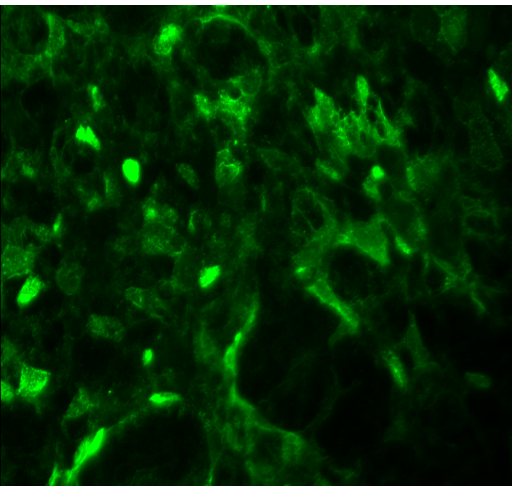
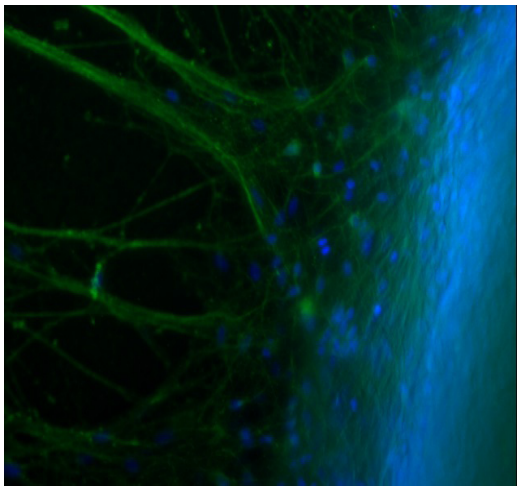
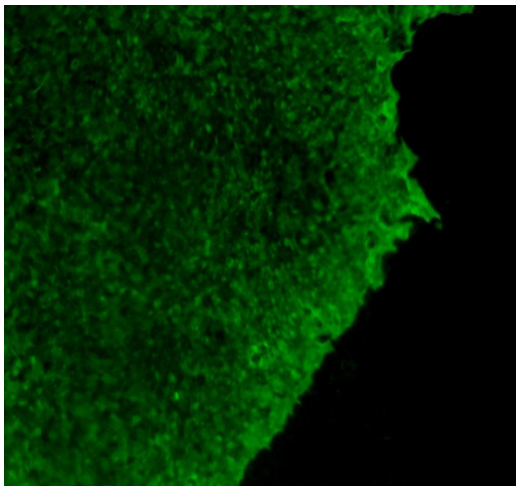
Cellular Engineering Technologies, Inc.

# Guide to Induced Pluripotent Stem Cell Culture

User Manual

2500 Crosspark Road
E110
Coralville, IA 52241
Phone: 319-665-3000
Email: orders@celleng-tech.com

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## Section 1: Transfecting and Reprogramming Target Cells

Cellular Engineering Technologies (CET) recommends transfecting and reprogramming target cells using our episomal reprogramming kit. CET's Episomal Reprogramming Kit contains a proprietary mix of vectors necessary to reprogram target cells into induced pluripotent stem cells (iPS). These vectors include the Yamanaka factors (Oct-4, Sox-2, Klf-4) along with p53 anti-sense, EBNA-1 and Red Fluorescent Protein (RFP). Vectors are optimized, pre-mixed and ready for transfection.
Section 1.1: Required Reagents, Cells and Media

1.1.1 Target Cells to be reprogrammed. CET recommends actively growing fibroblasts of low passage number or the nucleated fraction of whole blood. (Various Vendors)

1.1.2 Electroporator and Associated Equipment. This protocol is optimized for the Neon Electroporation System sold by ThermoFisher Scientific. You will have to optimize electroporation parameters for your own electroporator, which maximizes cell transfection while minimizing cell death. (ThermoFisher Scientific)

1.1.3 Lipofectamine 3000 (Optional). Although an electroporator is strongly recommended, especially for suspension cells, Lipofectamine 3000 may be used for adherent cells which are amenable to transfection. (Refer to manufacturer's directions to optimize for your own applications.) (ThermoFisher Scientific)

1.1.4 Target Cell Growth Media. Use the appropriate growth media specific to the target cell to grow and maintain target cell growth. (Various Vendors)

1.1.5 CET's Episomal Reprogramming Kit (CET.IPS.ERK-01)

1.1.6 CET's iPS Reprogramming Base Media (CET.IPS.GMK-500)

1.1.7 CET's iPS Reprogramming Supplement (CET.IPS.GMK-500)

**Section 1.2: Preparation of Target Cells for Transfection**

1.2.1 *Adherent Cells*
Examine target cells under microscope. If you are using fibroblasts, make sure the cells are actively growing in a logarithmic phase and are approximately 80% confluent. Do not use cells that are over-confluent or slow growing.

