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AlphaSTEM™ Protocol

INTRODUCTION

Minerva Biotechnologies is the first company to generate human naïve state induced pluripotent stem cells (iPSCs) using a single, naturally occurring human stem cell growth factor, $NME7_{AB}$. Naïve stem cells have several advantages over current stem cells, called 'primed' state cells. Scientists believe that because these earlier stem cells have a "clean slate", they are more easily directed to develop into functional mature cells. Using Minerva's AlphaSTEM™ Media System, iPSC generation is simpler and 50 times more efficient, cell expansion is faster and scalable, and differentiation efficiency is increased with the resulting cells having enhanced functionality.

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KEYS TO SUCCESS – PLEASE READ BEFORE STARTING

- AlphaSTEM™ Naive hPSC Medium media does not contain FGF2 and is not compatible with FGF2 media.
- Culture conditions: Hypoxic incubator (37°C, 5% CO_2, 5% O_2).
- Rho kinase I inhibitor
- AlphaSTEM™ Naive hPSC Medium is used with AlphaSTEM™ Culture Substrate coated plates. The medium can be used with other surfaces such as MEFs and Matrigel®; However, growth over these and other surfaces has been shown to compromise naïve characteristics of the resultant cells.
- The AlphaSTEM™ Culture System uses TrypLE™ dissociated single cells which grow in a uniform monolayer.
- Passage cells at 75-85% confluence (~3-4 days). Do not overgrow the cells.
- When first working with naïve cells, it is ideal to have existing naïve stem cells to use as a reference/control. Minerva offers both male and female naïve stem cells lines.

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MATERIALS NEEDED

PRODUCT	SUPPLIER	CATALOG #
TrypLE™ Express Enzyme (1X), no phenol red	Thermo Fisher Scientific	12604021
Gibco™ 2-Mercaptoethanol	Thermo Fisher Scientific	21985-023
MEM Non-Essential Amino Acids (100X)	Thermo Fisher Scientific	11140-050
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190250
Nunc™ Nunclon™ Vita 6-Well Multidish or Falcon® 6-Well TC-Treated Cell Culture Plate	Thermo Fisher Scientific Corning	145380 or 353046
Rho kinase I inhibitor Y-27632 2HCl	Selleck Chemicals	S1049
AlphaSTEM™ Naïve hPSC Medium	Minerva Biotechnologies	MN01500
AlphaSTEM™ Culture Substrate	Minerva Biotechnologies	MC01360
AlphaSTEM™ Differentiation Inducer	Minerva Biotechnologies	MD00312
AlphaSTEM™ Naïve iPSC Line, Male or AlphaSTEM™ Naïve iPSC Line, Female	Minerva Biotechnologies Minerva Biotechnologies	MSM0001 or MSF0001

CULTURING NAIVE STEMS CELLS IN THE ALPHASTEM™ CULTURE SYSTEM

Note: This section only applies to culturing AlphaSTEM™ Naïve iPSC Lines or cells already adapted to the AlphaSTEM™ Culture System. For adapting FGF grown lines see the next section.

1. AlphaSTEM™ Naive hPSC Medium:
    a. Aseptically remove 50ml of AlphaSTEM™ Basal Medium.
    b. To 450ml of AlphaSTEM™ Basal Medium, add 90ml AlphaSTEM™ Supplement 1, 5.4mL 100X Non-Essential Amino Acids, and 990uL of 55mM Beta-Mercaptoethanol.
    c. To the 546 mL, add (1) 110 uL aliquot of AlphaSTEM™ Supplement 2 and a Rho kinase I inhibitor (Y-27632) to a final concentration of 10uM.
2. Prepare plates or flasks at least 1 day prior to plating cells. Coat plates or flasks with 1X AlphaSTEM™ Culture Substrate (1.5mL per well of a 6 well plate diluted 1:100 in PBS minus calcium, minus magnesium), parafilm wrap and incubate at 4°C overnight; no rinse required prior to plating cells. Pre-coated plates can be stored at 4°C for up to 2 weeks before use.
3. Thaw a 1 mL aliquot of AlphaSTEM™ cultured cells (1M single cells) by placing cells in a 37°C water bath for one to two minutes.
4. When thawed, transfer suspension to a 50mL centrifuge tube and rinse cryovial with 1mL pre-warmed AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor.
5. Slowly add (drop-wise) 8 mL pre-warmed AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor at 37°C to the thawed cells for a total of 10mL in the same 50 mL centrifuge tube.
6. Centrifuge at 250 x g, 5 minutes to rid cells of DMSO used for freezing. Using a p1000, gently resuspend cells in 1 mL AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor. Add another 2 mL pre-warmed AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor, to bring the final volume to 3 mL.
7. Aspirate excess AlphaSTEM™ Culture Substrate solution from 1 well of the coated 6-well plate and immediately replace with the 3 mL cell suspension using a 5mL serological pipette.
8. Rock plates to distribute single cells evenly - this requires a rocking forward motion followed by a pause, then rocking a backing motion followed by a pause; do this several times. Leave plates undisturbed for 48 hours in a hypoxic incubator (37°C, 5% CO_2, 5% O_2).
9. After first 48 hours, change media daily and reduce media volume to 2 mL per well. Media changes must be as gentle as possible. Gently aspirate off old media and replace with 2 mL fresh AlphaSTEM™ Medium containing 10 µM Rho kinase I inhibitor.
10. Passage cells at 75-85% confluence (~3-4 days).
    --DO NOT OVERGROW THE CELLS--

