APR 20, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.6qpvr456bgmk/v1

Protocol Citation: Matthew Taylor, Francesca Tonelli, Dario R Alessi 2023. Organelle Tag Introduction in Mouse Embryonic Fibroblasts (MEFs). **protocols.io** https://dx.doi.org/10.17504/protocols.io.6qpvr456bgmk/v1

Protocol status: Working
We use this protocol and it's working

Created: Apr 19, 2023

Last Modified: Apr 20, 2023

PROTOCOL integer ID: 80758

Keywords: ASAPCRN, Organelle, LysoTag, GolgiTag, Mouse Embryonic Fibroblasts, MEF

Organelle Tag Introduction in Mouse Embryonic Fibroblasts (MEFs)

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ABSTRACT

We describe here a method to generate MEFs stably expressing a lysosome localized TMEM192-3×HA fusion protein (“LysoTag”), or a Golgi localized TMEM115-3×HA fusion protein (“GolgiTag”). The fusion proteins mark lysosomes or the Golgi apparatus, respectively, with a triple HA epitope, enabling the rapid isolation of intact tagged organelles from cells using an anti-HA antibody. The purified organelles can then be analysed with a variety of techniques including immunoblotting analysis and proteomic, lipidomic, or metabolomic analysis.This protocol can be adapted to introduce organelle tags in commonly cultured cells, such as HEK293 and A549 cells. For MEFs, we recommend performing a Simian Virus 40 (SV40) immortalisation of primary cells prior to the introduction of the organelle tag, which is also described here.

MATERIALS

1.1.Cell lines

1.1.1. HEK293FT for virus packaging and propagation (Invitrogen™ Catalog# R70007)
1.1.2. Mouse embryonic fibroblasts (MEFs) isolated according to the protocol described in dx.doi.org/10.17504/protocols.io.eq2ly713qlx9/v1.

1.2.Plasmids

1.2.1. pBabeD SV40 Large T Antigen (DU40867, available at MRCPPU Reagents and Services https://mrcppureagents.dundee.ac.uk).
Note: The plasmid should contain an antibiotic selection cassette for the selection of successfullytransduced cells (hygromycin, in this case).
1.2.2. pCMV VSV-G: Retrovirus envelope plasmid (Cell Biolabs RV-110).
1.2.3. pCMV Gag/Pol: Retrovirus Gag/Pol plasmid (Cell Biolabs RV-111).
1.2.4. pLJC5 TMEM192-3xHA (LysoTag) (DU68356, available at MRCPPU Reagents and Services https://mrcppureagents.dundee.ac.uk).
Note: The plasmid should contain an antibiotic selection cassette for the selection of successfully transduced cells (puromycin, in this case).
1.2.5. pLJC5 TMEM115-3xHA (GolgiTag) (DU68534, available at MRCPPU Reagents

and Services https://mrcppureagents.dundee.ac.uk).
Note: The plasmid should contain an antibiotic selection cassette for the selection of successfully transduced cells (puromycin, in this case).
1.2.6. pLJC5 HA-Empty (control) (DU70022, available at MRCPPU Reagents and Services https://mrcppureagents.dundee.ac.uk).
Note: The plasmid should contain an antibiotic selection cassette for the selection of successfully transduced cells (puromycin, in this case).

Note: We purify plasmids using a QIAGEN HiSpeed® Plasmid Maxi kit following the manufacturer's protocols and ensure sterile reagents are used and mixtures prepared in tissue culture hood to avoid contamination.

