APPLICATION NOTE No. 338

Low Oxygen Levels Enhance the Efficiency of Reprogramming Human Somatic Cells to Pluripotency

Rick Cohen
Rutgers University, Piscataway, NJ, USA
Address correspondence to: Ma Sha
Eppendorf, Inc., Enfield, CT, USA, sha.m@eppendorf.com

Abstract

In order to successfully reprogram human somatic cells to the pluripotent state, several variables must be considered and optimized. One of the most overlooked variables is the atmospheric composition in which the cells are cultured, reprogrammed, and expanded as induced Pluripotent Stem Cells (iPSCs). Many researchers optimize the types of starting populations of cells, media composition, growth factors, and small molecules, but fail to address the disadvantageous effects of normal atmospheric percentages of oxygen (O_2), which are about 21 %. In this study, we generated iPSCs in either standard norm-oxy conditions or under low O_2 (4 %) conditions. It is not surprising that, while both conditions were permissive for iPSC generation, the low O_2 condition allowed earlier detection of colonies, more mature looking colonies, and a larger and more robust number of colonies by 21 days post-electroporation. It is our finding that controlled O_2 conditions should be a vital part of optimized culture systems to generate iPSCs. Eppendorf incubators with optional O_2 control provide stable O_2 levels in a range of 0.1 – 19 %.

Introduction

Pluripotent stem cell research is rapidly expanding. In addition, iPSCs are now being used in drug screening, human embryology, and cancer research. Therefore, an efficient system of generating and culturing these stem cells is under intense investigation.

While advances in media optimization have recently provided several options for xeno-free culture methods [1 – 8], efficient, safe, and easy techniques to generate iPSCs are still evolving. Aside from media and reprogramming methods, another key element in developing robust cultures appears to be the atmosphere in which cells are generated and developed. Shinya Yamanaka, who together with John Gurdan was awarded the Nobel Prize for his work in stem cell research, demonstrated early on that hypoxic conditions, also referred to as low O_2 conditions, enhance cellular reprogramming [9]. Remarkably, since then, the majority of publications involving iPSCs continue to use ambient O_2 concentrations with 5 % carbon dioxide (CO_2) as the norm.

In order to effectively evaluate emerging methodologies, it is vital to optimize the variables considered above. In this study, we report an optimal reprogramming protocol for human foreskin fibroblasts using electroporation of a single Episomal vector [10 – 12] cultured in a small molecule cocktail-containing media similar to that reported by Yu and colleagues [10]. We aimed to determine whether or not the efficiency of a reprogramming method (in this case, the single Episomal method) could be improved by incorporating low O_2 conditions into the protocol. To accomplish this, we used two O_2 levels to precisely maintain the O_2 percentage at 4 %, and compared our reprogramming results in this

condition to standard atmospheric O_2. The low O_2 conditions allowed more proto-colonies to appear 10 – 12 days following electroporation. By day 21, the difference between norm-oxy and hypoxic conditions was more apparent. While norm-oxy conditions were able to generate iPSC colonies, they were fewer in number and smaller in size when compared to the 4 % low O_2 conditions created in the incubator.

Material	Supplier	Catalog No.
DMEM/F12, 270 mOsmo	PeproTech®	Custom
DMEM/F12, 340 mOsmo	PeproTech®	Custom
PeproGrow-hESC (Stem Cell Media)	PeproTech®	BM-hESC
Recombinant human FGF-basic	PeproTech®	100-18B
PBS-EDTA, high Osmolarity.	PeproTech®	Custom
KnockOut™ Serum Replacement	Life Technologies®	10828028
TrypLE™ Select Enzyme (1 X), no phenol red	Life Technologies®	12563029
Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibody	Life Technologies®	A-11008
Alexa Fluor® 594 Goat Anti-Mouse IgG1 (γ1)	Life Technologies®	A-21125
Alexa Fluor® 647 Goat Anti-Mouse IgM (μ chain)	Life Technologies®	A-21238
ProLong® Gold Antifade Mountant	Life Technologies®	P10144
Penicillin-Streptomycin (Pen/Strep: 10,000 U/mL)	Life Technologies®	15140-122
Neon® Transfection System 100 μL Kit	Life Technologies®	MPK10096
TRA-1-60 Monoclonal Antibody, Mouse (cl.A)	Life Technologies®	41-1000
Dispase II	Life Technologies®	17105-041
4',6-diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich®	D9542
2-Mercaptoethanol	Sigma-Aldrich®	M3148
Monoclonal Anti-Nanog antibody (mouse)	Sigma-Aldrich®	N3038
Fetal Bovine Serum, heat inactivated (HI-FBS)	Sigma-Aldrich®	12203C
Bovine serum albumin (BSA), fatty acid, IgG Free	Sigma-Aldrich®	A7030

Table 1 (continued on page 3): Consumable reagents used in this study.

