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Generation of HEK293 TMEM55B and TMEM55A CRISPR/Cas9 double knock-out cell line

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We use this protocol and it's working

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Abstract

The protocol described the strategy taken for knocking out TMEM55A and TMEM55B gene in HEK293 cells uisng CRISPR-Cas9 method.

Image Attribution

Figure panels A-D: TMEM55B gene details, Western blot analysis, and Sanger sequencing results for TMEM55B and TMEM55A KO clones. (Source: protocol authors)
Figure panel E: TMEM55A and B double KO clone confirmation by immunoprecipitation assay. (Source: protocol authors)
Panel E: Western blots and merged images for TMEM55A and TMEM55B double KO clone confirmation by immunoprecipitation assay. Probes and antibodies: TMEM55A Sheep Polyclonal Ab. DA241, Rabbit Polyclonal TMEM55A Ab. (Cusabio-Stratech), Sheep Polyclonal TMEM55A ab. (DA241), TMEM55B Sheep Polyclonal Ab. DA275 (Bleed-2), Rabbit Polyclonal TMEM55B Ab. (Proteintech), Sheep Polyclonal TMEM55B ab. (DA275). (Source: protocol authors)

Materials

1. Materials

1.1. Consumables

- Nunc™ Cell-Culture Treated 6-well plates (ThermoFisher Scientific #140675)
- Nunc™ Cell Culture/Petri Dishes (ThermoFisher Scientific #150318)
- Autoclaved 1.5 ml Eppendorf tubes (Eppendorf™ #0030120086)
- Spin-X Centrifuge tube filters, 0.22µm cellulose acetate membrane (Costar #8161) - 0.45µm syringe filter
- 1.5 ml Eppendorf tubes rack
- Marker pen
- Pipette set (1 ml, 200 µl, 20 µl, 10 µl)
- 15 ml falcon tubes
- Agar Plates
- Agarose (Molecular Biology Grade)
- LB Broth
- LB Agar
- Ampicillin
- 50 x TAE solution
- SYBR Safe
- Deionised water
- 0.2 µL PCR tubes

1.2. Reagents

- Rapid DNA Ligation Kit (ThermoFisher Scientific, K1422)
- BpiI (BbsI) restriction enzyme 10U/µL (ThermoFisher Scientific, ER1011)
- Lysis buffer: 50mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100, 10% Glycerol, 150mM NaCl, 1mM sodium orthovanadate, 10mM sodium glycerophosphate, 10mM sodium pyrophosphate, 1µg/ml mycocyctin-LR, complete EDTA-free protease inhibitor cocktail
- Anti-TMEM55A (sheep polyclonal antibody, MRC PPU Reagents and Services, #DA241), Anti-TMEM55A (Rabbit Antibody - # CSB-PA023855LA01HU, AB_2631310), Anti-TMEM55B antibodies (rabbit polyclonal antibody, Proteintech, #23992-1-AP)
- Protein A/G agarose beads
- IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (Licor # 926-32211)
- IRDye® 680LT Donkey anti-Goat IgG Secondary Antibody (Licor #926–68 024)
- DMEM, high glucose (ThermoFisher Scientific #11965092)
- Trypsin-EDTA (0.05%), phenol red (ThermoFisher Scientific # 25300054)
- PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000) (Polysciences #24765)
- Opti-MEM™ I Reduced Serum Medium (ThermoFisher Scientific #31985062)
- KOD Hot Start DNA Polymerase (Sigma #71086)
- StrataClone Blunt PCR Cloning Kit (Agilent #240218)
- FastDigest EcoRI (ThermoFisher Scientific #FD0274)
- DNeasy® Blood 6 Tissue Kit (Qiagen #69504)
- QIAprep Spin Miniprep Kit (Qiagen #27106)

1.3. Equipment

- Eppendorf Thermomixer
- PCR machine
- Incubator 37°C, supplemented with 5% CO_2
- Block heater
- Cold and room temperature centrifuge

Power pack
Gel tank
Gel casting tray and combs
37°C incubator
37C shaker incubator
Microwave oven
UV Transilluminator and camera
Pipettes – 20 µL, 200 µL and 1000 µL

Troubleshooting

Safety warnings

Note: lyse cells and immunoprecipitate TMEM55B from wild type HEK293 cells as a control.
Note: prepare 2 gels for immunoblotting, 1 gel will be used for anti-TMEM55A antibody, the other gel will be used for anti-TMEM55B antibody. The secondary antibody used are IRDye® 680LT Donkey anti-Goat (for TMEM55A) and IRDye® 800CW goat anti-rabbit (for TMEM55B).

