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Methods for Automated Single Cell Isolation and Sub-Cloning of Human Pluripotent Stem Cells

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Advances in human pluripotent stem cell (hPSC) techniques have led them to become a widely used and powerful tool for a vast array of applications, including disease modeling, developmental studies, drug discovery and testing, and emerging cell-based therapies. hPSC workflows that require clonal expansion from single cells, such as CRISPR/Cas9-mediated genome editing, face major challenges in terms of efficiency, cost, and precision. Classical sub-cloning approaches depend on limiting dilution and manual colony picking, which are both time-consuming and labor-intensive, and lack a real proof of clonality. Here we describe the application of three different automated cell isolation and dispensing devices that can enhance the single-cell cloning process for hPSCs. In combination with optimized cell culture conditions, these devices offer an attractive alternative compared to manual methods. We explore various aspects of each device system and define protocols for their practical application. Following the workflow described here, single cell–derived hPSC sub-clones from each system maintain pluripotency and genetic stability. Furthermore, the workflows can be applied to uncover karyotypic mosaicism prevalent in bulk hPSC cultures. Our robust automated workflow facilitates high-throughput hPSC clonal selection and expansion, urgently needed in the operational pipelines of hPSC applications. © 2020 The Authors.

Basic Protocol: Efficient automated hPSC single cell seeding and clonal expansion using the iotaSciences IsoCell platform
Alternate Protocol 1: hPSC single cell seeding and clonal expansion using the Cellenion CellenONE single-cell dispenser
Alternate Protocol 2: hPSC single cell seeding and clonal expansion using the Cytena single-cell dispenser
Support Protocol 1: Coating cell culture plates with Geltrex
Support Protocol 2: hPSC maintenance in defined feeder-free conditions
Support Protocol 3: hPSC passaging in clumps
Support Protocol 4: Laminin 521 coating of IsoCell plates and 96-well/384-well plates
Support Protocol 5: Preparation of medium containing anti-apoptotic small molecules
Support Protocol 6: 96- and 384-well target plate preparation prior to single cell seeding

CURRENT PROTOCOLS
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Support Protocol 7: Single cell dissociation of hPSCs
Support Protocol 8: IsoCell-, CellenONE-, and Cytena-derived hPSC clone subculture and expansion

Keywords: automation • hPSC • karyotyping • single cell isolation • sub-cloning

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INTRODUCTION

The combination of human induced pluripotent stem cell (hiPSC) reprogramming and CRISPR/Cas9 technology has generated new perspectives in the field of stem cell research that span developmental biology up to translational and therapeutic applications (Rossant & Tam, 2017; Wu & Izpisua Belmonte, 2015; Yamanaka, 2012). CRISPR/Cas9-based gene editing has made it possible to accurately correct or introduce disease-associated mutations in human pluripotent stem cells (hPSCs) to generate isogenic control/disease lines, overcoming the problem of genetic variability between individual hPSC lines. Similarly, transgenic hPSCs containing reporters or expression cassettes knocked into endogenous or safe harbor loci allow cell tracing experiments, functional studies, or gene induction (Doudna & Charpentier, 2014; Hsu, Lander, & Zhang, 2014). Although the combination of hPSC and CRISPR/Cas9 technologies is revolutionizing disease modeling and regenerative medicine, progress is hampered by the lack of standardization, low-throughput processes, and insufficient robustness.

One of the biggest challenges in the genome editing process for hPSCs is the isolation of single cells and derivation of clonal cell populations with the desired genetic modifications. This single-cell cloning bottleneck occurs because hPSCs are sensitive to environmental changes like pH, osmolarity and nutrient supply, mechanical stress/shear forces, and, most importantly, loss of cell-cell and cell-extracellular matrix (ECM) contact. In conventional 2D culture conditions, hPSC typically grow in densely packed colonies on surfaces coated with various ECM components. If these cell-cell and cell-ECM contacts are disturbed, an apoptotic program, known as anoikis, is induced, which causes a significant decrease in single cell survival rate (Chen, Hou, Gulbranson, & Thomson, 2010). A single cell isolation process combined with survival and proliferation enhancement using small molecules and optimized culture conditions is critical for the successful selection and expansion of genome-edited hPSCs. Approaches such as manual picking of single outgrown colonies and/or limiting dilution are not optimal, as they are labor-intensive and inefficient. In addition, these methods do not prove with certainty a successful single cell clonal outgrowth event following the isolation process. Moreover, gene editing protocols usually expose stem cells to harsh conditions that further lead to poor survival (e.g., electroporation); therefore, a gentle single cell isolation and dispensing technique would increase cloning efficiencies. Another challenge to overcome is the length of the process—a typical gene editing workflow takes 2 to 4 months, and a major part of the time is spent on hPSC sub-clone isolation, genotyping, selection, and expansion. Therefore, automated systems that offer quicker and more precise isolation of hPSCs with increased

single cell survival would be highly desirable for research and commercial applications. A variety of devices for single cell dispensing have been developed and are currently commercially available.

The authors of this article have tested three platforms with different working principles that can be applied within a complete hPSC sub-cloning workflow, including isolation, clonal expansion, and further characterization. The Basic Protocol that we describe here is coupled to the iotaScience IsoCell platform, a small-footprint device that enables miniaturization and therefore cost reduction (*https://www.iotasciences.com/*). We provide, in alternate protocols, two additional platforms: the CellenONE X1/F1 (*https://www.cellenion.com/*; see Alternate Protocol 1) and the Cytena c.sight/f.sight (*https://www.cytena.com/*; see Alternate Protocol 2) single cell dispensers. All three platforms have been developed to ensure high cell viability and low rates of contamination, and to increase the confidence of monoclonal efficiency. We describe the relevant cell culture methods and specific device protocols that enable robust and efficient automated sub-cloning of hPSCs for a variety of applications. Finally, we provide example data and highlight karyotypic stability and maintained pluripotency to validate the workflow for each device system.

STRATEGIC PLANNING

The methods described below have been selected and structured in order to ensure an easy workflow focusing on four critical steps (1) hPSC culture quality requirements as starting point, (2) preparation procedures before single cell seeding, (3) automated single cell seeding, and (4) culture and expansion of derived clones (Fig. 1).

Good practices to ensure a high-quality hPSC culture are described in Support Protocols 1, 2, and 3. A description of recommended culture characteristics can be found in Critical Parameters. Procedures such as target plate preparation and derivation of a single cell suspension prior to single cell seeding are described in Support Protocols 4, 5, 6, and 7. Automated single cell seeding is the central part of the workflow and is described for three alternate platforms in Basic Protocol 1 (IsoCell) and Alternate Protocols 1 and 2 (CellenONE and Cytena, respectively). Finally, a protocol for the expansion of the clones following the isolation process is provided in Support Protocol 8 (Fig. 1).

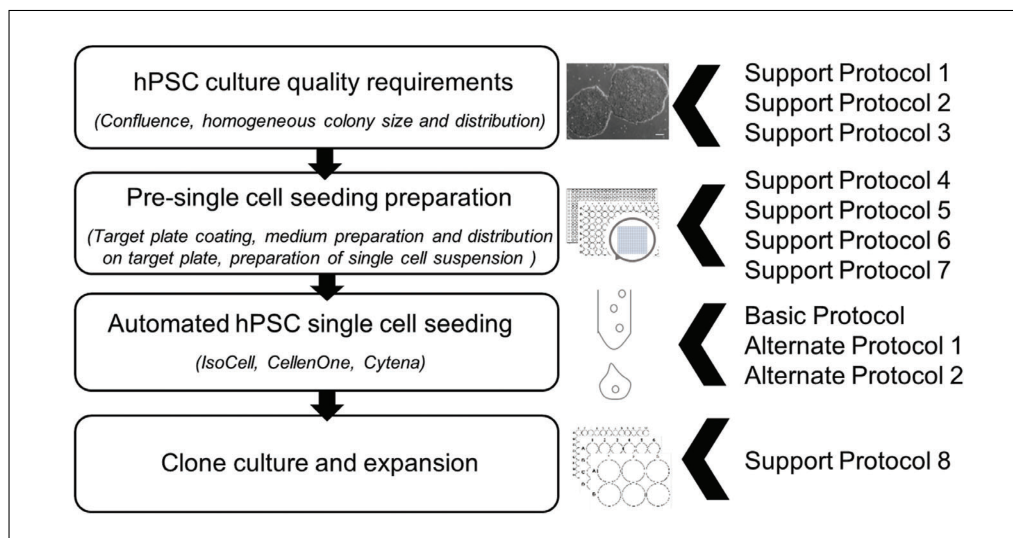


Figure 1 Strategic planning. Overview of the main working blocks and the related protocols to perform hPSC single cell isolation and sub-cloning. The picture next to the first block shows the desired morphology and confluence (70%-80%) that the hPSC cultures should have as starting material. Scale bar: 200 µm.

BASIC PROTOCOL

EFFICIENT AUTOMATED hPSC SINGLE CELL SEEDING AND CLONAL EXPANSION USING THE IotaSciences IsoCell PLATFORM

The IsoCell platform aims to automate single cell seeding and clonal expansion while decreasing hands-on time and scaling the procedure down to a minimal use of consumables and resources. The miniature workflow is based on the formation of a grid between two immiscible fluids. The grid contains chambers that enable cell culture in less than 1 µl of volume. The IsoCell exploits the principle of a fluid-shaping technology (Soitu et al., 2018, 2020; Walsh et al., 2017). Briefly, the IsoCell creates small-scale cell culture chambers on (optionally coated) polystyrene dishes using an aqueous phase (cell medium or substrate coating) and an immiscible liquid overlay (the fluorocarbon oil $FC40^{STAR}$). This results in an array of 256 cell culture reservoirs that are completely separated from each other by the immiscible $FC40^{STAR}$. The device applies a technology developed by Walsh and colleagues whereby grids are formed on top of a protein-coated surface of a cell culture dish, in a liquid phase overlaid with $FC40^{STAR}$ (Soitu et al., 2018). The integrated liquid-handling system automates cell isolation, feeding, and harvesting. Single cell seeding follows a Poisson distribution, limiting the number of single-cell chambers to 94 per grid (256 chambers). In-chamber verification of single cells is performed on a standard bright-field microscope using a 40× or 100× magnification. The contents of the chambers are stable, do not mix, and can be manipulated without extra care. The $FC40^{STAR}$ layer on top of the chambers does not mix with the aqueous phase of the culture medium, allows gas exchange, prevents evaporation of such small volumes, and remains in place for the entire cloning workflow.

This protocol describes a workflow in which the IsoCell platform is applied to enable robust, efficient, cost-effective, and easy-to-handle hPSC clone isolation and expansion (Fig. 2A).

NOTE: Special training on how to operate the instrument is recommended. Here we give an overview, but full details are beyond the scope of this protocol. Please contact the manufacturer for further information.

Materials

$FC40^{STAR}$ (Iota Sciences, cat. no. SKU10040)
Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS; e.g., ThermoFisher Scientific, cat. no. 10010023)
70% ethanol (e.g., Carl Roth, cat. no. T913.3)
hPSC culture (see relevant Current Protocols articles)
StemFlex medium (ThermoFisher Scientific, cat. no. A3349401)
CloneR (Stem Cell Technologies, cat. no. 05888; see Support Protocol 5 for use in medium preparation)
Cell culture−grade water (e.g., Corning, cat. no. 25-055-CM)
TrypLE Select Enzyme (ThermoFisher Scientific, cat. no. 12563011)
Essential 8 medium (ThermoFisher Scientific., cat. no. A1517001) or mTeSR (Stem Cell Technologies, cat. no. 85850)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
IsoCell device (Iota Sciences)
1.5-ml microcentrifuge tubes (e.g., Eppendorf, cat. no. 30120086)
Clone G kit-A—with tissue-culture treated 6-cm dishes (Iota Sciences, cat. no. SKU 10030)
P1000 repeat pipettor
200- and 1000-µl pipette tips (e.g., Eppendorf, cat. no. 3123000055 and 3123000063)
200- and 1250-µl pipette tips (e.g., Biozyme, cat. no. 770200 and 770600)
5- and 10-ml serological pipettes (e.g., Corning, cat. no. 357543 and 357551)