Figure 1: CellXpert® C170i CO_2 Incubator

Materials and Methods

Table 1 describes the consumable reagents and materials that were used in this study. If no specific instructions are presented here, then the Manufacturer's instructions were followed as recommended. A more detailed stem cell methodology handbook is available by email request for "Methods" from info@stemcellcourse.org.

Solutions

The dispase stock solution was prepared at 50 mg/mL in DMEM/F12 media, sterilized by 0.22 μm polyethersulfone (PES) filtration, and diluted to 0.5 mg/mL in the same DMEM/F12 media. All small molecules (#1 – 5), except sodium butyrate, were dissolved in DMSO and diluted in ethanol to 2000 X prior to sterilization using a 0.22 μm nylon syringe filter. Neutral buffered paraformaldehyde was made by mixing 40 g of paraformaldehyde powder into 800 mL ddH_20, adding drops of 1 N NaOH while mixing until visibly soluble before adding 100 mL of 10 X PBS and 50 mL of 1 M HEPES. After adjusting the pH to 7.4 using 1 N HCl, the solution was filtered and stored at -20 °C.

Fibroblast Culture

Human foreskin fibroblasts were cultured on 10 cm^2 uncoated TC-treated dishes in norm-oxy conditions in complete Fibrolife Serum Free medium containing 2 % HI-FBS and 1 % Pen/Strep. Routine expansion until passage 9 was carried out using TrypLE for cell dissociation.

Triton™ X-100 solution	Sigma-Aldrich®	93443
Phosphate buffered saline (PBS), Ca^{2+}/Mg^{2+} free	Cellgro®	21-040-CM
Matrigel®, hESC qualified	Corning®	354277
Costar® 24-well clear TC-treated dishes	Corning®	3524
Costar® 6-well clear TC-treated dishes	Corning®	3506
Falcon® 40 µm cell strainer	Corning®	352340
Normal Human Dermal Fibroblasts, Neonatal, Primary	Lifeline Cell Technology®	FC-0001
FibroLife® Serum Free Medium Complete Kit	Lifeline Cell Technology®	LL-0001
PS 48 (small molecule #1)	R&D Systems®	4087
A-83-01 (small molecule #2)	R&D Systems®	2939
Y-27632	R&D Systems®	1254
Sodium butyrate (small molecule #3)	Fisher Scientific®	AAA1107936
Paraformaldehyde, 96 %, extra pure	Fisher Scientific®	AC41678-5000
Rabbit Anti-Lin28A [EPR4640] antibody (DyLight® 488)	Epitomics®	4584-1
Goat Serum	EMD Millipore®	S26-Liter
P53 inhibitor, (small molecule #4), 1 µM final	Proprietary	N/A
Epigenetic Modifier (small molecule #5), 50 nM final	Proprietary	N/A
Episomal Reprogramming Plasmid, pERC-V1	Stemcellcourse.org	pERC-V1

Table 1 continued: Consumable reagents used in this study. Small molecules #1 – 5 were used to create the small molecule cocktail mentioned below.

Electroporation

Prior to electroporation procedures, target 6-well plates (one for norm-oxy and two for low O_2 conditions) were coated with Matrigel for 1 hour at 37 °C and then pre-equilibrated with Reprogramming Media Step 1. This media consisted of complete FibroLife Serum Free Medium, no TGF-beta 1, with 2 % FBS, and the small molecule cocktail described in Table 1. Once 90 % confluence was reached, the fibroblasts were rinsed once with PBS-EDTA and incubated with TrypLE for 2 – 3 min. Prior to detachment from the TC plate, the TrypLE was aspirated and the cells rinsed off the surface using FibroLife media. If the cells detached, then they were collected and diluted 1:1 with cell culture media. If clumps were observed, the solution was passed through a 40 µm cell strainer fitted into a 50 mL conical tube. A 10 µL aliquot was removed and enumerated using a standard bright line hemocytometer during which the remaining sample was centrifuged for 5 min at room temperature (RT) at 1000 rpm (150 x *g*). The pellet was suspended in PBS to achieve a density of 1 x 10^6 cells/mL, then 4 mL of the cell suspension were removed and re-centrifuged. The pellet was resuspended in electroporation solution to a density of 1.1 x 10^6 cells per 100 µL. The cells were mixed in duplicate with 5.5 µg (10 µL) of low endotoxin preparation of pERC-V1 DNA (Figure 2), and then 100 µL of cells/DNA were drawn into an electroporation tip and pulsed according to the Neon Transfection System instructions. Electroporation was conducted at 1700 V, 10 ms, 1 pulse. The 100 µL tip volume was diluted into 900 µL of equilibrated Reprogramming Media Step 1, and then the electroporation process was repeated with duplicate cells/DNA as previously prepared.

