Labbook

Wetlab - Protein purification, expression and ToxAssay

28.08.2018

Beginning of Tev (His-Tag) expression and purification.
Prepare buffer A HisTag (pH8. 0: 50mM NaP, 500mM NaCl, 10mM Imidazole)
Prepare buffer B HisTag (pH8. 0: 50mM NaP, 500mM NaCl, 350mM Imidazole)
Transformation of Rosetta bacteria with the Tev plasmid.
Overnight culture of the transformants.
LB Media autoclaved.

29.08.2018

LB Media mixed with Ampicillin and Chloramphenicol (1/1000).
Overnight culture in the LB media.
Place media in shaker (37°C, 100 rpm)
Measure OD600 until the value reaches 0. 6.
Cool addition of 900µl IPTG and shaker to 20°C.
5M HCL solution.

30.08.2018

Apply storage buffer DIA2x 2mM EDTA, 100mM Trisbase, 5mM DTT
Buffer A HisTag degassed
Bacteria Solution Centrifugation7k 15min
dry Pellet

Weigh wet mass: 7,7g

Lyse wet mass
Add 25mL buffer A Histag
Add 25µL DTT
Add spatula tip Lysozyme
Add DNAse: 2,75µl
Add Triton: 27,5µl

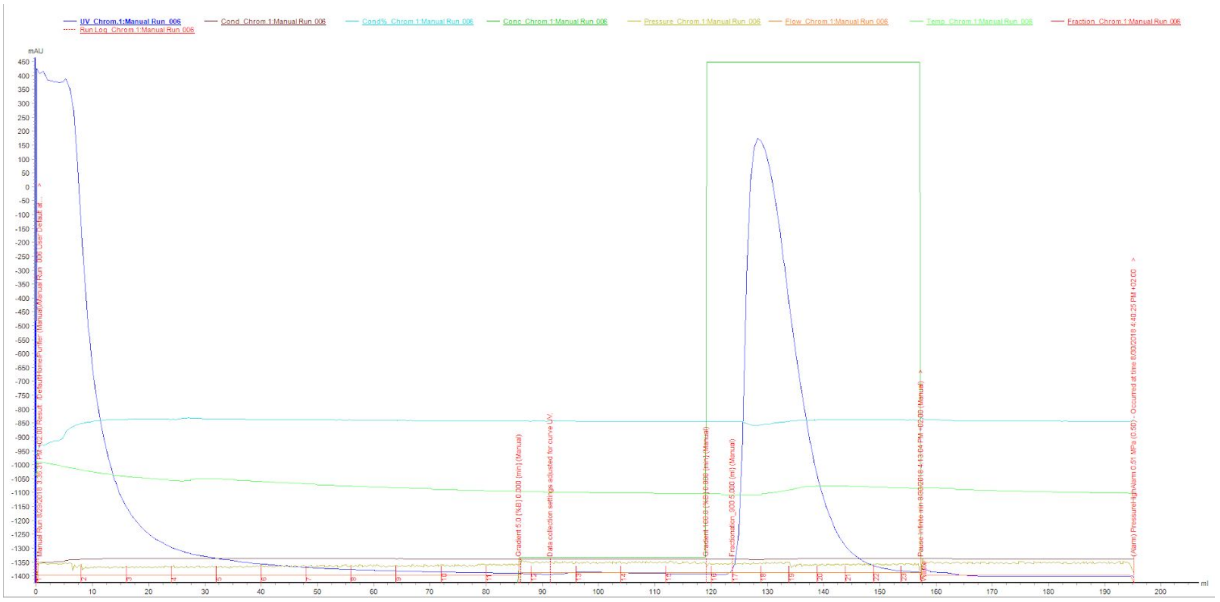
Storing lysate on ice

Sonification:
Amplitude 30% (large peak)
5:00 min
0. 5 Pulse on
2. 0 Pulse off

Lysate removed
Centrifugation 17k 50min
Transfer supernatant to piston

His column equilibrate
20min MilliQ Water
20min Buffer A Histag
45min Lysate circulate through column

ÄKTA
Connect buffer A and B Histag
Start PumpWash
Run buffer A through column
Add 5% buffer B
Add 100% buffer B



Fractions 17, 18, 19 united and dialyzed
Dialysis is performed with 1L storage buffer DIA2x.

31.08.2018

End dialysis and transfer contents to measuring cylinder

Nanodrop measurement (Tev Ext=31970; M=27731[g/mol])
Determination of protein concentration: 4. 42 mg/ml 240/260=0. 66

Dilute 15mL sample to 1 mg/mL
Add 1:2 glycerol tot he protein solution

Attention! Add glycerol while stirring

Freeze Aliquotes in liquid nitrogen and store at -80°C.

03.09.2018

Prep. PBS buffer (buffer A) for SNAP25 (20nM NaHP; 280mM NaCl; 6mM KCl) (V=2L)
Prep. 0.5 M sodium hydroxide solution to regenerate the Strep-Tag column (V=400mL)

Preparation of a 100mM Desthiobiotin Stoke solution
Watch your step! D-Biotin is sparingly soluble and should be added fresh to PBS as buffer B.

Buffer filtration and degassing

Apply 15% SDS gels:

Separating gel:
33.89mL 30% Acrylamide
16.89mL Tris/HCl pH8. 8
15.61mL distilled water
675µL SDS 10%
33.75µL TEMED
675µL APS

Collecting gel separating gel:
5. 85mL 30% Acrylamide
7.5mL Tris/HCl pH8. 8
27.45mL distilled water
450µL SDS 10%
45µL TEMED
450µL APS

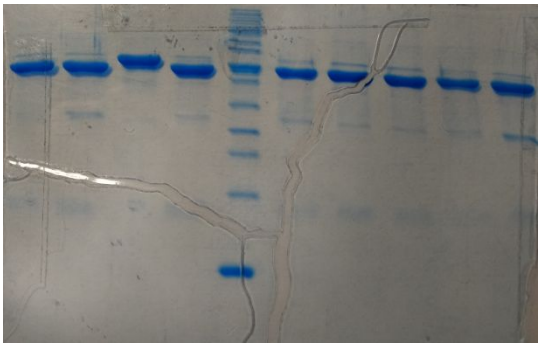
04.09.2018

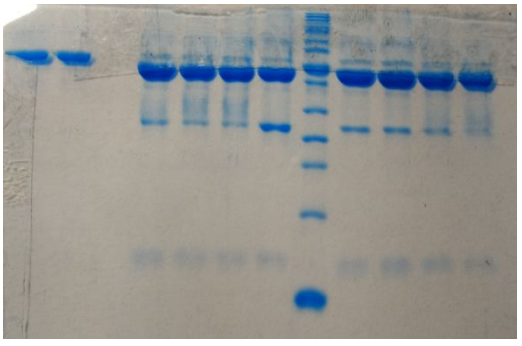
Activity Assay of Purified Tev Enzyme

Preparation of the approaches in a molar ratio of:
1:5; 1:10; 1:20; 1:50 Volume of the preparations is 20µL

Incubate control Tev enzyme and purified Tev with same substrate and apply to SDS gel.

