

PLURIPOTENT STEM CELL ASSAY**FIELD**

The invention relates to a method for detecting residual, undifferentiated pluripotent stem cells (PSCs) in a culture of cells differentiated from PSCs.

BACKGROUND

Pluripotent stem cells (PSCs), in particular human PSCs, which include Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs), provide the opportunity to develop novel cell-based therapeutic products.

Differentiated derivatives of iPSCs have therapeutic efficacy in a variety of disease applications (e.g. cirrhosis, Parkinson's disease, age-related macular degeneration, cardiac ischemia, diabetes, graft-versus host disease).

However, use in clinical trials of therapeutic compositions comprising PSC-derived cells poses several challenges, in particular the presence of residual undifferentiated PSCs in the final product that have a known potential for tumour formation. Such a challenge is especially pertinent to therapies that require high doses of differentiated, PSC-derived cells.

Accordingly, there is a need for a method, or an improved method, for detecting residual, undifferentiated PSCs in a culture of cells derived from PSCs, to be used in quality control when producing therapeutic compositions comprising PSC-derived cells.

Any publications mentioned in this specification are herein incorporated by reference. However, if any publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art in Australia or any other country.

SUMMARY

The inventors have addressed this need for quality control in manufacture of therapeutic compositions comprising PSC-derived cells by developing a method for detecting residual, undifferentiated PSCs in a culture of PSC-derived cells. The method relies on a cell

culture protocol for expansion of singularised, undifferentiated PSCs to increase sensitivity (true positive) and specificity (true negative) for residual undifferentiated PSCs.

Accordingly, a first aspect provides a method for detecting residual, undifferentiated pluripotent stem cells (PSCs) in a culture of cells differentiated from PSCs, the method comprising:

culturing the cells on a substrate coated with laminin-521 and E-cadherin in a medium comprising a Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor;

quantitating in the cultured cells expression of a marker of residual, undifferentiated PSCs; and

comparing the marker expression in the cultured cells with the marker expression in a reference culture of cells comprising a known proportion of PSCs,

wherein lower marker expression in the culture of cells than marker expression in the reference culture of cells indicates absence of residual, undifferentiated PSCs in the cultured cells or presence of residual, undifferentiated PSCs in the cultured cells at a proportion lower than the known proportion of PSCs in the reference culture of cells.

The inventors propose that this method provides an improved means for quality control and is an important contribution to the safety, and therefore advancement, of therapies relying on PSC-derived cells.

Accordingly, in one embodiment, the method further comprises formulating the culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion into a therapeutic composition. The method may further comprise treating or preventing a condition in a subject by administering the therapeutic composition to the subject.

A second aspect provides a method for manufacturing a therapeutic composition, the method comprising formulating into a composition for therapeutic administration to a subject a culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion when detected by the method of the first aspect.

A third aspect provides a method for treating or preventing a condition in a subject, the method comprising administering to the subject:

a culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion when detected by the method of the first aspect; or

a therapeutic composition when manufactured by the method of the second aspect.

An alternative form of the third aspect provides use of a culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion in the manufacture of a medicament, such as a therapeutic composition, for treating or preventing a condition in a subject, wherein residual, undifferentiated PSCs in the culture of cells differentiated from PSCs are detected by the method of the first aspect.

Another alternative form of the third aspect provides:

a culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion when detected by the method of the first aspect; or

a therapeutic composition when manufactured by the method of the second aspect,

for use in treating or preventing a condition in a subject.

The condition to be prevented or treated may be bone cysts, bone neoplasms, fractures, cartilage defects, osteoarthritis, ligament injury, osteogenesis imperfecta, osteonecrosis, osteoporosis, aplastic anaemia, graft versus host disease (GvHD), myelodysplastic syndrome, Type 1 diabetes, Type 2 diabetes, autoimmune hepatitis, liver cirrhosis, liver failure, dilated cardiomyopathy, heart failure, myocardial infarction, myocardial ischemia, Crohn's disease, ulcerative colitis, burns, epidermolysis bullosa, lupus erythematosus, rheumatoid arthritis, Sjogren's disease, systemic sclerosis, bronchopulmonary dysplasia, chronic obstructive airways disease, emphysema, pulmonary fibrosis, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, brain injury, ataxia, degenerative disc disease, multiple system atrophy, multiple sclerosis, Parkinson's disease, retinitis pigmentosa, Romberg's disease, spinal cord injury, stroke, muscular dystrophy,

limb ischaemia, kidney injury, lupus nephritis, endometriosis and complications of bone marrow or solid organ transplantation.