*Suspension Cells*
Examine target cells under microscope to ensure cell viability and cell growth. Make sure that cell density is at least $1x10_6$ cells/mL.

1.2.2 *Adherent Cells*
For each target cell type to be transfected, coat 2 wells of a 6-well tissue culture dish with Geltrex (Refer to manufacturer's directions). This step is to be completed 48 hours prior to transfection.

*Suspension Cells*
For each suspension cell type to be transfected, coat 1 well of a 6-well tissue culture dish with Matrigel (Refer to manufacturer's directions). This step is to be completed 48 hours prior to transfection.

**Section 1.3: Transfection of Target Cells**

1.3.1 *Adherent Cells*
Withdraw media and wash target cells once with Dulbecco's Phosphate Buffered Saline.
Add the appropriate amount of 0.25% Trypsin-EDTA. Incubate for 3-4 minutes in the incubator. When the cells are no longer adherent, add an equal volume of serum containing growth media. Make sure media contains NO antibiotics/antifungals. Count cells and adjust the density to 1x106 cells/mL.

*Suspension Cells*
Withdraw media containing suspension cells and pipette into a 15 mL conical. Count cells and adjust the density to $1x10_6$ cells/mL.

1.3.2 *Adherent and Suspension Cells*
Spin cells to pellet at 200 X G for 5 minutes in a swing bucket rotor.

1.3.3 *Adherent Cells*
Resuspend cell pellet in 100 microliters of Neon Electroporation Buffer R (ThermoFisher Scientific). Add 6 microliters of CET's Episomal Reprogramming Mix to the tube. This is approximately 3 micrograms of DNA. Mix gently using a micropipettor.

*Suspension Cells*
Resuspend cell pellet in 100 microliters of Neon Electroporation Buffer S (ThermoFisher Scientific). Add 6 microliters of CET's Episomal Reprogramming Mix to the tube. This is approximately 3 micrograms of DNA.

1.3.4 *Adherent and Suspension Cells*
Using a Neon Electroporation Tip-100 (ThermoFisher Scientific), introduce cells and DNA. Using Electroporation Buffer E2 (ThermoFisher Scientific) for the chamber buffer, electroporate the cells at 1650 V for 10 milliseconds for 3 cycles.

1.3.5 *Adherent Cells*
Immediately after electroporation, place the transfected cells in the original target cell's growth media on the Geltrex coated 6 well dish. It is critical that this media contains NO antibiotics/antifungals for the first 24 hours.

*Suspension Cells*
Immediately after electroporation, place the transfected cells in the original target cell's growth media on the Matrigel coated 6 well dish. It is critical that this media contains NO antibiotics/antifungals for the first 24 hours.

**Section 1.4: Reprogramming Transfected Cells**

1.4.1 *Adherent Cells*
At the end of 24 hours, withdraw the target cell growth media and conduct a complete media replacement with CET's iPS Reprogramming Media. CET recommends 3 mL of media per well of a 6 well tissue culture dish. At this time, the use of antibiotics/antifungals will not affect cell viability. Although exogenous gene expression should start within 12 hours, robust gene expression can be detected by RFP fluorescence at the end of 48 hours.

*Suspension Cells*
It will take approximately 72 hours post-electroporation for suspension cells to settle and become adherent. Therefore, it is critical NOT to aspirate suspension cells while conducting a media change. At the end of 24 hours, gently tilt the plate so cells settle to the bottom. Using a serological pipette, gently withdraw one half of the volume of the target cell growth media. Replace with a half volume of CET iPS Reprogramming Media.

1.4.2 *Adherent and Suspension Cells*
Perform a full CET iPS Reprogramming Media replacement every 48 hours. Suspension cells should be adherent at this point. All cells should start becoming more cuboidal or epithelial in appearance and start forming small, putative colonies. Repeat this step through day 14 of the reprogramming process.

