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## Expression and Purification of TMEM55B 80-166 2CysMUT

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**We use this protocol and it's working**

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## Abstract

We describe a method to express and purify TMEM55B residues 80-166 2CysMUT for the purpose of using it in protein crystallography and biochemical assays. The method also works for other related TMEM55B cytosolic-domain constructs. The protocol follows our standard procedure of recombinant protein expression, but since TMEM55B contains Zn<sup>2+</sup> ions in its structure, additional Zn<sup>2+</sup> is added to the expression medium.



## Materials

### Consumables

- 30 ml Universal tubes (Greiner 201172)
- 50 ml Centrifuge Tubes (Greiner 227261)
- 1.5 ml Reaction tubes (Greiner, 616201)
- Dialysis tube 12kDa MWCO (SIGMA D9777) and appropriate clamps

#### Note

Despite the construct having a MW of ~10kDa it does not escape the dialysis tube in the roughly 18 hours it is in it

- 3 or 10kDa MWCO Amicon Ultra-15 concentrator tube, Merck

### Reagents

- Autoclaved sterile 2xYT and LB media
- Cell stock (E. coli BL21 DE3, glycerol stock/agar plate), pET15b-TMEM55B 80-166 2CysMUT

#### Note

Sequence of the expressed construct: MGSSHHHHHHSSGLVPR\*GSHM**SP**DSGSAPMITCRVCQSLINVEGKMHQHV  
VKCGVCNEATPIKNAPPGKKYVRCPCNS**LLIA**KVTSQRIACPRPYCKRIINLGPV HPG

\* Thrombin cleavage, Bold: first residue of the TMEM55B sequence; bold italic: mutated Cysteines

- Ampicillin (100 mg/ml stock, sterile filtered)
- IPTG (1M stock, sterile filtered)
- ZnCl<sub>2</sub> (1M stock, sterile filtered)
- 20% Ethanol
- Distilled H<sub>2</sub>O
- **Extraction** Buffer: 20mM Tris pH 8, 300mM NaCl, 10mM β-mercaptoethanol (BME), 10 mM imidazole
- **Wash** Buffer: 20mM Tris pH 8, 300mM NaCl, 10mM BME, 40 mM imidazole
- **Elution** Buffer: 20mM Tris pH 8, 300mM NaCl, 10mM BME, 200 mM imidazole
- 13-14M β-Mercaptoethanol (BME) stock
- Bradford assay solution (Quick Start 1x, Bio-Rad #5000205)
- 10 IU Thrombin aliquots (Cytiva 27-0846-01)
- **Low salt** IEx buffer (10mM Tris pH8.0, 10mM NaCl, 1mM DTT)
- **High salt** IEx buffer (10mM Tris pH8.0, 1M NaCl, 1mM DTT)
- **Gel Filtration (GF) buffer** (20mM Tris pH8.0 at 4°C, 150mM NaCl, 1mM DTT)

### Equipment

- Shaking incubators (Infors: set at 18/37°C, 180 rpm)
- Photometer with disposable cuvettes
- Centrifuge Sorvall RC 5C plus, FiberLite F10-6×500y rotor



- Centrifuge Eppendorf 5810 R for concentrator tubes
- Glass homogenizer (Dounce homogenizer)
- Gravity flow columns (Bio-Rad Econocolumn) and suitable laboratory stand
- Sonicator equipped with a blunt probe (Branson Sonifier 250)
- Tabletop Vortex
- Centrifuge equipped with SS-34 rotor
- SDS-PAGE system (ATTO)
- FPLC system which allows gradients from two buffers, i.e. AKTA basic or AKTA purifier (Cytiva) equipped with a fraction collector for 1.5ml tubes
- Mono S 5/50 GL ion exchange column, Cytiva
- Superdex 75 10/300 GL size exclusion column, Cytiva

#### Note

Columns are stored in 20% Ethanol at 4°C. All buffers and liquids used on the FPLC system are filtered through 0.22µm filters to prevent particles entering and blocking the system. Proteins are centrifuged to remove precipitate and debris. The FPLC and columns were operated at either 4°C or room temperature. The pH of the buffers was adjusted to the respective temperature.