11. Cells will grow as a monolayer and do not form colonies (See Figure 1).
12. To harvest, aspirate media and wash wells with 2mL PBS minus calcium, minus magnesium. Dissociate cells using 1 mL TrypLE (~3-4 min. at 37°C) and neutralize with 1 mL of AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor per well. Collect 2 mL of cell suspension and transfer to a 15 mL centrifuge tube. Collect remaining cells by further washing the well with 1 mL AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor and transfer to same 15 mL centrifuge tube.
13. Centrifuge at 250 x g, 5 minutes. Resuspend in 1 mL AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor.
14. Harvested cells should be seeded onto fresh AlphaSTEM™ Culture Substrate coated plates at a density of 100,000 cells per well of a 6-well plate (10,000 cells/cm^2) in 3 mL of AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor.
    a. Count cells using a hemacytometer or an automated cell counter.
    b. Calculate the volume of cell suspension that contains 100,000 cells;
    c. Calculate the volume of media required for the desired number of wells;
    d. Resuspend cells in the calculated volume of media to give 100,000 cells per 3 mL media per well of a 6-well plate.
        i. *Note* - TrypLE™ will produce a mixture of single cells and clumps of 3-6 cells each; this mixture is fine for seeding new plates. However, cell clumps will make cell counting inaccurate. For accurate cell counts, withdraw 10 uL of cell suspension and add to 10 uL of Trypan Blue and vigorously pipet up and down with a P10 pipet tip until single cell suspension is attained. Use the single cell suspension for cell counts.
15. On room temperature AlphaSTEM™ Culture Substrate coated plates, aspirate AlphaSTEM™ Culture Substrate solution from wells and seed 3 mL of cell suspension per well of a 6-well plate (10,000 cells/cm^2 =100,000 cells per well of a 6-well plate). Rock plates to distribute single cells evenly - this requires a rocking forward motion followed by a pause, then a rocking backing motion followed by a pause; do this several times. Leave plates undisturbed for 48 hours in a hypoxic incubator (37°C, 5% CO_2, 5% O_2).
16. Repeat steps 12 through 15, expanding cells with each passage. Expect 10-20-fold expansion in 4 days after the cells have been cultured for two passages. However, pass cells when between 75-85% confluent, which typically happens on Day 3. Letting cell become overly confluent could lead to differentiation and/or karyotype abnormalities (See Figure 2).
17. If a large number of cells is required, see Table 1:

Table 1.

Plate/Flask	AlphaSTEM™ Substrate Vol. (mL)	Number Cells to Plate (10^6)	Expected Yield (10^6)
6-well plate	2/well	0.1	1.0
T-75	10	2	20
T-175	20	5	45
T-175 5-layer	100	20	125