Aktivitäts Assay





The enzyme activity of the freshly purified enzyme is the same with the positive control.
Conclusion Tev was successfully cleaned up.

05.09.2018

Gele konservieren

Fill Tupperware with distilled water (dH_2O)
Remove the SDS gel from the dye and transfer to Tupperware.
5 min shake
Then wash the gel with dH_2O.
Moisten a foil with dH_2O and place it on the plastic plate (the plate is on the frame).
Remove air bubbles between foil and plate
Place SDS Gel on foil
Moisten second foil with dH_2O and place carefully on gel and first foil (avoid air bubbles)
Place the second frame on the second foil and fix both frames over the clamps

Approaches of the LB medium
Add 45mg LB to 1.8L distilled water
Mix the solution
LB Medium autoclave

06.09.2018

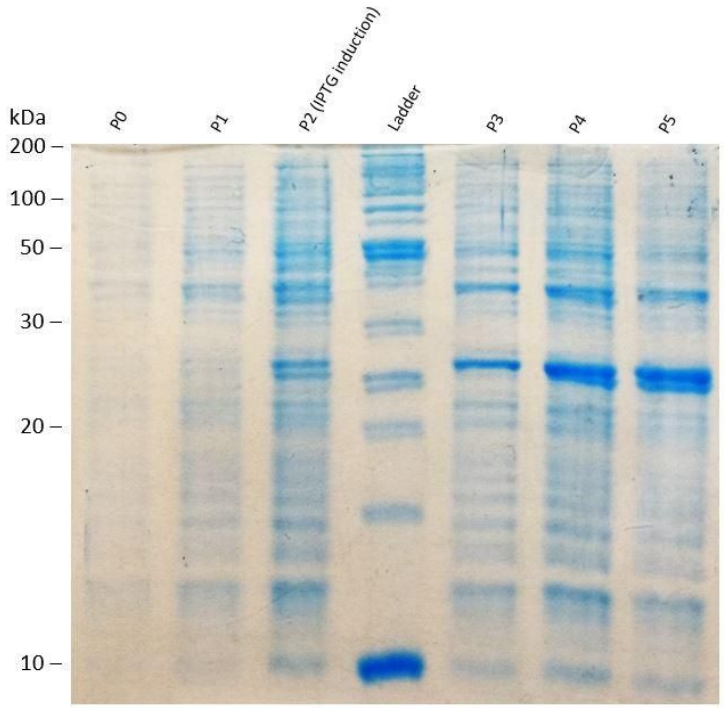
Beginning of SNAP25 (Strep-Tag) expression and purification.

Pour LB media into piston (1. 8L each)
add 1,8ml kanamycin + 20ml bacteria solution SNAP25
Shake at 37°C 100 rpm

OD Take samples:

SNAP25 P0: 0.052 (09:41)
SNAP25 P1: 0.153 (10:40)
SNAP25 P2: 0.34 (11:20)
SNAP25 P3: 0.597 (11:57) induction with 730µL IPTG switch to 17°C 100 rpm
SNAP25 P4: - (13:20)
SNAP25 P5: - (14:20)
SNAP25 P6: - (15:30)
SNAP25 P7: - (16:30)
SNAP25 P8: - (18:00)

Expression Assay



07.09.2018

Bacteria Solution centrifugate at 7k 12min
dry pallet
Weigh wet mass: 10,21g

Lysis
Add of 37mL buffer A Streptag
Add of spatula tip lysozyme
Add of DNAse: 3,7µl
Add of benzamidine: 185µl
Add of PMSF: 35µl

Sonification:
Amplitude 50% (small peak)
5:00 min
0. 5 Pulse on
2. 0 Pulse off

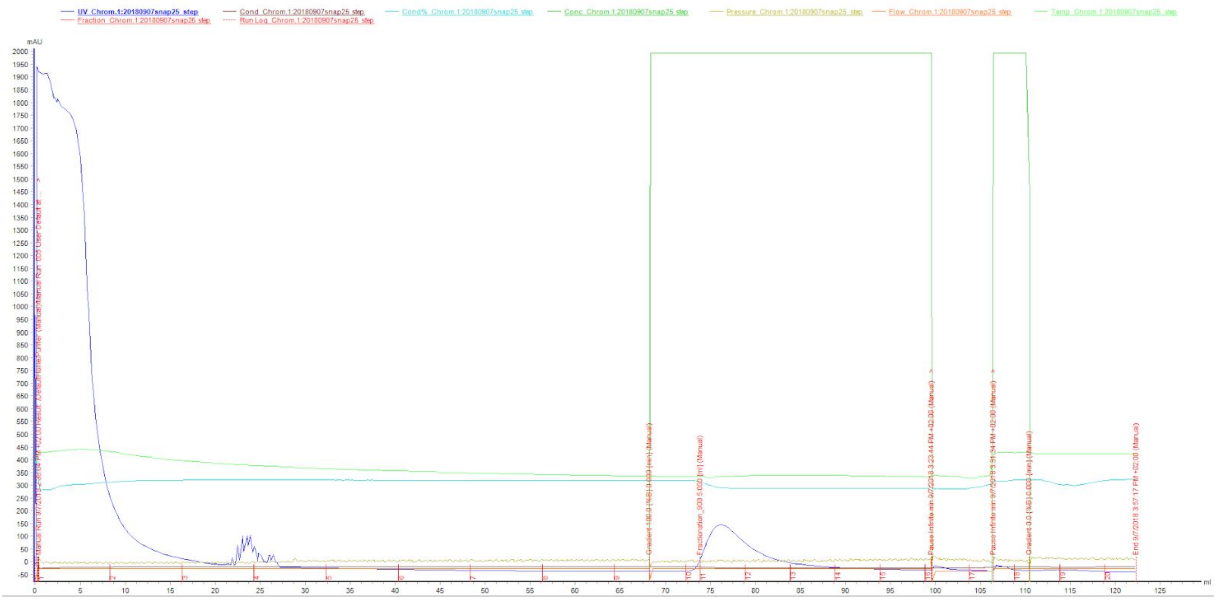
Lysate taken -> Sample "Lysis SNAP25".