1. Transfection for TMEM55B

1 HEK293 cells were transfected with the guide RNAs using PEI and Opti-MEM using following steps:

1.1 **Note:** Complementary oligonucleotides were designed to generate double-stranded DNA (dsDNA) inserts for ligation into BbsI digested destination vectors. To enhance expression, any 20 bp guide sequence which did not begin with a Guanine was extended with a ‘G’ nucleotide to ensure optimal expression from the U6 promoter driven RNA cassettes. Additional single stranded overhangs were included to facilitate directional ligation, with the upper strand oligo extended with additional 5′-CACC nucleotides and the lower strand oligos with 5′-AAAC nucleotides.

Optimal guides were identified in exon 2 and oligos designed as above; Complementary oligos for nickase paired guide RNAs, Sense A: 5′-GCAATCTCTCATCAACGTGGA-3′ and Antisense A: 5′- GACTCGGCAGGTGATCATAG-3′ unpaired guide G1: 5′-GCCCTTAACTAGCCCGGACAG-3′ and unpaired guide G2: 5′-GACTCGGCAGGTGATCATAG-3′ were subsequently annealed and used as dsDNA inserts for their respective expression vectors. The sense guide was cloned into pBabeD P U6 (DU48788), the antisense guide into pX335 (Addgene #42335) and the unpaired guides into pX459 (#62988). Derived clones were confirmed via sequencing with primer TM7016 (5′ CCTATTTCCCATGATTCC-3′).

1.2 Seed wild type HEK293 cells approximately 60 – 70% confluency in 3ml media/well (DMEM + 10% FBS (Foetal Bovine Serum) + 1% penicillin/streptomycin + 1% L-glutamine) in 6-well plates. Preferably plate 2 wells per guide (one well for cell growing and one for immunoblotting).

Note: prepare 2 extra wells as a control with no DNA transfection.

1.3 After 16 – 20 hours of cell seeding, do transfection using PEI.

Note: 1µg DNA used in total at a 1:1 ratio (sense guide 0.5µg: anti-sense guide 0.5µg). For single guides, 1 µg of DNA was used.

1.4 Mix 200µl of Opti-MEM and 3.5µl PEI (from 1mg/mL stock) gently and incubate at room temperature for 20-30 mins, add gently 200µl mix dropwise to cells.

1.5 After 24 hours of transfection, aspirate old media and add 2ml fresh media/well containing puromycin at 3µg/ml final concentration. Treat cells for 2 days before adding fresh media.

Note: Untransfected cells (control) should die, and transfected cells should have some cells
remaining due to puromycin resistance gene.

1.6 Allow cells to grow until confluent. Spit cells from 6-well plates into 10 cm petri dishes, then let cells grow until confluent. Cells from 1 dish are stored in freezing media (10% DMSO + 90% FBS) at -80°C for single cell sorting later, cells from the other dish are used for western blot to confirm the reduction of TMEM55B in the pool.

2. Single cell sorting

2 Using following steps single cell was sorted:

2.1 After confirming the absence of TMEM55B in transfected cells, re-grow cells stored at 80°C from the step 1.6 in 10 cm plate in 10 ml complete media (DMEM + 10%FBS + 1% penicillin/streptomycin + 1% L glutamine).

2.2 One day before cell sorting: add 200µl preconditioned media into a 96-well plate, keep in 37°C incubator supplemented with 5% CO_2.

Note: Preconditioned media was collected from HEK293 wild type cells which was complemented with 20%FBS + 1% penicillin/streptomycin + 1% L-glutamine and filtered using 0.45µm syringe filter.

2.3 On the day of cell sorting: Trypsinise cells (3ml/dish), stop trypsinisation with7ml media (DMEM + 10%FBS + 1% penicillin/streptomycin + 1% L-glutamine), transfer to a 15ml falcon tube.

2.4 Spin down at 300 x *g* for 3 mins. Remove media, resuspend cells in 10 ml of media (DMEM + 1%FBS + 1% penicillin/streptomycin + 1% L-glutamine).

2.5 Sort single cells into a 96-well plate prepared one day before.

Note: Wild type HEK293 cells used as a negative control for FACS.

