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Expression and Purification of the Human Cation-chloride Cotransporter KCC1 from HEK293F Cells for Structural Studies

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[Abstract] Cation-chloride cotransporters (CCCs) mediate the coupled, electroneutral symport of cations such as Na^+ and/or K^+ with chloride across membrane. Among CCCs family, K-Cl cotransporters (KCC1-KCC4) extrude intracellular Cl^- by the transmembrane K^+ gradient. In humans, these KCCs play vital roles in the physiology of the nervous system and kidney. However, mechanisms underlying the KCCs specific properties remain poorly understood, partly because purification of membrane proteins is challenging. Here, we present the protocol for purifying the full-length KCC1 from HEK293F cells used in our recent publication (Liu *et al.*, 2019). The procedure may be adapted for functional and structural studies.

Keywords: KCC, Mammalian cells, Purification, Bac-to-Bac baculovirus expression system, BacMam, Structural studies

[Background] The human Solute Carrier 12 (*SLC12*) gene family encodes the Cation-Chloride Cotransporters (CCCs) that mediate the electroneutral symport of Cl^- and cations Na^+ or (and) K^+ across plasma membranes. Defined by their transport properties and amino acid sequences, CCCs can be divided into several branches, including two Na-K-2Cl cotransporters (NKCC1 and NKCC2), one Na-Cl cotransporter (NCC) and four K-Cl cotransporters (KCC1-KCC4). CCCs play important roles in cell volume regulation, salt reabsorption in kidney, and the GABAergic modulation in neurons. The structural, biochemical and biophysical studies of CCCs involve challenges at the level of protein production and stabilization in the detergent-solubilized state. Baculovirus transduction of HEK293F cells (BacMam) system is a powerful method to heterologously express membrane proteins developed by Eric Gouaux (Goehring *et al.*, 2014). In this protocol, we describe the production of KCC1 with a C-terminal StrepII tag in HEK293F cells using the Baculovirus expression system. The purified protein can be applied for a variety of functional and structural studies.

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Materials and Reagents

1. 96-well plate
2. 6-well plate (Corning, catalog number: 3516)
3. Filter systems (250 ml, 0.22 μm; Corning, catalog number: 430767)
4. Syringe filters (33 mm, 0.22 μm; Millipore, catalog number: SLGPR33RB)
5. Amicon Ultra-4 centrifugal filters 100,000 MWCO (Millipore, catalog number: UFC810096)
6. pEZT-BM vector (Addgene, catalog number: 74099)
7. Sf9 cells (Thermo Fisher Scientific, catalog number: 11496015)
8. HEK293F cells (Thermo Fisher Scientific, catalog number: R79007)
9. DH10Bac Competent Cells (Thermo Fisher Scientific, catalog number: 10361012)
10. Penicillin-Streptomycin (Gibco, catalog number: 15140-122)
11. Fetal bovine serum (FBS) (Gibco, catalog number: 10270-106)
12. X-tremeGEN 9 DNA Transfection Reagent (Roche, catalog number: 06365787001)
13. SIM SF Expression Medium (Sino Biological, catalog number: MSF1)
14. SMM 293-TI Expression Medium (Sino Biological, catalog number: M293TI)
15. Sodium butyrate (Sigma-Aldrich, catalog number: 303410)
16. Baculovirus Envelope gp64 Antibody (Thermo Fisher Scientific, catalog number: 12699182)
17. N-dodecyl-β-D-maltopyranoside (DDM) (Anatrace, catalog number: D310S)
18. Cholesteryl Hemisuccinate Tris Salt (CHS) (Anatrace, catalog number: CH210)
19. Glyco-diosgenin (GDN) (Anatrace, catalog number: GDN101)
20. Strep-Tactin Sepharose resin (IBA, catalog number: 2-1201-010)
21. D-desthiobiotin (Sigma-Aldrich, catalog number: D1411)
22. Tris base (Sangon Biotech, catalog number: A610195)
23. KCl (Sangon Biotech, catalog number: A610440)
24. PMSF (Sangon Biotech, catalog number: A100754)
25. Leupeptin (Sangon Biotech, catalog number: A600580)
26. Aprotinin (Sangon Biotech, catalog number: A600153)
27. Pepstatin (Sangon Biotech, catalog number: A610583)
28. DNase I (Sangon Biotech, catalog number: A610099)
29. Resuspension buffer (see Recipes)
30. Washing buffer (see Recipes)
31. Elution buffer (see Recipes)
32. SEC buffer (see Recipes)
33. Sodium butyrate (2 M) (see Recipes)

Equipment

1. 37 °C, 5% CO_2 forced-air shaker incubator (Zhichu, model: ZCZY-CSV)

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2. 28 °C (shaker) incubator (Zhichu, model: ZCZY-CS9)
3. CytoFLEX S Flow Cytometer (Beckman Coulter Life Sciences)
4. Refrigerated centrifuge (Eppendorf, model: 5424R)
5. Sonicator (Scientz, model: IID)
6. High speed centrifuge (Thermo Fisher Scientific, model: Sorvall LYNX 6000)
7. ÄKTA Purifier chromatography system (GE Healthcare, model: ÄKTA Purifier)
8. SEC column Superose 6 Increase 10/300 GL (GE Healthcare, catalog number: 29-0915-96)
9. Nanodrop One (Thermo Fisher Scientific, catalog number: ND-ONE-W)