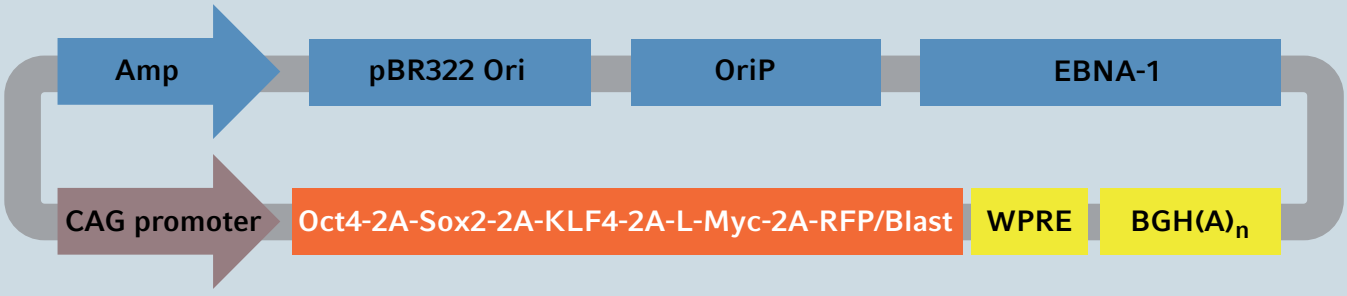

Figure 2: pERC-V1, a novel all-in-one episomal plasmid for cellular reprogramming. This plasmid expresses 5 genes connected by 4 canonical "2A" self-splicing motifs: Oct4, Sox2, KLF4, L-Myc, and a Red fluorescence (RFP)/Blasticidin resistance (Blast) fusion protein, under the control of the CMV early enhancer/chicken beta actin (CAG) promoter. The RFP/Blast fusion protein allows for easy visualization and selective enrichment of positively transfected cells.

The second electroporation was immediately pooled with the first, and then 18 identical 100 µL aliquots (500 ng of DNA/100,000 cells) were placed into all wells of the Matrigel-coated and pre-equilibrated 6-well plates.

The day after electroporation, the media was changed to Reprogramming Medium Step 2, which consisted of DMEM/F12, 270 mOsmo, supplemented with 20 % Knock-out Serum replacer, 20 ng/mL FGF-2, and the small molecule cocktail. Some cultures also received 1 % Pen/Strep. This media was refreshed every other day until colonies were visibly distinguished, then changed daily until passaging.

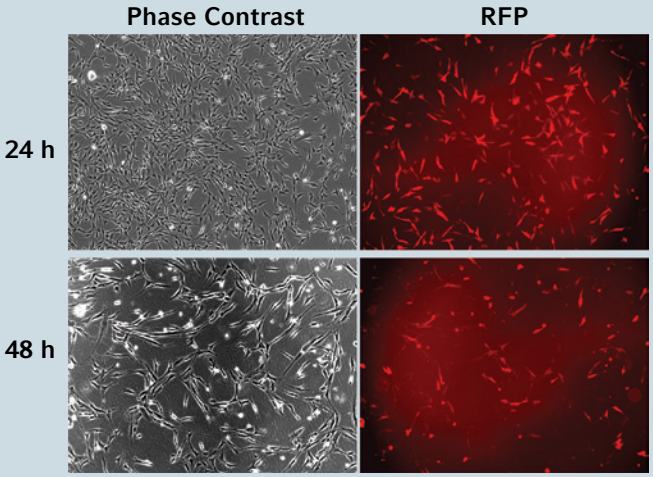

Figure 3: Human foreskin fibroblasts post-electroporation. Fibroblasts begin to express the genes encoded by pERC-V1 as reflected by the RFP signal within 24 h post-electroporation. Cell death is seen in both low O_2 and norm-oxy conditions during the culturing in Reprogramming medium step 1 with 5 µg/mL blasticidin. After 5 – 7 days of selection, the blasticidin is removed and cells are grown in Reprogramming medium step 1.

