Defined and xeno-free culture of human Pluripotent Stem Cells (hPSC)

PromoCell

Application Note

Background

The term pluripotency describes the ability of a stem cell to differentiate into all terminally mature cell types of the body, excluding extra-embryonic tissues, e.g. the placenta. This potential is progressively lost during the gradual multi-staged differentiation process of pluripotent stem cells towards terminally differentiated mature cells [1]. The two most well-known examples of pluripotent stem cells (PSC) are embryonic stem cells (ESC) and induced pluripotent stem cells (iPS) [1].

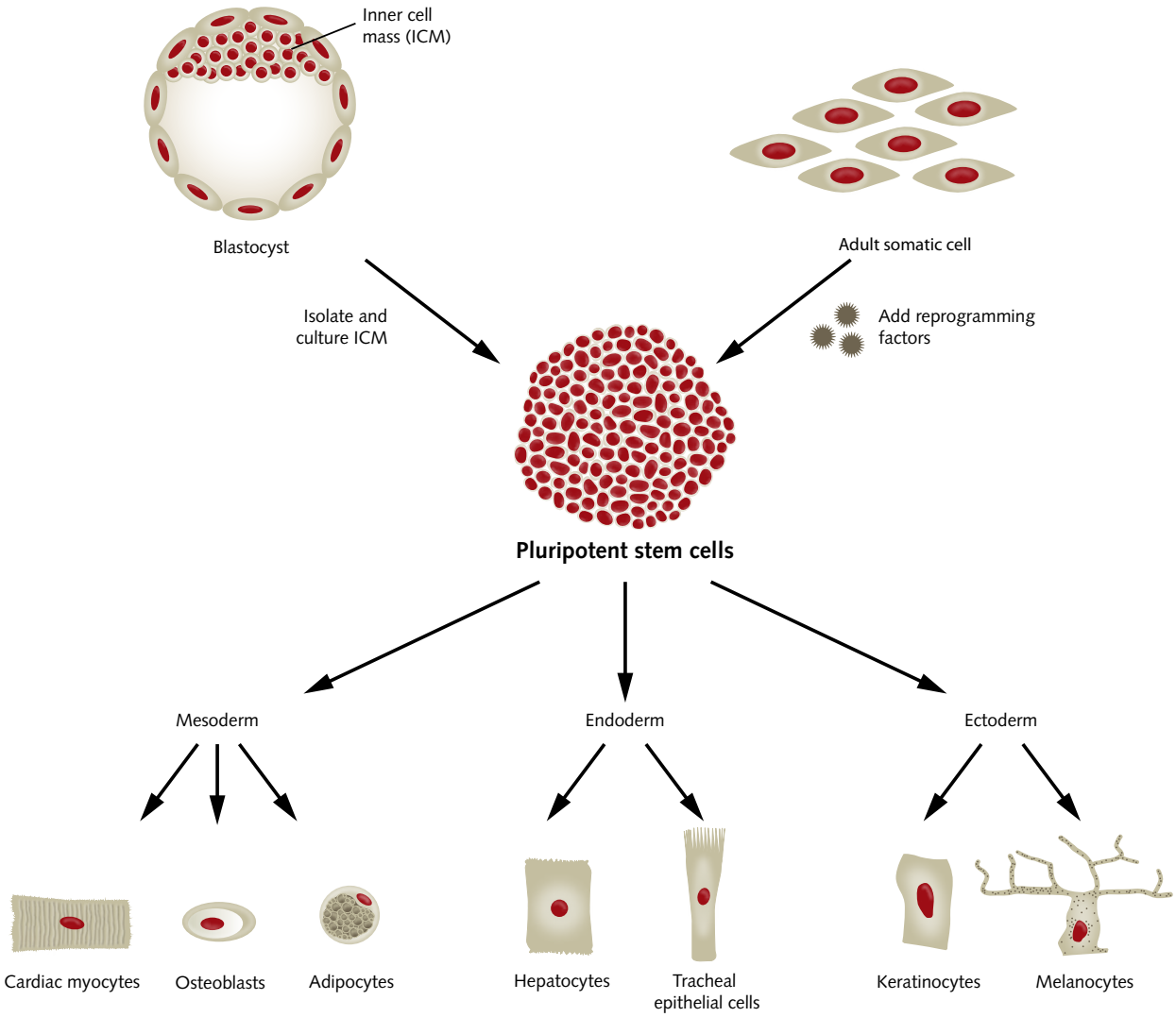

Fig. 1: Schematic overview on the origin and fate of pluripotent stem cells (PSC). While embryonic stem cells (ESC) are derived from the inner cell mass (ICM) of a developing blastocyst, induced pluripotent stem cells (iPS) are artificially generated by re-programming the nucleus of an adult somatic cell back to a pluripotent state. Alongside their virtually unlimited self-renewal potential, the extensive differentiation capabilities are another hallmark of pluripotent stem cells.

Embryonic stem cells were the first type of pluripotent stem cells isolated and taken into culture and were initially derived from the inner cell mass (ICM) of a developing mouse embryo in 1981 [see 2 for review]. These cells proliferate indefinitely and are genetically stable. The advent of human embryonic stem cells in 1998 [3], catapulted stem cell research to new heights. Embryonic stem cell research conquered science at a breath-taking pace, leading to major advances in cell and developmental biology [8]. Today, embryonic stem cells derived from various species represent versatile biological tools, e.g. for the directed differentiation into various cell types, gene targeting and generation of transgenic animals [8]. Importantly, their unlimited self-renewal capacity in combination with the ability to differentiate into cells with therapeutic value has given rise to new hope for the therapy of many non-treatable medical conditions.

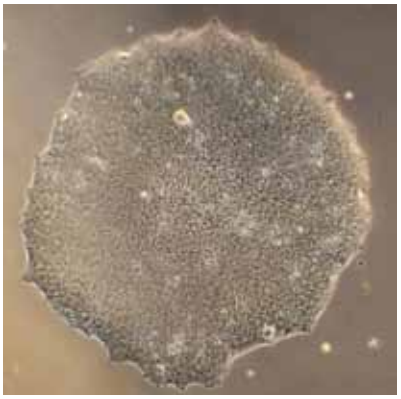