Procedure

A. Cell culture conditions
   1. Sf9 cells were cultivated in SIM SF Expression Medium containing 2% fetal bovine serum and 0.5% Penicillin-Streptomycin in shaker incubator at 28 °C, 130 rpm. Maintain the suspension Sf9 cells between 0.5 × 10^6-4 × 10^6 cells/ml.
   2. HEK293F cells were cultivated in SMM 293-TI Expression Medium containing 2% fetal bovine serum and 0.5% Penicillin-Streptomycin in shaker incubator at 37 °C, 130 rpm in the presence of 5% CO_2. Maintain the suspension HEK293F cells between 0.5 × 10^6-4 × 10^6 cells/ml. It is best to passage the cells by no more than two months.

B. Expression of KCC1
   1. The full-length human *SLC12A4* gene (NCBI accession NP_005063) and a C-terminal Strep tag connected by a GGSSGG linker were cloned into a pEZT-BM vector.
   2. 500 ng plasmid was added to 100 µl DH10Bac *E. coli* to Generate the recombinant bacmid.
   3. Isolation of bacmid DNA (page 20-22), transfection of Sf9 cells (page 25-28) and amplification of virus (page 30-31) were following methods from the Bac-to-Bac system (Invitrogen) (Reference 1).
   4. Measure the baculoviral titer for P2 stocks using flow cytometry with baculovirus envelope gp64 antibody (Figure 1). Briefly,
      a. In 96-well block, add 100 µl Sf9 cells/well (cell density = 2 × 10^6 cells/ml).
      b. Dilute virus stock 10× in an Eppendorf tube.
      c. In a 96-well plate, set up serial dilution of 10× diluted stock:
         i. 250× (4 µl 10× + 96 µl media)
         ii. 500× (50 µl 250× + 50 µl media)
         iii. 1,000× (50 µl 500× + 50 µl media)
         iv. 2,000× (50 µl 1,000× + 50 µl media)
      d. Transfer 20 µl of serially diluted samples from 96-well plate to 96-well block.
      e. Seal block and place in shaker for 18 h at 28 °C and 200 rpm.

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f. Stain Sf9 with anti-gp64-PE conjugated antibody for 20 min in the dark and analyze cell count by flow cytometry.

g. For infected values above 30%: IU = Total cell number × [-ln (1 - %infected value/100)] × [viral dilution factor]/(volume of inoculum); for infected values below 30% (more reliable): IU = Total cell number × (%infected value/100) × [viral dilution factor]/(volume of inoculum).

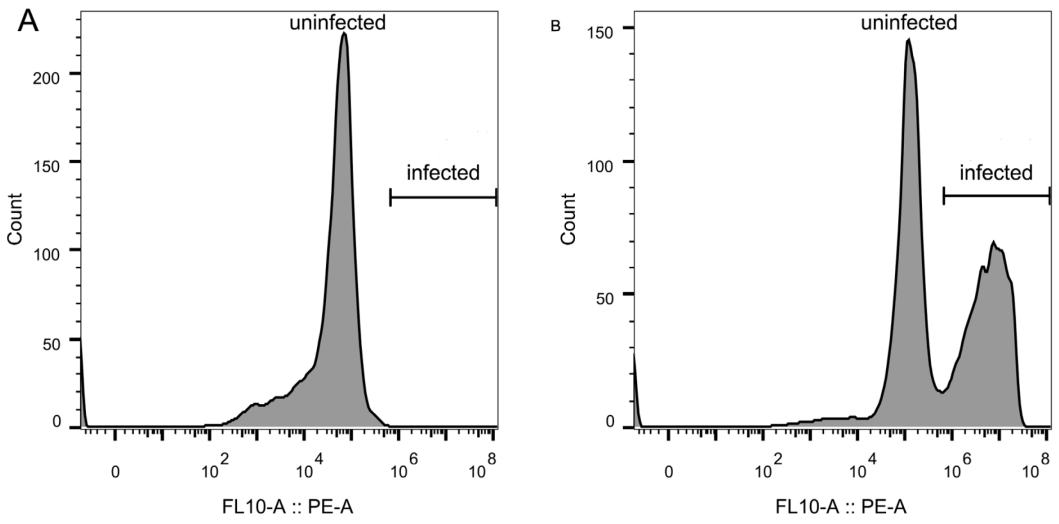

Figure 1. Estimation of baculoviral titer using flow cytometry. Histogram plots of cell count as a function of PE signal, indicating distinct populations for (A) uninfected Sf9 cells, as well as (B) PE-positive infected Sf9 cells.