A fourth aspect provides a kit for detecting residual, undifferentiated PSCs in a culture of cells differentiated from PSCs, the kit comprising:

laminin-521; and

E-cadherin; and

a ROCK inhibitor.

In one embodiment, the kit is used according to the method of the first aspect.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an example amino acid sequence of human laminin-521 α chain (SEQ ID NO: 1).

Figure 2 is an example amino acid sequence of human laminin-521 β chain (SEQ ID NO: 2).

Figure 3 is an example amino acid sequence of human laminin-521 γ chain (SEQ ID NO: 3).

Figure 4 is an example amino acid sequence of human E-cadherin (SEQ ID NO: 4).

Figure 5 is an example coding nucleotide sequence of human LIN28 (LIN28A) (SEQ ID NO: 5).

Figure 6 is an example coding nucleotide sequence of human OCT4 (POU5F1) (SEQ ID NO: 6).

Figure 7 is an example coding nucleotide sequence of human SOX2 (SEQ ID NO: 7).

Figure 8 is an example coding nucleotide sequence of human FOXD3 (SEQ ID NO: 8).

Figure 9 is an example coding nucleotide sequence of human NANOG (SEQ ID NO: 9).

Figure 10 is an example coding nucleotide sequence of human PODXL (SEQ ID NO: 10).

Figure 11 is an example coding nucleotide sequence of human REX1 (ZFP42) (SEQ ID NO: 11).

Figure 12 is an example coding nucleotide sequence of human SSEA1 (FUT4) (SEQ ID NO: 12).

Figure 13 is an example coding nucleotide sequence of human DPPA2 (SEQ ID NO: 13).

Figure 14 is an example coding nucleotide sequence of human DPPA3 (SEQ ID NO: 14).

DETAILED DESCRIPTION

Disclosed herein is a method for detecting residual, undifferentiated PSCs in a culture of cells derived from PSCs, where the PSC-derived cells are destined for therapeutic administration to a subject. Accordingly, the invention provides an improved method of quality control and risk minimisation, for example, risk of tumour formation from such residual, undifferentiated PSCs.

The method relies on specific culture conditions to expand the undifferentiated PSCs under conditions that support PSC clonal growth, then quantitation of expression of a gene that is highly expressed in PSCs, but is not expressed, or only minimally expressed, in PSC-derived cells that have undergone differentiation. The culture conditions may selectively support expansion of PSCs at the single cell level. In one embodiment, the PSCs may be cultured under conditions that support PSC growth from a single cell suspension. Such culture conditions are known in the art and include laminin-521 and E-cadherin as described herein, as well as commercially available systems that include the Cellartis iPSC Single-Cell Cloning DEF-CS Culture Media Kit, Gibco™ StemFlex™ Medium, and PluriQ™ G9™ Cloning Medium in conjunction with single cell growth on vitronectin.

Marker expression in a test culture at or below marker expression in a reference culture with a known proportion of undifferentiated PSCs indicates that the test culture comprises a lower proportion of undifferentiated PSCs than the reference culture.

Importantly, the method can detect residual, undifferentiated PSCs in a culture of PSC-derived cells at low proportions of the total cell number, for example 0.001% or 10 ppm.

Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly

understood by the person skilled in the art to which this invention belongs and by reference to published texts.

It is to be noted that the term "a" or "an" refers to one or more, for example, "a molecule," is understood to represent one or more molecules. As such, the terms "a" or "an", "one or more," and "at least one" may be used interchangeably herein.