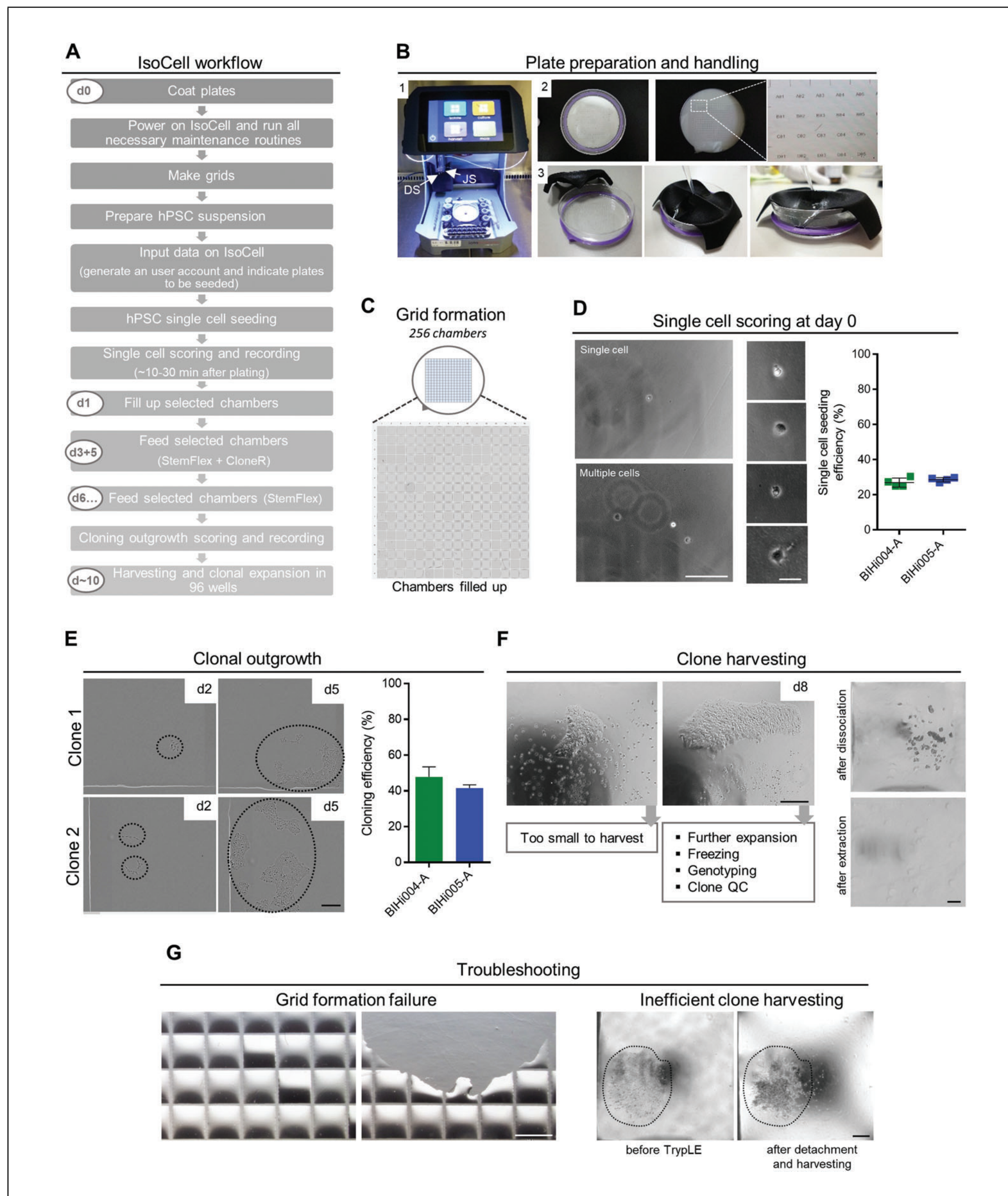


Figure 2 Basic Protocol: IsoCell hPSC single cell isolation and clonal expansion. (**A**) Step-by-step flowchart showing automated hPSC single cell seeding and clone expansion using the IsoCell platform. Time points are defined in days (d) or minutes (min). (**B**) (1) IsoCell device, arrows point to the needles from the dispensing (DS) and the jetting system (JS); (2) adaptor for microscopy counting with chamber coordinates; and (3) pipetting aid for achieving very gentle FC40^{STAR} filling of the plate. (**C**) Scheme of a 256-chamber grid generated in a 60-mm coated culture dish (upper part) and representative picture from a grid after selected chambers were filled up with medium (lower part). (**D**) Representative pictures showing chambers with single or multiple cells to be scored at day 0 (scale bar: 200 µm). On the right, different single cell morphologies that can be seen within 30 min after plating (scale bar: 50 µm). The graph shows single cell seeding efficiencies (mean ± SD, n = 4) for two different hiPSC lines. (**E**) Representative pictures of clone outgrowth at day 2 and 5 for two hiPSC clones (scale bar: 200 µm). Cloning efficiency (right) is calculated as relative to the number of chambers with single cells at day 0 (mean ± SD, n = 4). (**F**) Pictures showing examples for colony size in chambers at day 8 (left) and chambers after enzymatic dissociation and extraction (scale bars: 200 µm). (**G**) Troubleshooting: an example of grid formation failure is depicted on the left (scale bar = 2 mm). Inefficient harvesting due to short enzymatic treatment is shown on the right (scale bar: 200 µm). Cell3imager Duos was used for pictures in panel C, a Leica DMi8 microscope with phase contrast was used for pictures in panels D, E, F, and G, and an Olympus SZX16 stereomicroscope was used for grid pictures in panel G.

Inverted microscope with phase-contrast optics, 4×, 10×, and 20× objectives (e.g., DMi8, Leica)
Cell culture incubator (e.g., Binder)
Geltrex-coated 96-well tissue-culture plates (prepared as described in Support Protocol 1)

Additional reagents and equipment for laminin coating of dishes (Support Protocol 4), preparing single cell suspension of hPSCs (Support Protocol 7), preparation of medium containing anti-apoptotic small molecules (Support Protocol 5), and subculture and expansion of hPSC clones (Support Protocol 8)

Grid preparation and single cell seeding (day 0)

Initialize IsoCell device

1. Check FC40^{STAR} level in the reserve bottle on the back of the device.

   FC40^{STAR} level of the reservoir can be manually adjusted from the main display of the instrument ("more" > "FC40^{STAR} levels"). The instrument will then estimate the amount of FC40^{STAR} remaining for further operations and give an alert when the level may be too low. FC40^{STAR} bottle level is an estimation; therefore, it is important to verify it before starting any process.

2. Power on the IsoCell device.
3. Run the start-up routine by following the wizard on the display. 1.5-ml tubes containing sterile water, 70% ethanol, DPBS, and StemFlex medium must be prepared in advance and placed in the corresponding spots in the IsoCell tube rack.
4. If necessary, replace the dispenser needle: from the menu choose "more" > "replace dispenser," then follow the instructions (Fig. 2B).

   The dispenser needle must be changed at least every time a new Clone G kit-A is used.

5. Switch the heater on: from the menu choose "Settings" > "Options" > "heater on."

Prepare grid using FC40^{STAR}

6. Coat the necessary number of 6-cm culture dishes provided in the Clone G kit-A with laminin 521 as described in Support Protocol 4.
7. Carefully remove the coating solution from the 6-cm dish using a P1000 repeat pipettor with a 1000-µl pipette tip.
8. Very carefully add 1 ml of StemFlex medium by placing the pipette at the side wall of the dish and releasing the medium very slowly.
9. Remove the washing medium using the P1000 repeat pipettor with a 1000-µl tip.
10. Repeat step 3, avoiding any bubble formation, and incubate at room temperature for 5-10 min.
11. Remove the StemFlex medium from the dish carefully using the P1000 repeat pipettor with a 1000-µl tip.
12. Place the pipetting aid accessory on top of the culture dish and dispense 2 ml FC40^{STAR} into the center (Fig. 2B).

    This procedure ensures that FC40^{STAR} covers the surface of the dish slowly and without bubble formation. The procedure can be performed without the pipetting aid accessory using a P1000 pipet and slowly pipetting FC40^{STAR} against the side walls of the dish.

13. Proceed immediately to grid preparation on the IsoCell instrument: from the menu choose "isolate" > "Grid," then follow the wizard. A grid with 256 chambers can be seen on the dish (Fig. 2B and C).

    Prepared grids should be used within the same day on which they are generated.

Single cell seeding and scoring

14. Prepare an hPSC single cell suspension by following Support Protocol 7.

15. Re-suspend hPSCs in StemFlex supplemented with CloneR (preparation described in Support Protocol 5, i.e., 1:20 dilution) with a concentration of 10,000 cells/ml.

    The concentration of the cell suspension can be adjusted within a range of 5-10000 cells/ml to achieve optimal numbers of single-cell chambers; see Troubleshooting.

16. Place 0.5-1 ml of cell the suspension in a 1.5-ml tube, resuspend, and place the tube in the IsoCell rack in position that is depicted by the wizard.

    Immediately start the single cell dispensing to avoid cell sedimentation!

17. Perform single cell seeding with the IsoCell device: from the menu choose "Isolate," and follow the instructions.

    The IsoCell device will proceed with deposition of ~200 nl cell suspension per microchamber

18. *Scoring*: Immediately or at least within 10-20 min after seeding, visually identify and record chambers containing single cells using an inverted microscope (10× objective recommended) and the microscope plate adapter provided by Iota Sciences (Fig. 2B and D).

    Adjusting the focal plane up and down allows identification of the chamber or the coordinate of the chamber printed on the plate adapter. The focal plane of the chamber walls is roughly the same as that of the cells.

    It is important to quantify single cell chambers within 30 min of dispensing, when cells are not fully attached and have a round shape and the cell borders appear bright due to their refraction. 30 min to 1 hr after dispensing, mitotic events can occur and become false negative as duplets.

    The GRID is limited to around 94 single-cell chambers out of 256 due to Poisson distribution. With the protocol described here, ~60-80 chambers containing a single cell were achieved. Variations can occur depending on the cell preparation, counting method, and the experience of the operator (Fig. 2D).

19. Place the dish in the incubator at 37°C in a humidified environment, preferably under hypoxic conditions (5% O_2, 5% CO_2) for 24 hr.

    hPSC cultures are preferably maintained in hypoxic conditions to avoid spontaneous differentiation.

20. *Data input in IsoCell (single cell containing-well coordinates)*: First, create the experiment: from the menu choose "More" > "add dish" (name the experiment). Second, register the chambers containing a single cell: from the menu choose "Isolate" > "input data," then select coordinates and save. This allows the IsoCell to automate feeding and harvesting of only the relevant chambers during the subsequent workflow.

21. Shut down the IsoCell device by following the device wizard for shut-down routine.

Clonal culture maintenance in IsoCell chambers (d1 to ~d10)

22. Initialize IsoCell instrument as described in step 1.

23. On day 1 after single cell seeding, perform the fill up routine for the wells registered with a single cell. Follow the wizard: "Culture" > "Fill," then select the dish. Use StemFlex medium supplemented with CloneR.

    This procedure will add 600 nl of medium to each chamber containing a single cell that has been plated in 200 nl of medium. The resulting volume in one chamber is about

800 nl (Fig. 2C). Evaporation of the medium is prevented due to the FC40^{STAR} layer on top.

24. On day 3 and day 5, perform medium change, only in the preselected single-cell containing chambers, with StemFlex supplemented with CloneR following the "Culture" > "Feed" program and the wizard instructions (Fig. 2E)

    Cloning efficiency scoring (number of wells with outgrowth relative to number initially containing a single cell) can be performed at day 5 or later, and is an important quality control for the procedure (Fig. 2E).

25. From day 6 to day ~10, perform daily medium change with StemFlex medium without CloneR as explained in step 24.

    On day 7, cell death can be seen due to the CloneR removal; after that, colonies will re-shape to a more compact morphology with more defined borders, which helps the investigator select well-established cultures for further replating (Fig. 2F).

26. Regularly monitor clone growth and confluence in order to decide the time point for further expansion.

    The cloning efficiency is dependent on the specific hPSC line used. This has to be considered in order to estimate the number of dishes to be plated to isolate a sufficient number of clones.

27. Shut down the IsoCell device by following the device wizard for shut-down routine.

Clone harvesting and transfer to 96-well plate using the IsoCell (after day 10)

Clones with 50%-70% confluency can be re-plated for further expansion and downstream application (genotyping, freezing, etc.; Fig. 2F). The time point of adequate confluence may vary between clones, and is highly dependent on the hPSC line.

28. Prepare in advance 0.2-ml tube strips (provided in the Clone G kit-A) with 120 µl StemFlex medium supplemented with CloneR and five 1.5-ml tubes containing: water, 70% ethanol, DPBS, TrypLE, and StemFlex medium supplemented with CloneR.

29. Prepare a 96-well plate coated with Geltrex as described in Support Protocol 1

30. Remove coating solution from the 96-well plate and add 100 µl StemFlex medium supplemented with CloneR. Place in the incubator at 37°C until use.

31. Select the clones to be re-plated by groups of 8 or 16.

    Up to 16 clones can be detached and harvested per round according to the IsoCell program.

32. Initialize IsoCell device as described in step 1.

33. Cell harvesting should proceed following the wizard: "Harvest" > "Detach," then choose chambers.

    At this point is important to verify cell detachment by microscopy. Poor detachment results in expansion failure. Some hPSC clones may need longer enzymatic incubation (program can be paused) (Fig. 2F).

34. Proceed to "Harvest" > "Extract." IsoCell will collect each detached clone in a 0.2-ml tube already containing 150 µl of StemFlex medium supplemented with CloneR.

    This program can be run for up to 16 samples at the time.

35. Resuspend and manually transfer each cell suspension from step 34 to one well of a 96-well plate using a pipette.

36. Incubate the 96-well plate at 37°C in a humidified environment, preferably in hypoxic conditions (5% O_2, 5% CO_2).
37. Power off IsoCell device according to the shutdown instructions.
38. At day 1 after re-plating, monitor cell attachment.
39. At day 2 after re-plating, perform a 50% medium change with Essential 8 or mTeSR medium.