## Overnight Culture

18h

- 1 Overnight culture prepared to be ~1:50 of final volume (e.g. 1 × 10 ml LB in Universal tube + 100 µg/ml Ampicillin to inoculate 1/2l 2xYT medium).
- 2 Add colony from agar plate or scrape from glycerol stock using a sterile pipette tip or autoclaved toothpick. Leave lid loose to allow better aeration.
- 3 Incubate at 37°C, 180 rpm overnight in shaking incubator.

18h

## Induction of Protein Expression

21h

- 4 Add Ampicillin (1:1000, final conc. 100 µg/ml) and overnight culture (1:50) to a large flask containing autoclaved 2xYT medium.
- 5 Incubate at 37°C, 180 rpm (120-180) for ~1.5-2 hours.
- 6 Remove ~600 µl of sample into Eppendorf tube and check absorbance at 600 nm.
- 7 After the OD at 600 nm reaches 0.6-0.8, move flask to 18°C and continue shaking for ~1 hour.
- 8 Add 0.5 mM IPTG and 50 µM ZnCl<sub>2</sub> to the flask.
- 9 Continue to incubate cells at 18°C, 180 rpm overnight.

2h

1h

18h

## Bacterial Cell Collection Following Induction

25m

- 10 Fill bacterial suspension into 500ml centrifuge buckets.



11 Centrifuge at 3500 x g for 10 mins at 4°C.

10m

12 Bacteria with the expressed protein can be **stored** at -20°C.

12.1 Add ~25 ml cold PBS to the bacterial pellet, loosen the pellet with a spatula and vortex.

12.2 Transfer suspension to 50 ml centrifuge tubes.

12.3 Centrifuge at 3500 x g for 15 mins at 4°C.

15m

12.4 Decant supernatant, label tubes well and store pellets at -20°C.

13 **If purifying protein immediately:** Add ~25 ml extraction buffer, dislodge cell pellet with spatula and vortex to create a cell suspension.

## Cell Lysis and Sonication

58m

14 Prepare extraction buffer - aliquot 100ml buffer per 1L cell culture and add 10mM BME.

15 Take pellet from -20°C and keep on ice.

16 Add 20-30 ml extraction buffer to cell pellet and thaw quickly in a water bath.

17 Vortex to break up pellet and resuspend cells.

18 About 10 strokes are fine. Pour lysate into Dounce homogenizer and break up clumps.

**Note**

Gently move piston up and down until all cell clumps broken up and solution is uniform. About 10 strokes are fine.

19 Pour lysate into glass beaker and place in plastic beaker filled with ice.

20 Sonicate on ice. Settings: cycle 30%, output 4-5, 2 mins timer (Branson Sonifier 250).

8m

21 Repeat sonication 3 times leaving 1 min between sonications.

**Note**

Clean probe before & after with ethanol, ensure it does not touch the sides or bottom of beaker, should be just under the level of liquid to prevent bubbles.

22 Pour cell lysate into centrifuge tubes, balance within 0.1g (with extraction buffer), centrifuge at 20,000 x g (13,000rpm on rotor SS34), 45 mins, at 4°C.

45m

23 After centrifugation remove 15µl of supernatant for analysis by SDS PAGE gel. 15µl of sample added to 5ul 4x SDS-PAGE sample buffer in Eppendorf tube - labelled Lysate 'L' (boil 95°C for 5 minutes).

5m

## Ni-agarose Column Binding and Washing

24 Resuspend clean Ni-agarose stock by inverting several times.

25 Add 5 ml Ni-agarose (50% slurry in Extraction buffer) to a gravity flow column.

26 Load resin into column, open stopcock and let liquid flow through to create a resin bed. Bed volume of column is 2.5ml.

27 Wash column with 2ml extraction buffer and let flow through until ~0.5cm liquid left on top of resin.



28 Load supernatant into column and let it completely flow through.

29 Wash with ~3 ml extraction repeatedly until no more protein comes off.

#### Note

To test if there is still protein coming off, 30µl dots of Bradford reagent are placed on parafilm and 3µl flow through added - if protein is present, Bradford turns blue.

