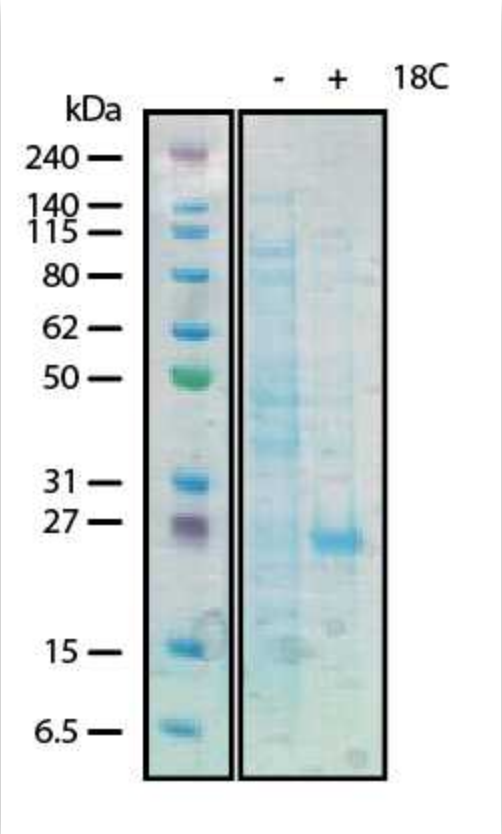
Discard the supernatant, Keep the pellet.

Resuspend pellet in 40 uL of the Urea loading dye.

Load 10 uL on to a NuPage 4-12% bis-Tris SDS-PAGE gel.

Expected result

Observed appearance of band near 25 kDa that would represent the induced protein



Induction gel

Prepare purification buffers

30m

6 From stock solutions prepare the following : 30m
Filter 0.22 or 0.45 um

	A	B	C
	Buffer	Component	Concentration (mM)
	Wash buffer (2 L)	NaCl	700
		HEPES, pH 7.5	20
	Elution buffer (1 L)	NaCl	700

	A	B	C
		HEPES, pH 7.5	20
		Imidazole, pH 7.5	500

Buffer lists for purification

Purification by Ni-NTA on an AKTA system

3h 30m

7 Resuspended pellets were supplemented with glycerol to 10% and DNase.
The mixture was then lysed using an Emulsiflex device by 3 passes until the lysate was visually not viscous. 30m

> **Note**
>
> Other lysis methods such as sonication and french press can also be used. We find it most efficient with an emulsiflex as due to the larger culture volumes, pellets are large and there is substantial amounts of nucleic acids that need to be sheared.

8 Total volume is around 100 mL. Centrifuge down the lysate at 20000 x g, 4°C, 00:40:00. 40m

9 Transfer the supernatant to a clean container, being sure not to transfer any liquid that does not contain visually turbid particles near the pellet. Filter the supernatant using a 0.45 um filter 15m

10 Supplement the filtered supernatant with [M] 1 Molarity (m) imidazole to a final concentration of [M] 25 millimolar (mM) imidazole 5m

11 1h 30m

Note

Bulk resin can also be used. One can program their AKTA system to equilibrate and load all automatically or be run manually as per one's lab methodology.

See the Materials for our recommendation for low-absorbance imidazole that has minimal interference with A280 measurements of protiens

Using an AKTA system, load the sample on to 3 x 5 mL HiFliq Ni-NTA (15 mL = 1 CV) columns equilibrated with 5% Elution buffer ([M] 25 millimolar (mM) imidazole).