    If there are only a few cells that are scattered throughout a well, change 50% medium with StemFlex supplemented CloneR daily until they recover.

After adapting the clones to Essential 8 or mTeSR medium (according to the original maintenance/expansion medium), clones may be subjected to further expansion as described in Support Protocol 8. For cell freezing, DNA collection for genotyping etc. standard procedures should be followed that are described elsewhere.

hPSC SINGLE CELL SEEDING AND CLONAL EXPANSION USING THE CELLENION CellenONE SINGLE-CELL DISPENSER

ALTERNATE PROTOCOL 1

The CellenONE instrument utilizes image-based cell detection and acoustic dispensing technology. The equipment performs automated image acquisition combined with fast processing using advanced algorithms in order to identify the cells of interest. Bright-field morphological parameters (diameter, circularity, and elongation) can be combined with up to four fluorescence signals to select specific cells for subsequent isolation. Therefore, application of live/dead dyes, fluorescently labeled antibodies, and fluorescence reporters enables the selection of specific cell subpopulations.

Single cell isolation is performed by drop-on-demand technology. Nanoliter drops are generated by piezo-acoustic technology, which ensures gentle cell isolation (*https://www.cellenion.com/*). Only single cell−containing drops are dispensed in each well of a target plate. Cells can be dispensed in a wide variety of target vessels, including any type of multi-well plate, e.g., 96- or 384-well plates. Empty drops or drops containing multiple cells are collected in a recycling tube for further use.

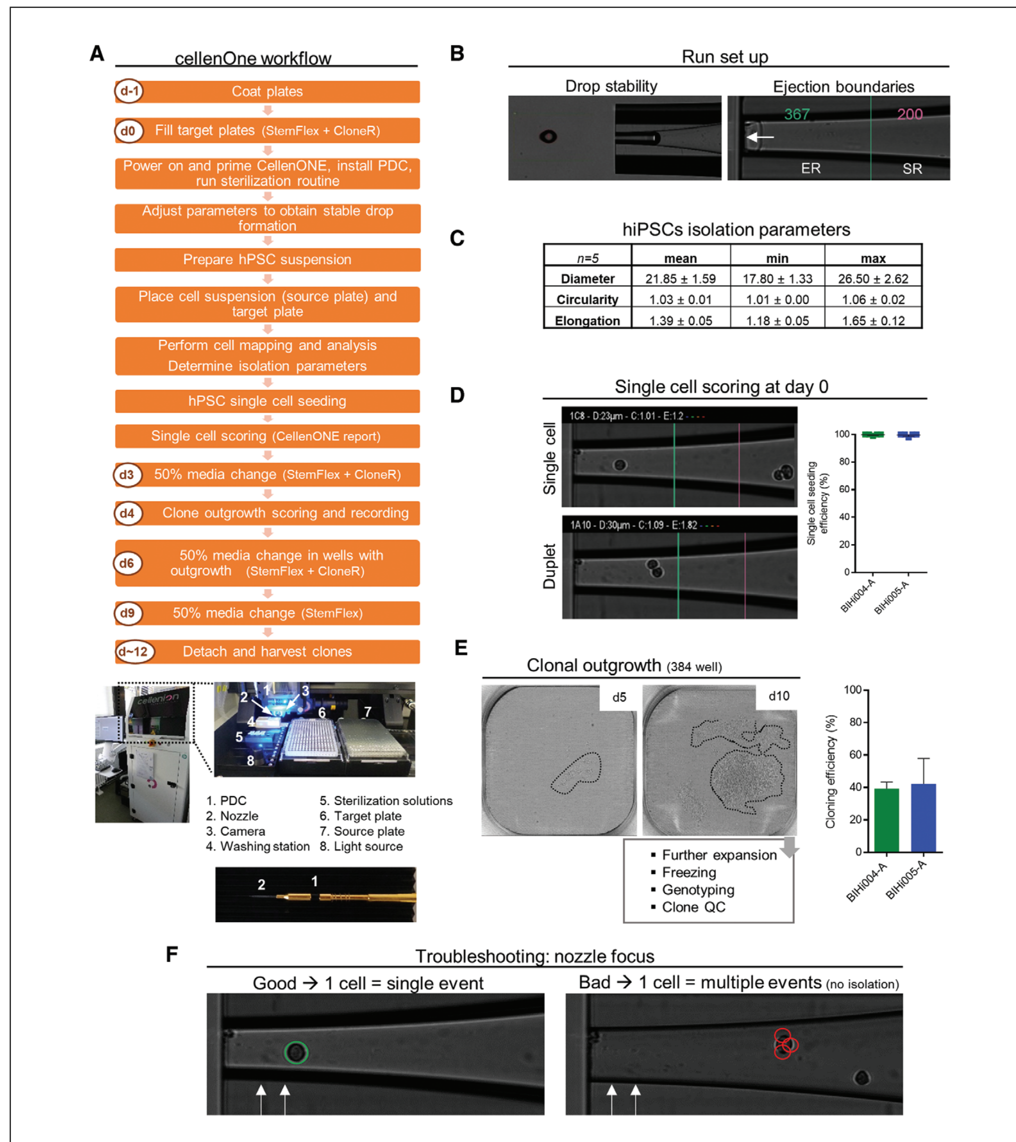
All acquired images and parameters of the isolated events are recorded by the software, and a final report with statistics can be automatically generated at the end of the process. Therefore, all information about the contents and corresponding parameters of each single well of the target plate is available.

In this alternate protocol, we provide a basic step-by-step procedure including all considerations, parameters, and important variables adapted to hPSC single cell isolation and posterior clonal expansion using CellenONE X1/F1 platform (Fig. 3).

NOTE: Special training on how to operate the instrument is recommended. Here we give an overview, but full details are beyond the scope of this protocol. Please contact the manufacturer for further information.

Materials

sciCLEAN8 (Scienion, cat. no. C-5283)
Bleach solution containing active Cl (e.g., Miltenyi, 130-093-663)
70% ethanol (e.g., Carl Roth, cat. no. T913.3)
Hydrogen peroxide (e.g., Carl Roth, cat. no. 8070.1)
hPSC suspension (see relevant Current Protocols articles)
StemFlex medium (ThermoFisher Scientific, cat. no. A3349401)
Y-27632 (Stem Cell Technologies, cat. no. 72305; see Support Protocol 5 for use in medium preparation)



n=5	mean	min	max
Diameter	21.85 ± 1.59	17.80 ± 1.33	26.50 ± 2.62
Circularity	1.03 ± 0.01	1.01 ± 0.00	1.06 ± 0.02
Elongation	1.39 ± 0.05	1.18 ± 0.05	1.65 ± 0.12

Figure 3 Alternate Protocol 1: CellenONE hPSC single cell isolation and clonal expansion. (**A**) Overview flowchart showing automated hPSC single cell seeding using CellenONE instrument. The instrument and its details are depicted in the lower part. (**B**) left: Representative picture showing a stable drop (no satellite drops or deviations should be visible); right: setting of the ejection boundaries delimiting the ejection and sedimentation regions (ER and SR, respectively), arrow shows the direction of drop ejection. (**C**) Table showing recommended isolation parameters for hPSC dispensing, obtained from the analysis of five independent experiments (**D**) Left: exemplary pictures from CelleONE dispensing report—a single cell event can be evaluated by review of the recorded images. Area between green and pink lines is the so called “safe space”; no element should be present within these boundaries, to ensure single cell isolation. Right: graph showing single cell seeding efficiency for two hiPSC lines using CellenONE (mean ± SD, n = 5). (**E**) Left: representative pictures showing clonal expansion in culture; dotted lines delimit the borders of the colonies (scale bars: 400 μm). Right: graph showing cloning efficiencies for two hiPSC lines (mean ± SD, n = 5). (**F**) Troubleshooting: panel showing the importance of the correct nozzle focus (pointed with white arrows) to guarantee correct isolation and high recovery rate. Cell3imager Duos was used for pictures in panel F. PDC = Piezo Dispense Capillary.

Propidium iodide, 1.0 mg/ml solution in water (ThermoFisher Scientific, cat. no. P3566)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
CellenONE F1.1 (1 channel: green) or F1.4 (4 channels: blue, green, orange, red)
Piezo Dispense Capillary (PDC; Cellenion, P-20-CM)

384-well sciSOURCE plate (Scienion, CPG-5501-1)
Serological pipettes, 5 and 10 ml (e.g., Corning, cat. no. 357543 and 357551)
Repeat pipettors, 200 and 1000 µl (e.g., Eppendorf, cat. no. 3123000055 and 3123000063)
Pipette tips, 200 and 1250 µl (e.g., Biozyme, cat. no. 770600)
Cell culture incubator (e.g., Binder)

Additional reagents and equipment for preparing target plates (Support Protocols 4 and 6), preparing single cell suspension of hPSCs (Support Protocol 7), preparation of medium containing anti-apoptotic small molecules (Support Protocol 5), and subculture and expansion of hPSC clones (Support Protocol 8)

CellenONE setup (day 0)

This protocol does not include full details for the handling of the device software or hardware. This protocol describes all steps relating specifically to hPSC single cell isolation (Fig. 3A).

1. Power on the instrument and follow the initial routine: filter and de-gas fresh autoclaved water and place it on the system bottle; re-fresh water for the washing procedure; empty waste; check water in the humidification system as well as the chiller.

2. Start the CellenONE software and follow the "prime" instructions.

3. Install the piezo dispenser capillary (PDC) according to the wizard (Fig. 3A).

4. Align the nozzle and correct the offset as recommended by the manufacturer.

5. Adjust pulse and voltage until a stable drop is achieved (Fig. 3B). Verify drop volume.

   Drop instability can be detected by drop deviation or presence of satellite drops.

6. Perform a Sci-Clean task.

   This task ensures that the nozzle is perfectly clean and helps to stabilize the drop. In this step, a diluted detergent and sonication are applied.

7. Perform a sterilization routine.

   This routine includes a serial clean in bleach solution, ethanol, and H_2O_2 to decontaminate the PDC.

8. Check drop stability.

   A stable drop is shown in Figure 3B. A deviation in the drop trajectory or appearance of satellite drops are signs of drop instability. Drop stability should always be validated before and after performing a dispensing task.

9. Set target plate platform at 4°C for the whole process.

   Setting temperature at 4°C ensures higher cell viability.

10. Set chamber humidity greater than 15%.

11. Open the sciSOURCE 384-well plate in a laminar flow hood, seal it with aluminum foil, and place it on the CellenONE source stage (Fig. 3A).

12. If drop is stable, the following steps can be performed.

Single cell isolation (day 0)

13. Prepare the target plates as described in Support Protocols 4 and 6.

14. Prepare hPSC cell suspension as described in Support Protocol 7.

15. Resuspend hPSCs in StemFlex medium supplemented with Y-27632 (preparation described in Support Protocol 5) at a concentration of 200 cells/µl.

    The cell suspension for spotting MUST be prepared with Y-27632 since CloneR is too viscous and generates a film at the end of the nozzle, which makes the drop unstable and therefore not suitable for isolation. In the event that StemFlex medium supplemented with Y-27632 also generates a film, DPBS containing Y-27632 can be used with similar results regarding cell survival.

16. If required, add propidium iodide (final concentration 2 µg/ml) for sorting viable cells by fluorescence discrimination.
17. Transfer 30 µl of cell suspension into one well of the SciSOURCE plate and register the well coordinates of the well in the software.
18. Place the target plate in the corresponding position (Fig. 3A).

    Do NOT forget to remove the lid of the target plate before the isolation procedure starts; not doing so will damage the PDC.

19. Take up 10 µl of hPSC suspension with the PDC and check drop stability.
20. Enter CellenONE (cell isolation) mode.
21. Create a folder for the set of experiments and name the run.
22. Choose continuous dispensing in transmission mode (T) until cells can be seen in the capillary.
23. Change to manual dispensing mode and dispense drops. When no cells are in the observed area, record a background image.
24. Perform a "mapping"; adjust ejection boundaries and save the selection (green and pink line shown in Fig. 3D).
25. Perform "analysis" on 100 events with wide detection parameters (default).
26. Create a gate that includes cells with the desired morphological parameters to ensure single cell isolation, and save the selection.

    Recommended morphological parameters for hPSC isolation are depicted in Figure 3C.

27. If additional fluorescence parameters are to be used for sorting, switch to Transmission>Fluorescence (T > F) mode.
28. Turn on desired channels (blue, green, orange, and/or red) and adjust power for a maximum fluorescence intensity 120.
29. Perform a background subtraction when no cells are in the field of view of the camera.

    If the background signal is high due to some artifact in the capillary, perform a washing task and a manual PDC cleaning with lint-free wipes (e.g., Kimwipes) and 70% ethanol.

30. Perform "analysis" in T > F mode using already selected morphological parameters, and adjust the fluorescence gate for the sorting.

    For propidium iodide staining, a negative gate must be selected for viable cells.

31. By continuously dispensing, verify that single cells with desired parameters are being positively selected (green ring around them), and that aggregates or duplets are shown in red (Fig. 3D).

    If too many events fit the parameters but are aborted (yellow ring around the event), try diluting the sample.