Primary iPSC picking

At 21 days post-electroporation, clearly passable colonies were seen in the low O_2 cultures whereas smaller, less abundant clones were available in the norm-oxy conditions (Figure 4). Some clones were picked onto new Matrigel-coated 12-well plates using mechanical dissociation with a flamed polished glass-picking tool. Some clones were picked after limited Dispase treatment (0.5 mg/mL for 3 – 5 min). Following both types of initial passaging techniques, replicates were plated in the presence of 2 µM Y-27632. The media in some cultures was changed from Reprogramming Media Step 2 to PeproGrow-hESC media the following day.

iPSC expansion

Once the primary selected colonies were of appropriate size, the cultures of presumptive iPSCs were selected and passaged using the Dispase technique, or cleaned of any visible fibroblasts using pick-to-remove techniques and then passaged using PBS/EDTA. Briefly, media was aspirated from the dishes, replaced with 1 – 2 mL of PBS/EDTA, and returned to the incubator for 3 – 5 min. Once the texture of the colonies turned from uniform and smooth to mostly phase bright and rough, the PBS/EDTA was aspirated and cells triturated off the surface by forceful pipetting with 5 mL PeproGrow-hESC media. The supernatant containing the cells was triturated once more and diluted to appropriate density prior to plating (1:10 to 1:100 dilution). For immunostaining techniques, cells were plated at a density of 10,000 to 20,000 cells per well.

Fluorescent marker staining

Following 5 to 6 passages, cells were plated onto Matrigel-coated 24-well culture plates and grown to sub-confluent density whereafter they were fixed and stained with three stem cell markers: Nanog (1:1000), Lin28 (1:500), and TRA-1-60 (1:250). These stem cell markers were chosen over other common markers such as Oct4, Sox2, KLF4, and L-Myc because Nanog, Lin28 and TRA-1-60 were not included on the DNA plasmid used to induce pluripotency. Therefore, their expression must be induced by genetic expression changes during adaptation. In addition, these markers are also widely accepted as a combination of epitopes clearly indicating iPSC phenotype. Briefly, the cultures were rinsed once with warmed DMEM/F12, fixed with 4 % neutral buffered paraformaldehyde for 20 min at RT, and rinsed 3 times with PBS. Samples were then treated with Blocking Solution (PBS containing 1 % BSA, 10 % goat serum and 0.1 % Triton X-100) for 1 h, and then incubated overnight at 4 °C with a cocktail of Nanog, Lin28, and TRA-1-60 antibodies diluted in 0.5 X Blocking Solution/PBS. The following day, the samples were rinsed 3 times with PBS and incubated for 1 h at RT with the following pre-clarified fluorescently-conjugated second step antisera diluted in 0.5 X Blocking Solution/PBS: 1:1000 Alexa Fluor 488 goat Anti-rabbit IgG, 1:500 Alexa Fluor 594 goat Anti-mouse IgG1, and 1:500 Alexa Fluor 647 goat Anti-mouse IgM. Samples were rinsed once for 5 min with PBS containing 500 ng/mL DAPI, twice more with PBS, and twice with distilled deionized water prior to drying and mounting under glass coverslips with Prolong Gold.

Results and Discussion

The day after electroporation, fibroblast cultures from low O_2 and norm-oxy conditions were observed for RFP fluorescence. Figure 3 shows a representative example of a culture brightly expressing RFP, indicating expression of the stem cell markers carried on the pERC-V1 plasmid (Figure 2). We estimated that roughly 40 % of the cells appeared to express RFP brightly. During the first several days of blasticidin selection, we observed cell death of many non-transfected and some RFP-expressing cells.

By 10 to 12 days (Figure 4), changes in the morphology of the normally spindle shaped fibroblasts were observed as many cells in the low O_2 conditions began to become cuboidal in shape and displayed a more compact cytoplasm. Fewer cells, if any, were seen during this time period in the norm-oxy conditions. By 15 – 16 days, morphological changes were very clear in the low O_2 cultures, and these alterations were starting to become apparent in the norm-oxy conditions (data not shown).

