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# Transfection and validation of BK channel expressing HEK-293 cells for the study of Mir-9 regulation. V.1

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

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## Disclaimer

There are no
competing interests financial or non-financial, professional, or personal.

## Abstract

Research has identified the large conductance voltage- and calcium-activated potassium channel (BK) as a key regulator of neuronal excitability genetically associated to behavioral alcohol tolerance. Sensitivity to ethanol at the molecular level is characterized by acute potentiation of channel activity. BK isoforms show variations in alcohol sensitivity and are differentially distributed on the plasma membrane surface in response to prolonged exposure. MiRNA targeting of alcohol sensitive isoforms coupled with active internalization of BK channels in response to ethanol are believed to be key in establishing homeostatic adaptations that produce persistent changes within the plasma membrane of neurons. In fact, MiR-9 upregulated expression is a key event in persistent alcohol tolerance mediating acute EtOH desensitization of BK channels. The exact nature of these interactions remains a current topic of discussion. To further study the effects of Mir-9 on the expression and distribution of BK channel isoforms we designed an experimental model by transfecting human BK channel isoforms ZERO and STREX heterologous constructs in HEK293 cells respectively expressing 2.1 (Mir-9 responsive), 2.2 (unresponsive) and control (no sequence) 3'UTR miRNA recognition sites. We used imaging techniques to characterize the stably transfected monoclonal cell lines, and electrophysiology to validate channel activity. Finally, we used immunocytochemistry to validate isoform responsiveness to MiR-9. Our findings suggest the cell lines were successfully transfected and have differential responses to miR-9 making them viable for use in future alcohol dependence studies.

## Guidelines

All cell culture procedures were performed inside a biological hood using a sterile cell culture technique. HEK-293 is a cell line exhibiting epithelial morphology that was isolated from the kidney of a human embryo commercially available through ATCC (American Type Culture Collection) a nonprofit, global biological resource center.

## Materials

*Cell transfection/Monoclonal selection*

- Human Embryonic Kidney Cells (HEK293) (ATCC, CRL-1573, VA, USA)

- OptiMEM (ThermoFisher, A41248-02, NY, USA)

- Lipofectamine 2000 (11668027 ThermoFisher, Lithuania)

- Blue Heron's plasmid cDNAs:

Protocol: Genemaker® Gene Synthesis Platform from Blue Heron, LLC
The company uses Genemaker® which is a fully automated gene synthesis platform designed to facilitate the entire gene synthesis process. The process began with inputting the desired gene sequence into the website ordering page. The company proceeded to optimize the gene build based on the entered sequence using a proprietary algorithm. The optimization aimed to balance complexity reduction and protein expression enhancement. Once the Oligonucleotides are synthesized the are amplified via PCR synthesis techniques, utilizing both solution and solid substrate technologies. The synthesized genes are then cloned into the vector of choice. The cloned gene is sequence verified to ensure 100% accuracy and ship to us.

| Project # | Vector Name | Common Name |
|---|---|---|
| 509802 | CCN139060 | ZERO RES |
| 509801 | CCN134655 | ZERO 2.2 |
| 509797 | CCN134654 | ZERO 2.1 |
| 509800 | CW101512 | STREX RES |
| 509802 | CW101512PA-BKalpha3UTR2 | STREX 2.2 |
| 509799 | CW101512PA-BKalpha3UTR | STREX 2.1 |

Table. Summary of Transfected Plasmids.

- miR-9 sequence and mimic (Sigma-Aldrich/oligolearning, USA)