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

The term "about" as used herein contemplates a range of values for a given number of $\pm 25\%$ the magnitude of that number. In other embodiments, the term "about" contemplates a range of values for a given number of $\pm 20\%$, $\pm 15\%$, $\pm 10\%$, or $\pm 5\%$ the magnitude of that number. For example, in one embodiment, "about 3 grams" indicates a value of 2.7 to 3.3 grams (i.e. 3 grams $\pm 10\%$), and the like.

Similarly, in other embodiments, periods of time may vary by $\pm 25\%$, $\pm 20\%$, $\pm 15\%$, $\pm 10\%$, or $\pm 5\%$ of that period of time. For example, "one day" may include a period of about 18 to about 30 hours. Periods of time indicated that are multiple day periods may be multiples of "one day," such as, for example, two days may span a period of about 36 to about 60 hours, and the like. In other embodiments, time variation may be lessened, for example, where: day 1 is 24 ± 3 hours from day 0; day 2 is 48 ± 3 hours from day 0; day 3 is 72 ± 3 hours from day 0; day 4 is 96 ± 3 hours from day 0; day 5 is 120 ± 3 hours from day 0, and so on. In some embodiments, about 3 days is 3 days ± 1 day, and about 5 days is 5 days ± 1 day.

As used herein, "pluripotent stem cell" or "PSC" refers to a cell that has the ability to reproduce itself indefinitely, and to differentiate into any other cell type. There are two main types of pluripotent stem cell: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

As used herein, "embryonic stem cell" or "ESC" refers to a cell isolated from a five to seven day-old embryo donated with

consent by patients who have completed *in vitro* fertilisation therapy, and have surplus embryos. The use of ESCs has been hindered to some extent by ethical concerns about the extraction of cells from human embryos.

Human PSCs suitable for manufacturing a therapeutic composition include H1 and H9 human ESCs.

As used herein, "induced pluripotent stem cell" or "iPSC" refers to an ESC-like cell derived from adult cells. iPSCs have very similar characteristics to ESCs, but avoid the ethical concerns associated with ESCs, since iPSCs are not derived from embryos. Instead, iPSCs are typically derived from fully differentiated adult cells that have been "reprogrammed" back into a pluripotent state.

Human iPSCs suitable for manufacturing a therapeutic composition include, but are not limited to, iPSC 19-9-7T, MIRJT6i-mND1-4 and MIRJT7i-mND2-0 derived from fibroblasts and iPSC BM119-9 derived from bone marrow mononuclear cells. Other suitable iPSCs may be obtained from Cellular Dynamics International (CDI; Nasdaq: ICEL) of Madison, WI, USA.

As used herein, "differentiating" refers to a process of a cell changing from one cell type to another, in particular a less specialised type of cell becoming a more specialised type of cell.

As used herein, the term "derived from" may encompass "differentiating" a PSC into another cell type.

Accordingly, an "undifferentiated PSC" is a PSC that has not differentiated into another cell type. With respect to the present disclosure, the undifferentiated PSC is present within a population of otherwise differentiated PSCs.

As used herein, "medium" or its plural "media" refers to a liquid or gel designed to support the growth, including expansion and differentiation, of cells. Such growth, including expansion and differentiation, of cells *in vitro* is referred to as "culturing" cells, or cell "culture".

However, cells cannot be held in culture indefinitely owing to the increasing concentration of toxic metabolites, decreasing concentration of nutrients, and, for dividing cells, an increasing number of cells. As used herein, "passaging" refers to the process of producing a new cell culture with refreshed concentrations of

nutrients, no toxic metabolites, and optionally a lower density of cells than the originating culture.

The number of passages for a cell culture, e.g. a PSC-derived cell culture to be tested by the present method, may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25. Preferably, the number of passages is 10 or fewer. More preferably, the number of passages is 5 or 6. In one embodiment, the PSC-derived cells have undergone 5 passages before being subjected to the present method.

In one embodiment, the PSC-derived cell is a mesenchymal stem (or stromal) cell (MSC).

In one embodiment, the ESC-derived cell is a MSC.

In a preferred embodiment, the iPSC-derived cell is a MSC, which may also be referred to as an iPSC-MSC.