32. Once all is set, select standby mode to avoid clogging of the nozzle by cells that have sedimented.

    Standby mode ejects picoliter drops in a continuous fashion.

33. Select the target plate and the positions to be spotted.
34. Check drop stability.
35. Start the run.

    If the cell suspension is adequate and the desired events frequent enough (high recovery rate), dispensing will take about 3-4 min for a complete 384-well plate.

36. When the run is finished, cover the target plate with the lid and place it in the incubator at 37°C in a humidified environment, preferably under hypoxic conditions (5% O_2, 5% CO_2).
37. Export data folders to be analyzed with the CellenONE report software.
38. Flush the PDC, perform a nozzle wash-removal task, and shut down the CellenONE instrument according to the manufacturer recommendations.

Single cell scoring and quality check

39. Obtain the PDF document name as CellenONE report using the data folders exported in step 37.

    Multiple parameters from the run can be revised and validated using this report. Statistical results can be compared between individual dispensing runs.

40. Review the nozzle images for each event isolated and validate the single cell isolation.

    Percentage of validated single cell events relative to the total spotted events is considered to be the single cell seeding efficiency score (Fig. 3D). This score can be used to calculate cloning efficiency as a quality control parameter for an experiment.

Clone culture maintenance (day 1 to ∼10)

41. On day 3 after seeding, perform a partial medium change with StemFlex medium supplemented with CloneR. In a well of a 96-well plate, remove 40 µl and add 50 µl per well; in the wells of a 384-well plate, remove 20 µl and add 30 µl.

    More medium is added than removed to compensate for medium evaporation, which is especially prevalent in the outer wells of the cell culture plates. A full medium change (100 and 50 µl, respectively) has also been tested and gives similar results.

42. On day 4, monitor clone outgrowth and score wells with a positive outcome (Fig. 3F).

    Cloning efficiency can be calculated as a proportion of wells with outgrowth in relation to the total number of wells containing a single cell directly after dispensing at day 0.

43. On day 6, repeat the partial medium change described step 41 only on wells with cell growth.

    This minimizes the use of reagents and requires less time.

44. On day 9, perform a partial medium change using StemFlex medium without CloneR.
45. From day 10 onwards, perform a full medium change daily with StemFlex medium until the clones are ready to be passaged (70% confluent).

46. Follow Support Protocol 8, describing further clonal expansion for later applications such as freezing, genotyping, and expansion for cell banking.

Standard protocols for such applications can be followed. Detailed procedures are out of the scope of the methods described in this article.

ALTERNATE PROTOCOL 2

hPSC SINGLE CELL SEEDING AND CLONAL EXPANSION USING THE CYTENA SINGLE-CELL DISPENSER

The working principle of the Cytena single-cell dispenser (c.sight/f.sight) or single cell “printer” is an inkjet-like technology using a silicon microfluidic chip within a proprietary disposable cartridge which generates free-flying, picoliter droplets that encapsulate the cells. The microfluidic chip is embedded in the dispensing cartridge. The lower part of the cartridge is called the nozzle, and is the interrogation point for the dispensing event. A microscopy system coupled to a camera detects the droplets and their composition. The cell-containing drop is assessed based on cell size and roundness; single cell−containing droplets are selected and ejected into multi-well plates (96 or 384 wells). The system contains a pneumatic shutter below the nozzle, which remains closed until a positive single cell event occurs, allowing droplets containing more than one cell, or empty droplets, to be aspirated away. The entire sorting procedure is automatically documented as a series of images for each positive droplet recorded by the camera system.

NOTE: Special training on how to operate the instrument is recommended. Here we give an overview, but full details are beyond the scope of this protocol. Please contact the manufacturer for further information.

Materials

Essential 8 medium (ThermoFisher Scientific., cat. no. A1517001)
CloneR (Stem Cell Technologies, cat. no. 05888; see Support Protocol 5 for use in medium preparation)
hPSC culture (see relevant Current Protocols articles)
StemFlex medium (ThermoFisher Scientific, cat. no. A3349401)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
Cytena c.Sight (no fluorescence option) or f.sight (1 channel = green) connected to computer running Cytena software
Cartridge (Cytena Celllink, cat. no: 42581/40-40 SHC)
Serological pipettes, 5 and 10 ml (e.g., Corning, cat. no. 357543 and 357551)
Repeat pipettors, 200 and 1000 μl (e.g., Eppendorf, cat. no. 3123000055 and 3123000063)
Pipette tips, 100, 200 and 1250 μl (e.g., Biozyme, cat. no. 770200 and 770600)
Cell culture incubator (e.g., Binder)

Additional reagents and equipment for preparing target plates (Support Protocols 4 and 6), preparing single cell suspension of hPSCs (Support Protocol 7), preparation of medium containing anti-apoptotic small molecules (Support Protocol 5), and subculture and expansion of hPSC clones (Support Protocol 8)

Cytena setup (day 0)

1. Start the instrument and the connected computer.
2. Start the Cytena software.
3. Choose the format of the target plate (96-well or 384-well).

Single cell printing (day 0)

4. Prepare the target plates as described in Support Protocols 4 and 6.
5. Prepare an hPSC single cell suspension by following Support Protocol 7.

6. Re-suspend hPSCs in Essential 8 medium supplemented with Y-27632 (preparation described in Support Protocol 5) at a concentration of 1000 cells/µl.

   Essential 8 medium must be used for the cell suspension because it has reduced protein content, which prevents blockage of the cartridge. Slightly longer trypsinization time and the use of a 40-µm cell strainer are recommended to remove cell aggregates from the suspension (see Support Protocol 7).

7. Take a new cartridge and unpack it under sterile conditions.

8. Add 70 µl of the cell suspension into the cartridge using a 200-µl repeat pipettor and pipette tip.

9. Open the lid of the Cytena instrument and mount the cartridge using the provided screw (Fig. 4A).

10. Attach a sterile 100-µl pipette tip to the pipetting arm in such a way that it sits in the cell solution within the cartridge (Fig. 4A).

11. Move arm to camera position.

12. Place the target plate on the substrate carrier.

    Carefully check that the plate is correctly placed, with the plate lid removed.

13. Close the lid and return to the software.

14. Open the droplet quality control (QC) tab to automatically start the process.

15. Ideally, a droplet should appear right away in the QC camera window. It should look as depicted in Figure 4B.

    The droplet should be stable. If the droplet is unstable, it may be necessary to adapt the stroke parameters by changing length and speed values. The settings we recommend are length "10" and speed "120."

16. When the droplet is stable, check the vacuum shutter. Use vacuum setting check box "turn on" to switch on the shutter. The droplet should disappear (Fig. 4B). Once the droplet has been reliably removed, proceed with the cell printing.

17. Set program settings as follows (Fig. 4C):

    Program: Printing
    Number cells/well: 1
    Cell size: 10-30 µm
    Cell roundness: 0.5-1.0.

18. Press the Play button to start the printing process

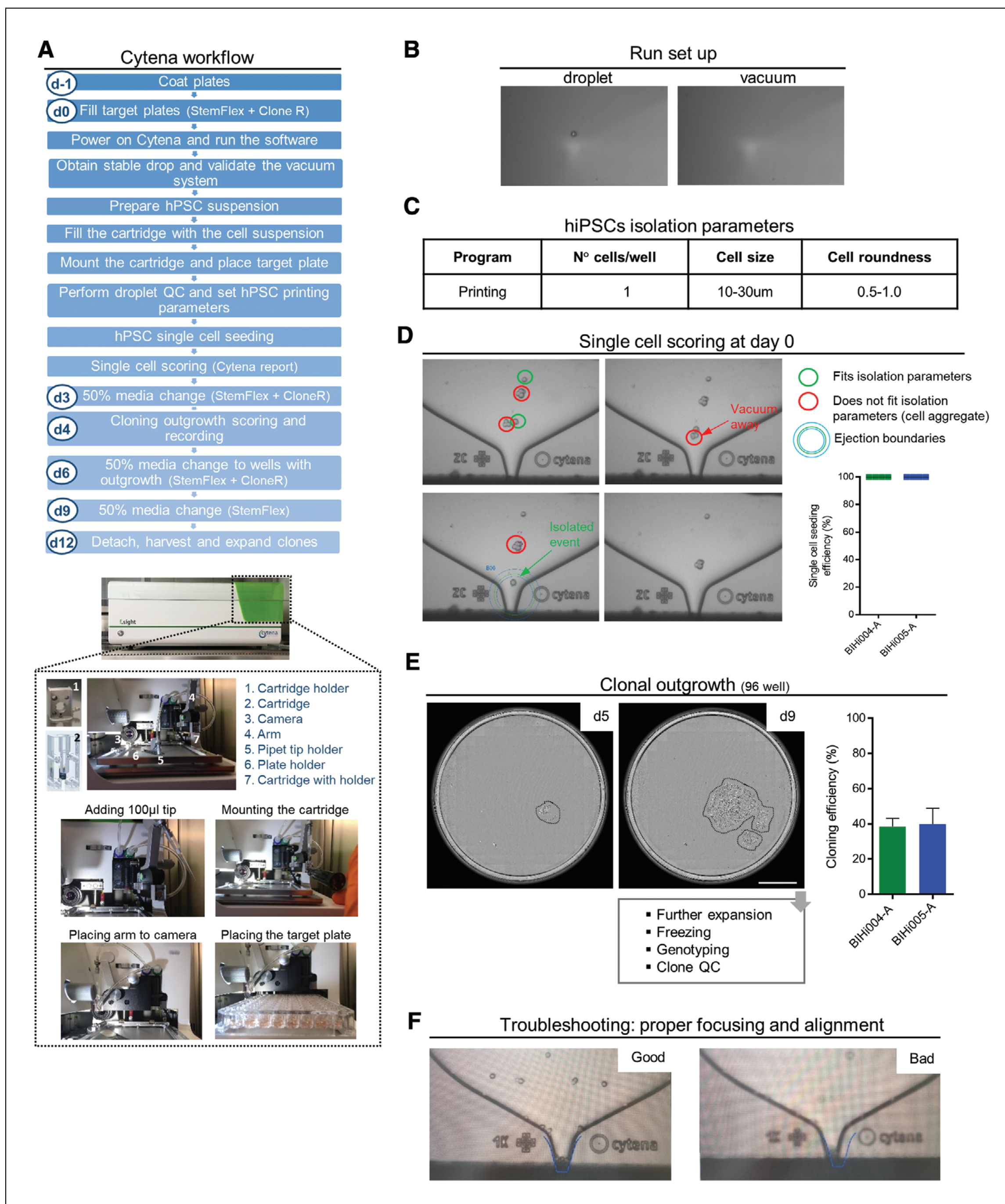
19. When the printing process has finished, place the plate in the incubator at 37°C in a humidified environment, preferably under hypoxic conditions (5% O_2, 5% CO_2).

Single cell scoring and quality check

20. Export the images generated for the run. A total of five pictures showing the nozzle during the isolation process are recorded for each positive single cell droplet.

21. Review the nozzle images for each event isolated and validate the single cell isolation.

    Percentage of validated single cell events in relation to the total number of spotted events is considered to be the single cell seeding efficiency score (Fig. 4D). This score can be used to calculate cloning efficiency as a quality control parameter for an experiment.



Program	N° cells/well	Cell size	Cell roundness
Printing	1	10-30um	0.5-1.0

Figure 4 Alternate Protocol 2: Cytena hPSC single cell isolation and clonal expansion. (**A**) Overview flowchart showing automated hPSC single cell seeding using Cytena (c.sight) instrument. Images of the instrument and its main components such as cartridge and pipetting arm are depicted in the lower part. (**B**) Pictures showing the proper droplet formation and the validation of the vacuum system performance. (**C**) Table showing the recommended morphological parameters to ensure single hPSC isolation. (**D**) Left: example of the nozzle images (lower part of the cartridge) taken before and after a single cell printing process; each dispensing event is documented. Right: graph showing single cell seeding efficiency for two hiPSC lines using Cytena (mean ± SD, n = 4) (right). (**E**) Left: Representative pictures showing clonal expansion in culture at different time points (scale bars: 1 mm). Right: graph showing cloning efficiencies for two hPSC lines (mean ± SD, n = 4). (**F**) Troubleshooting: panel showing the correct focus and alignment of cartridge to the camera (good and bad focus are depicted) for successful single cell printing. Incucyte imager was used for pictures in panel E. QC = quality control.

Clone culture maintenance (day 1 to ~10)

22. On day 3 after seeding, perform a partial medium change with StemFlex medium supplemented with CloneR (preparation described in Support Protocol 5). For 96-well plates: remove 40 µl and add of 50 µl per well; for 384-well plates remove 20 µl and add 30 µl.

    A full medium change (100 and 50 µl, respectively) has also been tested and gives similar results.

23. On day 4, monitor clone outgrowth and record wells with positive outcome.

    Cloning efficiency can be calculated as the proportion of wells with outgrowth in relation to the total number of wells containing a single cell on day 0.

24. On day 6, repeat partial medium change described in step 22 only on wells with cell growth.

    This minimizes the use of reagents and requires less time.

25. On day 9, repeat partial medium change from step 22 using StemFlex medium without CloneR.

26. From day 10 onwards, perform a full medium change daily with StemFlex medium until the clones are ready to be passaged (70% confluent).