2.6 After finishing cell sorting, spin down the 96-well plate at 200 x *g* for 1 min to ensure cells are at the bottom of the plate. Leave cells to grow for approximately 2-3 weeks in 37°C incubator supplemented with 5% CO_2.

3. Growing clones

3 The sorted single cells were then expanded.

3.1 Check every clone under microscopy. After approximately 2 weeks, a single cell will grow into a colony.

3.2 Split growing cells from single clones into 6-well plates (2ml media/well, DMEM + 10%FBS + 1% penicillin/streptomycin + 1% L-glutamine). Let them grow until confluent.

3.3 2.3.3. Split cells from single clones into 2 wells of 6-well plates, wait until they are confluent.

3.4 2.3.4. Lyse cells from one well for screening and store cells from another well in freezing media (10% DMSO + 90% FBS) at -80°C for short term storage.

Note: cell lysates and cells stored at -80°C from the same single clone should be labelled with the same name (ideally labelled as numbers, e.g.: 1,2,3, etc...)

4. Screening positive clones

4 Positive clones were screened following below steps:

4.1 Lyse cells from individual clones recovered from cell sorting with 150µl of lysis buffer.

4.2 Analyse the lysates by immunoblotting with anti-TMEM55B antibodies (Figure 1A). Wild type cells used as a control for wild type TMEM55B with molecular weight approximately 29 kDa.

4.3 Select clones not showing a band of wild type TMEM55B for further analysis.

5. Characterizing homozygous HEK293 TMEM55B knock-out cells

5 DNA sequencing was performed to characterise homozygous HEK293 TMEM55B knock-out cell line.

5.1 Extract genomic DNA from several positive clones using Qiangen kit according to the manufacturer's instructions.

5.2 Perform PCR using following primers: a pair of primers (*55BF: 5'GGCTTCCTTCCCTCCGTAATCG-3' and 55BR: 5'-CCAGAAGCCACTCACCAAAGCT-3'*) are used to amplify an amplicon with expected size at 520 bp.

Note: water and wild-type (WT) HEK293 should be included as negative controls.

5.3 KOD Hot Start Polymerase kit is used as described below.

Reagents	Volume (µl)
10X buffer	2
dNTP (2mM each)	2
25mM $MgSO_4$	1.2
DMSO	1.2
10 µM forward primer	0.6
10 µM reverse primer	0.6
Genomic DNA (75ng)	1
KOD polymerase	0.4
Nuclease-free water	11
Total volume	20

PCR Reaction setup

Step	Time	
98°C	2 min	
98°C	10 s	35 cycles
60°C	20 s	
70°C	20 s/kb	
70°C	5 min	
4°C	hold	

Cycling conditions

5.4 Select clones showing a band at expected size (520 bp) for further analysis. Clone PCR products from the positive clones into the StrataClone PCR cloning vector pSC-B-amp/kan according to the manufacturer's instructions (Stratagene).

5.5 After overnight incubation at 37°C, pick randomly 12-20 bacterial white colonies for plasmid DNA analysis and grow in 3ml LB amp media overnight. *Note*: do not pick blue colonies.

5.6 Prepare miniprep DNA plasmids from the selected colonies according to the manufacturer's instructions (Qiagen).

5.7 Identify plasmids containing the PCR product insert (1062bp) by restriction analysis with EcoRI digestion according to the manufacturer's instructions (ThermoFisher Scientific).

5.8 Send all the miniprep plasmids for DNA sequence with M13 forward primer (5′-GTAAAACGACGGCCAGTG) and M13 reverse primer (5′- GGAAACAGCTATGACCATG) to identify homozygous knock-out clones by sequencing.

5.9 Store homozygous HEK293 TMEM55B knock-out clones in liquid nitrogen for long-term storage.

6. Generation of TMEM55A knock-out in TMEM55B KO cells

6 Similar approach was taken to knock-out TMEM55A using the TMEM55B knock-out single sorted clone of HEK293 cells.

6.1 To target exon2 of TMEM55A, complimentary oligonucleotides encoding the following guide RNAs were designed, annealed and ligated into BbsI-digested vectors as described in section 1.1.1.

Paired guide RNAs Sense A: 5′-GTGCCATCATCAATCAATT-3′
Anti-Sense A: 5′ GTTTTATCTGGAATACCAC-3′
Single guide RNA G1: 5′-GCACACACGGCAGTTTATTAC-3′
Singel guide RNA G2: 5′-GTGATTATGTGATTGGCACACA-3′. The clones were confirmed by sequencing.