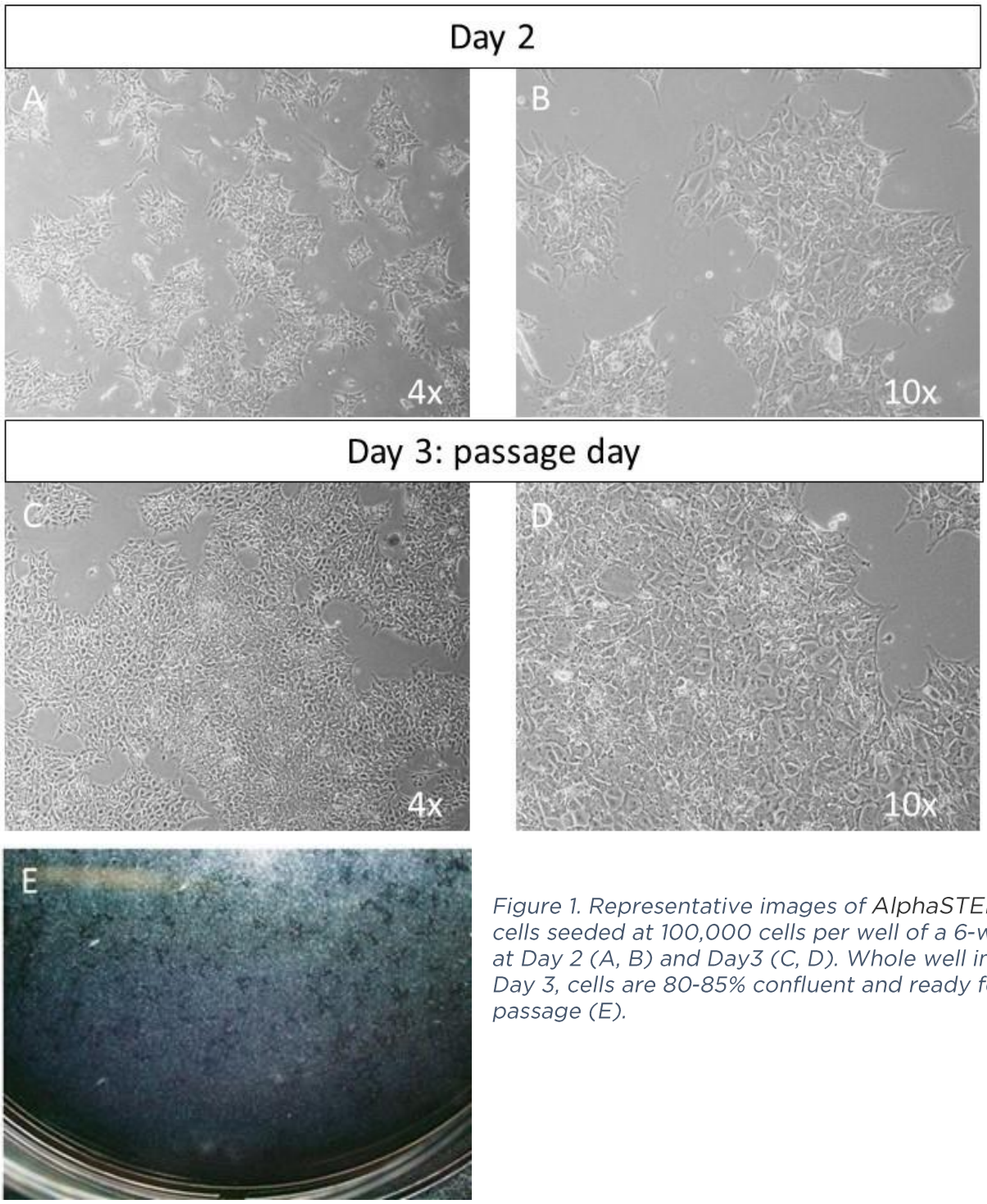

Figure 1. Representative images of AlphaSTEM™ grown cells seeded at 100,000 cells per well of a 6-well plate at Day 2 (A, B) and Day3 (C, D). Whole well image at Day 3, cells are 80-85% confluent and ready for passage (E).

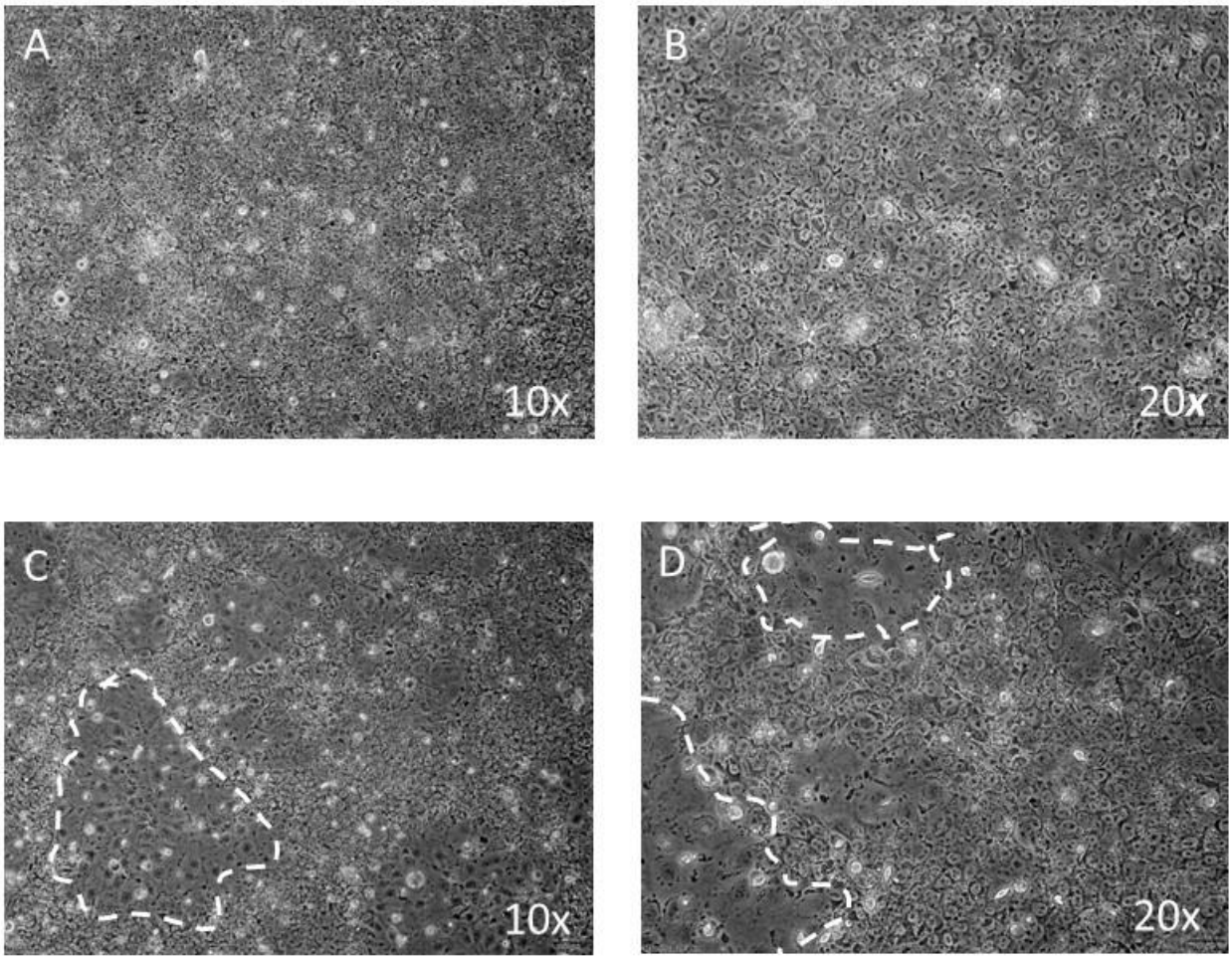

Figure 2. Cells should not be allowed to get over-confluent (A, B), otherwise, will lead to spontaneous differentiation (C, D). Areas indicated by the dashed line in C, D show spontaneous differentiation, which is characterized by large flat cells, with large black nuclei.