Centrifugation 17k 50min
Supernatant in flask -> Sample "Ü SNAP25"

Strep-Tag column equilibrate
20min MilliQ Water

20min Buffer A streptag
circulate 45min lysate through column -> sample "flow through

ÄKTA
Connect buffer A and B Streptag
Start PumpWash
Run buffer A through column until UV spec reaches basal line
Add 100% buffer B



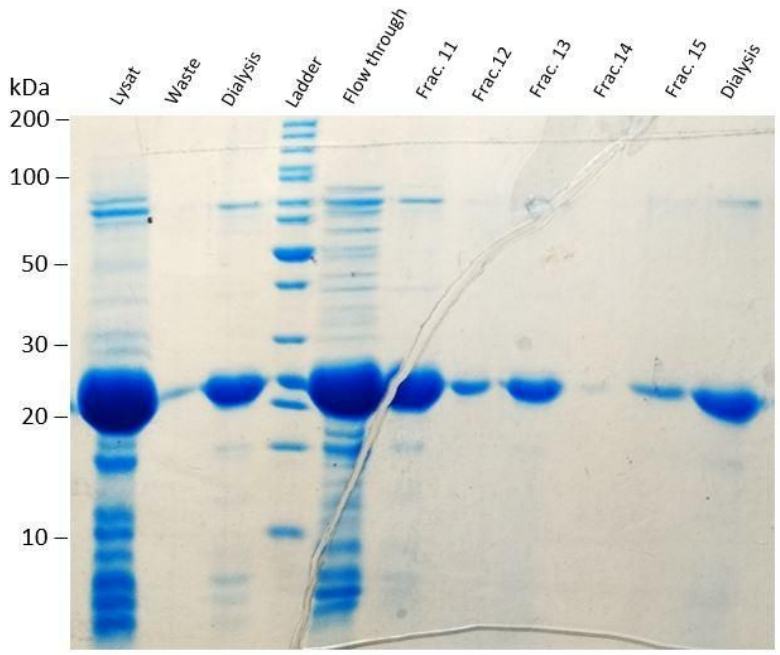
Fractions 11, 12 united and dialyzed
Dialysis is performed with 1L ToxAssay buffer.

10.09.2018

End dialysis and transfer contents to measuring cylinder

Nanodrop measurement (Tev Ext=74000; M=25 [g/mol])
Determination of protein concentration: 2.01 mg/ml 240/260=0.8

Preparing purification Assay



Result: SNAP25 got purified well

Beginning of Synthaxin1a (Strep-Tag) expression and purification.

Pour LB media into piston (1. 8L each)
add 1,8ml kanamycin + 20ml bacteria solution SNAP25
Shake at 37°C 100 rpm

OD Measurements:

Synthaxin1a P0: 0.014 (10:41)
Synthaxin1a P1: 0.065 (11:40)
Synthaxin1a P2: 0.33 (12:20)
Synthaxin1a P3: 0.6 (12:57) induction with 750µL IPTG switch to 17°C 100 rpm

11.09.2018

Bacteria solution centrifugate at 7k 12min
dry pallet
Weigh wet mass: (1) = 6,4g
(2) = 3,31g

(2) got stored by -80°C

Lysis of pallet (1)
Add of 18mL buffer A Streptag
Add of spatula tip lysozyme
Add of Benzoease: 3,7µl

Sonification:
Amplitude 50% (small peak)

5:00 min
0. 5 Pulse on
2. 0 Pulse off

Lysate taken -> Sample "Lysis Synthaxin1a".

Centrifugation 17k 50min
Supernatant in flask -> Sample "Ü Snynthaxin1a

Strep-Tag column equilibrate
20min MilliQ Water
20min Buffer A streptag
circulate 45min lysate through column -> sample "flow through

ÄKTA
Connect buffer A and B Streptag
forgot to start PumpWash
Eluated at frac. 3

The attempt to concentrate the sample on failed

12.09.2018

Second try to gain Synthaxin1a

Lysis of pallet (2)
Add of 18mL buffer A Streptag
Add of spatula tip lysozyme
Add of Benzoease: 3,7µl

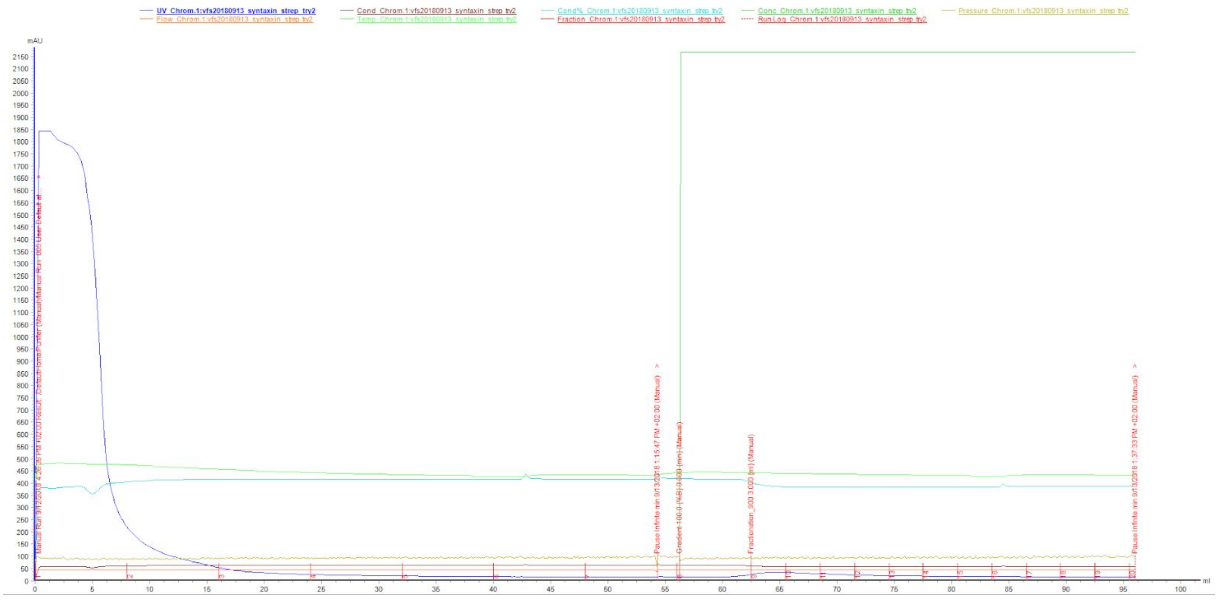
Sonification:
Amplitude 50% (small peak)
5:00 min
0. 5 Pulse on
2. 0 Pulse off

Lysate taken -> Sample "Lysis Synthaxin1a".

