iPSCs

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SELECTION AND ENRICHMENT OF TRANSGENIC CELL POPULATIONS (Basic Protocol 3)

In 1 collection

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1 Works for me dx.doi.org/10.17504/protocols.io.5fvg3n6

Neurodegeneration Method Development Community

ABSTRACT

While lipid-mediated transient transfection and expression of transgenes in hiPSCs is often quite high (> 50 %), even an optimal transfection with the best editing tools can result in very low genomic integration efficiency (< 1 %). For this reason, establishing pure cell populations is typically the major bottleneck of any transgenic workflow. Commonly, frontline selection and enrichment are used to facilitate later single-cell cloning, which remains necessary to ensure that transgene insertion did not result in mosaicism or off-target mutagenesis (See Basic Protocol 4).

In order to remove non-edited cells, positive selection markers such as genes coding for fluorescent proteins or antibiotic resistance are often included in the insert construct under constitutive promoters, and this protocol describes general methods for the former strategy. However, constant expression of a fluorophore interferes with immunocytochemistry, and antibiotic selection can incorrectly select for cells with multiple aberrant transgene insertions. To counter these shortcomings, we developed a platform for enrichment from heterogeneous cell populations through cell-surface affinity for magnetic streptavidin beads mediated by expression of a streptavidin binding peptide (SBP) fused to the truncated extracellular and transmembrane domains of low-affinity nerve growth factor receptor (LNGFR). The SBP tag is commonly used to facilitate co-immunoprecipitation with streptavidin-coated beads, and, with fusion to the LNGFR, it is efficiently localized to the extracellular surface where it can bind the beads more readily.

This construct was developed based on one initially designed for purification of transgenic T cells in suspension (Matheson, Peden, & Lehner, 2014), and it is included in Addgene cat. no. 105842 for direct use with hNIL or excision and insertion into other constructs. Here, we extend the original protocol in order to optimize enrichment following safe harbor locus insertion in iPSCs, and we present evidence to support its use as either a positive or negative selection marker in a variety of transgenic applications. However, we have found that the SBP-LNGFR construct should be the only gene expressed under a dedicated, highly active promoter, as it appears to interfere with 2A-mediated ribosome skipping. For example, a different construct, with the reverse tetracycline transactivator (rtTA) linked to SBP-LNGFR by a T2A sequence, led to disrupted differentiation due to reduced rtTA activity (see Support Protocol 6).

When structured correctly, however, SBP-LNGFR permits highly selective enrichment that does not require specialized equipment as in fluorescence-activated cell sorting (FACS) and does not require extensive tuning as in antibiotic selection. Notably, the efficiency of enrichment increases exponentially with a lower frequency of positive cells in the population. In addition, the protocol is easily modified to permit negative selection, e.g., for removing a floxed transgene with Cre recombinase or a gene knockout reporter. Furthermore, cells expressing SBP-LNGFR may be transiently labeled at the cell surface by incubating with any fluorophore conjugated to streptavidin. After washing to remove unbound fluorophore, these cells may be imaged, marked for clonal picking, or FACS-purified at an equivalent efficacy to cells constitutively expressing a fluorophore marker of insertion. Fluorescence then returns to undetectable levels within one passage and does not interfere with downstream immunocytochemistry applications.

Precise, label-free editing of endogenous loci may be enriched by FACS sorting for transient expression of the components necessary for editing 1 day after transfection [i.e., using a combined gRNA, Cas9, and GFP expression plasmid with an integration plasmid expressing RFP outside of the insertion sequence or by using functionalized RNPs such as S1mplex with Qdots or fluorescent streptavidin (Carlson-Stevermer et al., 2017)]. This can considerably increase the prevalence of correctly edited cells, but the combined stress of transfection and sorting can decrease cell survival. To compensate, it is recommended to transfect and sort (2.4 × 106 cells) in triplicate and to plate cells at high density following the sort. In addition, extensive downstream cloning and genotyping is often required due to the propensity for non-homologous end joining leading to mutagenesis at the target locus rather than homology-directed repair-mediated integration of the desired insert.

This protocol will continue with the assumption of integration of a transgene cassette into a safe-harbor locus as described in Basic Protocol 2. Label-free gene knockouts caused by NHEJ-mediated indel formation are typically prevalent (> 15 %), so frontline enrichment is optional for these applications. Following enrichment, small stocks of heterogeneous cell populations should be frozen, and the cells should be taken directly to Basic Protocol 4 for clonal isolation.