By 21 days post-electroporation, dozens of large, robustly growing colonies were observed in all three cell lines assayed. Whereas under norm-oxy conditions, less than 5 colonies were observed, and when present, often they were 1/3 or less in size as compared to the low O_2 counterparts (Figure 4). A summary of our assays are detailed in Table 2, and demonstrate that low O_2 conditions are far more favorable for generating iPSCs.

In addition to iPSC formation, it was also evident that initial steps to select a stable cell line were more easily

	Low O_2	Norm-Oxy
Proto-Colonies, day 12	+	-
Passable Colonies, day 21	+++	+
	13 – 21 , n = 3	2 – 4 small, n = 3

Table 2: Outcomes of reprogramming. Over the course of our investigation it was evident that low O_2 culture resulted in the observation of formation of proto-colonies as early as day 12 post-electroporation. In addition, low O_2 culture produced significantly more robustly growing colonies by day 21. Following passaging, the colonies grown in both low O_2 and norm-oxy conditions grew in a similar fashion.

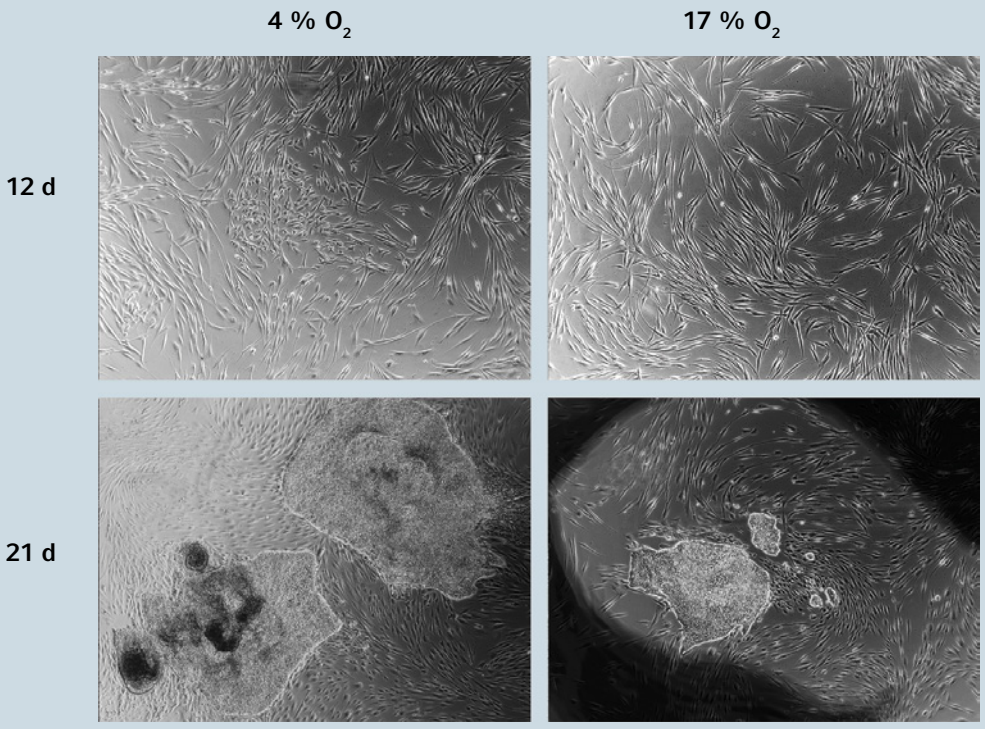

Figure 4: Generation of iPSCs under varying O_2 concentrations. After 12 days, early colony formation is seen in low O_2 cultures, whereas this is not observed as early in norm-oxy cultures. By 21 days post-electroporation, clear colonies are seen in both, however the size and number are more numerous in low O_2 cultures. This is consistently seen over 3 separate experiments.

accomplished in low O_2 conditions. To expand growing colonies, the cells were mechanically picked, with or without prior Dispase treatment, into fresh PeproGrow-hESC medium containing 2 µM Y-27632 and transferred onto fresh Matrigel-coated 6-well plates. In low O_2 conditions, many of the colonies attached well, and grew robustly, to form iPSC colonies (Figure 5, right panel) with typical morphology. Whereas, in norm-oxy conditions, many colonies had mixed morphological cell types. After 3 selective passages, seemingly stable iPSCs were obtained from norm-oxy conditions. After 4 to 5 passages, successfully reprogrammed colonies from both atmospheric conditions were seeded onto 24-well plates for standard indirect immunostaining techniques. Colonies replated after Dispase treatment tended to attach better than those that were mechanically picked. When treated with Y-27632, the colonies survived the process at a higher rate and formed colonies within 4 days. By 7 days post-passaging many colonies were observed. However, the morphology observed with low O_2 cultures resembled the more purified mature iPSC colonies.