TRANSITIONING CELLS FROM FGF2 TO THE ALPHASTEM™ CULTURE SYSTEM

Stem cells previously cultured in an FGF2-based media can be transitioned to AlphaSTEM™ Culture System by two different methods:

1. Method 1: Serial passaging
   Serial passaging takes about 4 passages before cells grow without differentiation and have a consistent 3-4 day passage schedule; 6-10 passages before 2nd X chromosome is re-activated and other characteristics of naïve cells are acquired. In anticipation of cell loss during transition period, the initial seeding density of cells is higher than usual and then stepped down in each successive passage.
2. Method 2: Sub-cloning
   Sub-cloning is a selection technique. Very low numbers of single cells are plated into AlphaSTEM™ Culture Substrate coated 96-well plates and only those cells that are readily able to adapt to the AlphaSTEM™ Culture System (~50%) survive. Within 5-10 days, growing stem cell clusters can be selected and passed onto larger wells. Within ~4 weeks selected "clones" will be at the 6-well plate stage and will have 2nd X chromosome re-activated and will also possess other naïve cell characteristics.

During the transition period, there will be cell loss. In addition to loss due to adjusting to new media, the AlphaSTEM™ Culture Substrate surface coating and AlphaSTEM™ Naïve hPSC Medium selectively promote the expansion of only those cells that have the ability to revert to a naïve state.

Follow the protocol for Culturing Naïve Stem is the AlphaSTEM™ Culture System with the following modifications:

Method 1: Serial passaging

1. Dissociate FGF2-grown stem cells to a single-cell suspension.
    a. For cells grown on MEF feeder layers, dissociate using 0.05% Trypsin/EDTA (incubate 5 min. at 37°C and neutralize with Defined Trypsin Inhibitor.
    b. For cells grown in FGF2-based media on a feeder-free surface, dissociate using 1 mL TrypLE (3-5 min. at 37°C) and neutralize with 1 mL of AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor.
2. Resuspend cells in AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor. For cells previously grown on MEFs, strain cells with 70 micron filter (to remove clumps) and count cells subtracting any feeder cells.
3. Seed cells in a single-cell suspension at 500,000 cells per well of a 6-well plate (50,000 cells/cm^2) onto AlphaSTEM™ Culture Substrate coated plates in 3 mL AlphaSTEM™ Naïve hPSC Medium containing 10 µM Y-27632 Rho kinase I inhibitor per well.
    a. *Note* - It is important to plate triplicate wells to ensure adequate cell numbers for replating. Depending on the cell line, it may be necessary to adjust the starting cell density for transitioning (range of 500K to 1M). During the first two passages, there may be significant cells loss because cells are not only being transitioned to AlphaSTEM™ Naïve hPSC Medium, but are also being selected for those cells that still have the ability to go back to the naïve state.
4. Rock plates to distribute single cells evenly - this requires a rocking forward motion followed by a pause, then a rocking backing motion followed by a pause; do this several times. Leave plates undisturbed for 48 hours in an incubator under same oxygen level as starting cells. If there is difficulty transitioning cells in 20% O_2 at different cell densities, we suggest transitioning cells in a hypoxic incubator (37°C, 5% CO_2, 5% O_2).
5. After first 48 hours, change media daily and reduce media volume from 3 mL to 2 mL per well. Media changes must be as gentle as possible. Replace media every 24 hours with 2 mL of fresh AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor.
6. Passage cells at 75-85% confluence (~3-4 days). However, during the first two passages, cells may need to be passed earlier, such as on Day 2 or Day 3, as they may reach 75-85% confluence earlier than expected. Overgrowing cells may lead to differentiation. Cells will grow as a monolayer and do not form colonies.
7. To harvest, aspirate media and wash wells with 2mL PBS minus calcium, minus magnesium. Dissociate cells using 1 mL TrypLE (~3-4 min. at 37°C) and neutralize with 1 mL of AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor per well. Collect 2 mL of cell suspension and transfer to a 15 mL centrifuge tube. Collect remaining cells by further washing the well with 1 mL AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor and transfer to same 15 mL centrifuge tube.
8. Centrifuge at 250 x g, 5 minutes. Resuspend in 1 mL AlphaSTEM™ Naïve hPSC Medium containing 10 uM Y-27632 Rho kinase I inhibitor.
9. Count cells and add to a volume of AlphaSTEM™ Naïve hPSC Medium containing 10 uM Y-27632 Rho kinase I inhibitor such that there is the desired number of cells per 3 mL media per well.

10. For the second passage, using a step-down approach, seed 400,000 cells in a single cell suspension per well of a room temperature AlphaSTEM™ Culture Substrate coated 6-well plate (40,000 cells/cm^2) in 3 mL media per well.
11. Repeat steps above but reduce seeding density gradually to achieve a 3- to 4-day passage schedule and expand the culture: (See Figures 3 and 4)
    a. Initial seeding – 500,000/well of a 6-well plate (50,000 stem cells/cm^2);
    b. Second passage – 400,000/well of a 6-well plate (40,000 stem cells/cm^2);
    c. Third passage – 300,000/well of a 6-well plate (30,000 stem cells/cm^2).
    d. Most stem cell lines have a stable 3- to 4- day passage time at 100,000-200,000 stem cells/well.
    e. During this transition, cells should be passaged when they reach optimal density (75-85% confluence), which can mean a passage at Day 2 for the higher plating densities.