30 Wash column with 40mM wash buffer until no more protein comes off.

#### Note

Don't overwash - 10-15 ml wash buffer is enough even if protein still coming off.

## Protein Elution

31 Prepare 20 ml elution buffer by adding 10mM BME to the 1x stock.

32 Place a collection tube (~30ml) on ice under the gravity flow column.

33 Add ~2ml elution buffer to column, open stopcock, check flow through for protein by adding 3µl to 30µl Bradford droplets on parafilm.

34 Add another 2-3ml elution buffer, collect flow through containing eluted protein and repeat until no more protein comes off (about 8-15 ml depending on cell culture vol).

#### Note

Check Bradford sample every ml until protein has been eluted and drops don't turn blue anymore.

35 Take a 15µl sample of elute and add to 5µl sample buffer. Boil 95°C for 5 minutes.



## Proteolytic Cleavage of His-tag

19h

- 36 Prepare 1l of extraction buffer. Use 1mM DTT instead of 10mM BME.

### Note

Proteins are cleaved in dialysis against extraction buffer as thrombin requires a buffer with less imidazole than the elution buffer.

- 37 Cut dialysis tube to an appropriate length (~1cm per ml + little extra for the clamp) and soak tube in dH<sub>2</sub>O.

- 38 Add eluted proteins to tube and place in beaker of buffer.

- 39 Incubate with gentle stirring for ~1 hr at 4°C.

1h

- 40 Thaw Thrombin protease, spin quickly and add to dialysis tubes (2×10μl =10UI per tube).

- 41 Dialyse overnight at 4°C.

18h

### Note

Protein precipitation will occur when the initial concentration of the eluted protein is high, which it usually is. The precipitate can be removed by centrifugation, and there is still more than sufficient protein left to proceed. The less salt a buffer contains, the more TMEM55B is prone to precipitation.

## Second Nickel Purification

20m

- 42 Transfer protein out of dialysis tube into 30ml universal tube on ice.

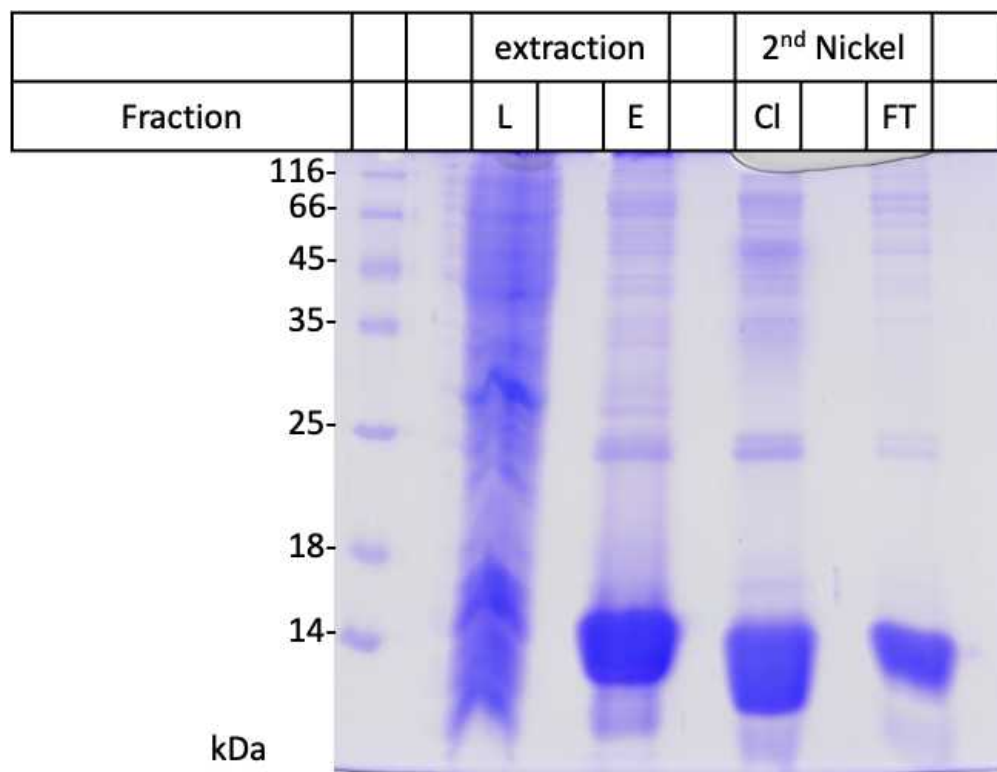
- 43 Take 15μl sample and mix with 5μl sample buffer - label cleaved 'CL'. Boil 95°C for 5 minutes.

