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ISOLATION AND VALIDATION OF CLONAL TRANSGENIC LINES (Basic Protocol 4)

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We use this protocol and it's working

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Abstract

While transgenic populations may be enriched to near-purity using the techniques described above, the derivation of a clonal iPSC line descended from a single parent cell is necessary for proper genotyping and for many downstream applications, and it is thus standard practice following any genetic edit. One common method for isolating such clones, as described below, is by serial dilutions on two 6-well plates, followed by manual picking. As with all single-cell dissociations of iPSCs, the use of Y-27632 ROCK inhibitor is required until colonies are properly established; wells with fewer cells may require treatment for 2 to 3 days. 6-well plates are used because their wells have a larger surface area than other multiwell dishes, facilitating downstream picking. For highly enriched populations, serial dilutions may be reduced to one 6-well plate loaded with 1×10^5 cells in the first well, as there is a higher probability of identifying a purely positive colony.

Following isolation, this protocol further describes basic genotyping in parallel with the gradual expansion of clonal lines, utilizing QuickExtract to prepare genomic DNA and PCR to test for the presence of a transgene of interest. Primer sequences and other specifics are included in more detail for the particular differentiation cassettes in Support **[Protocol 1](#)**. In general, basic genotyping by PCR should be performed as quickly as possible to screen out negative clones, while potentially positive clones should be confirmed by more stringent methods such as Sanger sequencing and western blotting as appropriate. To save culture reagents, negative clones may be immediately discarded, while all others should be expanded and frozen pending confirmation.

Attachments



[fernandopulle2018.pd...](#)

1.7MB

Materials

- Genomic DNA extraction kit (e.g., QuickExtract, Epicentre, cat. no. QE09050 or DNeasy kit, Qiagen, cat. no. 69504)

⊗ Epicentre QuickExtract™ DNA Extraction Solution **Epicentre Catalog #QE09050**

⊗ DNeasy Blood & Tissue Kit, QIAGEN **Catalog #Cat No./ID: 69504**

- Matrigel-coated 6- and 12-well plates (**Basic Protocol 1**, steps 1 to 4)
- Phase-contrast and fluorescent microscope
- Cell Dotter marking objective (SEO Enterprises, cat. no. MBW10020)

Equipment

Nikon Object Marker - Cell Dotter for Cytology

NAME

Object Marker

TYPE

Nikon

BRAND

MBW10020


SKU

<http://www.seoenterprises.com/shop/product.php?productid=16217&cat=0&page=1>^{LINK}

- Additional reagents and equipment for cell culture techniques (**Basic Protocol 1**) and counting cells (Phelan & May, 2015), PCR (Kramer & Coen, 2001), and agarose gel electrophoresis (Voytas, 2001)

Troubleshooting

Safety warnings



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

Serial Dilution



- 1 Prepare one well of 80 % confluent post-transfected cells from a 6-well plate as for an Accutase split ([see Basic Protocol 1](#), Step-case 'EDTA-mediated removal of spontaneously differentiating cells', step 20).



Note

Clonal isolation may be performed immediately after transfection; however, permitting outgrowth for one or two passages can improve subsequent growth rate and viability, and performing frontline enrichment ([Basic Protocol 3](#)) can dramatically improve the percentage of positive clones.



- 2 Aspirate the Matrigel solution from two 6-well plates and add  2 mL of E8 supplemented with  10 micromolar (μM) Y-27632 ROCK inhibitor to all but one well; leave the final well empty.

- 2.1 Aspirate the Matrigel solution from two 6-well plates.





- 2.2 Add  2 mL of E8 supplemented with  10 micromolar (μM) Y-27632 ROCK inhibitor to all but one well; leave the final well empty.

- 3 After centrifugation of the Accutase-dissociated cells, aspirate the supernatant and resuspend in  2 mL of E8 supplemented with  10 micromolar (μM) Y-27632 ROCK inhibitor.

- 3.1 After centrifugation of the Accutase-dissociated cells, aspirate the supernatant.

- 3.2 Resuspend in  2 mL of E8 supplemented with  10 micromolar (μM) Y-27632 ROCK inhibitor.



- 4 With a 5-ml pipet, add the full  2 mL of cells and medium to the first well (A1) and pipet up and down twice to mix.
- 5 With the same pipet, transfer  2 mL of this mixture into the second well and mix by pipetting up and down twice.
- 6 Continue pipetting and mixing for each well, performing a one-half dilution, and dispense the final  2 mL into the empty well.
- 6.1 Continue pipetting and mixing for each well, performing a one-half dilution.
- 6.2 Dispense the final  2 mL into the empty well.
- 7 Return the plates to the incubator and gently shake the plates front-to-back and side-to-side.
- 8 Track colony formation daily with a phase-contrast microscope.