As used herein, "mesenchymal stem cell" or "MSC" refers to a particular type of stem cell that may be isolated from a wide range of tissues, including bone marrow, adipose tissue (fat), placenta and umbilical cord blood. MSCs may differentiate into bone cells (osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and other kinds of connective tissue cells such as those in tendons

According to the present disclosure, MSCs may be formed from PSCs via ^{EMH}lin⁻KDR⁺APLN⁺R⁺PDGFR⁺alpha⁺ primitive mesoderm cells with mesenchymoangioblast (MCA) potential. "^{EMH}lin⁻KDR⁺APLN⁺R⁺PDGFR⁺alpha⁺ primitive mesoderm cell with mesenchymoangioblast (MCA) potential" refers to a cell expressing typical primitive streak and lateral plate/extra-embryonic mesoderm genes. These cells have potential to form mesenchymoangioblast (MCA) and hemangioblast colonies in serum-free medium in response to FGF2. The term ^{EMH}lin⁻ denotes lack of expression of CD31, VE-cadherin endothelial markers, CD73 and Cd105 mesenchymal/endothelial markers, and CD43 and CD45 hematopoietic lineage markers.

As used herein, "mesenchyme" or "mesenchymal" refers to to embryonic connective tissue that is derived from the mesoderm and that differentiates into hematopoietic tissue (lymphatic and circulatory systems) and connective tissue, such as bone and

cartilage. However, MSCs do not differentiate into hematopoietic cells.

MSCs secrete bioactive molecules such as cytokines, chemokines and growth factors and have the ability to modulate the immune system. MSCs have been shown to facilitate regeneration and effects on the immune system without relying upon engraftment. In other words, the MSCs themselves may not necessarily become incorporated into the host - rather, they may exert their effects and are then eliminated within a short period of time. However, MSCs may be engrafted into damaged tissues following administration and migration.

MSCs are currently in clinical trials for treating numerous conditions, diseases and disorders, including graft-versus host disease.

The ability of MSCs to exert immunomodulatory/ immunosuppressive effects, in particular by suppressing T cells, is believed to be central to the therapeutic effects of MSCs in a wide range of conditions, diseases and disorders, including graft-versus host disease, immune disorders including autoimmune disorders, cardiovascular disorders, orthopaedic disorders and rejection of transplanted solid organs. Some specific examples include bone cysts, bone neoplasms, fractures, cartilage defects, osteoarthritis, ligament injury, osteogenesis imperfecta, osteonecrosis, osteoporosis, aplastic anaemia, myelodysplastic syndrome, Type 1 diabetes, Type 2 diabetes, autoimmune hepatitis, liver cirrhosis, liver failure, dilated cardiomyopathy, heart failure, myocardial infarction, myocardial ischemia, Crohn's disease, ulcerative colitis, burns, epidermolysis bullosa, lupus erythematosus, rheumatoid arthritis, Sjogren's disease, systemic sclerosis, bronchopulmonary dysplasia, chronic obstructive airways disease, emphysema, pulmonary fibrosis, ALS, Alzheimer's disease, brain injury, ataxia, degenerative disc disease, multiple system atrophy, multiple sclerosis, Parkinson's disease, retinitis pigmentosa, Romberg's disease, spinal cord injury, stroke, muscular dystrophy, limb ischaemia, kidney injury, lupus nephritis, endometriosis and complications of bone marrow or solid organ transplantation,

MSCs are thought to perform a critical role in injury healing, and have been shown to be effective in treating tissue injury and degenerative diseases, including in the digestive system, for example in liver cirrhosis and liver failure, in the musculoskeletal system, in periodontal tissue, in diabetic critical limb ischemia, in osteonecrosis, in burn-related disorders, in myocardial infarction, in cornea damage, in the brain, in the spinal cord, in the lungs, and in treating radiation exposure.

MSCs have shown therapeutic outcomes in immune disorders, including graft-versus-host disease, systemic lupus erythematosus (SLE), Crohn's disease, multiple system atrophy, multiple sclerosis, amyotrophic lateral sclerosis, and stroke.