Centrifugation 17k 50min
Supernatant in flask -> Sample "Ü Snynthaxin1a

Strep-Tag column equilibrate
20min MilliQ Water
20min Buffer A streptag
circulate 45min lysate through column -> sample "flow through

ÄKTA
Connect buffer A and B Streptag
Start PumpWash
Eluated at 100% Buffer B



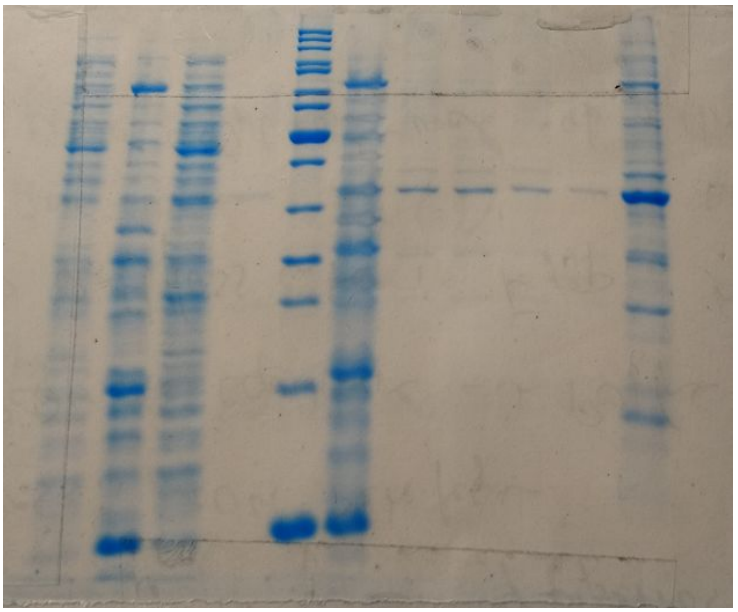
Collecting the fractions with Synthaxin1a.

13.09.2018

Several tries to concentrate Synthaxin1a

14.09.2018

Result of the purification Assay



17.09.2018

Pour LB media into piston (1. 8L each)
add 1,8ml kanamycin + 25ml bacteria solution LCmut and LCwt
Shake at 37°C 100 rpm

OD Take samples:

LCwt P0: 0.037 (11:51)
LCmut P0: 0.040 (11:51)

Lcwt P1: 0.133 (12:52)
LCmut P1: 0.126 (12:52)

LCwt P2: 0.475 (13:45)
LCmut P2: 0.518 (13:45)

LCwt P3: 0.682 (14:06)
LCmut P3: 0.744 (14:06)
Induktion of IPTG (730µL each)

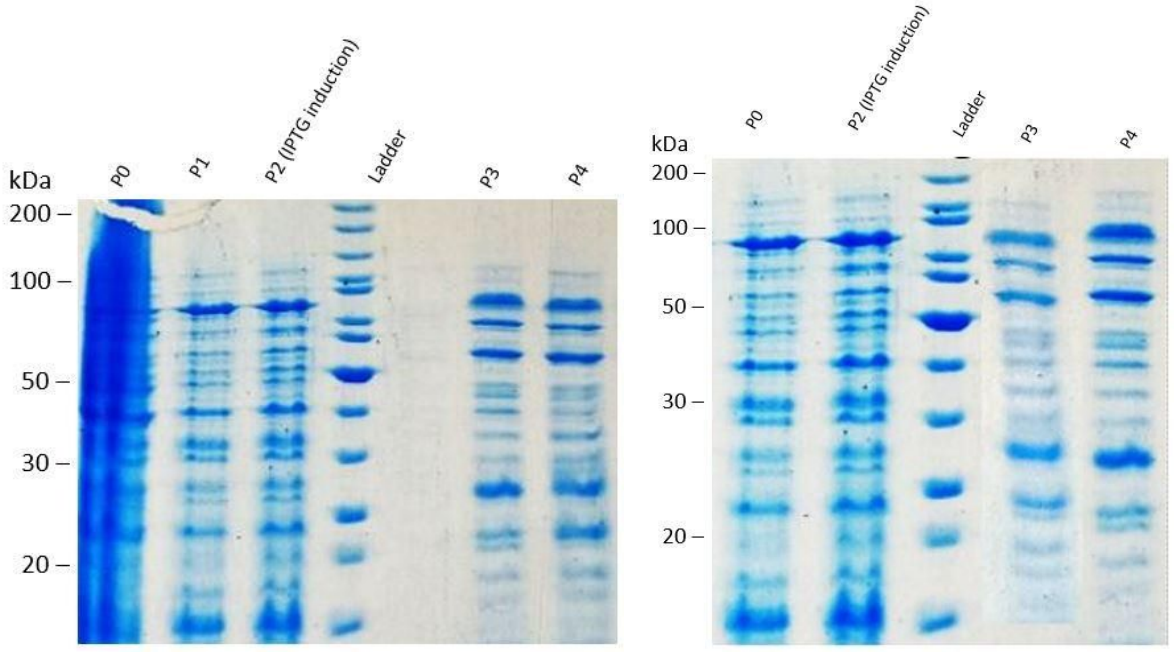
LCwt P4: 1.184 (15:56)
LCmut P4: 1.216 (15:56)

LCwt P5: 1.228 (16:51)
LCmut P5: 1.226 (16:51)

LCwt P6: (next Day)

LCmut P6: (next day)

Result of Expression Assay:
(LCmut on the left side, LCwt on the right side)



18.09.2018

Bacteria Solution LCmut and LCwt centrifuge 7k 12min
dry pellet

Lysis half of each pallet
Add of 30mL buffer A Histag
Add of spatula tip lysozyme
Add of DNAse: 3µl
Add of benzamidine: 150µl
Add of PMSF: 30µl