1.3.Media and Reagents

1.3.1. Growth Media (for HEK293FT): Dulbecco's Modified Eagle's Medium (DMEM), High Glucose, no glutamine (Gibco™, catalog number: 11960044, or equivalent) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Sigma #F7524, or equivalent), 2 mM L-glutamine (Gibco™, catalog number: 25030024, or equivalent), Penicillin-Streptomycin 100U/mL (Gibco™, catalog number: 15140122, or equivalent).
1.3.2. Growth Media (for MEFs): Dulbecco's Modified Eagle's Medium (DMEM), High Glucose, no glutamine (Gibco™, catalog number: 11960044, or equivalent) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Sigma #F7524, or equivalent), 2 mM L-glutamine (Gibco™, catalog number: 25030024, or equivalent), Penicillin-Streptomycin 100U/mL (Gibco™, catalog number: 15140122, or equivalent), 1 mM Sodium Pyruvate (Gibco™, 11360039, or equivalent), 1X Non-Essential Amino Acids Solution (Gibco™, 11140035, or equivalent).
1.3.3. 0.05% trypsin-EDTA (Gibco™, catalog number: 25300054, or equivalent)
1.3.4. Transfection media (for HEK293FT cells): Opti-MEM Reduced Serum Medium (ThermoFisher Scientific #31985062)
1.3.5. Linear polyethylenimine (PEI Max 40K. Polyscience #24765); 1 mg/ml (w/v) stock in de-ionised H_2O, pH 7.4; sterile filtered.
1.3.6. Polybrene Infection/Transfection reagent (Millipore #TR-1003-G) (10 mg/ml stock)
1.3.7. Selection Media (for MEFs after viral transduction): Growth Media with 200 µg/ml Hygromycin (InvivoGen #ant-hg-5) (for SV40 Immortalisation) or 2 µg/ml Puromycin (Sigma #P9620) (for Organelle Tag introduction).

1.4.Consumables

1.4.1. Tissue culture Petri dishes (100 mm) (BD Biosciences, Falcon®, catalog number: 351029, or equivalent)
1.4.2. Sterile plastic serological pipettes
1.4.3. Sterile conical centrifuge tubes (15 ml) (BD Biosciences, Falcon®, catalog

number: 352095, or equivalent)
1.4.4. Standard 1 ml and 200 µl Pipette tips (Greiner bio-one. Catalog# 686271 and 685261 respectively).
1.4.5. Syringe filter (0.45 µm. Sartorius, Item # ST16537-Q)
1.4.6. Syringes (10 ml) (Medicina. REF# IVS10).

1.5.Equipment
1.5.1. CO_2 incubator for cell culture (BINDER GmbH. Model: CB150, or equivalent), set at 37°C and 5% (v/v) CO_2.
1.5.2. Laminar flow hood for cell culture.

SAFETY WARNINGS

All steps should be performed under sterile conditions in a CATEGORY 2 biological safety cabinet.

1.1 Packaging the SV40 large T antigen plasmid into a Retr...

1 1.1.1) Grow HEK293FT cells to 60% confluency in Growth media in a 10 cm Petri Dish.

2 1.1.2) Prepare a transfection mix in a sterile 1.5ml Eppendorf tube, containing:
- 3.8 µg pCMV Gag/Pol plasmid
- 2.2 µg pCMV VSV-G plasmid
- 6 µg pBabeD SV40 Large T Antigenplasmid
- 36 µl 1mg/ml PEI Max 40K
- 500 µl OptiMem

3 1.1.3) Mix by vortexing and incubate at room temperature for 20 mins.

4 1.1.4) Add the mixture dropwise to the cells from step 1.1.1 using a P1000 sterile pipette.

5 1.1.5) Incubate cells at 37°C for 24 h.

6 1.1.6) Replace media with 10 mL of fresh Growth Media and incubate cells for a further 24 h at 37°C.

7 1.1.7) Collect the culture media from step 1.1.6 (that now contains the retroviruses) and pass through a 0.45 µm syringe filter.
Note: The retrovirus infection media from step 1.1.7 can be used immediately (Step 1.3.1) or stored at -80°C for subsequent use.

1.2 Packaging the Organelle Tag (LysoTag or GolgiTag) pla...

8 1.2.1) Grow HEK293FT cells to 60% confluency in Growth media in a 10cm Petri Dish.

