Nov 09, 2020

iNGN iPS Cell Differentiation to Neurons for Extracellular Vesicle Isolation

Dima Ter-Ovanesyan[1], Roey Lazarovits[1], Wendy Trieu [1], Maia Norman[2], Emma Kowal[1], George Church[3], David Walt[2]

[1]Wyss Institute for Biologically Inspired Engineering;

[2]Wyss Institute for Biologically Inspired Engineering, Brigham and Women's Hospital;

[3]Wyss Institute for Biologically Inspired Engineering, Harvard Medical School Department of Genetics

1 *Works for me* dx.doi.org/10.17504/protocols.io.bn63mhgn

Walt Lab

Roey Lazarovits

ABSTRACT

We present a protocol for differentiation of human induced pluripotent stem (iPS) cells into neurons in large quantities *in vitro* in large quantities for isolation of extracellular vesicles (EVs). We employ iNGN (induced Neurogenin) cells expressing the transcription factors Neurogenin1/2 under the control of a doxycycline-inducible promoter. EVs from the conditioned media of these cells can be isolated by differential ultracentrifugation.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Busskamp, Volker et al. "Rapid neurogenesis through transcriptional activation in human stem cells." Molecular systems biology vol. 10,11 760. 17 Nov. 2014, doi:10.15252/msb.20145508

DOI

dx.doi.org/10.17504/protocols.io.bn63mhgn

PROTOCOL CITATION

Dima Ter-Ovanesyan, Roey Lazarovits, Wendy Trieu , Maia Norman, Emma Kowal, George Church, David Walt 2020. iNGN iPS Cell Differentiation to Neurons for Extracellular Vesicle Isolation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bn63mhgn

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Busskamp, Volker et al. "Rapid neurogenesis through transcriptional activation in human stem cells." Molecular systems biology vol. 10,11 760. 17 Nov. 2014, doi:10.15252/msb.20145508

CREATED

Oct 28, 2020

LAST MODIFIED

Nov 09, 2020

PROTOCOL INTEGER ID

43963

MATERIALS TEXT

mTeSR media (05850, StemCell Technologies)
Matrigel hESC-qualified Matrix (354277, BD Biosciences)
InSolution™ Y-27632 Rho Kinase inhibitor (688001, EMD Millipore)
Doxycycline (D9891-5G, Sigma)
ReLeSR (05872, StemCell Technologies)
DMEM basal medium, high glucose, GlutaMAX (10569-010 ThermoFisher Scientific)

ABSTRACT

We present a protocol for differentiation of human induced pluripotent stem (iPS) cells into neurons in large quantities *in vitro* in large quantities for isolation of extracellular vesicles (EVs). We employ iNGN (induced Neurogenin) cells expressing the transcription factors Neurogenin1/2 under the control of a doxycycline-inducible promoter. EVs from the conditioned media of these cells can be isolated by differential ultracentrifugation.

Preparing Reagents

1 Matrigel

1.1 Thaw matrigel stock in ice overnight.

1.2 Make aliquots of matrigel to be stored at <-20 °C. Aliquots should be volumes that, when diluted to 12 mL, have a working concentration of 1 mg/mL. Stock concentrations of matrigel will differ depending on lot number, but aliquot volumes can be calculated as follows:

Matrigel Stock Concentration (mg/mL) x Aliquot volume (ul) = 1 mg/mL x 12,000 ul

The matrigel stock concentration can be found on the manufacturer's website.

2 mTeSR Media

2.1 Thaw mTeSR supplement at 4 °C overnight.

2.2 Make mTeSR media by combining 100 mL of thawed supplement with 400 mL of basal medium. Mix by inverting and store in the fridge. Before using mTeSR in cell culture, warm by leaving an aliquot at room temperature for 30 minutes. NOTE: Do not warm mTeSR media in 37 °C water bath.

3 DMEM 1x B-27, 1% Penicillin/Streptomycin

3.1 Thaw a vial of B-27 50x serum free supplement on ice. Add to 500 mL bottle of DMEM basal medium.

Citation: Dima Ter-Ovanesyan, Roey Lazarovits, Wendy Trieu , Maia Norman, Emma Kowal, George Church, David Walt (11/09/2020). iNGN iPS Cell Differentiation to Neurons for Extracellular Vesicle Isolation. https://dx.doi.org/10.17504/protocols.io.bn63mhgn

3.2 Thaw 5 mL Penicillin/Streptomycin and add to 500 mL bottle DMEM basal media.

Thawing Cells

4 Pre-chill P1000 tips at -20 °C.

5 Prepare an aliquot of 12 mL of cold DMEM basal medium. Thaw an aliquot of matrigel on ice. Using your pre-chilled pipet tips and a P1000, transfer the full contents of the matrigel into the cold DMEM and use the cold basal medium to wash the matrigel tube once. Work swiftly to prevent the matrigel from solidifying. Mix the matrigel solution by inverting.

Note: Matrigel can be reused up to two times, or within two weeks when stored at 4 °C.

6 Transfer 1.5 mL of matrigel to each of two wells in a six-well plate. Incubate at room temperature for at least one hour.

7 Prepare an aliquot of mTeSR medium and allow to warm by leaving at room temperature for at least thirty minutes.

8 Prepare a subaliquot of mTeSR media and supplement Rock Inhibitor for a working concentration of 6 ug/mL.

9 Remove matrigel from six-well plate wells and add 1.5 mL of room temperature mTeSR stem cell medium with Rock Inhibitor. Prepare a falcon tube with 8 mL of mTeSR basal medium.