Fig. 2: Colony morphology of human foreskin fibroblast-derived iPS cells cultured in the PromoCell hPSC Growth Medium DXF using PromoCell's hPSC-ECM DXF as an extracellular matrix.

First described in 2006 for the murine system [12] and 2007 in man [9,10], induced pluripotent stem cells (iPS) cells are artificially generated by re-programming mature somatic cells back into a pluripotent state. The principle is based on inducing the expression of distinct pluripotency genes by means of various technical approaches [11]. This elegant and revolutionary technique allowed for the circumvention of the ethical concerns associated with blastocyst-derived hESC and opened new possibilities for the generation of patient-specific stem cells and disease models. At first glance, iPS share many features with ESC, e.g. morphology, pluripotency and marker expression. Detailed analysis, however, revealed some fundamental differences between hESC and their artificially generated counterpart, e.g. variance in epigenetic modifications/DNA-methylation ("imprinting") and accumulation of mutations during the somatic stage of the cell [4].

It is important to note that pluripotent cell lines can also be established either directly from epiblast cells or indirectly from their unipotent descendants, the primordial germ cells (PGC), by *in vitro* dedifferentiation into pluripotent embryonic germ cells (EGC).
Additional, yet rarely used techniques to reprogram mature somatic cells back into a pluripotent state are somatic-cell nuclear transfer (SCNT) and cell fusion [1]. SCNT achieves the reprogramming process by transfer of a somatic nucleus into an enucleated oocyte. This technique generates a fully functional embryo and is sometimes used for cloning of (transgenic) animals, e.g. "Dolly the sheep" ("reproductive cloning") [5] but can also be used for the establishment of patient specific pluripotent cell lines ("therapeutic cloning").
Lastly, cell fusion employs the generation of tetraploid or polyploid pluripotent cell hybrids by fusion of a pluripotent stem cell with a somatic cell.