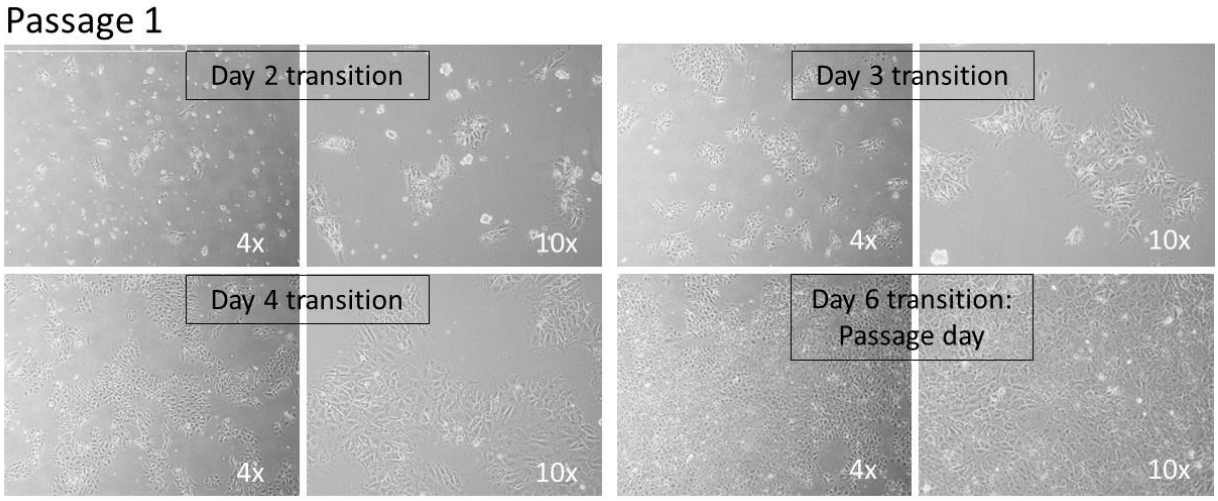

Figure 3. Representative images of cells previously grown in an FGF2-based media undergoing transition to AlphaSTEM™ Cell Culture System. The initial seeding density was 500,000 cells/well of an AlphaSTEM™ Culture Substrate coated 6-well plate (50,000 stem cells/cm2), which results in pluripotent stem cell growth and positive selection over approximately 2-6 days. Some cell lines may transition more efficiently, requiring passage as early as Day 2, when they reach 75-85% confluence. Do not let the cells become over-confluent otherwise the cells may undergo spontaneous differentiation.