27. Follow Support Protocol 8, describing further clonal expansion for later applications such as freezing, genotyping, and expansion for cell banking.

    Standard protocols for such applications can be followed. Detailed procedures are out of the scope of the methods described in this article.

COATING CELL CULTURE PLATES WITH GELTREX

SUPPORT PROTOCOL 1

Soluble forms of basement membrane extract purified from murine Engelbreth-Holm-Swarm tumor cells, such as Geltrex (ThermoFisher Scientific) or Matrigel (Corning), are widely used as a feeder-free alternative to murine embryonic fibroblast feeder cells for hPSC cultures. Here, we briefly describe the coating procedure for standard plastic tissue culture–treated plates using Geltrex.

Materials

Geltrex (ThermoFisher Scientific, cat. no. A1413302)
KnockOut DMEM/F-12 (ThermoFisher Scientific, cat. no. 12660012)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
50-ml tubes (e.g., Corning Falcon, cat. no. 430290)
Multi-well culture vessels (e.g., 96 well, Corning, cat. no. 353072; see Table 1)
Serological pipettes, 5 and 10 ml (e.g., Corning, cat. no. 357543 and 357551)

Table 1 Coating Solution and Medium Volumes for Different Cell Culture Vessels

Plate	Area (cm^2)/well	Coating solution/well	Culture medium/well
6-well	9.5	1 ml	2 ml
12-well	3.8	0.6 ml	1 ml
24-well	1.9	0.3 ml	0.5 ml
48-well	1	0.2 ml	0.3 ml
96-well	0.32	0.05 ml	0.1 ml
384-well	0.056	0.02 ml	0.06 ml

1.5-ml tubes (e.g., Eppendorf, cat. no. 30120086)
Parafilm M sealing film (Sigma, P6543-1EA)
Cell culture incubator (e.g., Binder)

1. Thaw a frozen Geltrex vial by placing it in ice.

   Geltrex in its concentrated form will start to polymerize and form a gel within a few minutes when warmed up to room temperature; hence, all media and tubes should be pre-cooled to 4°C, and solutions should be kept on ice during handling. It is suggested to thaw stock solutions of Geltrex overnight on ice, aliquot small volumes (0.1-1 ml) into pre-cooled tubes, and re-freeze at −20°C to avoid multiple freeze-thaw cycles. Follow manufacturer's guidelines for handling and dilution where available.

2. Pre-cool a 50-ml Falcon tube containing 49.5 ml DMEM/F12 by placing in ice for 30 min.

3. Add 0.5 ml Geltrex to the 49.5 ml of cold DMEM/F-12 and invert tube gently several times to mix.

   Geltrex dilutions ranging from 1:100 to 1:120 can be used for hPSC culture with similar results.

4. Add appropriate volume of diluted Geltrex solution to desired wells of culture vessels (see Table 1 below).

5. Incubate culture vessels with Geltrex solution for at least 30 min at 37°C before use.

6. For preparation and storage of coated plates for up to 2 weeks, add 1.5× the volume recommended in Table 1 of Geltrex/DMEM/F12 solution, then seal lids on plates using Parafilm and store at 4°C.

   The increased volume is to prevent drying of the plates due to evaporation, which will cause the coating to degrade.

SUPPORT PROTOCOL 2

hPSC MAINTENANCE IN DEFINED FEEDER-FREE CONDITIONS

The quality of the hPSC cultures has a major impact on the downstream application of the cells. Daily medium change and monitoring of cell density and morphology are required to preserve self-renewal, pluripotency, and differentiation capacities.

Materials

hPSC culture (see relevant Current Protocols articles)
Geltrex-coated plates (e.g., 6-well; see Support Protocol 1)
Essential 8 medium (ThermoFisher Scientific., cat. no. A1517001) or mTeSR (Stem Cell Technologies, cat. no. 85850)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
Inverted microscope with phase-contrast optics, 4×, 10×, 20× objectives (e.g., DMi8, Leica)
Serological pipettes, 5 and 10 ml (e.g., Corning, cat. no. 357543 and 357551)
Pipette tips, 10 μl (e.g., Biozyme, cat. no. 770020)
50-ml tubes (e.g., Corning Falcon, cat. no. 430290)
Glass Pasteur pipette (e.g., VWR, cat. no. 612-1701)
Cell culture vacuum pump (e.g., Integra)
Cell culture incubator (e.g., Binder)
Water bath (e.g., VWR)

Additional reagents and equipment for passaging of hPSC (Support Protocol 3)

1. Most hPSC cultures can be regularly maintained in 6-well Geltrex-coated plates (Support Protocol 1).
2. Use an inverted microscope with phase-contrast optics (4× to 10× objective) to visually monitor the growth and morphology of the hPSC colonies.

   Regions identified as differentiated should be mechanically removed by scraping the bottom of the plate with a plastic pipette tip (10 µl).

3. *For medium change*: Prepare an aliquot of Essential 8 or mTeSR medium in a 50-ml tube with the volume needed for the medium change according to the number of wells (2 ml/well), and warm it to 37°C using a water bath.

   Avoid warming up excess medium, in order to preserve growth-factor activity for a longer period of time.

4. Aspirate medium from the wells using a vacuum system with sterile tip (e.g., glass Pasteur pipette).
5. Add pre-warmed fresh Essential 8 or mTeSR medium according to the hPSC line specification, using 5- or 10-ml pipettes.

   Recommended culture medium volumes for different culture vessels are given in Table 1.

6. When cultures reach 70%-80% confluency, passage according to Support Protocol 3.

hPSC PASSAGING IN CLUMPS

SUPPORT PROTOCOL 3

Standard hPSC cell culture practices include the regular passaging of the cells to maintain self-renewal and pluripotent capacities. Classical enzymatic splitting is harsh for the cells, and the survival rate is lower than with passaging in aggregates or clumps. Here we describe a standard procedure for hPSC splitting using EDTA to "loosen" cell-cell and cell-plate contact without exposing hPSC to single cell dissociation.

Materials

hPSC culture (see relevant Current Protocols articles)
Essential 8 medium (ThermoFisher Scientific., cat. no. A1517001) or mTeSR (Stem Cell Technologies, cat. no. 85850)
0.5 M EDTA, pH 8.0 (ThermoFisher Scientific., cat. no. 15575020)
Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS; e.g., ThermoFisher Scientific, cat. no. 10010023)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
Geltrex-coated plates (e.g., 6-well; see Support Protocol 1)
Inverted microscope with phase-contrast optics, 4×, 10×, 20× objectives (e.g., DMi8, Leica)
Cell culture vacuum pump (e.g., Integra)
Glass Pasteur pipette (e.g., VWR, cat. no. 612-1701)
Serological pipettes, 5 and 10 ml (e.g., Corning, cat. no. 357543 and 357551)
50-ml tubes (e.g., Corning Falcon, cat. no. 430290)
Cell culture incubator (e.g., Binder)
Water bath (e.g., VWR)

Additional reagents and equipment for maintaining hPSC in culture (Support Protocol 2)

1. hPSC confluence before splitting should be 70%-80%. Support Protocol 2 provides details on keeping hPSC in culture.

2. Prepare Geltrex-coated tissue culture−treated 6-well plate(s) as described in Support Protocol 1.

3. Aspirate the Geltrex coating solution from the prepared 6-well plate and add 2 ml per well of Essential 8 or mTeSR medium. Keep the plate in the incubator at 37°C until it will be used.

4. Dilute 0.5 M EDTA to a final concentration of 0.5 mM in DPBS

   For example, dilute 500 µl 0.5 M EDTA in 49.5 ml DPBS.

5. Aspirate culture medium from the wells to be split.

6. To wash the wells, add 1 ml of 0.5 mM EDTA to each well and immediately remove it by aspiration.

7. Add 1 ml 0.5 mM EDTA to each well and incubate for 3-4 min at room temperature.

   Monitor the process with an inverted phase-contrast microscope; when cells are rounding up and separate from the neighboring cells, the incubation should be stopped. Cells should not detach!

8. Remove EDTA solution from the wells by aspiration and add 2 ml of Essential 8 or mTeSR medium using a 5-ml serological pipette.

9. Triturate gently using a 1000-µl pipette tip against the culture surface three times to detach the hPSC clumps.

10. Collect the clump suspension and distribute dropwise in the plate prepared in step 3.

    The splitting density will determine how quickly cells will reach confluency for passaging again. Typically, we split 1:6 to 1:20, which would be 333-100 µl volume per well according to the volume in which the clumps were triturated (see step 8), providing about 3-6 days outgrowth.

11. Incubate at 37°C, preferably under hypoxic conditions (5% O_2, 5% CO_2).

12. Change medium every day and monitor the culture as described in Support Protocol 2.

SUPPORT PROTOCOL 4

LAMININ 521 COATING OF IsoCell PLATES AND 96/384-WELL PLATES

hPSC feeder-free cultures need extracellular matrix (ECM) components to successfully attach and generate colonies while keeping pluripotency and self-renewal capacities. Recombinant Laminin 521 is widely used, and in practice increases single cell cloning efficiency compared to commonly used ECMs such as Matrigel or Geltrex. In addition, the purity of Laminin 521 is an advantage for imaging purposes.

Materials

Human recombinant Laminin 521 (Bio Lamina, cat. no. LN521-05)
DPBS with calcium and magnesium (e.g., ThermoFisher Scientific, cat. no. 14040117)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
Clone G kit-A—with tissue-culture treated 6-cm dishes (Iota Sciences, cat. no. SKU 10030)
96-well tissue-culture treated plates (e.g., Corning, cat. no. 353072)
384-well cell culture plate (e.g., Greiner, 781182)
Serological pipettes, 5 and 10 ml (e.g., Corning, cat. no. 357543 and 357551)

Repeat pipettors, 200 and 1000 µl (e.g., Eppendorf, cat. no. 3123000055 and 3123000063)
Multichannel pipette (e.g., Eppendorf, cat. no. 3125000052)
Pipette tips, 200 and 1250 µl (e.g., Biozyme, cat. no. 770200 and 770600)
15-ml tubes (e.g., Corning Falcon, cat. no. 352096)
50-ml tubes (e.g., Corning Falcon, cat. no. 430290)
Sterile disposable plastic pipetting reservoirs (e.g., ThermoFisher Scientific., cat. no. 95128095)
Parafilm M sealing film (e.g., Sigma, P6543-1EA)
Cell culture incubator (e.g., Binder)

1. Thaw Laminin 521 on ice.

2. Prepare the coating solution by diluting Laminin 521 in DPBS with calcium and magnesium to a final concentration of 5 µg/ml. Total volume depends on number of plates to be coated.

   Prepare 8 ml coating solution per 384-well plate, 5 ml coating solution per 96-well plate, or 2 ml coating solution per 6-cm IsoCell culture-treated dish provided in the Clone G kit-A (Iota Sciences).

3. Add 20 µl coating solution per well of a 384-well plate, 50 µl coating solution per well of a 96-well plate, or 2 ml coating solution per IsoCell culture-treated dish.

   The use of multichannel pipettes or dispensing robots for multi-well plates simplifies the task and reduces the hands-on time.

4. Distribute the coating solution evenly

   For 96- and 384-well plates, centrifuge 1 min at 300 × g to ensure that the bottoms of all wells are covered.

5. Incubate Laminin 521 at least 2 hr at 37°C for multiwell plates before using them, or maximum of 2 hr for 6-cm dishes.

   Best results can be obtained if the plates are prepared fresh. If the plates are not going to be used the same day, they can be sealed with Parafilm and stored immediately after coating at 4°C for up to 1 week, but with a significant reduction in performance.

PREPARATION OF MEDIUM CONTAINING ANTI-APOPTOTIC SMALL MOLECULES

SUPPORT PROTOCOL 5

The use of Rho-kinase (ROCK) inhibitors such as Y-27632 or supplements enhancing cell survival such as CloneR has been shown to play an important role in at least partially blocking apoptotic events in hPSC, among other cell types. We have established the single cell seeding and clonal expansion described in this method paper using these small molecules and supplements to enhance hPSC survival. Here we describe the preparation of culture medium containing anti-apoptotic factors.

Materials

Y-27632 (Stem Cell Technologies, cat. no. 72305)
DMSO (e.g., Sigma-Aldrich, cat. no. D2650)
CloneR (Stem Cell Technologies, cat. no. 05888)
Essential 8 medium (ThermoFisher Scientific, cat. no. A1517001)
mTeSR (Stem Cell Technologies, cat. no. 85850)
StemFlex medium (ThermoFisher Scientific, cat. no. A3349401)

1. Dissolve Y-27632 in DMSO to prepare a 10 mM stock solution.

   Aliquots can be kept at −20°C until use.

Table 2 Anti-Apoptotic Small Molecules and Supplement Dilutions in Medium

Medium	Small molecule stock concentration	Small molecule final concentration	Dilution
StemFlex + CloneR	10×	0.5×	1:20
StemFlex + Y-27632	10 mM	10 µM	1:1000
Essential 8 + Y-27632	10 mM	10 µM	1:1000

2. Thaw a ready-to-use vial of CloneR or aliquot of Y-27632.
3. Calculate the final volume needed of medium containing small molecules.
4. Refer to Table 2 for medium preparation.