Note

Typically, the first plate will rapidly become confluent and may be EDTA split and combined for other uses, such as freezing a heterogeneous population. Meanwhile, the second plate should be kept for picking (see below).

- 9 Once colonies are properly established, aspirate medium and replace with fresh culture medium.

Note

Medium should only be changed after a majority of cell colonies contain at least 8 cells. As the rate of cell division is typically faster in wells with a higher cell density, any wells seeded with at least 50,000 cells should have their medium changed after 1 day, while wells seeded with fewer than 5,000 cells will likely require 3 days.

Marking and picking clones

- 10 Using the plates seeded above, identify the wells at which colonies are well spaced and of a standard size and shape.

Note

Look for compact, round colonies to indicate single-cell origin, adequate separation from other colonies to permit further outgrowth, and fluorescence (if applicable).

- 11 Mark colonies on day 3 to 4 with a Cell Dotter.

Note

For fluorescent markers, allow colonies to grow to at least 500 μm in diameter in order to be easier to discern. Ten to twenty clones should be sufficient; however, marking and picking more clones can help ensure proper genotyping and avoid the need to re-pick. For label-free editing, frontline enrichment is highly recommended (**Basic Protocol 3**), and it is often necessary to screen 50 to 100 clones. The Cell Dotter attaches directly to the objective turret, enabling rapid marking of the underside of the plate with 1.8-mm rings. Center the colony of interest in the field of view using a 10 \times objective, then switch to the Cell Dotter and gently extend the marker to make contact with the plate.

- 12 Pick-to-remove other cells in close proximity to marked colonies (See **Basic Protocol 1**).


Note

Any cells within or immediately surrounding the ring formed by the marking objective should be removed.

- 13 Check cells daily for growth and pick-to-keep around days 5 to 7.






Note

Pick when colonies are about as large as the interior of the marking ring.

- 14 Before picking, rinse wells once with PBS and add at least  5 mL medium to each well to allow picking of multiple clones in succession.

- 14.1 Before picking, rinse wells once with PBS.






- 14.2 Add at least  5 mL medium to each well to allow picking of multiple clones in succession.
- 15 Aspirate the Matrigel solution from a 24-well plate and load each well with  250 μ L of E8 supplemented with  10 micromolar (μ M) Y-27632 ROCK inhibitor.
- 15.1 Aspirate the Matrigel solution from a 24-well plate.
- 15.2 Load each well with  250 μ L of E8 supplemented with  10 micromolar (μ M) Y-27632 ROCK inhibitor.
- 16 Pick-to-keep each colony into an individual well of the 24-well plate (see **Basic Protocol 1**).
- 17 Grow cells in 24-well plates for 5 to 7 days until colonies are large (at least 2 mm in diameter).

Note

Be wary of spontaneous differentiation, especially at the center of colonies which tend to grow upwards and turn brown. It is possible to pick away these areas for better splitting, or continue to expand and perform later EDTA-mediated removal (see **Basic Protocol 1**), but clones with excessive spontaneous differentiation may harbor genetic abnormalities and should simply be discarded.

Expansion and genotyping

- 18 Prepare clones in the 24-well plate as for an EDTA split (using  250 μ L of  0.5 millimolar (mM) EDTA in PBS).
- 19 During incubation, for each clone, prepare a microcentrifuge tube labeled with the clone number for genomic DNA collection and load a new well for growth loaded with one half volume E8 supplemented with  10 micromolar (μ M) Y-27632 ROCK inhibitor.

**Note**

Ideally, use one well of a 12-well plate to maximize surface area and promote outgrowth.

If cells are precious, e.g., if only a few clones were successfully derived, use two wells of a 24-well plate to immediately have a backup well. This well may also be used for later genomic DNA collection if desired.

If colonies are small, use one well of a 24-well plate to improve survival. Genomic DNA collection may also be postponed until this well is split.

19.1 During incubation, for each clone, prepare a microcentrifuge tube labeled with the clone number for genomic DNA collection.

19.2 Load a new well for growth loaded with one half volume E8 supplemented with **10 micromolar (μM)** Y-27632 ROCK inhibitor.

20 Aspirate EDTA and gently dissociate with a P1000 pipet tip by pipetting **1 mL** of medium two to three times.

21 Deposit half of the medium in the microcentrifuge tube.

22 Deposit the other half of the medium in the new well.

23 Return recipient plates to the incubator and gently shake the plates front-to-back and side-to-side.

24 Spin down the microcentrifuge tube **00:05:00** at **500 x g**, **Room temperature**.

Note

Arrange all the tubes in the same orientation so the cell pellet forms in the same location for each tube.

