Purification of TEVS219V\_L56V\_S135G and activity test

6xHisTEVS219V\_L56V\_S135G-MBP expression: MBP will be cut off by TEV itself during the purification steps

# Day 1

* Media preparation for GST-Tagged protein (*Erwina amylovora* levansucrase, Lsc, in this case) and TEV: 3 x 500 ml 2xYT
* Preparation buffers for Lsc purification:
  + PBS pH 7.3, 150 mM NaCl: 250/500 ml
  + Elution buffer: 50 mM TrisHCl pH 8.0, 10mM Red-Glu: 100 ml
* Preculture Lsc (Kan 30mg/l): 6 ml medium

# Day 2

* Culture Lsc (1x500 ml 2xYT, Kan 30mg/l): Preculture 1/100 culture volume(5 ml). 37°C 220 rpm ~ 2.5h (OD600~1.2) (chill on ice if OD higher) and grow for another hour at 20°C. Induce at 20°C with 1 mM IPTG, o/n (~16h)
* Preparation buffers for TEV purification
  + 25 mM PBS pH 8.0, 200 mM NaCl, Glycerol 10% (v/v), 25 mM Imidazol 25: 250 ml
  + 25 mM PBS pH 8.0, 200 mM NaCl, Glycerol 10% (v/v), 500 mM Imidazol: 100 ml
  + 25 mM PBS pH 8.0, 200 mM, Glycerol 10% (v/v), 5 mM DTT: 500 ml
* Preculture of TEV\_L56V/S135G (Amp 100 mg/l+Cam 34/40 mg/l): 20 ml

# Day 3

* TEV culture (2x500 ml A.I.M, Amp 200 mg/l+Cam 34/40 mg/l): Preculture 1/50 culture volume (10 ml), 37°C 220 rpm for ~5/6h, then at OD600 ~2/3 chill on ice and put at 20°C
* Lsc GSTrap:
  + Centrifugation (4500g, 20’, 4°C)
  + Resuspension 100 ml buf
  + Centrifugation
  + Resuspension in 50 ml Lysis buffer (Lysozime 0.25 mg/ml, DNase 20 μg/ml and P.I. AEBSF 0.5 mM final)
  + Sonication: 10s sonication/50s pause for > of 2’ of a sonication
  + Centrifugation: 18000 g 20’ 4°C
  + Filter the sample: before with 0.45um membrane, then 0.2um
  + GSTrap chromatography: 5 ml column, 1 step elution.
* SDS-PAGE of Lsc only if chromatogram is unclear
* Pull the fractions and aliquot Lsc: ~1 mg/ml aliquots of 1 ml each (εc~ 86.5, MW~73). Fresh freeze in LN and put at -80°C
* Put S75 under water and equilibration o/n (program)

# Day 4

* TEV 1st purification step: IMAC
  + Centrifugation (4500g, 20’, 4°C)
  + Resuspension 100 ml buf
  + Centrifugation
  + Resuspension in 50 ml Lysis buffer (Lysozime 0.25 mg/ml, DNase 20 μg/ml and P.I. AEBSF 0.5 mM final)
  + Sonication: 10s sonication/50s pause for > of 2’ of a sonication
  + Centrifugation: 18000 g 20’ 4°C, collect surnatant
  + Filter the sample: before with 0.45um membrane, then 0.2um
  + IMAC chromatography: 5 ml column, direct elution with 400mM on
* TEV 2nd step purification: SEC S75
  + Concentration: to 6 ml with 10KDa centricon.
  + Filtration through 0.2 um membrane
  + S75 run
  + Pull fractions, measure concentration (εc~33, MW~30)
  + Aliquot TEV (volume and concentration variable), fresh freeze in LN and put at -80°C.

# Day 5

* Thaw 1 aliquot Lsc (1ml 1mg/ml) and 1 aliquot of TEV.
* Divide the Lsc aliquot in 2 x 500 μl
* Add TEV (1:10 TEV protein ratio). “Lsc 1:10 cut” sample
* Add to the second aliquot TEV (1:100 TEV protein ratio). “Lsc 1:100 cut” sample. Keep the leftover TEV for SDS-PAGE
* Collect 13 μl from sample 1:10 and 10.7 μl (in order to have same quantity of 1:10) from sample 1:100 at intervals of 1, 2, 3h and heat inactivate TEV by preparing the SDS-PAGE sample (thus by sample buffer, DTT and a ~90°C step, better if using the Incubate option of the PCR machine!)
* Stocks of DTT and Sample loading buffer should be 10X (1M) and 4X, respectively. Prepare 20 ul SDS-PAGE samples (for 10 μl loads, thus keeping ~10 μl as back up): 2 μl DTT, 5 μl Sample loading buffer and 13 μl Sample+H2O.
* Make at least a SDS-PAGE of
  + TEV after IMAC
  + TEV after SEC/GF (you should see only pure TEV without the MBP protein part)
  + Lsc-GST
  + Lsc 1:10 cut 1h
  + Lsc 1:100 cut 1h (10.7 ul + 2.3 ul H2O)
  + Lsc 1:10 cut 2h
  + Lsc 1:100 cut 2h (10.7 ul + 2.3 ul H2O)
  + Lsc 1:10 cut 3h
  + Lsc 1:100 cut 3h (10.7 ul + 2.3 ul H2O)
* MWs expected:
  + Lsc-GST6xHis ~76 KDa
  + Lsc ~46 KDa
  + GST6xHis ~29 KDa
  + TEV-MBP ~72 KDa (TEV self digests the linker with MBP; MBP is probably used to help TEV getting its proper fold)
  + TEV ~ 30 KDa
  + MBP ~ 42 KDa
* Make vial good for 10/20 mg of protein to digest o/n at RT