Despite the significant therapeutic potential that pluripotent cells hold, at present science has not reached the threshold required for safe and efficient therapies based on pluripotent cells. Ironically, the virtually unlimited possibilities of these cells represent the main obstacle for their use in a broad range of therapeutic applications: residual pluripotent cells in cell preparations for transplantation pose a high risk for developing cancer/tumors and there are no safe techniques to guarantee the absence of such dangerous cell impurities at the moment [4]. In addition, even after 15 years of hESC-culture, overall optimized differentiation protocols that give functionally transplantable cells remain scarce [6].

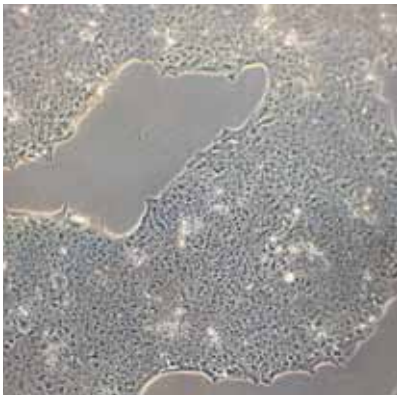

Fig. 3: Culture of human adipose stem cell-derived iPS cells in the PromoCell hPSC Growth Medium DXF using PromoCell hPSC-ECM DXF as an extracellular matrix.

The pluripotency of PSC can be assessed by various direct and indirect technical approaches. The gold standard for animal cells is the generation of chimeras and testing for contribution to all tissues including germline transmission [13,14]. For human pluripotent stem cells these rigorous tests for pluripotency can not be performed for obvious ethical reasons. Therefore, indirect methods (e.g. the formation of teratomas in immunocompromized mice or embryoid bodies with differentiation into all three germ layers) are used as alternative methods of pluripotency testing of human PSC [13]. These approaches are laborious, expensive/time-consuming and technically challenging. As such, testing for pluripotency marker expression patterns, e.g. Oct-3/4, Nanog, SSEA and Tra antigens is also currently widely accepted as an indirect proof of a pluripotent state [see 7 for review]. Indeed, to date no technique is available to ultimately verify the pluripotency of existing hPSC lines by functional means.

In the first PSC research, embryonic stem cells were cultured on feeder cell layers, mostly murine embryonic fibroblasts (MEF), in non-defined culture media containing FCS [8]. Later on, FCS could be replaced by somewhat more defined serum replacements. The introduction of extracellular matrix coatings supporting self-renewal of ESC allowed for feeder-free culture using feeder-cell conditioned medium. However, the culture process remained time-consuming, laborious and poorly defined.

In recent years PSC-culture has experienced significant technical progress. Chemically defined media compositions became a standard, followed by the first xeno-free defined formulations. However, all these culture systems still depended on non-defined, xenogenic and poorly standardized extracellular matrix (ECM) preparations – a contradiction in terms when using defined/xeno-free media. Indeed, research just recently overcame this hurdle and identified natural/synthetic ECM molecules and peptides supporting undifferentiated PSC-expansion. Scientists are now capable of completely defined/humanized derivation and culture of hPSC.

Despite these recent technical advancements, the established hPSC culture systems still share one or more of the following unfavourable properties: use supra-physiologically high amounts of growth factors, contain substances purified from human or animal origin, rely on animal-derived and/or non-defined ECM.

The PromoCell hPSC Growth Medium DXF consequently eliminates these disadvantages. The formulation is not only chemically defined/xeno-free, but also completely excludes substances purified from human or animal origin. In addition, the medium works with growth factor concentrations in the lower physiological range. The optimal culture environment allows for a well-controlled culture process, consistent and reproducible performance, robust support of pluripotency and improved cloning efficiency.

In combination with the extracellular matrix hPSC-ECM DXF and the hPSC Dissociation Buffer DXF PromoCell provides a defined and xeno-free complete culture system for human pluripotent stem cells. The hPSC-ECM DXF is a defined and xeno-free extracellular matrix of recombinant origin. The non-enzymatic chemically defined and xeno-free hPSC Dissociation Buffer DXF was designed for gentle but efficient subculture of hPSC. It supports clump as well as single cell passage.