## Section 2: Growing and Maintaining IPS Cells

iPS cells are fastidious cells, require regular maintenance and should only be grown by users who have extensive tissue culture experience. Instructions should be carefully followed or cells risk differentiation, slow growth or cell death. These are irreversible events and will require the investigator to begin the transfection process again.

**Section 2.1: Required Reagents, Cells, and Media**

2.1.1 CET iPS Growth Base Media (CET.IPS.GMK-500)

2.1.2 CET iPS Growth Supplement (CET.IPS.GMK-500)

**Section 2.2: Growing and Maintaining iPS Cells**

2.2.1 *Adherent and Suspension Cells*
Day 14 onwards, perform a full media replacement with CET's Complete iPS Growth Media every 48 hours. Although it is difficult to predict when mature IPS colonies will emerge, this process should take approximately 17 days post-electroporation. Monitor the iPS colonies daily. Mature iPS colonies are ready to be passaged when they have sharp, distinct edges.

2.2.2 From this point forward, once iPS colonies have formed, adherent and suspension colonies will be cultured and grown in the same manner.

## Section 3: Passaging iPS Cells

It is critical to passage iPS cells at regular intervals using good technique. Delays in passaging can result in differentiated iPS colonies, senescent cells or contamination of the culture. While there are two techniques used in iPS literature, CET recommends using enzymatic passaging if your iPS culture has less than 15% differentiated colonies. If your IPS culture has more than 15% differentiated colonies, then CET recommends that you use the "pick to keep" technique so that you can reduce or eliminate differentiated colonies. Desired undifferentiated colonies have sharp, distinct borders (Figure 1 of Appendix). Differentiated colonies have irregular borders that are not sharp or have interior voids that do not contain adjacent cells (Figure 2 of Appendix). These colonies must be removed.

### Section 3.1: Required Reagents, Cells and Media (Enzymatic Passaging)

3.1.1 Vitronectin XF Coating Matrix (StemCell Technologies)

3.1.2 Cell Adhere Dilution Buffer (StemCell Technologies)

3.1.3 CET iPS Growth Base Media (CET.IPS.GMK-500)

3.1.4 CET iPS Growth Supplement (CET.IPS.GMK-500)

3.1.5 Y-27632 Rock Kinase Inhibitor (Various Vendors)

3.1.6 D-PBS without Calcium and Magnesium (Various Vendors)

3.1.7 CET iPS Cell Passaging Solution (CET.IPS.PASS-100)

### Section 3.2: Enzymatic Passaging of IPS Colonies

3.2.1 A day prior to passaging iPS cells, 6 well dishes must be coated with Vitronectin XF (See manufacturer's instructions).

3.2.2 An hour and a half before passaging, aspirate off Vitronectin XF and wash each well with Cell Adhere Dilution Buffer. Aspirate off the buffer and replace with an appropriate amount of CET's Complete iPS Growth Media that contains 10 micromolar Y-27632 (final concentration).

3.2.3 Examine the iPS colonies to be passaged visually under a microscope. Colonies should have distinct border and should be uniform. If you have non-uniform colonies, determine the percent of these colonies. It should be less than 15%. If it is greater than 15%, proceed to Sections 3.3 and 3.4.

3.2.4 Aspirate all of CET's Complete iPS Growth Media and wash 1X with pre-warmed D-PBS by gently swirling the plate. Be extremely careful when washing since colonies will be loosely adherent and will detach if the washing is too rigorous. CET recommends using 5 mL D-PBS per 100 mm dish or 2 mL per well of a 6-well tissue culture dish.

3.2.5 Immediately aspirate D-PBS and add CET's iPS Passaging Solution. We recommend using 2 mL per well of a 6 well tissue culture dish and 5 mL per 100 mm dish. Do not let the plates or wells dry. Return the plates to an incubator at 37°C, 5% $CO_2$, and 95% humidity. Monitor colonies under a microscope at 5X magnification every 5 minutes and return to the incubator. iPS colonies will begin to curl. When the outer edge of the colony is fully curled, they are ready.

3.2.6 In a laminar flow hood, carefully aspirate the passaging solution and cell debris. It is natural for some iPS colonies to detach and get aspirated with the cell debris.