EXTERNAL LINK

https://doi.org/10.1002/cpcb.51

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Fernandopulle, M. S., Prestil, R., Grunseich, C., Wang, C., Gan, L., & Ward, M. E. (2018). Transcription-factor mediated differentiation of human iPSCs into neurons. Current Protocols in Cell Biology, e51. doi:https://doi.org/10.1002/cpcb.51

fernandopulle2018.pdf

MATERIALS TEXT

- Heterogeneous post-transfection iPSC population (Basic Protocol 2)
- 1 M EDTA stock
- 10% (w/v) bovine serum albumin (BSA; Miltenyi, cat. no. 130091376) stock

MACS BSA Stock Solution
by Miltenyi Biotec
Catalog #: 130-091-376

- autoMACS Rinsing Solution (optional; Miltenyi, cat. no. 130091222)

autoMACS Rinsing Solution
by Miltenyi Biotec
Catalog #: 130-091-222

- Magnetic streptavidin beads (Dynabeads MyOne Streptavidin C1; Thermo, cat. no. 65001)

Dynabeads™ MyOne™ Streptavidin C1
by Thermo Fisher Scientific
Catalog #: 65001

- Biotin (optional; Sigma, cat. no. B4639)

Biotin
by Merck Millipore Sigma
Catalog #: B4639
CAS Number: 58-85-5

- Fluorescence-activated cell sorter (FACS)
- Vacuum source
- DynaMag-2 magnetic tube rack (Thermo, cat. no. 12321D)

DynaMag™-2 Magnet
Magnetic Separator
Invitrogen 12321D

- Cell strainer, 40-µm pore diameter (Corning, cat. no. 352340)

Falcon® 40 µm Cell Strainer
Cell Strainer
Falcon 352340

- Rotator Genie benchtop spinner (Scientific Industries, SI-2100)

Rotator Genie
Rotator
Scientific Industries SI-2100

- Additional reagents and equipment for general iPSC culture (Basic Protocol 1) and counting cells (Phelan & May, 2015)

SAFETY WARNINGS

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

Cell Preparation

1 Culture cells after transfection and expand to at least one full 6-well plate or one 10 cm dish by EDTA split (see Basic Protocol 1).

Post-transfection recovery and expansion should take about 1 week; this enables the degradation / dilution of transient plasmids and permits expansion of edited cells to ensure enough cells are present for effective selection. Genomic DNA may be collected from unpurified cells to test for integration efficiency, and extra heterogeneous cells should be frozen throughout the purification process to provide a low-passage backup should contamination occur or should validation fail at any point.

step case

For fluorescent proteins (FACS)

Protocol for fluorescent proteins (FACS)

Cell Sorting

2 When cells reach **70 % to 80 % confluency** (day 3 to 4 after plating), dissociate with Accutase (see Basic Protocol 1).

3 Transfer cells and Accutase solution to conical tube, rinse with DMEM/F12, and spin **00:05:00** at **200 x g** , **Room temperature** .

4 Aspirate supernatant and resuspend in E8 supplemented with **10 Micromolar (µM)** Y-27632 ROCK inhibitor.

Using medium at this step helps to improve cell viability; however, if FACS is not performed rapidly, cells can clump and reduce purification efficacy. Cold PBS with 3 % BSA can be substituted to reduce clumping.

5 Transfer cell solution to FACS tube and place on ice.

6 Perform FACS using appropriate fluorophore gating and nozzle diameter (see Critical Parameters and Troubleshooting for more detail).

Sort directly onto a Matrigel-coated cell culture plate loaded with E8 supplemented with **10 Micromolar (µM)** Y-27632 ROCK inhibitor (even if PBS is used in step 4 instead of E8) since typically small volumes are deposited. Alternatively, positive cells can be deposited into a separate tube, centrifuged, and replated. Some iPSC lines tolerate single cell/well sorting into 96- or 384-well dishes containing E8 supplemented with **10 Micromolar (µM)** Y-27632 ROCK inhibitor, which can further expedite clonal isolation and expansion. Survival of a polyclonal population can then be maximized by depositing any additional positive cells into the remaining well.

7 Expand selected cells for clonal isolation.

Colonies of positive cells may be marked and picked to keep, and/or negative cells may be marked and removed.

step case

Magnetic streptavidin bead affinity purification

Protocol for magnetic streptavidin bead affinity purification.

2 **Prepare bead incubation buffer:**

3 Prepare bead incubation buffer by supplementing PBS with [M]**2 Milimolar (mM)** EDTA and 0.5 % BSA.

For example, 47.4 ml PBS, 100 µl of 1M EDTA stock, and 2.5 ml of 10 % BSA stock.
Buffer may be formulated by the consumer or purchased (e.g., autoMACS buffer).