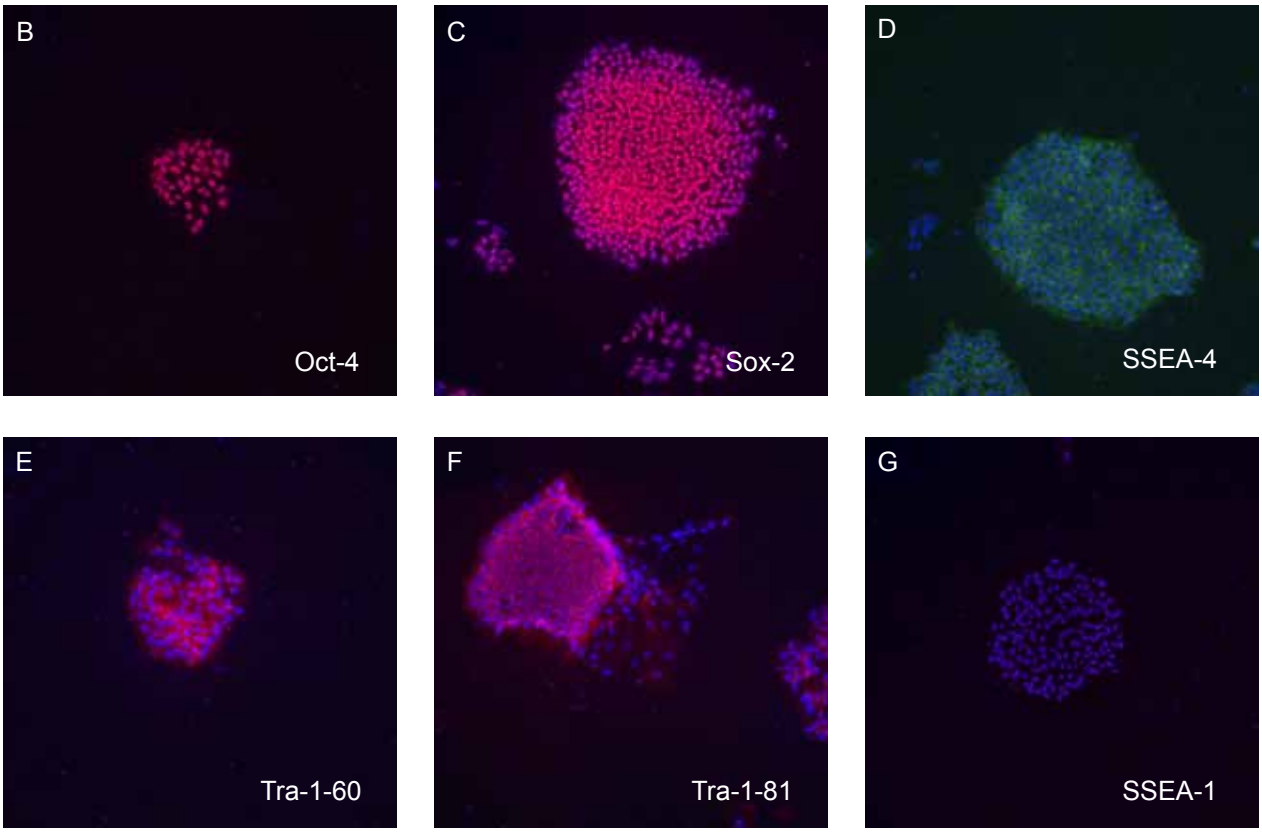

Fig. 4: hESC line cultured in the PromoCell hPSC Growth Medium DXF using PromoCell hPSC-ECM DXF as an extracellular matrix. A) Phase contrast image of hESC colony. B-G) ICC stainings of common pluripotency markers. Nuclei appear blue by DAPI-counterstain.