5m

- 44 Add 3 ml 50% slurry of Ni-agarose resin in extraction buffer to the tube and incubate on rotator for 15 minutes, 15 rpm, 4°C.

15m

- 45 Transfer Ni-agarose and cleaved protein into a gravity flow column as described previously and allow resin to settle.
- 46 Open stopcock and collect flow through in a 30ml tube.
- 47 Wash column with 2-3ml extraction buffer to wash off un-specifically bound cleaved proteins.
- 48 Take 15µl sample of flow through and add 5µl sample buffer - label Flow through "FT". Boil 95°C for 5 minutes.
- 49 Analyse and verify purification by running the samples on a 15% SDS-PAGE (Figure 1).



**Figure 1.** Expression and extraction of TMEM55B 80-166 2CysMUT from *E. coli*. L: superlatant Lysate after extraction, E: Eluted from Ni-Agarose, Cl: protein cleaved with Thrombin, FT: 2<sup>nd</sup> Nickel flow through. 15µl protein samples were mixed with 5µl 4xLD, 95°C, 5min. Load 5µl. Stain with Coomassie Brilliant Blue

## Ion Exchange Chromatography (IEx)

4h 10m



- 50 Ion Exchange chromatography helps to remove unwanted proteins left over from the extraction and is an important step to get crystallography-grade TMEM55B.

**Note**

Ion exchange chromatography is used to separate TMEM55B from impurities according to their charges. TMEM55B 80-166 2CysMUT has a theoretical pI of 9.47 determined by using the “protparam” tool (<https://web.expasy.org/protparam/>). At a pH of 8.0 it is positively charged. Therefore, the strong cation-exchange chromatography column MonoS is used to separate the second Nickel flow-through from impurities.

51 **Preparation of the MonoS 5/50 GL column.**

- 51.1 Connect column to the FPLC system.

- 51.2 Run column into dH<sub>2</sub>O for 5 column volumes (CV) at 1ml/min.

5m

**Note**

One CV for the MonoS 5/50 GL column is 1 ml. The pressure limit of the column (4MPa) might make it necessary to run it at a smaller flow rate.

- 51.3 Run the column into low salt buffer for 5 CV at 1 ml/min.

5m

- 51.4 Run the column into high salt buffer for 5 CV at 1 ml/min.

5m

- 51.5 Run the column into low salt buffer for 15 CV at 1 ml/min.

15m

**Note**

The absorption at 280nm and conductivity baselines must be stable.

must

52 **Loading the column.**

- 52.1 The protein to be purified by ion exchange chromatography is dialysed into 500 ml low salt buffer for a maximum of 2 hours.

2h

**Note**

The volume of the dialysed protein is usually between 2-5 ml. Higher volumes require repeated loading steps as the loading loop of the FPLC system has a maximum capacity of 5 ml. TMEM55B 80-166 2CysMUT precipitates under low salt conditions. Therefore, it is crucial to keep the dialysis time as short as possible. When precipitation becomes obvious, the amount of salt is usually low enough to allow binding of the protein to the column matrix even if the dialysis is rather incomplete.

- 52.2 Spin down protein after dialysis to remove precipitated protein from the sample.

- 52.3 Attach the 5ml loading loop to the FPLC system.

- 52.4 Fill the loop with the centrifuged protein solution into the loading loop.

- 52.5 Inject the protein to the ion exchange column at a flow rate of 0.5 ml/min. Keep loading running for 15 ml until the absorption and conductivity baselines are stable.