3.2.7 Immediately add a sufficient amount of CET's Complete iPS Growth Media that contains 10 micromolar Y-27632 (final concentration). Dislodge the colonies by pipetting media repeatedly but do so in a gentle manner. Collect media and colonies and pipette into a 15 mL conical tube.

3.2.8 Spin the colonies at 200 X G for 5 minutes in a swing bucket rotor. Colonies will settle to the bottom but the pellet will be loose so do not bump or shake the tube. Aspirate the supernatant. Do not let the pellet dry.

3.2.9 Resuspend the colonies in 1 mL of CET's Complete IPS Growth Media. Do so by gently using a 1,000 microliter micropipette tip and pipetting 5 times.

3.2.10 At this point, the colonies are ready to be plated. CET recommends a passage ratio of 1:6 at the most or the iPS cells will differentiate.

3.2.11 iPS cells should be left undisturbed in an incubator at 37°C, 5% $CO_2$, and 95% humidity for 24 hours to allow the cells to attach.

3.2.12 At the end of 24 hours, perform a complete media replacement and feed iPS cells with pre-warmed CET Complete iPS Growth Media. CET recommends 10 mL per 100 mm dish and 2 mL per well of a 6 well tissue culture dish.

3.2.13 Monitor iPS colony growth by microscopy. Colonies will take 3-4 days to be easily visualized. Continue to perform a complete media replacement with CET's Complete iPS Growth Media every 24 hours.

3.2.14 iPS cells are ready for passaging when the media color turns yellow, indicating an increase in metabolites and a drop in pH. Usually this occurs around 6-8 days depending on the plating density. Waiting longer than this time period risks colony differentiation and loss of the iPS cell line.

**Section 3.3: Required Equipment, Reagents and Media ("Pick to Keep" Passaging Method)**

3.3.1 Dissecting Microscope with 5X magnification

3.3.2 20-200 microliter micropipettor and sterile micropipette tips

3.3.3 Sterile 1.5 mL microcentrifuge tubes

3.3.4 CET iPS Growth Base Media (CET.IPS.GMK-500)

3.3.5 CET iPS Growth Supplement (CET.IPS.GMK-500)

3.3.6 Y-27632 Rock Kinase Inhibitor (Various Vendors)

3.3.7 Microcentrifuge tube rack

**Section 3.4: Mechanical Passaging of iPS Colonies: "Pick to Keep" Method**

3.4.1 Since you will be working in a laminar flow hood and not an incubator, perform these steps as quickly as possible since iPS cells are not viable at room temperature for extended periods of time. Also, steps should be performed with sterility in mind, as you will have to open tissue culture lids to isolate the colonies.

3.4.2 Examine dishes to be mechanically passaged. Using a dissecting microscrope and 5X magnification, select colonies with distinct borders and cells that have a larger nucleus to cytoplasm ratio (Figure 1 of Appendix). Use a sharpie or marker to mark the borders of the colonies to be picked.

3.4.3 Place the dish with the colonies to be picked under the oculars of the microscope and fasten it with double sided tape or a plate/dish holder designed for holding dishes securely. Do not aspirate the media.

3.4.4 Place a sterile 20-200 microliter pipette tip on a 20-200 micropipettor. Depress the plunger handle on the micropipettor before introducing the tip to the plate. Gently suck the colony up and place in a 1.5 mL microcentrifuge tube that contains 1 mL of pre-warmed CET Complete iPS Growth Media containing 10 micromolar Y-27632 (final concentration).

3.4.5 Replate individual colonies on previously coated Vitronectin XF coated plates (1 well of a 6 well dish). Pick at least 12 colonies and expand each individually. Passage only the colonies that show iPS morphology and appropriate growth characteristics.

## Section 4: Freezing and Recovering iPS Cells

It is imperative to properly cryopreserve and thaw out iPS cells. Improper freeze/thaw of cells will lead to a marked loss in viability and potential loss of the cell line. CET strongly recommends that you keep back up plates or dishes of the cell line you plan to freeze in case you experience problems.