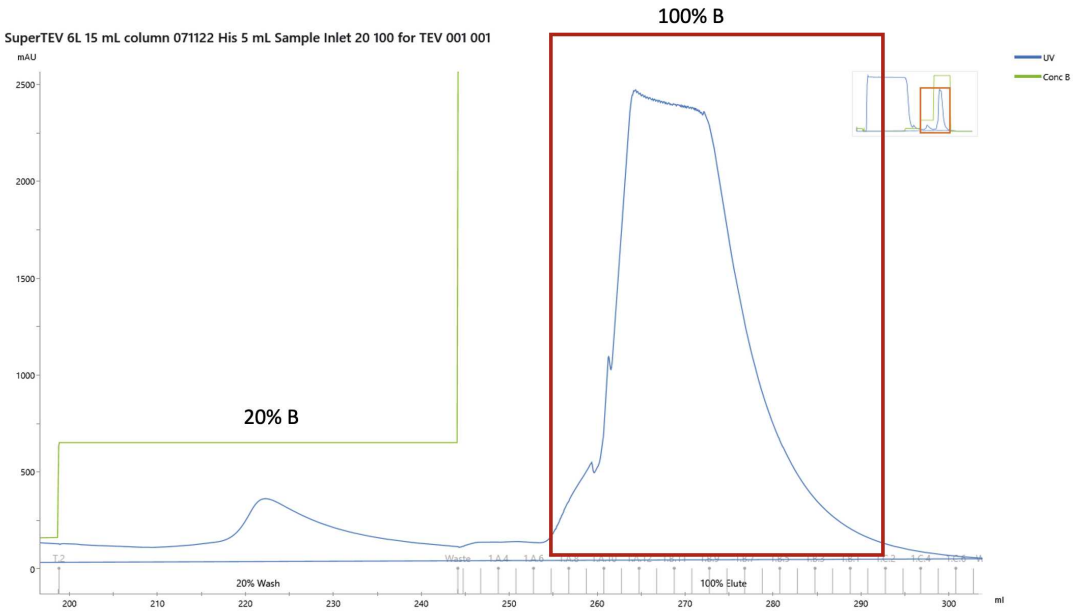
The loaded sample was then washed extensively and eluted as a step gradient as follows

	A	B	C
	Step	Elution buffer concentration (%B)	Column Volumes (CV)
	Wash + 25 mM imidazole	5	8
	Wash + 100 mM imidazole	20	5
	Wash + 500 mM imidazole	100	10