30m

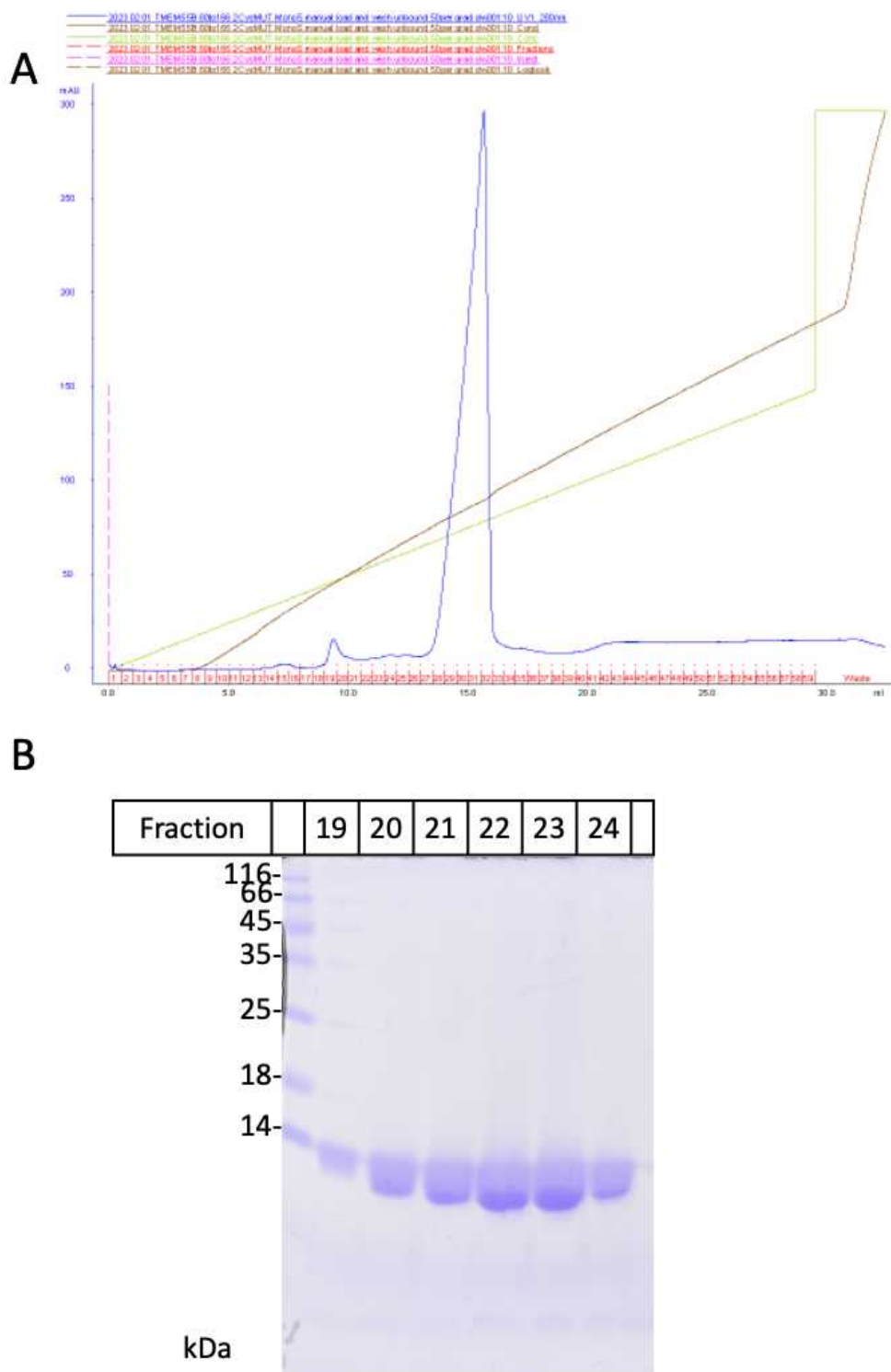
**53 Elution of the protein.**

- 53.1 Select an appropriate method in the FPLC control software:
- Flow rate 1 ml/min if pressure limit of the column allows.
  - Set linear gradient 0% high salt buffer to 50% high salt buffer over 30CV. This increases the salt concentration over the course of the run from 10mM to 500mM. The TMEM55B construct is eluted at about 25% high salt buffer = 250mM NaCl.
  - Collect fractions of 0.5ml over the gradient.