9 1.2.2) Prepare a transfection mix in a sterile 1.5ml Eppendorf tube, containing:
- 3.8 µg pCMV Gag/Pol plasmid
- 2.2 µg pCMV VSV-G plasmid
- 6 µg pLJC5 TMEM192-3xHA (LysoTag), or pLJC5 TMEM115-3xHA (GolgiTag), or pLJC5 HA-Empty (control)
- 36 µl 1 mg/ml PEI Max 40K
- 500 µl OptiMem

10 1.2.3) Mix by vortexing and incubate at room temperature for 20 mins.

11 1.2.4) Add the mixture dropwise to the cells from step 1.2.1 using a P1000 sterile pipette.

12 1.2.5) Incubate cells at 37°C for 24 h.

13 1.2.6) Replace media with 10 mL of fresh Growth Media and incubate cells for a further 24 h at 37°C.

14 1.2.7) Collect the culture media from step 1.2.6 (that now contains the lentiviruses) and pass through a 0.45µm syringe filter.
Note: The retrovirus infection media from step 1.2.7 can be used immediately (Step 1.4.2) or stored at -80°C for subsequent use.

1.3 Viral transduction and selection of MEFs stably express..

15 1.3.1) Mix 5 ml of retrovirus infection media from step 1.1.7 with 5 ml of fresh Growth Media in a sterile 15 ml conical centrifuge tube.

16 1.3.2) Add Polybrene to a final concentration of 10 µg/ml and mix by vortexing.

17 1.3.3) Add dropwise to a 10 cm plate of primary MEFs at ~70% confluency.

18 1.3.4) Incubate at 37°C for 24 h.

19 1.3.5) Change media to Growth Media and incubate for another 24 h at 37°C.

20 1.3.6) To select cells stably expressing SV40, replace media with 10 ml of freshly prepared Selection Media containing 200 µg/ml Hygromycin.
Note:

- ***MEFs that have not been infected should be included as a control for the efficiency of the selection agent.***
- ***MEFs that have not been successfully transduced should start dying 24 h after the addition of selection media.***

21 1.3.7) Change Selection Media every 24 h for 3-5 days to remove dead cells. After 5 days, cells stably expressing SV40 should have reached 100% confluency.
Cells can now be passaged and plated for further experiments (Step 1.4.1), or frozen down for long term storage in liquid nitrogen (Freezing media: growth media added with 10% v/v DMSO).

1.4 Viral transduction and selection of MEFs stably express..

22 1.4.1) Plate SV40 immortalised MEF cells from Step 1.3.7 in 10 cm dishes to give a 70% confluency the following day.

23 1.4.2) Mix 5 ml of lentivirus infection media from step 1.2.7 with 5 ml of fresh Growth Media in a sterile 15 ml conical centrifuge tube.

24 1.4.3) Add Polybrene to a final concentration of 10 µg/ml and mix by vortexing.

25 1.4.4) Add dropwise to cells from Step 1.4.1.

26 1.4.5) Incubate at 37°C for 24 h.

27 1.4.6) Change media to Growth Media and incubate for another 24 h at 37°C.

28 1.4.7) To select cells stably expressing the organelle tag, replace media with 10 ml of freshly prepared Selection Media containing 200 µg/ml Hygromycin and 2 µg/ml Puromycin.
Note:
- ***MEFs that have not been infected should be included as a control for the efficiency of the selection agent.***
- ***MEFs that have not been successfully transduced should start dying 24 h after the addition of selection media.***

29 1.4.8) Change Selection Media every 24 h for 3-5 days to remove dead cells. After 5 days, cells stably expressing the organelle tag should have reached 100% confluency.
Cells can now be passaged and plated for experiments, or frozen down for long term storage in liquid nitrogen (Freezing media: growth media added with 10% v/v DMSO).

Note: We recommend confirming the expression of organelle tags by detection of HA-Tag by performing quantitative immunoblotting analysis as described in dx.doi.org/10.17504/protocols.io.bsgrnbv6.