- miR-9/sponge (Sigma-Aldrich/oligolearning with Mission Synthetic micro-Inhibitor (#cat. NCSTUD001) USA)
- Dulbecco's modified Eagle's medium (DMEM) (D5796, Milwaukee, WI, USA)
- 10% fetal bovine serum (FBS) (F-4135), 2.52 mM
- Penicillin/Streptomycin (Pen/Strep)(P4333, Saint Louis, MO, USA)
- Na-pyruvate (Sigma-Aldrich, S8636 Milwaukee, WI, USA)
- Tripsin-EDTA 10X (ThermoFisher, 15400-054 New York, NY, USA)
- Phosphate Buffered Saline (PBS) (Sigma Aldrich, 806552 Milwaukee, WI, USA)
- Trypan Blue dye (BioRad, 64315131, UK)
- G418 antibiotic (Sigma Aldrich, A1720 Milwaukee, WI, USA)
- 35mm petri dish (Corning 353001, NY, USA)
- Counting slides (BioRad, 64302432, USA)
- 96-well plates (Millipore SIGMA, CLS3922)

*Electrophysiology*

- Borosilicate glass (VWR Micropipettes)

- NaCl – sodium chloride
- KCl – potassium chloride
- HEPES - N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
- $CaCl_2$ - calcium chloride
- $MgCl_2$ - magnesium chloride
- 150 K-gluconate

*Immunocytochemistry*

- 100mm dishes (Millipore sigma, Z358762)
- 35mm glass bottom dishes (MATTEK Life Sciences, P35G-1.5-20-C MA, USA)
- miR-9 or pBabe-puro-miR-9 sponge (Addgene, 2504).
- Paraformaldehyde solution 4% in PBS (PFA) (Santa Cruz Biotechnology, CAS 30525-89-4 TX, USA)
- Cholera Toxin Subunit B Alexa Fluor 594 conjugate (C22842, ThermoFisher, New York, NY, USA)
- Sodium Azide (Sigma Aldrich, S2002 S2002)
- Aqueous Mounting Medium with DAPI (Santa Cruz Biotechnology, sc-24941 CA, USA)

- Square coverslip size 22 × 22 -1 (Fisher Scientific,12-548-B USA)

*Equipment*

- Sanyo CO2 incubator MCO-18AC (Sanyo Electric Co., Japan)
- Purifier Biological Safety Cabinet Class//typeA2 (LABCON Co.)
- Vacuum pump p-79202-00 (Cole Parmer Instrument Co.)
- HEKA EPC10 amplifier controlled with PATCHMASTER program (all by HEKA Elektronik, Dr. Schulze GmbH, Wiesenstraße 71, D-67466 Lambrecht/Pfalz, Germany).

## Troubleshooting

## Safety warnings

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

## Cell Culture

1 Thaw and plate the cells as follows:

1.1 Resuspend in modified DMEM media composed of Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 2.52 mM; 10 units and 10µg/mL of Penicillin/Streptomycin (Pen/Strep), and 1mM Na-pyruvate.

1.2 Pipette 20µL of the resuspended solution onto 35 mm petri dishes.

1.3 Flood with modified DMEM up to a final volume of 2mL.

2 Place cells in a Sanyo CO2 incubator (37.0$^{o}$C/5% CO2 level) and allowed to reach full confluency changing the media every 3-5 days.

3 Once the cells are fully confluent suction media from the plates and add Trypsin-EDTA 10X diluted in PBS to solubilize the cells.

4 Add an equal volume of modified DMEM to prevent cell lysis.

5 Pipette suspended cells onto 10mL tubes.

6 Remove 10mL tubes from the hood and centrifuge at 3,300rpm for 60 seconds.

7 Place inside the hood and aspirate the supernatant.

8 Resuspend in modified DMEM.

9 Extract 10µL of each tube and mix with equal volume of Trypan Blue Dye.

10 Add a single droplet of the Trypan Blue mixture to a counting slide and place in an automated cell counter.

11 Once counted add the appropriate seeding density (*50- 80% confluent*) was to the 35 mm plate to being transfections.

## Transfection

12 Once cells reach 90% confluence place the plates in the hood.

13 Wash the plates 3 times with PBS for 30 seconds with gentle swirling.

14 Place the cells in serum deprived DMEM (no FBS) for 24 hours in the incubator.

15 The next day prepare solution containing the DNA of interest by diluting DNA in 250ml of Opti-MEM I - Reduced Serum Medium, mixing gently, adding lipofectamine 2000, then diluting the appropriate amount in 250µl of Opti-MEM I Medium, and incubating for 5 minutes at room temperature. See attached Protocol.