35m

- 53.2 Run the method.

- 53.3 The fractions containing the TMEM55B were analysed by SDS-PAGE for their purity and then pooled accordingly (Figure 2).



**Figure 2.** Ion Exchange purification of TMEM55B. (A) Elution profile from FPLC. (B) SDS-PAGE analysis of the fractions from the IEx. Fractions 20-24 were pooled for further processing.

## 54 Cleaning of the column.



54.1 Run column in 5CV high salt buffer at 1ml/min to remove all bound proteins.

5m

54.2 Run column into 15CV low salt buffer.

15m

#### Note

If another cycle of loading and elution is required, the column can be loaded again after this step.

54.3 Run column into dH<sub>2</sub>O for 5CV at 1ml/min.

5m

54.4 Run Column into 20% Ethanol for 5CV at 0.5ml/min.

10m

54.5 Store column at 4°C.

## Size Exclusion Chromatography (=gel filtration)

1h 30m

55 Connect the superdex 75 10/300 GL column to the FPLC system and exchange the 20% Ethanol it is stored in for dH<sub>2</sub>O (30ml).

1h

56 Equilibrate the column in GF buffer for 30ml.

30m

57 Concentrate the pooled ion exchange fractions of TMEM55B 80-166 2CysMUT in a 10 kDa MWCO concentrator tube (Amicon Ultra-15, Merck) to a volume of 500-1000 µl.

#### Note

The loading loop for this column should be <1ml, else the quality of the separation is poor.

58 Load the concentrated protein into the 1ml loading loop of the FPLC.

59 Select the method for the FPLC run.

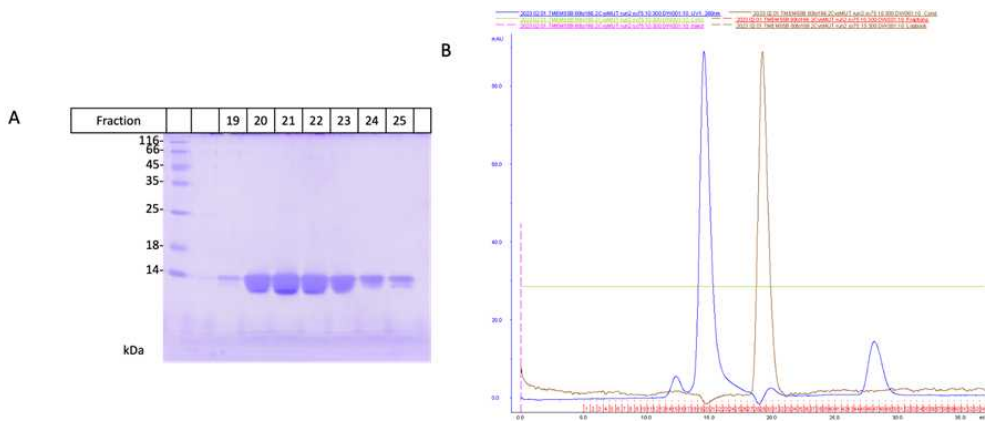
- 0.8 ml/min flow rate if pressure limit allows.

- protein injection for 3ml.
- 30ml elution volume.
- Collect 0.5ml fractions after 3ml injection.

60 Execute the run.

61 Collect the fractions corresponding to the peak in absorbance at 280nm containing the eluted TMEM55B 80-166 2CysMUT protein.

62 Analyse the fractions by 15% SDS-PAGE to confirm the correct protein and purity (Figure 3).



**Figure 3.** Size Exclusion Chromatography (SEC) of TMEM55B 80-166 2CysMUT. (A) SDS-PAGE analysis of the fractions from the SEC depicted in B. Fractions 20-24 were pooled for crystallography. (B) Elution profile from FPLC.

63 The protein can now be used in crystallography or biochemical analyses like ITC or direct pulldowns.

#### Note

If the protein is purified solely for biochemical analyses, the ion exchange step can be cancelled, as this step usually leads to a massive loss of protein due to precipitation. The purity is still very good.