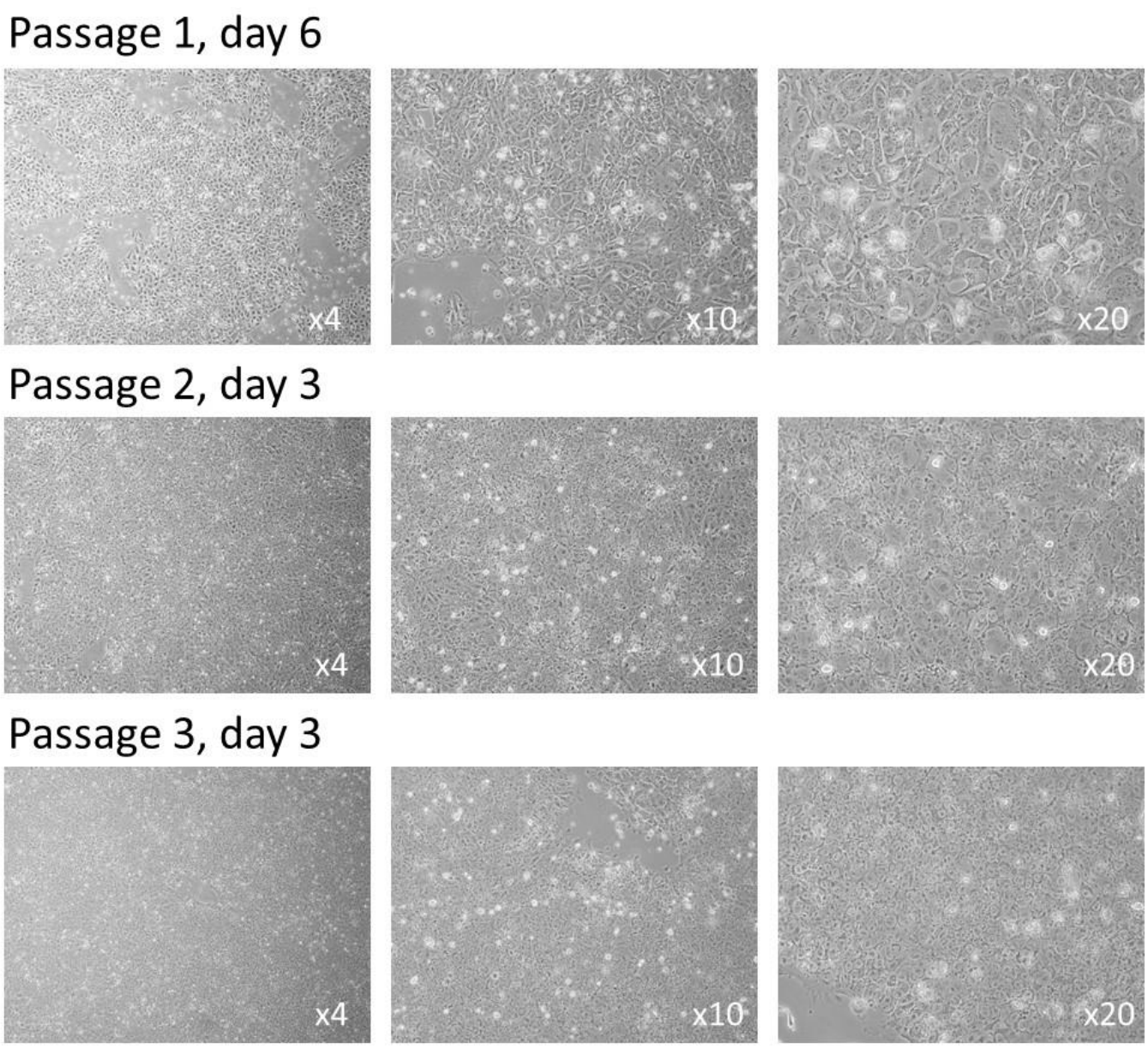

Figure 4. Images show serial passaging of stem cells undergoing transition to the AlphaSTEM™ Cell Culture System. Initial seeding density of 500,000 cells per well (Passage 1), stepping down to 400,000 cells (Passage 2), then 300,000 cells (Passage 3), to 100,000 – 200,000 cells for subsequent passages. Depending on cell line, the initial passages may take 2-6 days to reach 75-85% confluence. However, after this initial transition, cells should be seeded at a density that results in a consistent 3-4 day passing schedule: typically 100,000-200,000 cells per well.

Method 2: Sub-cloning

1. Dissociate FGF2-grown stem cells to a single-cell suspension.
    a. For cells grown on MEF feeder layers, dissociate using 0.05% Trypsin/EDTA (incubate 5 min. at 37°C and neutralize with Defined Trypsin Inhibitor).
    b. For cells grown in FGF2-based media on a feeder-free surface, dissociate using 1 mL TrypLE (3-5 min. at 37°C) and neutralize with 1 mL of AlphaSTEM™ Naïve hPSC Medium.

2. Resuspend cells in AlphaSTEM™ Medium containing 10 uM Y-27632 Rho kinase I inhibitor. For cells previously grown on MEFs, strain cells with 70 micron filter (to remove clumps) and count cells subtracting any feeder cells.
3. Culture FGF2 grown source cells for 1 passage in AlphaSTEM™ Naïve hPSC Medium on an AlphaSTEM™ Culture Substrate coated 6-well plate, seeding cells at 500,000 cells per well, as described in Method 1: Transition by Serial Passaging). Cells should be cultured in incubator at the same concentration of O_2 used prior to transition.
4. **Passage 2:** Coat 96-well tissue culture plate with AlphaSTEM™ Culture Substrate and incubated at 4°C overnight.
5. Dissociate cells as usual but before plating, dilute cells to 900 live cells/mL in AlphaSTEM™ Naïve hPSC Medium containing 10 µM Y-27632 Rho kinase I inhibitor.
6. On room temperature AlphaSTEM™ Culture Substrate coated 96-well plates, aspirate excess AlphaSTEM™ Culture Substrate solution from wells and seed 100 µL per well of cell suspension, which should be ~90 cells per well, in AlphaSTEM™ Naïve hPSC Medium containing 10 µM Y-27632 Rho kinase I inhibitor. Rock plates to distribute single cells evenly - this requires a rocking forward motion followed by a pause, then a rocking backing motion followed by a pause; do this several times. Leave plates undisturbed for 48 hours in incubator.
7. Check plates daily for growth starting at Day 3, and change media (100 µL) every 48h after that. Successful clones should cover 50-70% of wells after 7-10 days. Typically 50% of wells will contain growing clones.
8. **Passage 3:** Coat 24-well tissue culture plate with AlphaSTEM™ Culture Substrate (200 µL/well, 12.5 µg/mL) and incubated at 4°C overnight.
9. From room temperature 24-well plate, aspirate excess AlphaSTEM™ Culture Substrate solution from wells and fill with 1 mL per well AlphaSTEM™ Naïve hPSC Medium containing 10 µM Y-27632 Rho kinase I inhibitor.
10. Gently wash selected wells of the 96-well plate (containing the growing clones) once, with 200 µL PBS and then dissociate cells with 100 µL TrypLE (~3 min. at 37°C).
11. Add 50 µL AlphaSTEM™ Naïve hPSC Medium containing 10 µM Y-27632 Rho kinase I inhibitor to each dissociated well.
12. Using a 200 µL pipette, dissociate cells from each well and transfer the suspension directly to the 24-well plate. Rock plates to distribute single cells evenly - this requires rocking forward motion followed by a pause, then rocking backing motion followed by a pause; do this several times. Leave plates undisturbed for 48 hours in incubator.
13. After 48 hours, begin daily media changes at 0.5 mL/well with AlphaSTEM™ Naïve hPSC Medium containing 10 µM Y-27632 Rho kinase I inhibitor. When clone covers 50-70% of well, passage to 12-well plates.