Use aseptic techniques and a laminar flow bench.

hPSC Culture Protocol

I. Materials and media/solutions

- hPSC Growth Medium DXF (C-28060)
- hPSC Dissociation Buffer DXF (C-41322)
- hPSC-ECM DXF, 20x conc. (C-43070)
- Dulbecco's PBS, w/o Ca^{++}/Mg^{++} (C-40232)

Optionally needed for thawing of frozen hPSC or single cell subculture:
- Y-27632 (PK-CA577-1596-1) *or alternatively*
- Thiazovivin (PK-CA577-1681-1)

II. Initiation of the culture

The protocol describes the steps for switching existing cultures to the PromoCell hPSC Growth Medium DXF. Ensure that cultures are in good condition. Pluripotency should be ≥90% for optimal results.
For media performance comparison tests, please refer to Section IV "How to perform a hPSC Media Performance Comparison?".

1. **Coat the culture vessel with Extracellular Matrix (ECM)**
   Dilute the thawed ECM stock solution of hPSC-ECM DXF 1:20 with Dulbecco's PBS, w/o Ca^{++}/Mg^{++}. Use 100 µl per cm^2 of culture surface to coat the closed tissue culture vessel with the diluted ECM solution and leave for 2 hours at room temperature. Make sure that the ECM solution covers the complete vessel. If not to be used immediately, the sealed vessel may be stored for up to 7 days at 2 to 8°C for later use. Aspirate the ECM solution just before seeding the cells. Diluted ECM solution may be stored for up to 2 weeks at 2 to 8°C protected from light.
   Note: For best results PromoCell recommends using of the hPSC-ECM DXF. However, other established types of appropriate ECM can be used.

2. **Prepare the complete hPSC Growth Medium DXF**
   Thaw the SupplementMix in your hands and mix thoroughly. Make sure there is no precipitate left. Prepare the complete hPSC Growth Medium DXF by adding the thawed SupplementMix aseptically to the Basal Medium. Swirl gently to obtain a homogeneous mixture.
   Note: Use the completely supplemented hPSC Growth Medium DXF within 10 days. For use, pre-warm only an aliquot of the complete medium and keep the remaining medium refrigerated at 2 to 8°C protected from light.

3. **Plate the cells (day 0)**
   For existing proliferating cultures perform a clump passage as described below in step III. A. Seed the cells in a 1:2 to 1:3 ratio into the ECM coated tissue culture vessel using an appropriate amount of hPSC Growth Medium DXF. For an example, use 2 - 3 ml per well for 6 well plates and 15-25 ml for T-75 flasks.
   For cryopreserved cells, add a ROCK-Inhibitor (10µM Y-27632 or 2µM Thiazovivin) to the thawing medium as well as to the hPSC Growth Medium DXF.
   Note: Adaption of hPSC to the PromoCell hPSC Growth Medium DXF is not necessary. However, allow up to 5 passages to reach full performance.

4. **Let the cells attach (day 0)**
   After plating let the subcultured cells attach over night (12 - 24 hours).

Use aseptic techniques and a laminar flow bench.

5. Medium change (day 1)

Perform a medium change: aspirate the medium including non-attached cells, wash once with Dulbecco's PBS, w/o Ca^{++}/Mg^{++} and provide the cells with fresh hPSC Growth Medium DXF (w/o ROCK-Inhibitors).

Note: Do not add ROCK-Inhibitors.

6. Cell expansion (day 1+)

Perform daily media changes. Let the cells expand until the single colonies touch each other (usually after 4 - 7 days) or colonies show the first signs of differentiation evoked from their large size. Proceed with step III) for subculture of the cells.

Note: For established hPSC cultures, the hPSC Growth Medium DXF supports extended feeding intervals for up to 48 hours as an exception. However, this is not recommended for the initiation phase of the culture.

The PromoCell hPSC Growth Medium DXF can rescue differentiating cultures. Perform a 1:1 ratio clump passage (see step III. A) and change medium twice daily until the differentiated cells are lost and cell/colony morphology has normalized.