5. The baculovirus was used to infect HEK293F cells of a density of 2 × 10^6-3.5 × 10^6 cells/ml at a ratio of multiplicity of infection (MOI) 20.
6. HEK293F cells were supplemented with 10 mM sodium butyrate to boost protein expression in 8-12 h.
7. Cells were cultured in suspension at 37 °C and 130 rpm for 48 h.
8. Harvest the cells by centrifugation at 4,600 × *g* for 15 min.

C. Purification of KCC1

1. The cell pellet was re-suspended in 15-20 ml per liter of expression culture resuspension buffer and homogenized by sonication (2 s sonication and 5 s of pause in 40 cycles, 300 kW) on ice.
2. Add 2% (w:v) n-Dodecyl-β-D-Maltopyranoside (DDM) supplemented with 0.2% (w:v) cholesteryl hemisuccinate (CHS), shake on ice for 2 h to extract membrane proteins.
3. Centrifuge at 38,000 × *g* for 30 min to remove the pellet.
4. The supernatant was incubated with 2 ml per liter of expression culture Strep-Tactin Sepharose resin (IBA) with gentle agitation for 1 h.
5. The resin was collected on a disposable gravity column, washed in washing buffer for 10 column volumes.
6. KCC1 was eluted with elution buffer. Analyze on SDS-PAGE gel (Figure 2). Typical yield at this

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step is ~2-3 mg per liter of expression culture.

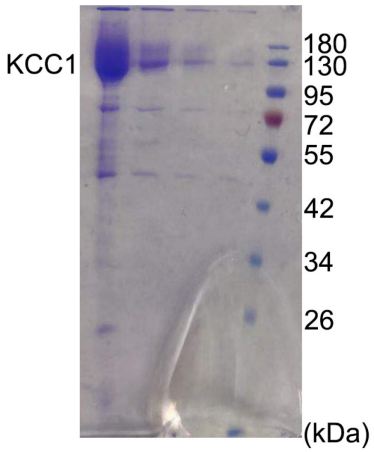

Figure 2. 12% SDS-PAGE analysis of purified KCC1 stained with Coomassie Brilliant Blue

7. Load the sample into a Superose 6 Increase 10/300 GL column pre-equilibrated with 1.2 column volume of SEC buffer, at a flow rate of 0.5 ml/min at 4 °C, collect 0.5 ml fractions.
8. Pool the fractions based on the gel filtration chromatogram (Figure 3) and concentrate to 2 to 7 mg/ml using an Amicon Ultra-4 centrifugal filters (100 kDa cut-off) for structural analysis.

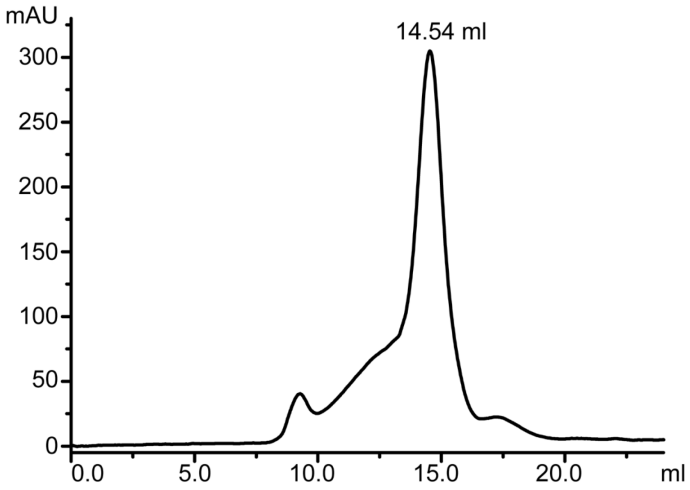

Figure 3. Purification of KCC1 by size-exclusion chromatography. The purified KCC1 is monodisperse.

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Recipes

1. Resuspension buffer
   20 mM Tris (pH 8.0)
   150 mM KCl
   0.1 mM PMSF
   2 μg/ml DNase I
   0.5 μg/ml pepstatin
   2 μg/ml leupeptin
   1 μg/ml aprotinin
   Chill the buffer to 4 °C. Add DNase I immediately before use, PMSF, Aprotinin, Leupeptin and Pepstatin A
2. Washing buffer
   20 mM Tris (pH 8.0)
   150 mM KCl
   0.1% (w/v) n-dodecyl-β-D-maltopyranoside
   0.02% (w/v) cholesteryl hemisuccinate
   Store at 4 °C for up to 1 week
3. Elution buffer
   20 mM Tris (pH 8.0)
   150 mM KCl
   0.06% glyco-diosgenin (GDN)
   10 mM d-desthiobiotin
   Store at 4 °C for up to 1 week
4. SEC buffer
   20 mM Tris (pH 8.0)
   150 mM KCl
   0.06% glyco-diosgenin (GDN)
   Filter using a 0.22 μm filter
   Store at 4 °C for up to 1 week
5. Sodium butyrate (2 M)
   Dissolve 11 g of sodium butyrate with water to a final volume of 50 ml and filter-sterilize using a 0.22 μm filter inside the biological safety cabinet. Store at -20 °C for at least 1 month

Acknowledgments

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Competing interests

There are no conflicts of interest or competing interest.

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