Passage cells to 12-well then 6-well plates using the same technique as described above. Volumes change as follows:

- 12-well: 500 µL TrypLE
- 6-well 1 mL TrypLE

Once the clones are expanded to 6-well plates, the cells can be grown as described in the section “Culturing Naive Stems cells in the AlphaSTEM™ Culture System” ex

- Exception: if cells were transitioned to the AlphaSTEM™ Culture System in 20% O2, then continue at that O2 concentration

IPSC GENERATION

Reprogramming Fibroblasts

Plate Human Fibroblasts 2 days prior to Reprogramming:

1. Dissociate cells to a single-cell suspension and seed at 100,000/well in an uncoated BD-Falcon 6-well plate in human fibroblast media. Culture at 37°C, 5%CO_2, ambient O_2 for 24 hours. Be sure to plate 2 extra wells for cell counting.
2. After 24 hours, replace media with AlphaSTEM™ Naïve hPSC Medium without Rho kinase inhibitor. Culture for another 24 hours.
3. Target density is 50-90% confluency, after 48 hours in culture - over-dense cells have reduced reprogramming efficiency.

Reprogram Human Fibroblasts (Day 0):

1. Trypsinize and count cells from 2 extra wells to determine the number of cells per well.
2. Pre-warm 2 mL per well of AlphaSTEM™ Naïve hPSC Medium without Rho kinase inhibitor to 37°C for media change. Pre-warm 1 mL per well AlphaSTEM™ Naïve hPSC Medium without Rho kinase inhibitor in a separate tube for reprogramming.
3. Aspirate spent media and replace with 2 mL/well AlphaSTEM™ Naïve hPSC Medium without Rho kinase inhibitor. Return plates to incubator until needed.
4. Deliver reprogramming factors as directed by manufacturer, in AlphaSTEM™ Naïve hPSC Medium without Rho kinase inhibitor.
5. Incubate for a full 24 hours, then replace with fresh AlphaSTEM™ Naïve hPSC Medium without Rho kinase inhibitor.
6. Culture cells for 6 more days, feeding with fresh media every other day.
7. On day 5 or 6 post-reprogramming, coat AlphaSTEM™ Culture Substrate surfaces for clonal isolation as described above. Store at 4°C until needed.

Isolate reprogrammed iPSC Clones (day 7 through 28):

1. On day 7 post-reprogramming, dissociate cells with TrypLE Express and count.
2. Plate cells to 6-well AlphaSTEM™ Culture Substrate plates at 50, 100, and 150 thousand cells per well in AlphaSTEM™ Naïve hPSC Medium with Rho kinase inhibitor, 3 mL/well. Transfer cells to 37°C, 5% CO_2, 5% O_2 incubator and leave undisturbed for 48 hours.

   NOTE: From this point onward, AlphaSTEM™ Naïve hPSC Medium with Rho kinase inhibitor (10 µM Y-27632) will be used. Cells will be cultured in 5% CO_2, 5% O_2 from this point forward.

3. Days 9 – 28 post-reprogramming: Feed cells every 24 hours, and check for colony formation every other day. Colony picking during the third week is optimal (See Figure 5).
4. On the day before colony picking, coat 96-well flat-bottom BD-Falcon TC plates with AlphaSTEM™ Culture Substrate, 50 µL/well overnight at 4°C.
5. Pick colonies manually and transfer into 30 µL TrypLE Express in a 96-well round-bottom plate. Incubate for ~4 min. at 37°C. Using a P-200, neutralize with 100 µL AlphaSTEM™ Naïve hPSC Medium with Rho kinase inhibitor and triturate gently to dissociate cells. Transfer to a 96-well flat bottom dish coated with AlphaSTEM™ Culture Substrate. Wash well out with an additional 100 µL of media and combine with the first 100 µL in the destination well.
6. NOTE: This step work best in manageable groups of 8 or 12 colonies at a time (one row or column of the 96-well plate).
7. Leave cells in 96-well plate undisturbed (5% CO_2, 5% O_2) for 48 hours, then change media daily and monitor clones for growth.

8. As clones grow, passage up to the next larger plate well size using TrypLE – neutralize with AlphaSTEM™ Naïve hPSC Medium with Rho kinase inhibitor and transfer directly to the destination well. Wash source well with additional media and combine in the destination well.

Use the following volumes for passaging:

Source Plate	PBS	TrypLE	Neutralize/Wash
96-well	200 µL	50 µL	100 µL / 100 µL
24-well	400 µL	100 µL	200 µL / 200 µL
12-well	800 µL	250 µL	500 µL / 500 µL
6-well	2 mL	500 µL	3 mL / 3 mL (1:3)