Sonification:
Amplitude 50% (small peak)
5:00 min
0. 5 Pulse on
2. 0 Pulse off

Lysate taken -> Sample "Lysis LCwt".
"Lysis LCmut"

Centrifugation 17k 50min
Supernatant in flask -> Sample "Ü LCwt”
“Ü LCmut”

Apperently we took the wrong collums and thus lost all expressed protein

19.09.2018

Lysis of other half of pallet LCmut
Add of 30mL buffer A Histag
Add of spatula tip lysozyme
Add of DNAse: 3µl
Add of benzamidine: 150µl
Add of PMSF: 30µl

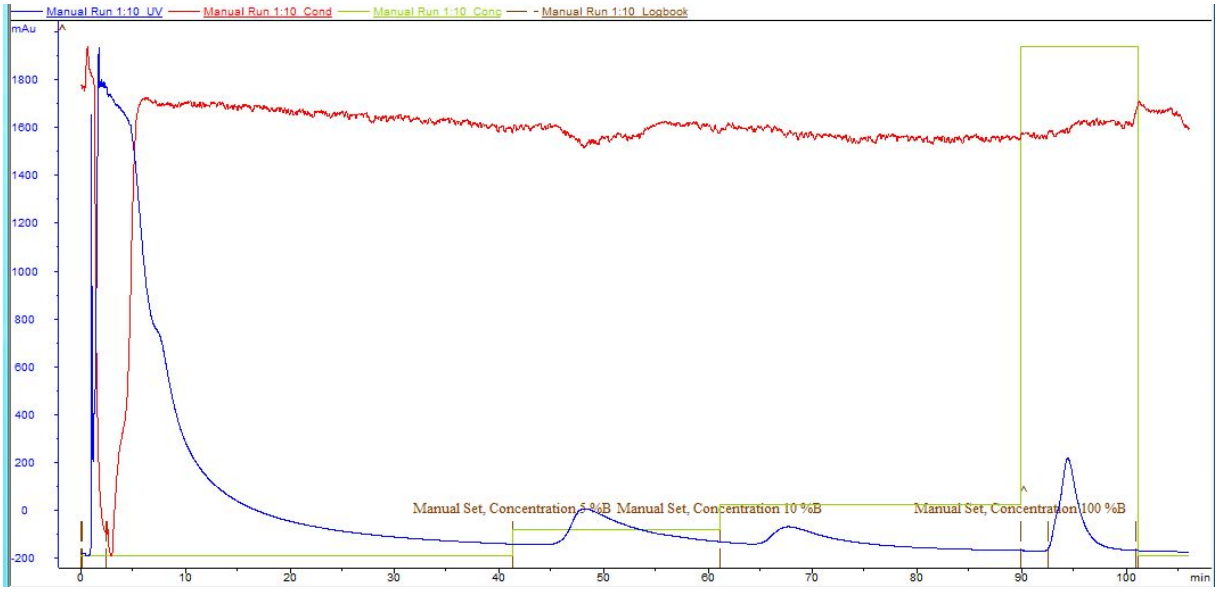
Sonification:
Amplitude 50% (small peak)
5:00 min
0. 5 Pulse on
2. 0 Pulse off

Lysate taken -> Sample "Lysis LCmut".

Centrifugation 17k 50min
Supernatant in flask -> Sample "Ü LCmut”

His column equilibrate
20min MilliQ Water
20min Buffer A Histag
circulate 45min lysate through column -> sample "flow through

ÄKTA
Connect buffer A and B Histag
Start PumpWash
Run buffer A through column
Add 10% buffer B
Add 20% buffer B
Add 100% buffer B



22.09.2018

Lysis of other half of pallet LCwt
Add of 30mL buffer A Histag
Add of spatula tip lysozyme
Add of DNAse: 3µl
Add of benzamidine: 150µl
Add of PMSF: 30µl

Sonification:
Amplitude 50% (small peak)
5:00 min
0. 5 Pulse on
2. 0 Pulse off

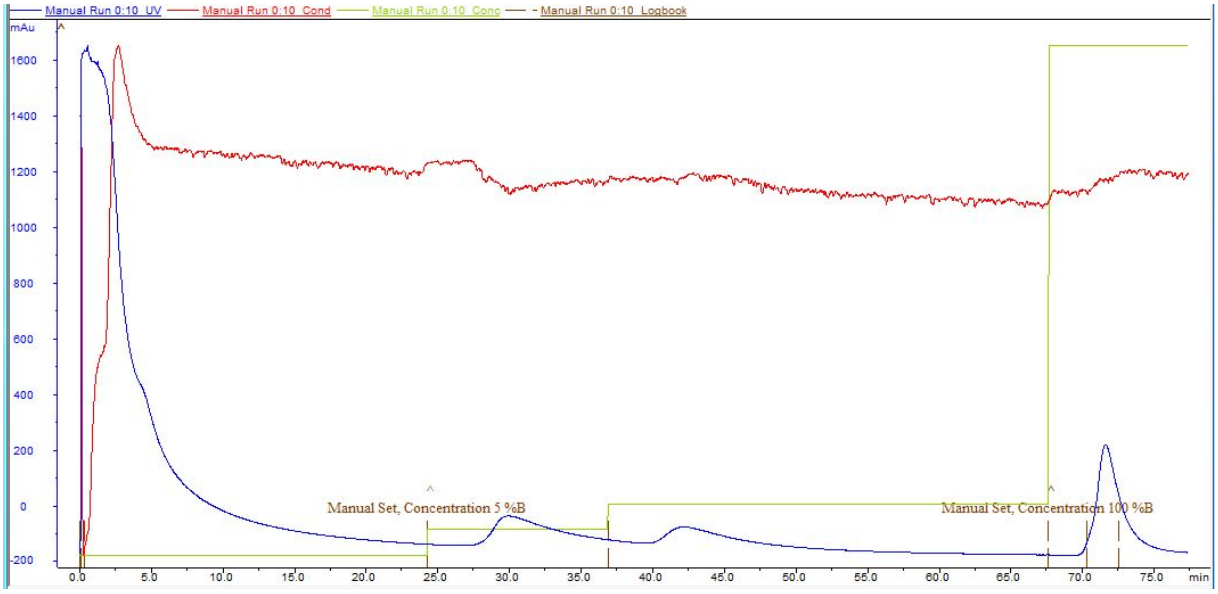
Lysate taken -> Sample "Lysis wt

Centrifugation 17k 50min
Supernatant in flask -> Sample "Ü LCwt”