Gradient for Elution

Expected result

A typical elution profile looks like this

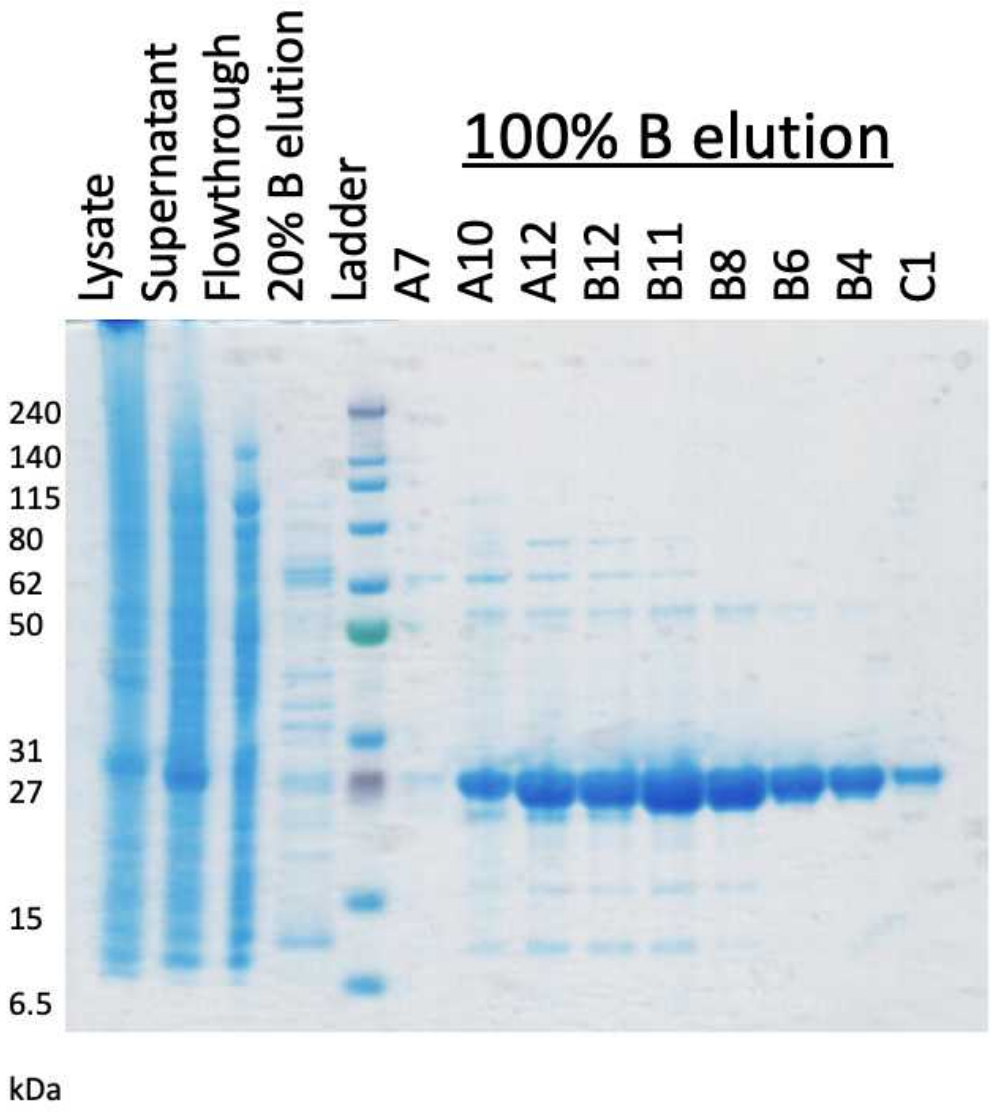


Expected peak during elution

Collect fractions as appropriate for verification on an SDS-PAGE gel.

Expected result

Typical SDS-PAGE gels of all fractions during purification



SDS-PAGE gel of Ni-NTA purification

12 The protein elutes at [M] 500 millimolar (mM) imidazole. Fractions containing the purest protein are pooled. It is advised to measure the absorbance at A280 to determine the concentration. 10m

1 mg/mL of SuperTEV = *A280, 1.2*

13 Dilute with wash buffer to around [M] 2-3 mg/mL or as desired. 5m

14 Transfer the protein to dialysis bags for dialysis in 2 L of dialysis buffer ([M] 150 millimolar (mM) NaCl , [M] 20 millimolar (mM) HEPES, pH7.5 , [M] 5 millimolar (mM) DTT , [M] 10 % (v/v) glycerol) at 4C overnight 15m