7. Immunoprecipitation of TMEM55A and TMEM55B

7 To validate the TMEM55A and TMEM55B knock-out clones, immunoprecipitation assay was performed.

7.1 Aspirate media from 10cm dish, wash cells twice with PBS. Add 300µl lysis buffer into the dish and scrape it using a suitable scrapper. Transfer the lysate into a 1.5ml Eppendorf tube.

7.2 Centrifuge the lysates at 17,000g for 10mins at 4°C. Transfer supernatant into a new 1.5ml Eppendorf tube.

7.3 Quantify protein concentration using Bradford assay.

7.4 Immuno-precipitate TMEM55A and TMEM55B using anti-TMEM55A and antiTMEM55B coupled A/G Sepharose beads. 500µg of lysates is incubated with 10µl of beads for 2 hours in the cold room. Note: anti-TMEM55B antibody is covalently coupled with A|G agarose beads at a ratio 1:1 (1µg antibody: 1µl resin).

7.5 Wash the beads 3 times with PBS, then add 20µl of 2X SDS-PAGE loading buffer, incubate for 10 mins at 70°C. Note: lyse cells and immunoprecipitate TMEM55B from wild type HEK293 cells as a control.

7.6 Collect the supernatant by centrifuging through a 0.22µm Spin-X Centrifuge tube filters.

7.7 Add ß-mercaptoethanol to the eluate to a final concentration 1%.

7.8 Boil the eluate at 95°C for 5 mins, then subjected to immunoblot analysis. Note: prepare 2 gels for immunoblotting, 1 gel will be used for anti-TMEM55A antibody, the other gel will be used for anti-TMEM55B antibody. The secondary antibody used are IRDye® 680LT Donkey anti-Goat (for TMEM55A) and IRDye® 800CW goat anti-rabbit (for TMEM55B).