Triple staining of cultures revealed that, as expected, low O_2 conditions were favorable for the expansion and development of iPSC colonies with stable typical morphology (Figure 6). Similar staining was observed in norm-oxy conditions (data not shown).

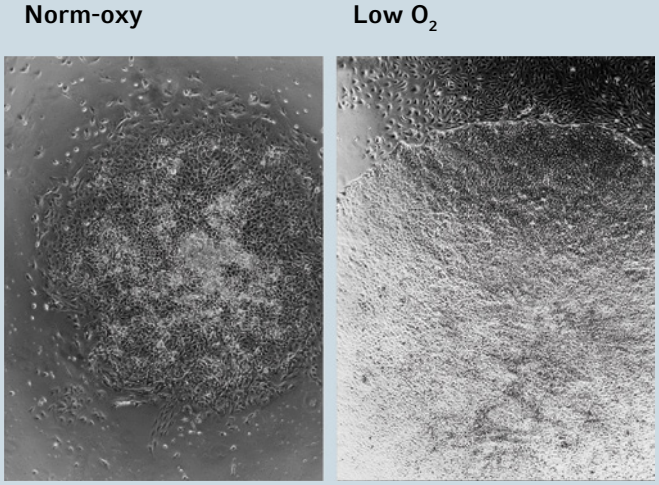

Figure 5: Primary passaging of pERC-V1 reprogrammed iPSCs. Colonies replated after Dispase treatment tended to attach better than those mechanically picked. When treated with Y-27632, colonies survived the process more efficiently and formed colonies within 4 days. By 7 days post-passaging, many colonies were observed, however the morphology observed with low O_2 cultures tended to resemble more purified mature iPSC colonies.

Conclusion

Here we have demonstrated that we were able to increase the efficiency of the generation of stable iPSC lines using a non-optimized and novel reagent. Without the regulation of O_2, this method yielded marginal results and could have been discarded as inefficient. However, as compared to norm-oxy conditions, we were consistently able to observe morphological changes in fibroblast cultures cultivated in low O_2 conditions within 12 days post-electroporation, and clearly robust colonies after 3 weeks. While other labs may not use the same reprogramming methodology, or starting cell types, it is clear that the data presented here demonstrates the advantage of maintaining a low O_2 environment during reprogramming. Our future goals are to determine whether or not low O_2 can also play a role in the differentiation of iPSCs into the three germ layers.

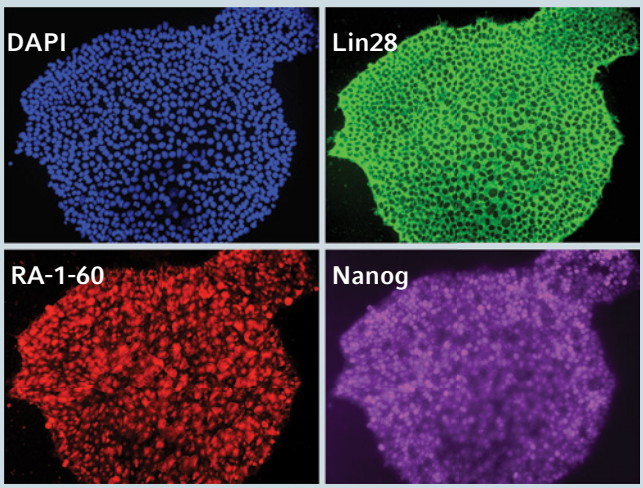

Figure 6: Immunostaining of colonies generated using low O_2 culture. iPSCs plated on 24-well dishes were immunostained with a cocktail of antibodies recognizing Nanog, Lin28, and TRA-1-60. Following indirect immunostaining procedures, the cells were counterstained using DAPI and mounted. As expected, those cells cultured in low O_2 conditions have immunostaining patterns resembling other published iPSC lines.

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CellXpert® C170i, 4 inner door, right door handle, O_2 control	6731 001.041	6731 001.042	6731 001.043	6731 001.044	6731 011.045
CellXpert® C170i, 8 inner door, right door handle, O_2 control	6731 001.081	6731 001.082	6731 001.083	6731 001.084	6731 011.085

*This study was performed using the Galaxy® 170 R incubator. The CellXpert C170i incubator replaces the Galaxy 170 R model.

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