SUPPORT PROTOCOL 6

96- AND 384-WELL TARGET PLATE PREPARATION PRIOR TO SINGLE CELL SEEDING

This protocol describes how to prepare the target multi-well culture plates for automated single cell seeding using CellenONE and Cytena instruments. This is a manual procedure; however, the use of liquid-handling robots reduces the hands-on time and standardizes the workflow.

Materials

StemFlex medium containing CloneR, prepared as described in Support Protocol 5
Penicillin/streptomycin (ThermoFisher Scientific, cat. no. 15140122; optional)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
96- or 384-well tissue-culture treated plates pre-coated with Laminin 521 as described in Support Protocol 4
Multichannel pipette (e.g., Eppendorf, cat. no. 3125000052)
Sterile disposable plastic pipetting reservoirs (e.g., ThermoFisher Scientific., cat. no. 95128095)
Pipette tips, 200 and 1250 µl (e.g., Biozyme, cat. no. 770200 and 770600)
Pipette tips, 300 µl (e.g., Eppendorf, cat. no. 10497221)
Cell culture vacuum pump (e.g., Integra)
Multichannel adaptor for the cell culture pump (e.g., Integra)
Centrifuge (e.g., Eppendorf) with plate adapter

1. Aspirate the coating solution from the plate (see Support Protocol 4, step 5) using the a multichannel pipette and 200-µl pipette tips with adapter connected to the cell culture vacuum pump system.
2. Add StemFlex medium supplemented with CloneR (see Support Protocol 5) using a multichannel pipette.

   Volumes: 60 µl/well (384-well plate); 100 µl/well (96-well plate).

   Optional addition of antibiotics to the medium (penicillin/streptomycin, 1×) for the single cell seeding minimizes the risk of bacterial contamination, especially when using the CellenONE device, and does not affect cell viability.

3. If using 384-well plates, centrifuge plates for 1 min at 300 × *g*, to ensure even surface coverage by the medium and remove any bubbles. If bubbles can be observed after filling of a 96-well plate centrifugation can also be applied.
4. Keep the multi-well plate at 4°C until use within 24 hr.

SINGLE CELL DISSOCIATION OF hPSCs

SUPPORT PROTOCOL 7

This protocol describes a procedure, optimized for cell viability, to obtain a homogeneous single cell suspension from hPSC cultures, to be used for automated single cell isolation. Before this point, standard culture conditions for feeder-free hPSC culture using Essential 8 or mTeSR medium should be maintained, as described in Support Protocols 1, 2, and 3, in order to ensure good-quality starting cultures. The procedure is described for hPSCs cultured in 6-well plates. Volumes have to be adapted for other formats taking into account the culture surface, as described in Support Protocol 1 and Table 1.

Materials

hPSC culture (see Support Protocols 1, 2, and 3)
Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS; e.g., ThermoFisher Scientific, cat. no. 10010023)
TrypLE Select Enzyme (ThermoFisher Scientific, cat. no. 12563011)
Essential 8 medium (ThermoFisher Scientific, cat. no. A1517001) or mTeSR (Stem Cell Technologies, cat. no. 85850)
StemFlex medium (ThermoFisher Scientific, cat. no. A3349401)
CloneR (Stem Cell Technologies, cat. no. 05888; see Support Protocol 5 for use in medium preparation)
Y-27632 (Stem Cell Technologies, cat. no. 72305; see Support Protocol 5 for use in medium preparation)
0.04% Trypan blue (e.g., Sigma-Aldrich, cat. no. T8514-20ML)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
Inverted microscope with phase-contrast optics, 4×, 10×, 20× objectives (e.g., DMi8, Leica)
Multichannel pipette (e.g., Eppendorf, cat. no. 3125000052)
Sterile disposable plastic pipetting reservoirs (e.g., ThermoFisher Scientific., cat. no. 95128095)
Pipette tips, 200- and 1250-µl (e.g., Biozyme, cat. no. 770200 and 770600)
Pipette tips, 300-µl (e.g., Eppendorf, cat. no. 10497221)
Cell culture vacuum pump (e.g., Integra)
Multichannel adaptor for the cell culture pump (e.g., Integra)
6-well tissue-culture treated plates (e.g., Corning, cat. no. 353046)
Serological pipettes, 5 and 10 ml (e.g., Corning, cat. no. 357543 and 357551)
15-ml tubes (e.g., Corning Falcon, cat. no. 352096)
1.5-ml tubes (e.g., Eppendorf, cat. no. 30120086)
Centrifuge (e.g., Eppendorf)
Automated cell counter with counting chambers (e.g., Countess II, ThermoFisher Scientific) or hemocytometer (Neubauer counting chamber)
Cell culture incubator (e.g., Binder)
Water bath (e.g., VWR)

Additional reagents and equipment for preparation of medium containing anti-apoptotic small molecules (Support Protocol 5) and cell counting (see Current Protocols article: Phelan & May, 2015)

1. Verify 70%-80% hPSC culture confluency using inverted microscope with phase contrast (4× objective).
2. Aspirate and discard medium from the well to be harvested.
3. Wash the well with 1 ml DPBS.
4. Aspirate and discard DPBS. Add 1 ml TrypLE.

Gentle enzymatic dissociation reagents comparable to TrypLE such as Accutase can also be used.

5. Incubate at room temperature until cells are loose (typically 3-5 min). Monitor this step with the microscope. Remove the TrypLE carefully. Make sure the cells are still attached to the bottom of the plate while TrypLE is being removed.

   Unnecessarily long incubations decrease cell viability.

6. Add 1 ml Essential 8 or mTeSR medium to the well to neutralize the enzymatic action, and gently homogenize the cells suspension using a 1000-µl pipette tip.
7. Transfer the cell suspension to a 15-ml tube and centrifuge 5 min at 300 × *g*, room temperature.
8. Aspirate supernatant.
9. Re-suspend the cell pellet with 1 ml of medium.

   Medium for resuspension will be different according to downstream applications: hPSC single cell isolation by IsoCell (StemFlex medium containing CloneR), CellenONE (StemFlex medium or DPBS containing Y-27632), or Cytena (Essential 8 medium containing Y-27632). See Support Protocol 5 for preparation of these media.

10. Prepare an aliquot for cell counting 1:1 in 0.04% trypan blue (e.g., 10 µl cell suspension + 10 µl trypan blue).
11. Homogenize the aliquot and load 10 µl in the cell counting chamber.

    Dedicated chambers are provided specifically for automated counters. A Neubauer chamber (hemocytometer) can also be used, as described in Current Protocols article Phelan & May (2015).

12. Express the result in number of viable cells/ml.

    This value is used for further calculations regarding cell suspension concentrations required for each single cell dispensing method.

SUPPORT PROTOCOL 8

IsoCell-, CellenONE-, AND CYTENA-DERIVED hPSC CLONE SUBCULTURE AND EXPANSION

After hPSC clones derived from single cells have reached 60%-70% confluence, they can be dissociated and further expanded for downstream genotyping, characterization, or freezing. Initial seeding with CellenONE and Cytena instruments will have already been performed in 96- or 384-well plates. For IsoCell, the first expansion step into 96-well plates is described in the Basic Protocol. Here we describe how to passage these clones into larger plate formats. The decision on the plate format to scale up the culture will depend on the several factors: how many clones have to be expanded, workload, possibility of automation, amount of cells needed for downstream purposes, etc.

Materials

StemFlex medium (ThermoFisher Scientific, cat. no. A3349401)
CloneR (Stem Cell Technologies, cat. no. 05888; see Support Protocol 5 for use in medium preparation)
hPSC clones in wells of 96-well plate or 384-well plate (Basic Protocol)
Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS; e.g., ThermoFisher Scientific, cat. no. 10010023)
TrypLE Select Enzyme (ThermoFisher Scientific, cat. no. 12563011)
Essential 8 medium (ThermoFisher Scientific, cat. no. A1517001) or mTeSR (Stem Cell Technologies, cat. no. 85850)

Laminar flow hood (e.g., Herasafe, ThermoFisher Scientific)
12-well tissue-culture treated plates (e.g., Corning, cat. no. 353043)
Serological pipettes, 5 and 10 ml (e.g., Corning, cat. no. 357543 and 357551)
Repeat pipettor, 200 and 1000 µl (e.g., Eppendorf, cat. no. 3123000055 and 3123000063)
Pipette tips, 200 and 1250 µl (e.g., Biozyme, cat. no. 770200 and 770600)
Cell culture vacuum pump (e.g., Integra)
Inverted microscope with phase-contrast optics, 4×, 10×, 20× objectives (e.g., DMi8, Leica)
Cell culture incubator (e.g., Binder)
48-well tissue-culture treated plates (Corning, cat. no. 353078)
15-ml tubes (e.g., Corning, cat. no. 352096)
Water bath (e.g., VWR)

Additional reagents and equipment for Geltrex coating of cell culture plates (Support Protocol 1) and preparation of medium containing anti-apoptotic small molecules (Support Protocol 5)

To split a 96-well plate of clones

1a. Coat in advance, with Geltrex (Support Protocol 1), the number of wells of 12-well plates according to the number of clones to be passaged (1 × 96-well to 1 × 12 well).

2a. Aspirate and discard the coating solution from the 12-well plate and add 1 ml of StemFlex supplemented with CloneR per well (see Support Protocol 5). Keep the plate in the incubator at 37°C until it will be used.

3a. Aspirate and discard the medium from a well of the 96-well plate with a clone of interest.

4a. Add 200 µl DPBS per well and subsequently discard it to wash the well.

5a. Add 30 µl TrypLE to the well, distribute it evenly, and monitor cell detachment at room temperature using an inverted microscope.

TrypLE incubation time may vary from clone to clone within the range of 3-10 min

6a. Neutralize the TrypLE with 150 µl StemFlex supplemented with CloneR. Pipette up and down three times using a P200 tip on a repeat pipettor set at 150 µl.

7a. Collect the suspension and directly transfer it into one well of the previously prepared 12-well plate (step 2a)

8a. Distribute the cell suspension in the 12-well plate well by a gentle cross movement of the plate.

9a. Incubate at 37°C, preferably under hypoxic conditions (5% O_2, 5% CO_2).

To split a 384-well plate of clones

1b. Coat in advance, with Geltrex (Support Protocol 1), the number of wells in a 48-well plate according to the number of clones to be passaged (1 × 384-well to 1 × 48 well).

2b. Aspirate and discard the coating solution from the 48-well plate and add 0.3 ml per well of StemFlex supplemented with CloneR (see Support Protocol 5). Keep the plate in the incubator at 37°C until it will be used.

3b. Aspirate and discard the medium from a well of the384-well plate with the clone of interest.

4b. Add 80 µl DPBS per well and subsequently discard it to wash the well.

5b. Add 15 µl TrypLE to the well, distribute it evenly, and monitor cell detachment at room temperature using an inverted microscope.

TrypLE incubation time may vary from clone to clone.

6b. Neutralize the TrypLE with 80 µl StemFlex supplemented with CloneR. Pipette up and down three times using a P200 tip on a repeat pipettor set at 80 µl.

7b.. Collect the suspension and transfer it directly to one well of the previously prepared 48-well plate (step 2b)

8b. Homogenize the cell suspension in the 48-well plate by gentle cross movement of the plate.

9b. Incubate at 37°C, preferably under hypoxic conditions (5% O_2, 5% CO_2).

Culture maintenance after clone splitting

10. On day 2 after splitting, adapt clones to the regular cell culture medium (Essential 8 or mTeSR) by changing medium to a 1:1 mixture of StemFlex:Essential 8 or mTeSR.

11. Change medium every day from here on.

12. Further culture expansion follows standard procedures for hPSC culture maintenance and splitting, as described in Support Protocols 1-3.

Other procedures including clone genotyping, characterization, or freezing can be performed using standard protocols that are out of the scope of this article and described in the literature.

COMMENTARY

Background Information

The current standard approaches for hPSC isolation and sub-cloning involve either manual or FACS-based methods. Although there have been improvements in the workflows over the last years, both methods still have limitations, including relatively low survival rates. Various approaches using different recombinant ECM proteins or hydrogels (Higuchi et al., 2016; Rodin, Antonsson, Hovatta, & Tryggvason, 2014), protein inhibitors (Valamehr et al., 2012; Watanabe et al., 2007), mouse embryonic fibroblasts (MEFs; Yang et al., 2013), and human serum-derived protein (Pijuan-Galitó et al., 2016) have shown improved survival of single-cell derived hPSC clones. Recently, FACS sorting in combination with different ECM proteins such as Laminin 521 or with MEF co-culture showed an increased single cell survival (Chen & Pruett-Miller, 2018; Singh, 2019). However, the usability of these methods is often severely compromised due to high cost, the need for MEFs, poor reproducibility, or the requirement for a dedicated operator/facility to maintain and operate the FACS equipment.