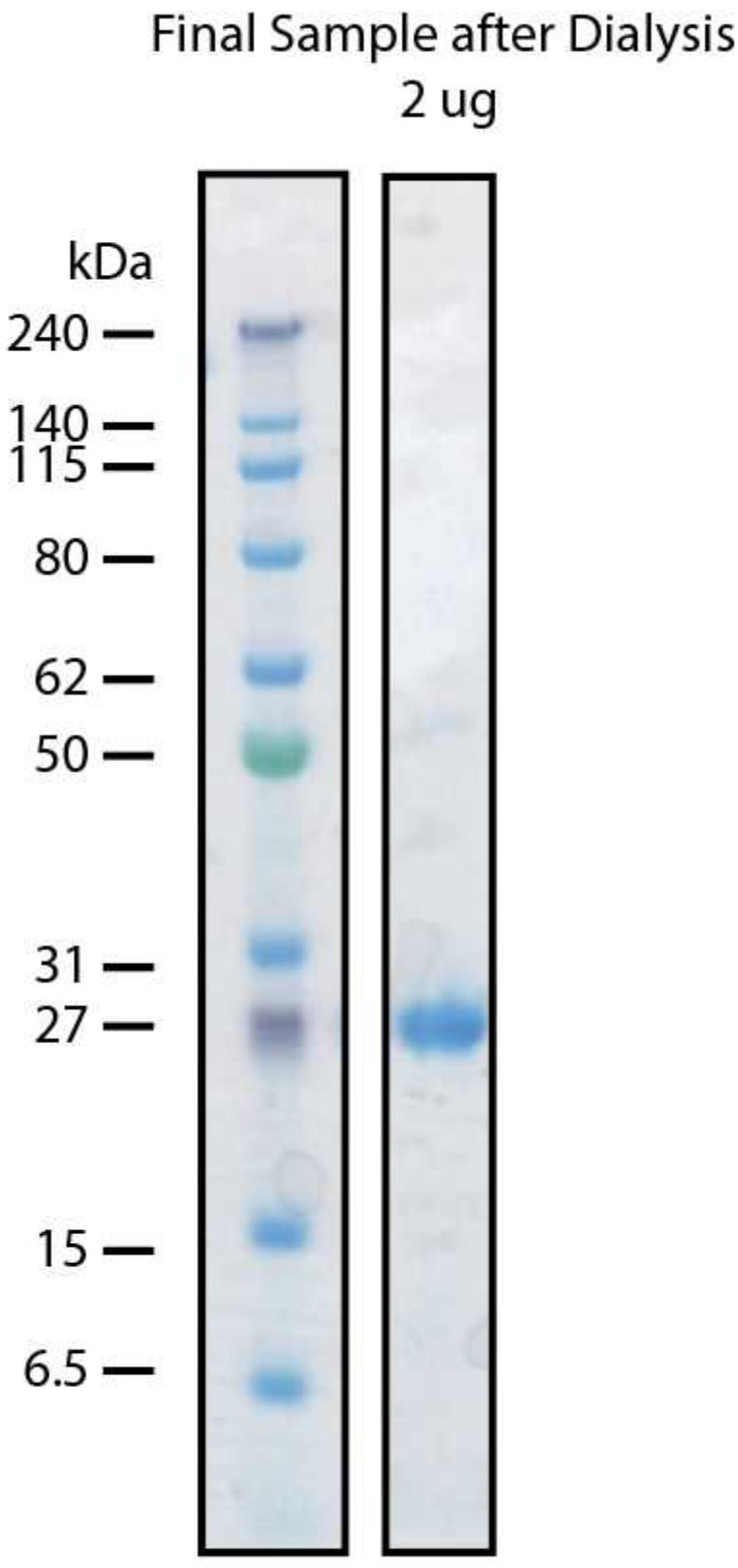
15 The next day precipitation will be expected. Transfer the dialyzed material to 50 mL tubes and centrifuge 20000 x g, 4°C, 00:10:00 . The supernatant should be clear. Once again determine the concentration by measurement at A280. 30m

We have never required to concentrate our TEV protease. We make aliquots that correspond to 1-2 mg per tube. Aliquots are flash frozen under liquid nitrogen and then store indefinitely at -80 °C

Our final yield is typically in the 10s of mg/L (on average 50 mg/L)