16 After the incubation period syphon the serum deprived media from the plates.

17 Wash three time (30sec each) with PBS and gentle swirling.

18 After the washes Add Opti-MEM I solution for 16 hours.

19 Wash 3 additional times with PBS and place in modified DMEM for 24 hours in the incubator.

## Monoclonal Selection

20 After transfections the cells wash 3 times with PBS and place in modified DMEM supplemented with 400µg/mL of G418 antibiotic.

21 Replaced half of the volume with modified DMEM for 10-14 days or until visually resistant colonies where visible.

22 Plate onto 96-well plate by limited dilution (to create monoclonal cell lines) as follows:

22.1 In a 96 well plate pipe 12 µL of cell culture media into all except A1.

22.2 Pipette 200 µL of the cell suspension into well A1.

22.3 Pipette 100 µL from well A1 to A2 and gently resuspend gently.

22.4 Repeat this process for the entire row.

22.5 Then Pipette 100 µL from all wells on Row A too wells on row B and resuspend gently.

22.6 Repeat this process for pair of rows (B-C, C-D...).

22.7 Add 100 µL of media to each well.

22.8 Incubate for 4 to 5 days.

23 Observe daily to ensure colonies were formed by a single cell.

24 Allow cells to reach 40% confluence at which point they were expanded for storage in liquid nitrogen or for use in experiments.

## Electrophysiology

25 Remove transfected cells from the incubator and place in Normal Locke's media.

26 Then perform single channel recordings in the cell-attached mode using the following conditions:

26.1 External bath solution: 145 NaCl, 2.5 KCl, 10 HEPES, $CaCl_2$, $1MgCl_2$.

26.2 Internal (pipette) conditions: 150 k-gluconate, $1MgCl_2$, 10 HEPES, PH 7.4.

27 Correct for the effective applied voltage by estimating correction deviations using the leak currents during cell-attached recordings

28 Aquire Data using a HEKA EPC10 patch-clamp amplifier controlled by a Macintosh-based computer system equipped with Patch Master acquisition software.

29 Sample Data at 25 µs per sample and filtered at 10kHz low bandpass

## Electrophysiology Data Analysis

30 Perform data analysis with IgorPro graphing and curve fitting software as follows:

30.1 Determine NPo for each voltage using the following:
$NP_o = (\sum_i (iA_i))/(\sum_i (A_i))$
where N is the number of observed channels (levels), i the ith level number, and $A_i$ the area of a Gaussian fit to the ith level's in all-points-histogram from each voltage.

31 When seeking the half-action voltage ($V_{1/2}$) from NPo versus voltage curves, determine the values were from sigmoidal fits assuming the following:
$NPo = N/(1 + e^(k(V(1/2) - V))$
where $V_{1/2}$ is the half-action voltage and V the applied voltage. The $V_{1/2}$'s were thus obtained from the half height of the sigmoidal fits.

## Immunocytohemistry (ICC)

32 For ICC place plates in the hood and wash 3 times with PBS as previously described.

33 Fix in a 4% paraformaldehyde (PFA) solution for 1 hour with gentle shaking.

34 Remove excess PFA with 3 washes with ice cold PBS.

35 Incubate for 1 hour with Cholera Toxin B Alexa Fluor 594 conjugate (CtB) antibody 864ng/mL to label GM1.

36 Wash 3 additional times with PBS.

37 Following the final wash, remove all PBS.

38 Add Fluoromount-G™ Mounting Medium, with DAPI 00-4959-52 (Thermo Fisher) to the wells prior to cover slipping with Premium Cover Glass 12-548-B (Fisher Scientific USA).

39 Image using Nikon Instruments A1 Confocal Laser Microscope using a 60X objective (Refractive Index=1.51 and Numerical Aperture=1.40) (Maintain imaging parameters between treatments).

40 Analyze Mean intensity fluorescence and colocalization by Nikon Software NIS Elements v5.30 – Software, Automated Measurements – Module.

## Protocol references

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