### Section 4.1: Required Reagents, Cells and Media

4.1.1 CET iPS Growth Base Media (CET.IPS.GMK-500)

4.1.2 CET iPS Growth Supplement (CET.IPS.GMK-500)

4.1.3 D-PBS without Calcium and Magnesium (Various Vendors)

4.1.4 CET iPS Cell Passaging Solution (CET.IPS.PASS-100)

4.1.5 Y-27632 Rock Kinase Inhibitor (Various Vendors)

4.1.6 CET iPS Freezing Medium A (CET.IPS.FZ-200)

4.1.7 CET iPS Freezing Medium B (CET.IPS.FZ-200)

### Section 4.2: Cryopreserving iPS Cells

4.2.1 Remove the iPS cells from the incubator and aspirate off the media.

4.2.2 Replace the media with CET's Complete iPS Growth Media that contains 10 micromolar Y-27632 (final concentration). CET recommends 10 mL per 100 mm tissue culture dish and 2 mL per well of a 6 well tissue culture dish. Return cells to a tissue culture incubator and incubate for 1 hour.

4.2.3 Aspirate the media and wash cells once with D-PBS. Aspirate the D-PBS, working quickly so the cells do not dry.

4.2.4 Add CET iPS Passaging Solution and follow the instructions listed above for passaging IPS cells.

4.2.5 Once the iPS cells are in CET's Complete iPS Growth Media, centrifuge the cells at 200 X g for 5 minutes.

4.2.6 Carefully aspirate the supernatant without disturbing the cell pellet.

4.2.7 Prepare a sufficient quantity of cryovials.

4.2.8 Gently flick the tube to fully dislodge the cell pellet from the bottom of the tube and resuspend the cells in CET's iPS Freezing Medium A by gently pipetting up and down using a 5 mL serological pipette while simultaneously gently agitating the tube back and forth. Following uniform suspension of cell clumps, add equal volume of CET's iPS Freezing Medium B in a drop wise manner, while gently swirling the cell suspension to mix. At this point, the cells are in contact with DMSO and work should be performed quickly and efficiently. Once cells are in contact with DMSO they should be aliquoted and frozen within 2-3 minutes.

4.2.9 Aliquot 1 mL of the cell suspension into each cryovial.

4.2.10 Quickly place the cryovials in a Mr. Frosty and transfer to a -80°C freezer overnight.

4.2.11 After overnight storage at -80°C, transfer the cells to a liquid nitrogen tank for long-term storage.

**Section 4.3: iPS Vial Thaw and Cell Recovery**

4.3.1 Carefully remove the iPS cell vial from the liquid nitrogen storage tank or dry ice.

4.3.2 Rapidly thaw the frozen vial of cells by immersing the vial in a 37°C water bath without submerging the cap. Swirl the vial gently. The vial is considered to be thawed when 80% of the contents of the vial are liquid and there is a small ice pellet remaining. Spray the outside of the vial with reagent grade alcohol and introduce into the laminar flow hood.

4.3.3 Aseptically transfer the entire contents of the vial into a 15 mL conical tube using a 1000 microliter micropipettor.

4.3.4 Rinse the vial with 1 mL of pre-warmed CET Complete iPS Growth Media and add this to the 15 mL conical tube.

4.3.5 Slowly add 8 mL of pre-warmed CET Complete iPS Growth Media drop wise to the cells in the 15 mL conical tube. While adding the medium, gently move the tube back and forth to mix the cells. (This reduces the osmotic shock to the cells.)

4.3.6 Centrifuge the 15 mL tube with the cell suspension at 200 X g for 5 minutes at room temperature.

4.3.7 Carefully aspirate and discard the supernatant without disturbing the cell pellet.

4.3.8 Resuspend the cell pellet in pre-warmed CET Complete iPS Growth Media containing 10 micromolar Y-27632 (final concentration) using a 5 mL pipette and gently pipette the cells up and down until the cell pellet is fully dispersed but still as clumps. Plate on previously Vitronectin XF coated plates.

4.3.9 Return cells to a tissue culture incubator and incubate at 37°C, 5% $CO_2$, and 95% humidity.

4.3.10 Gently perform a complete media replacement 24 hours post-thaw and every 24 hours thereafter with CET's Complete iPS Growth Media to remove cell debris and to provide fresh nutrients until the dish is 70-80% confluent. For continued culture and passaging of iPS cells, refer to Section 3.