His column equilibrate
20min MilliQ Water
20min Buffer A Histag
circulate 45min lysate through column -> sample "flow through

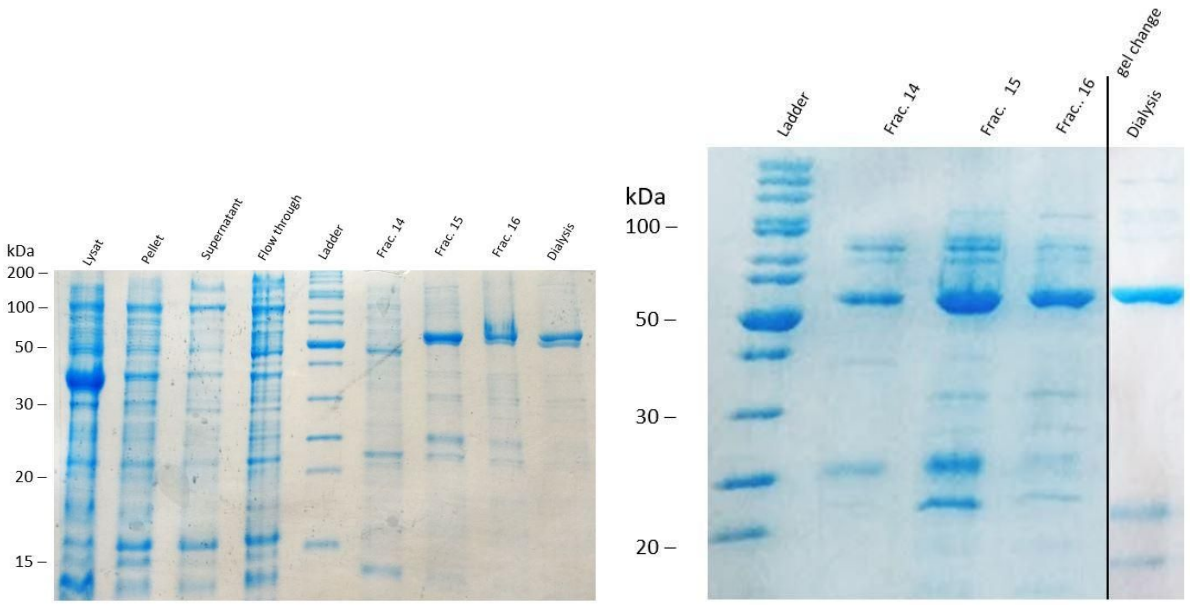
ÄKTA
Connect buffer A and B Histag
Start PumpWash
Run buffer A through column
Add 10% buffer B

Add 20% buffer B
Add 100% buffer B



23.09.2018

Results of Purification LCwt on the right side LCmut, on the left side



As we could see, our LC were purified but instabel so that weh ad to dialys quickly.

24.09.2018

ToxAssay first approuch

	1mut LC=1.6µM	1wt LC=1.6µM	2mut LC=3.2µM	2wt LC=3.2µM	3mut LC=4.8µM	3wt LC=4.8µM	Blanc SNAP25	Blanc LCmut	Blanc LCwt
V_{SNAP25}	38.4µL	38.4µL	38.4µL	38.4µL	38.4µL	38.4µL	38.4µL	-	-
V_{LCmut}	42.7µL	-	83.4µL	-	85.8µL	-	-	85.8µL	-
V_{LCwt}	-	28.6µL	-	57.36µL	-	128.1µL	-	-	128.1µL
ToxPuffer	238.9µL	253µL	196.2µL	224.24µL	195.8µL	153.5µL	281.6µL	234.2µL	191.9 µL
V_{total}	320mL	320mL	320mL	320mL	320mL	320mL	320mL	320mL	320mL

Already at the beginning, sampling of the ToxAssay (100µL) preparations and desalting of the samples
Place the C18 occupied pipette tips in the reaction vessel.

Pipit 20µL methanol into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Pipit 20µL formic acid into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Piping protein sample into the C18 tip
Centrifuge 10 minutes at 5000 rpm

Pipit 20µL formic acid into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Place tip in new reaction vessel

Pipit 10µL methanol into the C18 tip
Centrifugation 12 minutes at 1000 rpm

Freeze samples at -80°C und beschriftet mit P0

25.09.2018

Already at the beginning, sampling of the ToxAssay (100µL) preparations and desalting of the samples
Place the C18 occupied pipette tips in the reaction vessel.

Pipit 20µL methanol into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Pipit 20µL formic acid into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Piping protein sample into the C18 tip
Centrifuge 10 minutes at 5000 rpm

Pipit 20µL formic acid into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Place tip in new reaction vessel

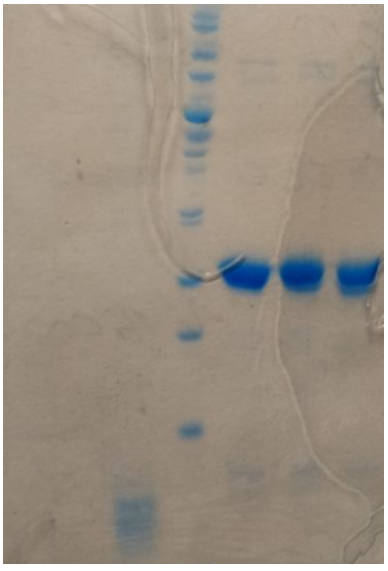
Pipit 10µL methanol into the C18 tip
Centrifugation 12 minutes at 1000 rpm

Freeze samples at -80°C und beschriftet mit P1

Results were negative
Conclusion maybe there were no substrate in the Assay

26.09.2018

SDS Gel der Expression von SNAP25
15µl Probe + 10µl Probe-Puffer (1SNAP 5°C/2SNAP 5°C/M/1SNAP -80°C/2SNAP -80°C/3SNAP -80°C)



SNAp25 Test
Results: SNAP wasnt in the ToxAssay No1

Western Blot
Vortest
Antibodys dont bind tot he Strep tag

27.09.2018

Repetition of the Western Blots

28.09.2018

SDS gel prepared ToxAssay components
Result: desired proteins are in Aliquot

ToxAssay second approach launched:

	1mut LC=1.6µM	1wt LC=1.6µM	2mut LC=3.2µM	2wt LC=3.2µM	3mut LC=4.8µM	3wt LC=4.8µM	Blanc SNAP25	Blanc LCmut	Blanc LCwt
V_{SNAP25}	38.4µL	38.4µL	38.4µL	38.4µL	38.4µL	38.4µL	38.4µL	-	-
V_{LCmut}	42.7µL	-	83.4µL	-	85.8µL	-	-	85.8µL	-
V_{LCwt}	-	28.6µL	-	57.36µL	-	128.1µL	-	-	128.1µL

ToxPuffer	238.9µL	253µL	196.2µL	224.24µL	195.8µL	153.5µL	281.6µL	234.2µL	191.9 µL
V_{total}	320mL	320mL	320mL	320mL	320mL	320mL	320mL	320mL	320mL

Already at the beginning, sampling of the ToxAssay (100µL) preparations and desalting of the samples
Place the C18 occupied pipette tips in the reaction vessel.

Pipit 20µL methanol into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Pipit 20µL formic acid into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Piping protein sample into the C18 tip
Centrifuge 10 minutes at 5000 rpm

Pipit 20µL formic acid into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Place tip in new reaction vessel

Pipit 10µL methanol into the C18 tip
Centrifugation 12 minutes at 1000 rpm

Freeze samples at -80°C und beschriftet mit P0

29.09.2018

After 18. 5 hours of sampling the ToxAssay (100µL) Preparations and desalting of the samples
Place the C18 occupied pipette tips in the reaction vessel.

Pipit 20µL methanol into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Pipit 20µL formic acid into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Piping protein sample into the C18 tip
Centrifuge 10 minutes at 5000 rpm

Pipit 20µL formic acid into the C18 tip
Centrifuge 2 minutes at 1300 rpm

Place tip in new reaction vessel

Pipit 10µL methanol into the C18 tip
Centrifugation 12 minutes at 1000 rpm

Freeze samples at -80°C und beschriftet mit P1

30.09.2018

After 45. 5h, remove the ToxAssay preparations and freeze at -80°C.
10µL samples taken for MALDI with label P2

02.10.2018

15% SDS gels perpetrated

Eurofins contacted about Minipräp -> Samples lost

03.10.2018

LB Medium prepared:
Media autoclaved

Preparation of buffer:
Buffer A HisTag pH8. 0: 50mM NaP, 500mM NaCl, 10mM Imidazole
Buffer B HisTag pH8. 0: 50mM NaP, 500mM NaCl, 350mM Imidazole
PBS pH 7. 3: 20 mM sodium phosphate, 280 mM NaCl, 6 mM potassium chloride

S1 Form for pHluorin2 completed

Meeting with Kalbacher about ELSIA and its preparation

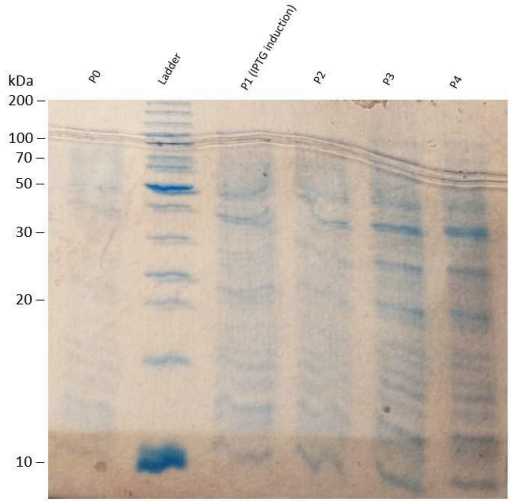
04. 10. 2018

Pour LB media into piston (1. 8L each)
add 1,8ml kanamycin + 25ml bacteria solution
Shake at 37°C 100 rpm

OD Take samples:
P0: 0,039 (10:48)
P1: 0,079 (11:41)
P2: 0,189 (13:11)
P3: 0,527 (13:25)
P4: 0. 710 (16:21) 730µl addition of IPTG
P5: 1,140 (16:21)
P6: 1,348 (16:21)
P7: 1,402 (16:21)
P8: 1,406 (16:57)

Prepare Kanamycin stock solution
1g Kan + 20mL dH2O
1,8mL Create aliquots

SDS gel of expression of phluorin2
10µl sample + 30µl sample buffer (P0/ P1/ P2/ P3/ P4/M/ P5/ P6/ P7/ P8



Expression-Assay pHluorin2

05.10.2018
Bacteria Solution centrifugate at 7k 12min
dry pallet
Weigh wet mass: 28,27g
Half frozen -80°C

Half lysed
Add of 30mL buffer A Histag
Add of spatula tip lysozyme
Add of DNAse: 3µl
Add of benzamidine: 150µl
Add of PMSF: 30µl

Sonification:
Amplitude 50% (small peak)
5:00 min
0. 5 Pulse on
2. 0 Pulse off

Lysate taken -> Sample "Lysis pHluorin2".

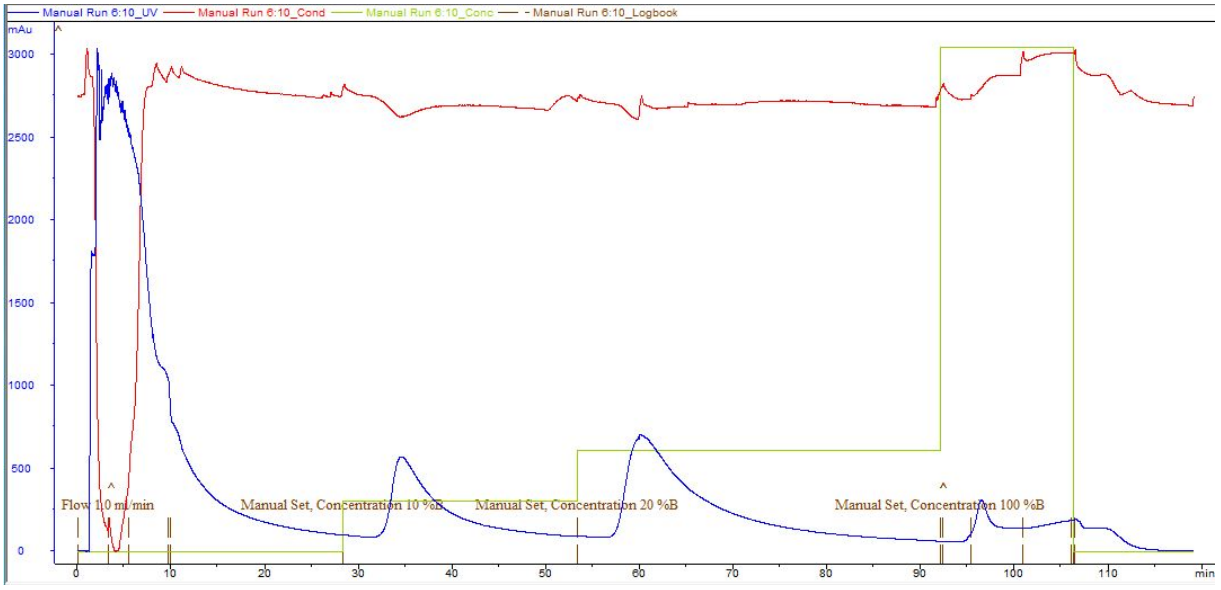
Centrifugation 17k 50min
Supernatant in flask -> Sample "Ü pHluorin2"