10 Thaw a vial containing one million iNGN iPS cells by submerging in a 37 °C water bath until only a small piece of solid ice remains. Quickly transfer contents into a Falcon tube with 8 mL of mTeSR basal medium using a P1000 pipet. To ensure all cells are transferred, wash the cell tube once with basal medium and add the wash into the Falcon tube.

11 Centrifuge cells at 300 x *g* for 5 minutes. Being careful not to disturb the cell pellet, aspirate the supernatant and resuspend cells in 3 mL mTeSR media, mixing well but gently to avoid breaking clusters.

12 Aliquot 1.5 mL of cells into each well of the six-well plate. Firmly rock the plate back and forth to distribute cell clusters evenly along the surface of the well. Leave cells undisturbed in an incubator (37 °C, 5% CO2) overnight before moving the plate.

Expanding Cells

13 Feed cells daily by replacing media with fresh mTeSR.

Warm an aliquot of mTeSR at room temperature for at least 30 minutes.

Citation: Dima Ter-Ovanesyan, Roey Lazarovits, Wendy Trieu , Maia Norman, Emma Kowal, George Church, David Walt (11/09/2020). iNGN iPS Cell Differentiation to Neurons for Extracellular Vesicle Isolation. https://dx.doi.org/10.17504/protocols.io.bn63mhgn

13.1

13.2 Aspirate media from each well taking care not to touch the bottom of the well (so as not to disturb the matrigel coating).

13.3 Add 3 mL of mTeSR to each well by pipetting gently down the side of the well.

14 Cells should be passaged when they reach 70% confluency (up to five days after initial thawing). To passage into two new 15 cm dishes:

14.1 Coat two 15 cm dishes using 20 mL of diluted matrigel per plate, similarly to above. Leave plates at room temperature for at least one hour.

14.2 Warm an aliquot of mTeSR at room temperature.

14.3 Remove media from each well in the six-well plate. Add 3 mL of sterile PBS per well and aspirate to wash. Add 1 mL of ReLeSR to wells being passaged, and aspirate ReLeSR , leaving only a thin film behind. Incubate for 5-7 minutes at 37 °C, until the layer of cells starts to dissociate from the plate.

14.4 Resuspend cells by adding 1 mL mTeSR to each well and pipetting forcefully to release cells. Transfer cells to a 15 mL falcon tube, and wash each well by pipetting an additional 1 mL of mTeSR into each well. Transfer wash to same 15 mL falcon tube.

14.5 Remove matrigel from 15 cm dishes and replace with 18 mL mTeSR media.

14.6 In the 15 mL falcon tube, use a P1000 to pipette up and down to break clusters into small pieces (be careful not to pipette too much, or clusters will break down into single cells). Ideal cluster size should be between 50-200 um.

14.7 Ensure the cell solution is well mixed, and aliquot 2 mL of cells into each 15 cm dish evenly across the dish surface. Firmly rock the plate back and forth to evenly distribute cell clusters. Leave overnight undisturbed in an incubator.

14.8 Replace media daily until cells reach 70% confluency (3-5 days)

Differentiating Cells

15 Prepare 250 mL of mTeSR (with mTeSR supplement) and doxycycline at a working concentration of 0.5 ug/mL.

Note: Doxycycline is sensitive to light and should be stored in the dark.

Citation: Dima Ter-Ovanesyan, Roey Lazarovits, Wendy Trieu , Maia Norman, Emma Kowal, George Church, David Walt (11/09/2020). iNGN iPS Cell Differentiation to Neurons for Extracellular Vesicle Isolation. https://dx.doi.org/10.17504/protocols.io.bn63mhgn

16 Prepare 250 mL of matrigel at a working concentration of 1 mg/mL as described in step 1.2.

17 Coat 14 15 cm dishes with matrigel by adding 20 mL of matrigel per plate and incubating at room temperature for at least one hour.

18 Resuspend cells from 15 cm dishes by aspirating media, adding 20 mL sterile PBS per dish, removing PBS, adding 5 mL of ReLeSR per dish, aspirating the ReLeSR (leaving behind a thin film), incubating for 5-7 minutes at 37 °C, and resuspending cells into 14 mL of mTeSR with doxycycline per dish. The total volume of resuspended cells from your 2 starting dishes will be 28 mL.

19 Remove matrigel from the 14 matrigel coated dishes from step 17 and replace with 18 mL of mTeSR with doxycyline per dish.

20 Ensuring cell clusters from cells resuspended in step 18 are sufficient in size, and that cells are homogenously mixed, begin aliquoting 2 mL of cells into each of the 14 15 cm dishes. Be sure to distribute cells equally across the surface of each dish.

21 Rotate plates back and forth to evenly distribute cells and leave undisturbed in an incubator for 4 days. Cells will immediately begin differentiating into neurons.

22 On Day 4 after splitting the cells, replace media with DMEM GlutaMAX (1% Pen/Strep) with B27.

23 On Day 6, collect media in 50 mL Falcon tubes and proceed to EV isolation, as described in our protocol titled "Isolation of Extracellular Vesicles from Cell Culture Media by Differential Ultracentrifugation" on protocols.io. Discard neurons, or proceed to optional next step.

24 Optional: replace media with fresh DMEM GlutaMAX with B27 and 1% Pen/Strep for an additional Day 7 EV isolation. Add media to the side of the plate very slowly/gently to avoid disturbing the cells (as neurons are prone to peeling off of the plate at this stage).

Citation: Dima Ter-Ovanesyan, Roey Lazarovits, Wendy Trieu , Maia Norman, Emma Kowal, George Church, David Walt (11/09/2020). iNGN iPS Cell Differentiation to Neurons for Extracellular Vesicle Isolation. https://dx.doi.org/10.17504/protocols.io.bn63mhgn