## Appendix: Figures

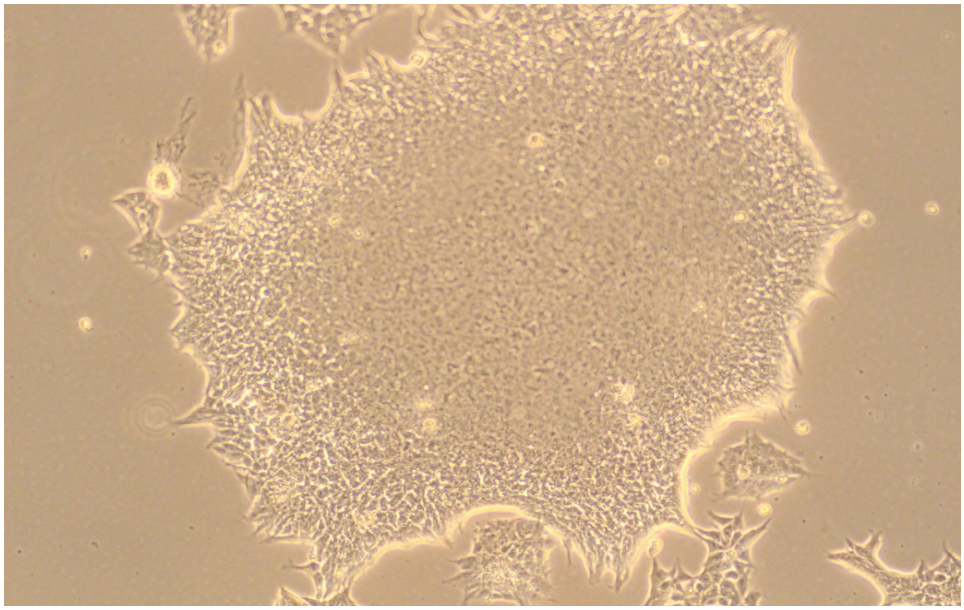

**Figure 1:** Figure represents a mature iPS colony growing on Vitronectin XF coated plate in CET's Complete iPS Growth Media.

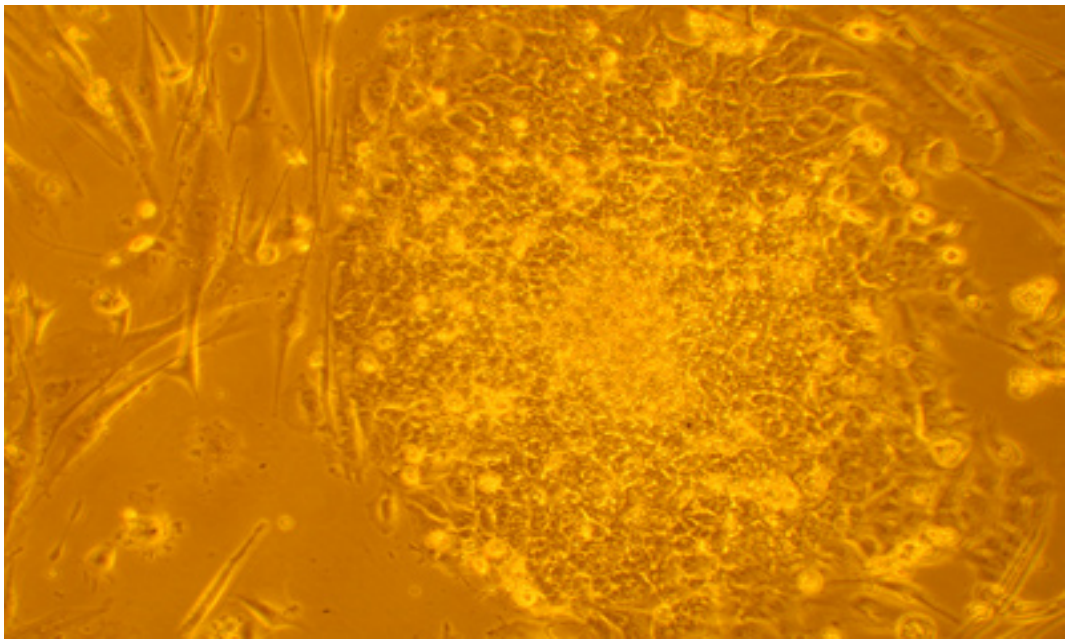

**Figure 2:** Figure represents an example of a differentiated iPS colony that needs to be avoided during the "Pick to Keep" passaging method. Notice the irregular borders, gaps between the cells and heterogeneous cellular morphology.

### Warranty and Disclaimer

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