III. Clump- or single cell subculture of hPSC

A. Clump passage (recommended for routine culture)

1. Aspirate culture medium and wash cells twice with Dulbecco's PBS, w/o Ca^{++}/Mg^{++}.
2. Add 200 - 300 µl/cm² of hPSC Dissociation Buffer DXF and incubate for 5-8 minutes in the incubator at 37°C and 5% CO_2.
   Note: Other established enzymatic dissociation/subculture procedures can also be used.
3. Carefully aspirate the Dissociation Buffer and add 1 - 5 ml of fresh hPSC Growth Medium DXF. Using a serological pipet, flush the colonies away from the surface. Avoid flushing more than 4 - 5 times in order to maintain cell clumps of sufficient size.
4. Dispense cell clumps in new ECM-coated culture vessels with fresh hPSC Growth Medium DXF.
5. Proceed according to the cell attachment step II. 4.

B. Single cell passage

1. Aspirate culture medium and wash cells twice with Dulbecco's PBS, w/o Ca^{++}/Mg^{++}.
2. Add 200 - 300 µl/cm² of hPSC Dissociation Buffer DXF and incubate for 5-8 minutes in the incubator at 37°C and 5% CO_2.
   Note: Other established enzymatic dissociation/subculture procedures can also be used.
3. Aspirate the Dissociation Buffer and replace by 50 - 100 µl/cm² fresh hPSC Dissociation Buffer DXF supplemented with a ROCK-Inhibitor (10µM Y-27632 or 2µM Thiazovivin).
4. Detach the cells by flushing the culture surface several times using a serological pipet. Triturate by pipetting up and down an additional 5-10 times.
5. Spin the single cell suspension (5 min, 200 x g, room temperature).
6. Aspirate and discard the supernatant and resuspend the cells in fresh hPSC Growth Medium DXF supplemented with a ROCK-Inhibitor (10µM Y-27632 or 2µM Thiazovivin).
   Note: Alternatively, resuspend the cells in a buffer of choice and use them for your experiments, e.g. immunostaining for flow cytometry analysis.
7. Plate the cells in an ECM-coated vessel and proceed with the attachment according to step II. 4.

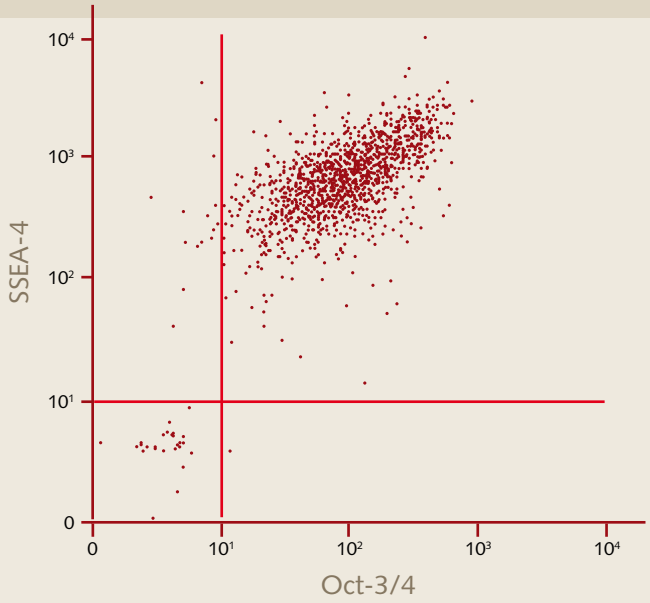

Fig. 4: Flow-cytometry analysis of human foreskin fibroblast-derived iPS cells. The cells were cultured for 17 passages in the PromoCell hPSC Growth Medium DXF using the PromoCell hPSC-ECM DXF as an extracellular matrix. Subculture was performed by clump-passage with the PromoCell hPSC Dissociation Buffer DXF. More than 98% of the cells in the culture stain positive for the human pluripotency markers Oct-3/4 and SSEA-4. More than 97% of the cells are negative for SSEA-1, a differentiation marker for human pluripotent stem cells (not shown).