Based on preliminary results (data not shown) and the literature, we have included key factors in our workflows such as growth factor−stabilized medium (StemFlex medium) that ensure the activity of growth factors for longer periods of time, as well as a supplement that enhances cell survival (CloneR) and a coating matrix suitable for better hPSC single cell survival, outgrowth, and imaging (Laminin 521) (Chen & Pruett-Miller, 2018). Using these conditions, automated single cell dispensing and follow-up culture were optimized. Other cell isolation and culture conditions than the ones described here can potentially be used as well, but they need to be tested. Classical media used for hPSC culture such as Essential 8 or mTeSR contain unstable components and need to be replaced every day. These medium changes cause stress to the hPSCs and significantly affect the single cell outgrowth potential of the developing clones. The less the cultures are manipulated during the first days after single-cell seeding, the better. In addition, a reduced number of medium changes in high-throughput experiments translates into lower maintenance cost and less hands-on time.

Addition of CloneR to the medium has shown better hPSC single cell survival in our experiments compared to the widely used ROCK inhibitor Y-27632, but, other options available on the market such as RevitaCell (ThermoFisher Scientific) could also be explored. It is important to keep CloneR in the culture medium for at least 5 days. Earlier removal leads to a reduction of cloning efficiency by 5%-7% (data not shown). Regarding the coating of the culture surface, other options such as vitronectin, Geltrex, or Matrigel at various dilutions can be considered; however, vitronectin has shown interference with IsoCell grid formation, and after Geltrex or Matrigel coating, certain debris-like deposits are present that affect imaging procedures and make the single cell scoring process difficult. We have applied three automated systems to generate hPSC sub-cultures with proven mono-clonality, reaching efficiencies of 30%-60%. This percentage refers to the outgrowth observed in wells where a single cell event was registered. However, we also want to point out that the cloning efficiency is cell-line dependent and that it might be important to optimize the conditions for other cell lines. Knowing the cloning efficiency allows estimation of the number of plates and format (96 or 384 wells, or 256 chambers) to be seeded in order to isolate a desired clone. For example, if the frequency of occurrence of a certain homologous directed recombination (HDR) event using CRISPR/Cas9-based gene editing is about 1%-5% and the hPSC line used for this experiment shows 50% cloning efficiency after single cell seeding, about 1-10 clones containing the desired edit can be isolated from 384 initially seeded single cells. This estimation is valid for single cell dispensing with an efficiency equal or greater than 95%, which can be reached using the CellenONE or Cytena instruments (see Fig. 3E and Fig. 4E). Notably, single cell seeding efficiencies reported for CellenONE and Cytena rely on images that show the content of the nozzle at the moment of dispensing, which is a good validation considering the difficulty of finding a single isolated cell in a well post-seeding; however, this is not 100% proof of mono-clonality (Yim & Shaw, 2018). The IsoCell instrument yields lower single cell seeding efficiencies (25%-35%) which results in 60-80 chambers out of 256 containing a single cell (Fig. 2D). This lower single cell seeding efficiency is due to the fact that the instrument relies on a Poisson distribution and the experience of the operator for scoring the wells containing the single cells using an inverted microscope. Therefore, with the IsoCell instrument, a higher number of chambers/dishes must be seeded to isolate a desired clone. This is not necessarily a disadvantage of this platform, as the consumable costs are reduced in comparison to CellenONE or Cytena due to the minuscule medium volumes used in the IsoCell from seeding to clone outgrowth.

Critical Parameters

There are several important parameters that influence the efficient derivation of single cell clones from hPSCs. One of the most important parameters is the quality of the hPSC starting culture from which single cell clones are isolated. Preferably, hPSC in log phase of growth at 70%-80% confluency should be used. To keep the cells in log phase, the culture split ratios must be optimized. In most cases, splitting ratios between 1:4 and 1:8 are optimal, and one should standardize the time between passages at 4 and 5 days. However, the optimal split ratio varies between individual cell lines. The confluency and morphology of the cultures should be observed daily. hPSC colonies should have similar sizes and be distributed evenly in the culture vessel. Variability observed between different hPSC lines makes it necessary to adapt several parameters/procedures to achieve optimal sub-cloning outcomes. These parameters/procedures could include adaptation to specific ECM coating (e.g., Laminin 521) and culture medium (e.g., culture in StemFlex medium supplemented with CloneR for some time prior to the dispensing procedure), or obtaining more homogeneous cultures in terms of hPSC colony size and distribution by performing a single cell split 1 or 2 days before automated single cell seeding. We have not exhaustively explored these alternatives; however, preliminary data indicate that further improvement is possible.

Other critical parameters to pay attention to are possible mycoplasma or other microbial contaminations. In general, the growth kinetics of hPSCs infected with mycoplasma are slower and might eventually reduce the single cell survival rate. Mycoplasma testing at regular intervals is highly recommended. Adding antibiotics to the culture medium during critical steps such as open single cell seeding (CellenONE, Cytena) helps to reduce the risk of microbial contamination and does not affect the culture outcome.

An additional critical step in the protocols is the preparation of the single cell suspension

prior to the dispensing procedure. Here, either too-long or too-short enzymatic incubations affect the outcome of single cell sub-cloning experiments. Insufficient enzymatic dissociation leads to cell aggregates in the suspension, which will interfere with the single cell dispensing process. Prolonged enzymatic incubation as well as harsh pipetting lead to increased cellular stress, resulting in a decreased single cell survival rate.

Furthermore, the time between preparation of the single cell suspension and dispensing should kept as short as possible (maximum 15 min) to preserve hPSC viability and quality. Therefore, the materials needed have to be ready for use before cell harvesting. These include solutions, reagents, and target plates; in addition, equipment must be cleaned, sterilized, and primed as described in the corresponding protocols.

Troubleshooting

Common problems the user may encounter, with possible causes and potential solutions, in all three single cell seeding protocols are described in Table 3.

Understanding Results

In the present protocols, we provide a complete automated workflow to efficiently derive hPSC monoclonal cultures using three alternative platforms—IsoCell, CellenONE, and Cytena—dedicated to the single cell seeding step. Each platform (Table 4) has different characteristics that may be more appropriate depending on the laboratory setup (throughput, operator experience, space, GMP, cost, etc.) or application (gene editing, hPSC mosaicism, etc.). Here we describe application examples using the different technologies—the first two examples aim to derive clones with a normal karyotype from a mosaic hPSC culture consisting of mixed hPSC populations of normal cells and cells with karyotypic abnormalities. In a third example, we demonstrate how the genetic stability and genotype composition of hPSC cultures can be analyzed.

Genetic integrity in hPSC cultures is one of the most important quality controls that must be validated before performing other downstream applications. Methods such as genome-wide SNP analysis using microarrays and G-banding karyotyping are employed to assess genetic integrity in hPSC cultures. Both methods have certain technical limitations that result in the non-detection of certain karyotypic abnormalities. SNP arrays, for example, cannot detect balanced chromosomal relocations or aberrations that are under-represented (below 15% of the entire population) in an overall cell population. G-band karyotyping can be more informative in this regard, since it visually identifies metaphase-stage chromosomal defects and is therefore able to detect balanced translocations. However, G-band karyotyping cannot detect loss of heterozygosity (LOH) or abnormalities below 5 MB, and, due to the low number of cells analyzed, does not have a good threshold of detection. Therefore, the combination of both methods can compensate for the shortcomings of each, and makes it possible to detect a wide variety of chromosomal abnormalities in hPSC cultures.

During our routine analysis of the karyotypic stability of our cell lines, we detected a large common duplication of chromosome 20 (Chr20q11, around 5 MB) in the BIHi050-A line by SNP array analysis (Table 5; Markouli et al., 2019). In an attempt to investigate as well as resolve this large copy number variation, we applied the Basic Protocol utilizing the IsoCell platform following a slightly modified version of Support Protocol 1 (preadaption of the hPSCs to Laminin 521 and StemFlex). We established and subsequently analyzed 18 single cell−derived clonal populations. Out of these clones, 16 showed a karyotype comparable to the parental hPSC (Table 5). The detected copy number differences of the clones are in the range of naturally occurring variants within the human population (Liang & Zhang, 2013). Interestingly, within the region duplicated on Chr20q11 are genes like *BCL2* or *DNMT3* having key roles in pluripotency and cell survival (Markouli et al., 2019). This might give a growth advantage to the cells carrying the aberration, and therefore increase the probability of acquiring compromised clones with the subsequent passages.

In a second application, we used Alternate Protocol 1 and the CellenONE technology to derive a clone with a normal karyotype from an hPSC population (BU3 NG hiPSC line) with a mosaicism of a trisomy of chromosome 12 that had been detected by G banding. Even though 3/21 (14%) of the karyograms analyzed by G banding exhibited this trisomy, the aberration could not be detected using SNP array. Therefore, we estimated that the rate of mosaicism was probably below 10%. We isolated several clones and analyzed the karyotype of five clones using SNP arrays (Table 5). None of the clones showed the chromosome 12 trisomy; however, three of them

Table 3 Troubleshooting for Single Cell Isolation and Sub-Cloning of Human Pluripotent Stem Cells

Problem	Possible causes	Potential solutions
Failure in grid formation and/or grid merging (Fig. 2G) (IsoCell)	• Jetting system is not creating adequate pressure to make the grids • Bubbles in jetting system • Bubbles in a dish • Not enough protein content on the dish surface	• Repeat the startup routine or perform a jetting flush routine • Change the jetting system • Lay solutions on the dish carefully • Use fresh coatings (within 24 hr) • Wash plate 2-3 times with StemFlex medium and perform 5-10 min medium incubation at RT before adding FC40^{STAR}
Low number of chambers with a single cell and difficulty in counting the chambers with a single cell (IsoCell)	• Initial density of cell suspension is not optimal • Cell counting varies from lab to lab or person to person • Cell suspension contains clumps • Excessive cell debris • Long incubation after plating • Extra drops or bubbles are seen in the chamber, which confuses the counting	• Optimize the right cell density for plating using 5000, 7500, and 10000 cells/ml • Optimize TrypLE dissociation time for the each hPSC line (range = 7-10 min at RT) • Use 40-μm cell strainer to filter single cell suspensions • Count within 10-20 min after plating • Change the dispense system
Few cells (or none) are transferred to 96-well plate after enzymatic dissociation from chambers(IsoCell)	• Too short enzymatic incubation time	• Monitor cell detachment using the inverted microscope • Increase enzymatic incubation times needed (automatic program can be paused) • Include an initial wash step with 0.5 mM EDTA in DPBS
High cell abortion rate during the run (CellenONE)	• Cell suspension is too concentrated • Isolation parameters are not adequate • Image focus at the nozzle is not correct (Fig. 3F) • Dirt or fluorescent artifacts in the nozzle are detected as cells	• Dilute cell suspension and perform a new mapping • To improve the adequate cell morphology parameters adequate for the present run, perform a new analysis and generate a gate on the main population • Use nozzle off-set tab to adjust the camera focus and save the new parameters • Take a new background image to avoid the identification of background artifacts as cells
Film formation at the tip of the PDC(CellenONE)	• PDC is dirty • Viscosity of the medium interferes with the drop formation	• Perform wash and SciClean tasks • Gently wipe the PDC with a particle-free tissue soaked with ethanol 70% or SciClean solution • Avoid viscous medium or supplements, e.g., DPBS containing Y-27632 can be used
No cells visible in the nozzle during continuous dispensing (CellenONE)	• An aggregate of cells or debris has blocked the PDC • Bubble formation in the PDC • Uptake sample task was not performed	• Use always standby mode to avoid blockings • Flush out 2-3 μl • Perform AirEx task or flush PDC, perform a wash step, and uptake sample (homogenized cell suspension) from the source plate
Printing does not start (Cytena)	• Cartridge is not well aligned • Focus is not set (Fig. 4F)	• Repeat the alignment process • Adjust the focus

(*Continued*)

Table 3 Troubleshooting for Single Cell Isolation and Sub-Cloning of Human Pluripotent Stem Cells, *continued*

Problem	Possible causes	Potential solutions
Low survival rate of single cells (all devices)	• Missing CloneR supplement in the culture medium • Long/harsh dissociations to obtain single cell suspension • Long time between obtaining single cell suspension and dispensing • Too-sensitive cell lines • Crystal structures in grids or plate due to evaporation	• Make sure you add CloneR supplement (1:20) • Keep CloneR in the medium until day 6 of culture • Optimize right dissociation time • Avoid dissociation times longer than 10 min • Avoid high confluency (>80%) or heterogeneous colony size of initial culture • Dispense the cells immediately • Pre-adapt the culture conditions to StemFlex medium containing CloneR and Laminin 521 coated plates • Make sure incubator conditions (humidity and temperature) are correct

Table 4 Automated Single Cell Dispensers' Characteristics and Utilities for Applications on hPSC Field

Aspect/equipment	IsoCell	CellenONE (F1.1/F1.4)	Cytena (c.sight/f.sight)
Training	Half day	3-5 days	1 day
Deposition volume/cell culture volume	Nanoliter/0.2-0.9 µl	Picoliter/60-100 µl	Picoliter/60-100 µl
Embedding in bigger automated platform	No	Yes	Yes
Sample recovery	No	Yes	No
Proof of clonality	In-chamber verification (manually, microscopy)	Before-chamber verification (automated/imaging)	Before-chamber verification (automated/imaging)
Sterility	Yes	Yes	Yes
	(can be place inside cell culture cabinet)	(open system, uses sterilization routine)	(closed system)
Documentation	Manual	Automated (PDF report)	Automated
Mean number of clones generated	20-40	15-30 (96 well)	30-50 (96 well)
	per dish	70-100 (384 well)	
Isolation using fluorescence	No	Yes (up to 4 channels)	Yes (1 channel)
Isolation using morphological parameters	No	Yes	Yes
Equipment maintenance requirements	Low	Demanding	Low
Handling	Easy	Difficult	Moderate
Hardware acquisition cost	Medium	High	High
Consumable cost (per dispensing run)	Low	Low	High

were carrying an additional deletion in chromosome 18, depicting once more the mosaicism prevalent within hPSC bulk populations, as well as the contrasting features of G banding versus SNP array karyotyping analysis.