His column equilibrate
20min MilliQ Water
20min Buffer A Histag
circulate 45min lysate through column -> sample "flow through

ÄKTA
Connect buffer A and B Histag
Start PumpWash
Run buffer A through column
Add 10% buffer B

Add 20% buffer B
Add 100% buffer B

Note: pHluorin does not bind strongly specifically, eluts already at 20% bufferB -> directly 100% buffer



pHluorin2 ÄKTA

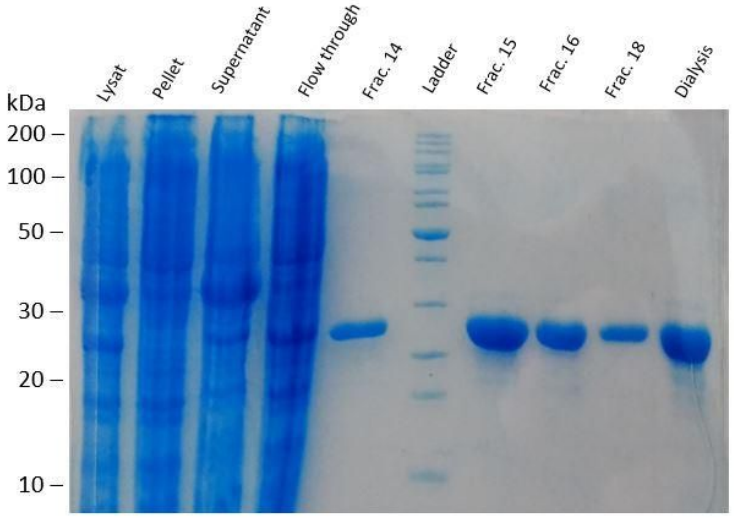
DatenNanodrop measurement of fractions
Frac 9: 2. 19 mg/ml 260/280: 1. 00
Frac 14: 1. 22 mg/ml 260/280: 0. 87
Frac 15: 3. 39 mg/ml 260/280: 0. 72
Frac 16: 2. 05 mg/ml 260/280: 0. 75
Frac 18: 0. 92 mg/ml 260/280: 0. 80
8ml of fraction 15 is taken for dialysis rest is frozen. (-80°C)

Dialysis with 1L PBS buffer.

08. 10. 2018

Dialysis taken -> sample "Dialysis pHluorin2
Nanodrop measurement: 3. 38mg/ml 260/280: 0. 73
Check whether pHluorin2 is cold-stable.
Defrost sample fresh freeze afterwards
Centrifuge sample and nanodrop measurement: 3. 27mg/ml
Note: pHluorin2 is still stable.

SDS gel of purification of phluorin2
5µl sample + 15µl sample buffer (lysate/soil/surfactant/flow/Fr14/M/Fr15/Fr16/Fr18/dialysis)



Purification Assay pHluorin2

Gel preservation: SDS ProbeTox(28. 9), pHluorin2 Etest (5. 10), iGEM LCMut (18. 9), SNAPtest (26. 9)

09. 10. 2018

2L PBS buffer (pH: 7. 4; 20mM NaP; 280mM NaCl; 6mM KCl) prepared

IPTG 1M aliquots applied

Kanamycin aliquots set

2mL benzamidine 1M prepared

4x 1. 8L LB medium prepared and autoclaved

10. 10. 2018

Preserved gels "Cleaning pHluorin2; Etest pHluorin2; Etest2pHluorin2".

11. 10. 2018

SDS gel prepared ToxAssay components

LCwt/LCmut/SNAP25/Ladder/Ladder/SNAP/LCmut/Lcwt

Induction LB Meida with Hibit/BoNT constructs

12. 10. 2018

Strep-Tag columns regenerated

15 CV 1mM HABA in PBS buffer

30 CV PBS buffer

5 CV distilled water

Harvesting and lysis of the Hibit/BoNT construct

Induction and inoculation with Omomyk/BoNT constructs

13. 10. 2018

Pour LB media into piston (1.5L each)
add 1,5ml kanamycin + 25ml bacteria solution Hibit/BoNT
Shake at 37°C 100 rpm

OD Take samples:
Induced 730µl IPTG at OD 0.67

14. 10. 2018

Harvesting the Hibit/BoNT construct and purify it.

Protein was to unstable, so we lost it in the column

15.10.2018

Pour LB media into piston (1.5L each)
add 1,5ml kanamycin + 25ml bacteria solution Omomyc/BoNT
Shake at 37°C 100 rpm

OD Take samples:
Induced 730µl IPTG at OD 0.58

16.10.2018

Harvesting the Omomyc/BoNT construct and purify it.

Protein was not expressed (we found that out while purifying)

17.10.2018

Applied 15% SDS gels:

Separating gel:
33.89mL 30% Acrylamide
16.89mL Tris/HCl pH8. 8
15.61mL distilled water
675µL SDS 10%
33.75µL TEMED
675µL APS

Collecting gel:

5.85mL 30% Acrylamide
7. 5mL Tris/HCl pH8. 8
27.45mL distilled water
450µL SDS 10%
45µL TEMED
450µL APS

Set up LB medium
Add 45mg LB to 1.8L distilled water
Mix the solution
Autoclave LB Medium