IV. How to perform a hPSC Media Performance Comparison?

hPSC Culture Protocol

In order to compare the performance of PromoCell hPSC-GM DXF culture medium with your hPSC line(s), optimal growth conditions for each culture are essential for reliable test results.
Therefore, before comparing the performance of the PromoCell hPSC-GM DXF with your cells to other established hPSC-media, consider the following notes:

1. The PromoCell hPSC culture system is not only xeno-free and chemically defined, it also completely excludes substances purified from human origin.
   An explicit adaption of your cells to our culture system is not necessary. However, it will take your cells up to 5 passages to reach full performance under these highly defined culture conditions.

2. Use a 1:2 split ratio at 90% confluency for switching hPSC from other hPSC-Medium to the PromoCell hPSC culture system.

3. Direct comparison tests against other PSC culture media should only be evaluated after a minimum of 5 adaption passages of your cells in the PromoCell hPSC culture system

4. Use the same type and brand of tissue culture vessels for all samples.

5. Use the same ECM for both cultures. Preferentially use the PromoCell hPSC-ECM DXF.
   Note: PromoCell hPSC-GM DXF will also work with Matrigel and the like. However, as a chemically defined and xeno-free system, the medium was optimized for our defined hPSC-ECM DXF and performs best in this culture environment. Vitronectin may also be used.

hPSC Culture Protocol

6. Refer to the manufacturers´ instructions for media handling. Do not pre-warm the whole bottle of PromoCell hPSC-GM DXF, rather remove the needed amount and immediately put the remaining media back into the refrigerator. Do not use the complete medium longer than recommended.

7. Perform media changes according to the recommendations of the supplier of each hPSC culture medium. The cells should be culture in optimal conditions for each media tested.

8. Cells should be split according to the actual state of the culture, i.e. passage the test samples individually when they reach optimal condition for passage. Do not passage cultures at parallel time points in suboptimal conditions, i.e. too early or too late, which will be detrimental.

9. Use the split ratio recommended by the manufacturer of each medium.

10. Use the same passaging technique and reagent(s) for each test culture. The PromoCell hPSC-GM DXF is optimized for the use with the hPSC-Dissociation Buffer DXF and clump passage as described in section III.A of this Application Note.
Note: Use of enzymatic passaging reagents in defined culture systems is prone to detrimental over-digestion.

References

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Products

Product	Size	Catalog Number
hPSC Growth Medium DXF	500 ml	C-28060
hPSC-ECM DXF (20x)	2 ml	C-43070
hPSC Dissociation Buffer DXF	100 ml	C-41322
Cryo-SFM	30 ml 125 ml	C-29910 C-29912

Related Products

Product	Size	Catalog Number
Dulbecco's PBS, w/o Ca^{++}/Mg^{++}	500 ml	C-40232
A 83-01	1 mg	PK-CA577-1725-1
BIX 01294	25 mg	PK-CA577-1678-25
BIX 01294	5 mg	PK-CA577-1678-5
Cardiogenol C hydrochloride	25 mg	PK-CA577-1926-25
Cardiogenol C hydrochloride	5 mg	PK-CA577-1926-5
Forskolin	5 mg	PK-CA577-1531-5
PS48	100 mg	PK-CA577-1869-100
PS48	25 mg	PK-CA577-1869-25
PS48	5 mg	PK-CA577-1869-5
Pyrintegrin	1 mg	PK-CA577-1729-1
Pyrintegrin	5 mg	PK-CA577-1729-5
RepSox	25 mg	PK-CA577-1894-25
RepSox	5 mg	PK-CA577-1894-5
RG108	10 mg	PK-CA577-1679-10
RG108	30 mg	PK-CA577-1679-30
Stauprimide	500 µg	PK-CA577-1743-500
Thiazovivin	1 mg	PK-CA577-1681-1
Thiazovivin	5 mg	PK-CA577-1681-5
Valproic Acid, Sodium Salt	200 mg	PK-CA577-1647-200
Y-27632, dihydrochloride	1 mg	PK-CA577-1596-1
Y-27632, dihydrochloride	5 mg	PK-CA577-1596-5
Y-27632, dihydrochloride	50 mg	PK-CA577-1596-50

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