25 Aspirate as much of the medium as possible.


Note

The cell pellet may not be readily visible, so use a filterless pipet tip on the end of an aspirating pipet if suction is used, or use a P1000 for better control.

- 26 Extract genomic DNA from the cell pellet as per manufacturer's instructions.

Note

QuickExtract (Epicentre) is recommended due to its scalability and ease-of-use.


 30 μL is usually sufficient for small cell pellets (<50,000 cells), and any samples which are overly viscous after thermocycling may be diluted with DNase-free water.

- 27 Test the extracted genomic DNA by PCR (Kramer & Coen, 2001).

Note

For inserts at a safe-harbor locus, testing for integration at the correct locus can be accomplished by using one primer beyond the homology arm and one primer unique to the insert cassette.

Insertion zygosity can also be determined by using primers on either side of the insertion locus, as insertion will prevent amplification of that allele.

Use  1 μL of the QuickExtract solution in a 10- μL PCR preparation; additional cleanup is typically not necessary for amplification (See **Supporting Protocol 1**).

- 28 Run each PCR sample on an agarose gel (Voytas, 2001) in order to confirm amplification.

Note

Clones that fail to amplify the test fragment but properly amplify a positive control fragment should be immediately discarded to save culture reagents.

The genomic DNA of clones with inconclusive genotyping results (i.e., variable band intensities, multiple bands, etc.) should be checked for quality and either re-amplified with different PCR conditions or re-extracted.

Clones with positive initial genotyping should be PCR purified and Sanger sequenced to confirm integration. In particular, further selection may be desired for scarless integration and/or retaining an unedited wild-type allele.



- 29 Expand clones with positive PCR results via EDTA splitting 1:4 into larger wells at **50% to 60% confluency**.

Polyclonal populations may be discarded or combined and subcloned if desired.

Note

Split the entire population at low ratio to enhance growth rate and outcompete differentiation.

For example, split one well of a 24-well plate to two wells of a 12-well plate, one of which can be split into two wells of a 6-well plate.

- 30 For each clone in two wells of a 6-well plate at **80% confluency**, prepare both wells as for an EDTA split (see **Basic Protocol 1**).

- 31 During incubation, prepare a recipient plate with E8 medium supplemented with **10 micromolar (μM)** Y-27632 ROCK inhibitor, prepare a 15-ml conical tube with **1 mL** medium containing the components for **3 mL** cryopreservation medium, and label three cryovials with the clone number.

Note

For example, combine 300 μL DMSO and 700 μL E8 medium.

- 31.1 During incubation, prepare a recipient plate with E8 medium supplemented with **10 micromolar (μM)** Y-27632 ROCK inhibitor.

- 31.2 Prepare a 15-ml conical tube with **1 mL** medium containing the components for **3 mL** cryopreservation medium.





- 31.3 Label three cryovials with the clone number.

- 32 Detach the cells from one well with **3 mL** medium and transfer **0.5 mL** to two wells to accomplish a 1:6 split for maintenance. Use the remaining **2 mL** medium and cells to detach the second well and add to the 15-ml conical tube to constitute 1 \times cryopreservation medium. Add **1 mL** to each of the three cryovials and freeze.

**Note**

If cells are not fully dissociated from the plate, rinse the wells again with the full cryopreservation medium to maximize cell density.

Maintenance cultures should be further split 1:12; if sequencing results are not readily available, reduce reagent use by freezing all of the cells and not retaining a maintenance culture. All clones should be kept frozen until a few sequenced clones are confirmed, and any clones which sequence incorrectly may be discarded.

- 32.1 Detach the cells from one well with  3 mL medium.
- 32.2 Transfer  0.5 mL to two wells to accomplish a 1:6 split for maintenance.
- 32.3 Use the remaining  2 mL medium and cells to detach the second well.
- 32.4 Add to the 15-ml conical tube to constitute 1× cryopreservation medium.
- 32.5 Add  1 mL to each of the three cryovials and freeze.
- 33 Clones confirmed by sequencing should be further expanded to a full 6-well plate and frozen into six to eight vials, in addition to keeping the clone in culture for other characterization assays.

Note

While only one clone is necessary to move forward, isolating at least three sequence-confirmed clones is ideal in case off-target effects or other genetic abnormalities are found later.

Common options for further validation include: (1) western blot to confirm transgene expression; (2) karyotype to ensure no chromosomal abnormalities have arisen; (3) sequencing of potential CRISPR off-target loci; and (4) exome or whole-genome sequencing of pre- and post-edited cells to search for genetic drift or CRISPR off-target effects.