Expected result

Final sample should look like this



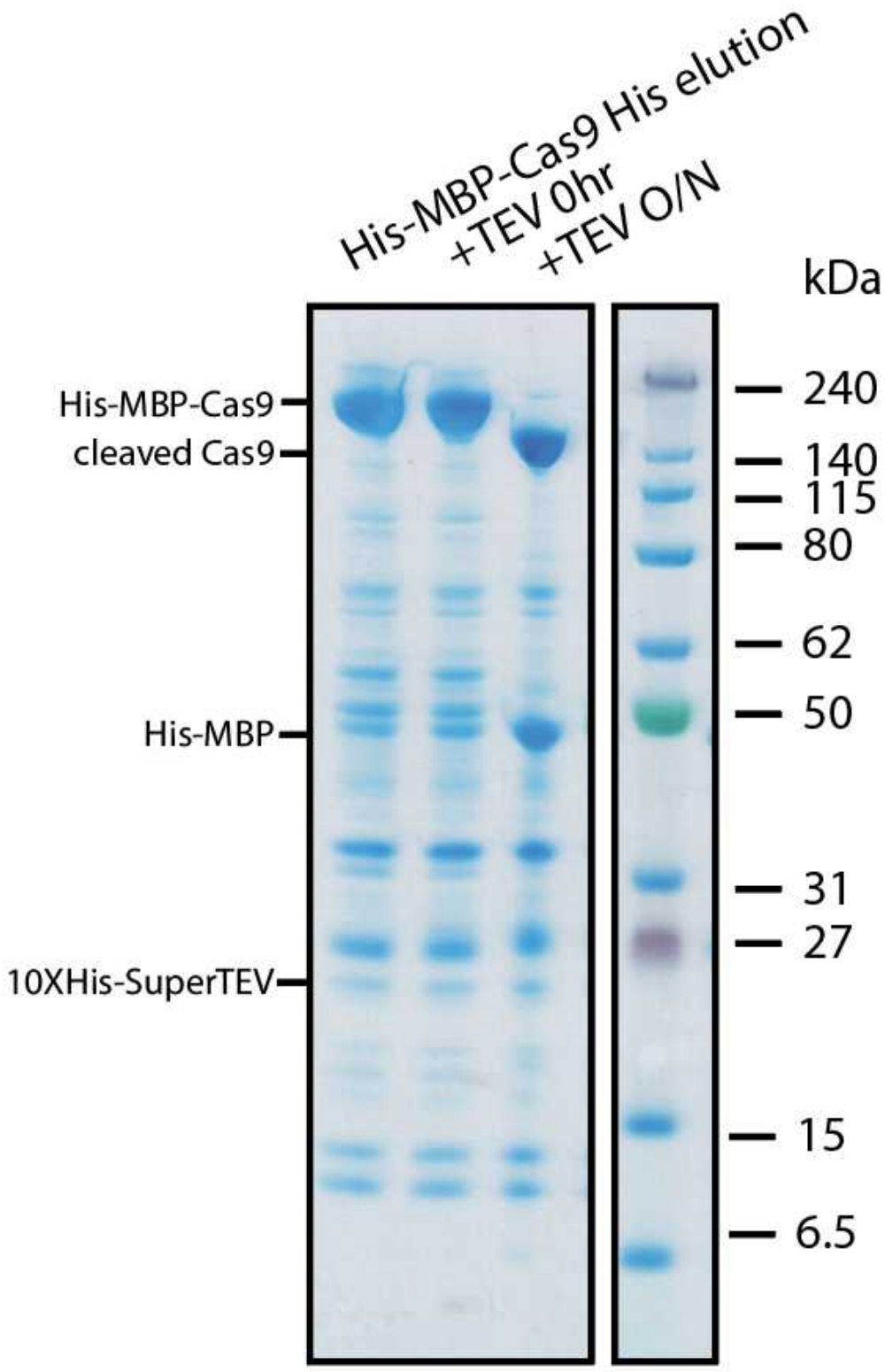
Final sample loaded on a gel

Use of 10xHis-SuperTEV Protease

16 We have tested cleavage of substrates containing the TEV protease site of ENLYFQ/GS. We typically use the protease in a 1:50-1:100 (protease:protein mass ratio). However we have used it also at significantly higher ratios. The protease performs well during dialysis in PBS or HBS buffers at Room temperature or 4 °C overnight

Expected result

His-MBP-Cas9 was purified from Addgene #69090 and cleaved with SuperTEV in a 1:100 ratio. (100 mg total crude protein from elution : 1.4 mg SuperTEV) overnight at 4C



Sample cleavage with SuperTEV protease