8

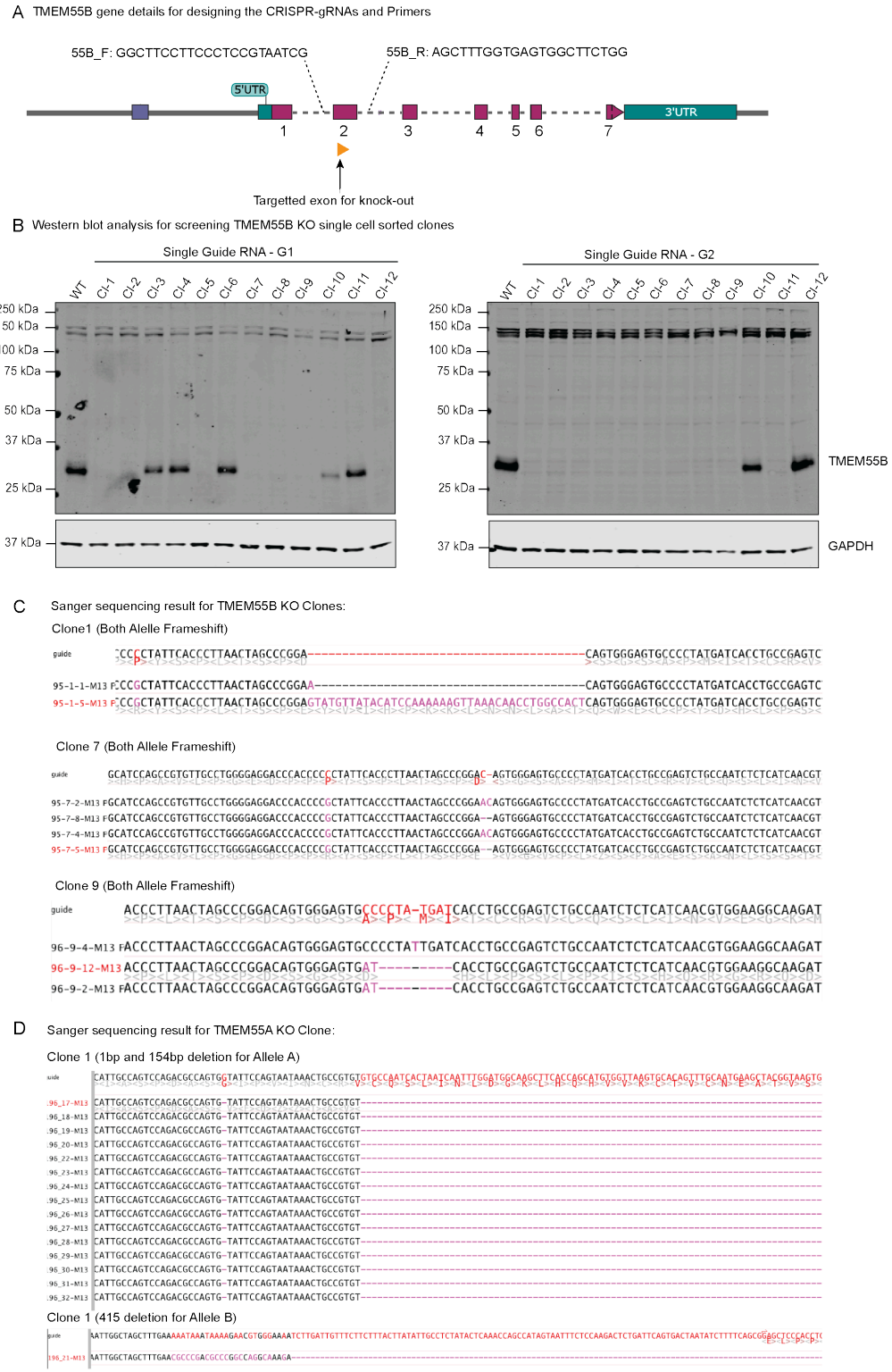

Figure 1. A. Schematic diagram and details of target region of TMEM55B knock-out. B. Screening of TMEM55B knock-out clones. C and D. Sequencing result of selected knock-out clones of TMEM55B and TMEM55A.

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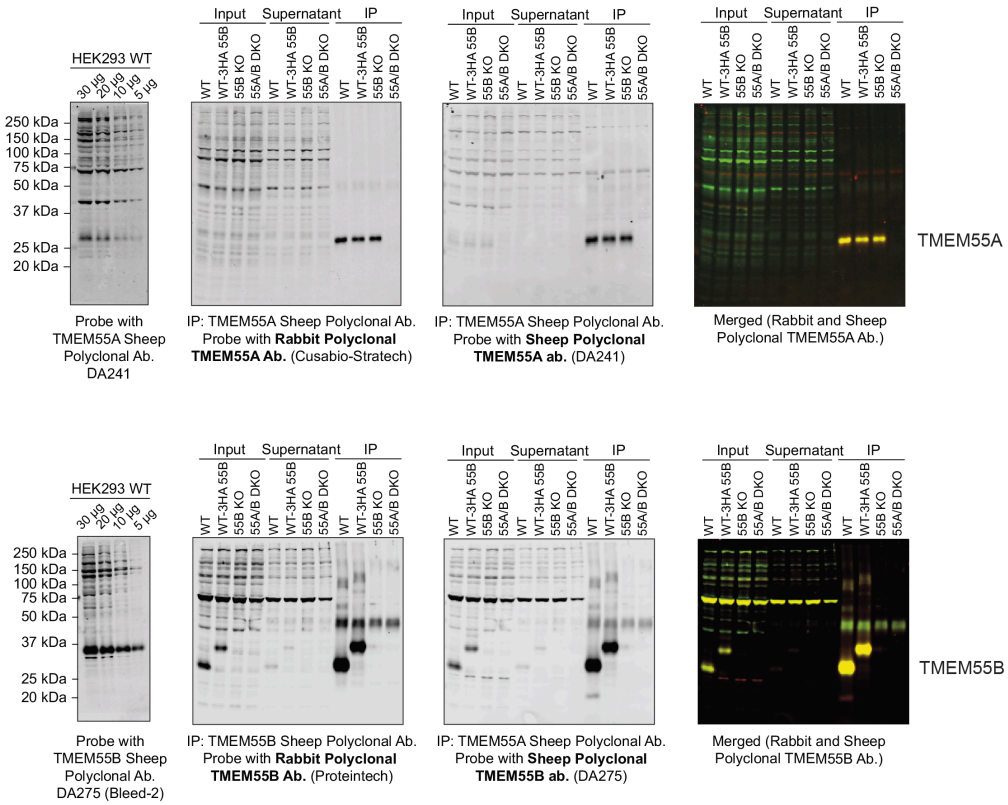

Figure 2. Knock-out clone verification by immunoprecipiation assay