Reliable large-scale production of Good Manufacturing Practice (GMP)-compliant hPSC banks is a crucial starting point for cell therapies: therefore, it is important to maintain a stable karyotype and homogenous

Table 5 Single Cell Seeding and Clone Derivation Application Examples

Protocol/ technology	Aim	Parental hPSC	Finding/ reference	Cloning efficiency (%)	n° clones analyzed	n° clones with normal karyotype	Conclusion
Basic Protocol (IsoCell)	Clean up karyotype abnormality	BIHi050-A	Chr20q11, large duplication (detected by SNP array)	68	18	16	Reference hPSC showed mosaicism. Clones with a normal karyotype could be isolated by single cell sub-cloning
Alternate Protocol 1 (CellenONE)	Clean up karyotype abnormality	BU3 NG	Chr12 trisomy (detected by G-banding)	21	5	2	Reference hPSC showed mosaicism. Clones with a normal karyotype could be isolated by single cell sub-cloning
Alternate Protocol 2 (Cytena)	Readout of homogeneity and genetic stability under cGMP conditions	BIHi005-A	Parental hPSC cultured in cGMP conditions show normal karyotype	48	3	2	Single cell subcloning and clone genotyping reveals mosaicism in the parental hPSC

cell population under cGMP culture conditions. It is necessary to develop quality control assays that can efficiently and reproducibly monitor growth kinetics, random differentiation, heterogeneity, and chromosomal integrity during either the adaptation to GMP compliant-hPSC culture conditions or large-scale production, processing, and storage of hPSC banks. More quantitative karyotype analysis of derivative single cell sub-clones would provide an excellent readout of homogeneity and genetic stability of hPSCs derived and cultured under GMP compliant conditions. As a proof of principle, we have studied the control cell line BIHi005-A following adaptation to GMP-grade culture conditions (i.e., using GMP-grade reagents), and analyzed sub-clones derived according to Alternate Protocol 2 using Cytena technology. Compared to the parental bulk line, karyotype analysis indicated that two out of three sub-clones were comparable, with a third exhibiting an additional copy-number variation (Table 5). Although the detected aberration in this particular clone is relatively small, below 3 MB in size, it once more shows the latent frequency of mosaicism within a bulk culture of hPSCs, which should be kept in mind for further downstream applications of hPSC-derived clinical products.

Our experimental data reiterate several important points with regard to an automated single cell isolation workflow. First, they highlight hPSC mosaicism; second, the detection threshold and different characteristics of G banding and SNP array karyotype analysis; and finally the ability to maintain, quantify, or even “clean up” the karyotypes of hPSC sub-clonal lines. Importantly, we find that the automated single cell seeding and subsequent clonal expansion described here results in hPSC clones that preserve pluripotency markers and classical colony morphology, as exemplified in Figure 5.

In summary, the protocols presented here utilizing automated single cell seeding and further clonal expansion enable efficient, precise, reproducible, and quality-controlled derivation of monoclonal hPSCs, and can be

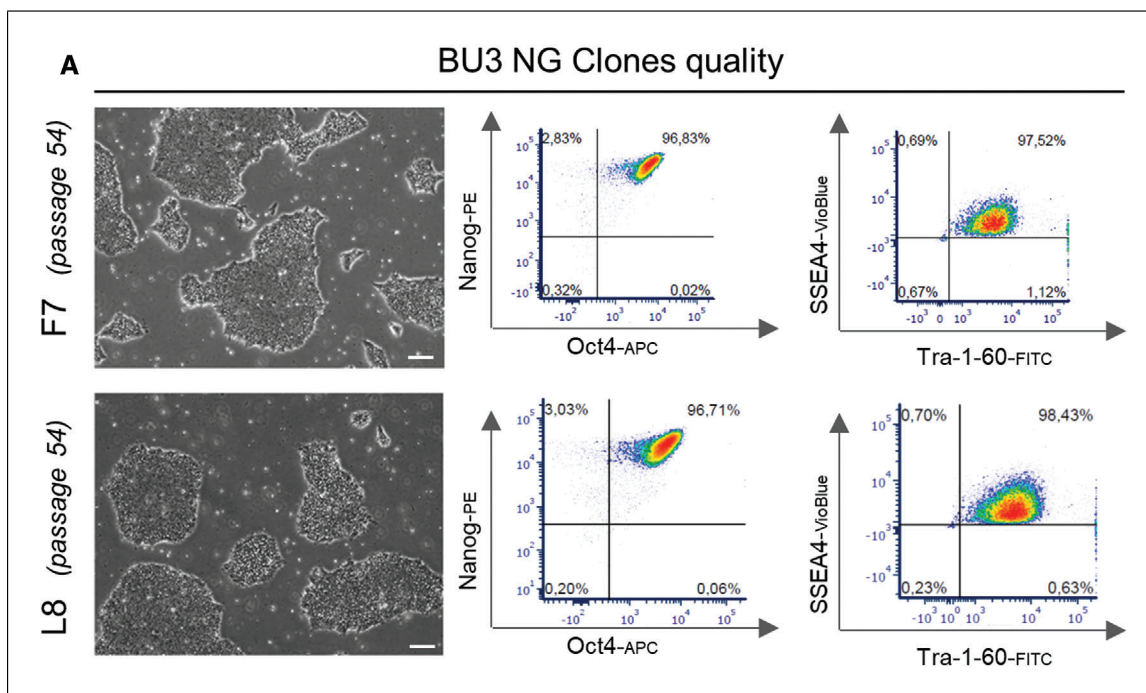


Figure 5 Example data for the validation of hPSC pluripotent morphology and marker expression following automated single cell sub-cloning. (**A**) Pictures showing colonies with typical hPSC morphology of two clones isolated using Alternate Protocol 1 (scale bar: 200 μM). (**B**) FACS analysis showing pluripotent marker expression (Nanog, Oct4, SSEA-4, and Tra-1-60) by almost 100% of the cells in the clones. A Leica DMi8 microscope with phase contrast was used for pictures.

Table 6 Time Considerations

Time required for /equipment	IsoCell	CellenONE (F1.1/F1.4)	Cytena (c.sight/f.sight)
Equipment setup	5-15 min	30-40 min	10-15 min
Single cell seeding	1-2 min per dish	1-2 min per 96-well plate 2-3 min per 384-well plate	2-4 min per 96-well plate
Single cell validation	10-15 min	5-10 min	5-10 min
Feeding routine	4-6 min per dish	10-30 min per plate[a]	10-30 min per plate[a]
Until first clone can be expanded	8-10 days	10-12 days	10-12 days
Clone harvesting	15-20 min (8 clones)	5-7 min per clone	5-7 min per clone

[a]Depends on the number of wells with clonal outgrowth selected.

used for stem cell engineering and future stem cell therapy applications.

Time Considerations

Time considerations for key steps and comparing the different platforms are shown in Table 6.

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Author Contributions

Valeria Fernandez Vallone: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing-original draft. **Narasimha Swamy Telugu:** Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing-original draft. **Iris Fischer:** Conceptualization; data curation; formal analysis; investigation; methodology; software; validation; visualization; writing-review & editing. **Duncan Miller:** Data curation; formal

analysis; investigation; methodology; visualization; writing-review & editing. **Sandra Schommer:** Investigation. **Sebastian Diecke:** Conceptualization; data curation; methodology; project administration; supervision; validation; writing-review & editing. **Harald Stachelscheid:** Conceptualization; Data curation; methodology; project administration; supervision; validation; writing-review & editing.

Literature Cited

Chen, G., Hou, Z., Gulbranson, D. R., & Thomson, J. A. (2010). Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. *Cell Stem Cell*, *7*(2), 240–248. doi: 10.1016/j.stem.2010.06.017.

Chen, Y.-H., & Pruett-Miller, S. M. (2018). Improving single-cell cloning workflow for gene editing in human pluripotent stem cells. *Stem Cell Research*, *31*, 186–192. doi: 10.1016/j.scr.2018.08.003.

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213), 1258096. doi: 10.1126/science.1258096.

Higuchi, A., Kao, S.-H., Ling, Q.-D., Chen, Y.-M., Li, H.-F., Alarfaj, A. A., … Umezawa, A. (2016). Long-term xeno-free culture of human pluripotent stem cells on hydrogels with optimal elasticity. *Scientific Reports*, *5*(1), 18136. doi: 10.1038/srep18136.

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, *157*(6), 1262–1278. doi: 10.1016/j.cell.2014.05.010.

Liang, G., & Zhang, Y. (2013). Genetic and epigenetic variations in iPSCs: Potential causes and implications for application. *Cell Stem Cell*, *13*(2), 149–159. doi: 10.1016/j.stem.2013.07.001.

Markouli, C., Couvreu De Deckersberg, E., Regin, M., Nguyen, H. T., Zambelli, F., Keller, A., … Spits, C. (2019). Gain of 20q11.21 in human pluripotent stem cells impairs TGF-β-dependent neuroectodermal commitment. *Stem Cell Reports*, *13*(1), 163–176. doi: 10.1016/j.stemcr.2019.05.005.

Phelan, K., & May, K. M. (2015). Basic techniques in mammalian cell culture. *Current Protocols in Cell Biology*, *66*, 1.1.1–1.1.22. doi: 10.1002/0471143030.cb0101s66.

Pijuan-Galitó, S., Tamm, C., Schuster, J., Sobol, M., Forsberg, L., Merry, C. L. R., & Annerén, C. (2016). Human serum-derived protein removes the need for coating in defined human pluripotent stem cell culture. *Nature Communications*, *7*(1), 12170. doi: 10.1038/ncomms12170.

Rodin, S., Antonsson, L., Hovatta, O., & Tryggvason, K. (2014). Monolayer culturing and cloning of human pluripotent stem cells on laminin-521−based matrices under xeno-free and chemically defined conditions. *Nature Protocols*, *9*(10), 2354–2368. doi: 10.1038/nprot.2014.159.

Rossant, J., & Tam, P. P. L. (2017). New insights into early human development: Lessons for stem cell derivation and differentiation. *Cell Stem Cell*, *20*(1), 18–28. doi: 10.1016/j.stem.2016.12.004.

Singh, A. M. (2019). An efficient protocol for single-cell cloning human pluripotent stem cells. *Frontiers in Cell and Developmental Biology*, *7*, 11. doi: 10.3389/fcell.2019.00011.

Soitu, C., Feuerborn, A., Tan, A. N., Walker, H., Walsh, P. A., Castrejón-Pita, A. A., … Walsh, E. J. (2018). Microfluidic chambers using fluid walls for cell biology. *Proceedings of the National Academy of Sciences*, *115*(26), E5926–E5933. doi: 10.1073/pnas.1805449115.

Soitu, C., Stovall-Kurtz, N., Deroy, C., Castrejón-Pita, A. A., Cook, P. R., & Walsh, E. J. (2020). Jet-printing microfluidic devices on demand [Preprint]. *Bioengineering*. doi: 10.1101/2020.05.31.126300.

Valamehr, B., Abujarour, R., Robinson, M., Le, T., Robbins, D., Shoemaker, D., & Flynn, P. (2012). A novel platform to enable the high-throughput derivation and characterization of feeder-free human iPSCs. *Scientific Reports*, *2*(1), 213. doi: 10.1038/srep00213.

Walsh, E. J., Feuerborn, A., Wheeler, J. H. R., Tan, A. N., Durham, W. M., Foster, K. R., & Cook, P. R. (2017). Microfluidics with fluid walls. *Nature Communications*, *8*(1), 816. doi: 10.1038/s41467-017-00846-4.

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., … Sasai, Y. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology*, *25*(6), 681–686. doi: 10.1038/nbt1310.

Wu, J., & Izpisua Belmonte, J. C. (2015). Dynamic pluripotent stem cell states and their applications. *Cell Stem Cell*, *17*(5), 509–525. doi: 10.1016/j.stem.2015.10.009.

Yamanaka, S. (2012). Induced pluripotent stem cells: Past, present, and future. *Cell Stem Cell*, *10*(6), 678–684. doi: 10.1016/j.stem.2012.05.005.

Yang, L., Guell, M., Byrne, S., Yang, J. L., De Los Angeles, A., Mali, P., … Church, G. (2013). Optimization of scarless human stem cell genome editing. *Nucleic Acids Research*, *41*(19), 9049–9061. doi: 10.1093/nar/gkt555.

Yim, M., & Shaw, D. (2018). Achieving greater efficiency and higher confidence in single-cell cloning by combining cell printing and plate imaging technologies. *Biotechnology Progress*, *34*(6), 1454–1459. doi: 10.1002/btpr.2698.

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