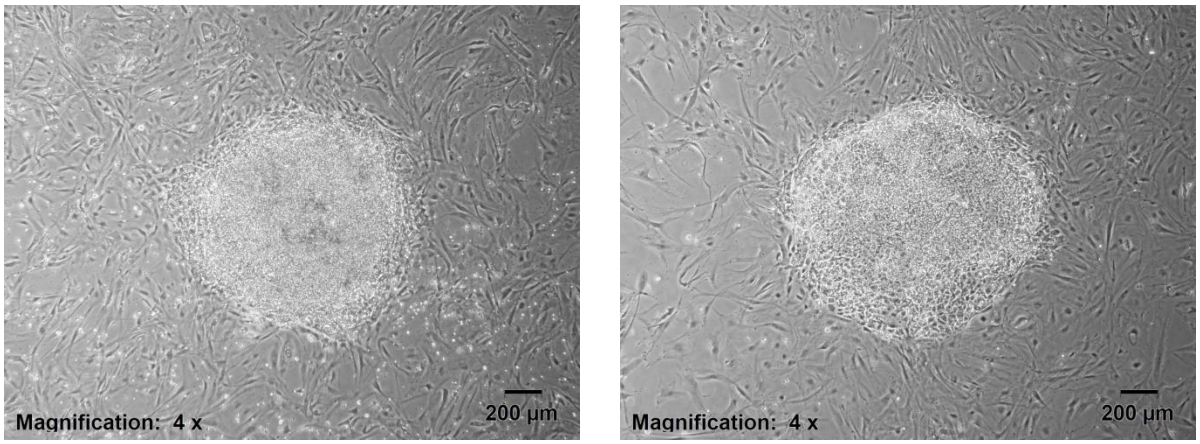

Figure 5. Representative AlphaSTEM™ hiPSC clones 21 days post-transduction. These colonies are ready to be picked for clone expansion.

DIFFERENTIATION

AlphaSTEM™ Differentiation Inducer should only be added when differentiation is desired, and should be not be added to AlphaSTEM™ Naive hPSC Medium which maintains cells in their naïve state.

Use AlphaSTEM™ Differentiation Inducer (1 x 12µl vial diluted 1,000X into 12mL of differentiation media) at 1X concentration, 1-time, at the onset of directed differentiation. AlphaSTEM™ Differentiation Inducer should be added into media that does not contain $NME7_{AB}$ – this can be any media used to initiate differentiation in a specific differentiation protocol. AlphaSTEM™ Naive hPSC Medium should not be used when differentiation is initiated.

Other factors that induce or direct differentiation to a specific cell type can be added to the same media that contains AlphaSTEM™ Differentiation Inducer. There is no need to remove AlphaSTEM™ Differentiation Inducer at any specific time point. For example, if a differentiation protocol calls for 3-day incubation with growth factor-free media that contains certain inhibitors, but no media changes, the presence of the AlphaSTEM™ Differentiation Inducer will not hurt the cells or their differentiation.

CHARACTERIZATION

Cells are characterized for pluripotency markers as detailed in Minerva's paper entitled "A Primitive Growth Factor, $NME7_{AB}$, Is Sufficient to Induce Stable Naive State Human Pluripotency; Reprogramming in This Novel Growth Factor Confers Superior Differentiation" found at this link: Carter, Smagghe, Stewart et al 2016

CRYOPRESERVATION

1. Prepare AlphaSTEM™ Freezing Media A by combining 5 mL KOSR with 5 mL AlphaSTEM™ Naïve hPSC Medium containing 10 µM Y-27632 Rho kinase I inhibitor. Prepare AlphaSTEM™ Freezing Media B by combining 8 mL AlphaSTEM™ Naïve hPSC Medium containing 10 µM Y-27632 Rho kinase I inhibitor with 2 mL optimally containing DMSO such as Sigma-Aldrich #D2650. Prepare both on the day of use and store at 4°C until needed.
2. Dissociate cells as described above. Re-suspend cells at 2 – 2.5x10^6 cells/mL in cold freezing Media A. Add, drop wise, an equal amount of cold freezing Media B.
3. Transfer 1 mL of cell suspension each into cryo-vials. Freeze cells slowly to -80°C at a rate of -1°C/min by using either a programmable cooler or a container designed for controlled cell cryopreservation. Transfer to LN_2 storage the next day.

TROUBLESHOOTING

- Do not add other growth factors or cytokines to AlphaSTEM™ Naïve hPSC Medium; the effect is not additive as $NME7_{AB}$ stimulates a different pathway than FGF2 or TGF-beta. Stem cells cultured in AlphaSTEM™ Naïve hPSC Medium grow as a monolayer, not colonies.
- Initial problems with stem cells attaching to the AlphaSTEM™ Culture Substrate are cell line specific. Poor initial attachment could mean that your cell line needs a Rho kinase Inhibitor to enhance adhesion. Some stem cell lines only require Rho kinase inhibitors for the first 48 hours, while others require it throughout.
- Plates with a VITA™ (ThermoFisher) 6-well plates or BD Falcon™ type surfaces work best with our AlphaSTEM™ Culture Substrate and AlphaSTEM™ Naïve hPSC Medium.
- Harsh or excessive pipetting prior to plating will cause failure to attach.
- Leaving stem cells at room temperature for extended periods of time will cause failure to attach.
- Disturbing plates within initial 48 hours will cause failure to attach.
- Detachment of established monolayer is usually due to mechanical or temperature shock to the plate.
- If your cell line requires use of a Rho kinase Inhibitor during first 48 hours only, but cells begin to detach, addition of the inhibitor without changing the media usually makes cells re-attach.
- Non-uniform plating of cells can lead to detachment of overly dense regions of cells.

REFERENCES

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2. Carter, M.G., Smagghe, B.J., Stewart, A.K., Rapley, J.A., Lynch, E., Bernier, K.J., Keating, K.W., Hatziioannou, V.M., Hartman, E.J. and Bamdad, C. C. (2016), A Primitive Growth Factor, NME7AB, Is Sufficient to Induce Stable Naïve State Human Pluripotency; Reprogramming in This Novel Growth Factor Confers Superior Differentiation. STEM CELLS. doi: 10.1002/stem.2261

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