MSCs have been shown to exert immunosuppressive activities against T cells, B cells, dendritic cells, macrophages, and natural killer cells. While not wishing to be bound by theory, the underlying mechanisms may comprise immunosuppressive mediators, for example nitric oxide, indoleamine 2,3, dioxygenase, prostaglandin E2, tumour necrosis factor-inducible gene 6 protein, CCL-2, and programmed death ligand 1. These mediators are expressed at a low level until stimulated, for example by an inflammatory cytokines, such as IFN γ , TNF α , and IL-17.

iPSC-derived MSCs (iPSC-MSCs) have a unique advantage over directly sourced MSCs, i.e. derived from tissues such as bone marrow, umbilical cord blood, adipose tissue, because *in vitro* expansion of iPSCs can provide a virtually unlimited supply of MSCs.

iPSC-MSCs can be produced according to example 1.

PSCs are able to differentiate into any cell type of any of endoderm, ectoderm, and mesoderm. PSCs undergo differentiation into multipotent progenitor cells that differentiate further into functional cells. Therefore, the description above of MSCs is exemplary and not to be construed as limiting, and other examples of PSC-derived cells may include: hematopoietic stem cells that give rise to all the types of blood cells including red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, macrophages, and platelets; neural stem cells in the brain that give rise to its three major cell types being nerve cells (neurons) and two categories of non-

neuronal cells, astrocytes and oligodendrocytes; epithelial stem cells in the lining of the digestive tract that give rise to absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells; and skin stem cells that occur in the basal layer of the epidermis give rise to keratinocytes and that occur at the base of hair follicles and give rise to both the hair follicle and to the epidermis.

As used herein, a "substrate" is any material suitable for culturing PSCs and/or cells derived from PSCs. Examples include plastic culture ware such as dishes, multi-well plates, and flasks.

All proteins described herein are known to the person skilled in the art and are available commercially.

Laminin-521 is a heterotrimeric protein secreted by human PSCs. Laminin-521 comprises five α chains, two β chains and one γ chain (i.e. $\alpha 5\beta 2\gamma 1$). In one embodiment, the α chain, the β chain and the γ chain have the amino acid sequences represented by SEQ ID NOs: 1, 2 and 3 (Figures 1, 2 and 3), respectively.

E-cadherin is a calcium-dependent cell adhesion protein associated with epithelial cell function. In one embodiment, human E-cadherin has the amino acid sequence represented by SEQ ID NO: 4 (Figure 4).

In performing the present method, the substrate may be coated with laminin-521 using about 0.1 $\mu\text{g/mL}$, 0.2 $\mu\text{g/mL}$, 0.3 $\mu\text{g/mL}$, 0.4 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, 0.6 $\mu\text{g/mL}$, 0.7 $\mu\text{g/mL}$, 0.8 $\mu\text{g/mL}$, 0.9 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, 3 $\mu\text{g/mL}$, 4 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 6 $\mu\text{g/mL}$, 7 $\mu\text{g/mL}$, 8 $\mu\text{g/mL}$, 9 $\mu\text{g/mL}$, or 10 $\mu\text{g/mL}$ and/or about 0.1 $\mu\text{g/cm}^2$, 0.2 $\mu\text{g/cm}^2$, 0.3 $\mu\text{g/cm}^2$, 0.4 $\mu\text{g/cm}^2$, 0.5 $\mu\text{g/cm}^2$, 0.6 $\mu\text{g/cm}^2$, 0.7 $\mu\text{g/cm}^2$, 0.8 $\mu\text{g/cm}^2$, 0.9 $\mu\text{g/cm}^2$, 1 $\mu\text{g/cm}^2$, 2 $\mu\text{g/cm}^2$, 3 $\mu\text{g/cm}^2$, 4 $\mu\text{g/cm}^2$, 5 $\mu\text{g/cm}^2$, 6 $\mu\text{g/cm}^2$, 7 $\mu\text{g/cm}^2$, 8 $\mu\text{g/cm}^2$, 9 $\mu\text{g/cm}^2$, or 10 $\mu\text{g/cm}^2$.

The substrate may be coated with E-cadherin using about 0.1 $\mu\text{g/mL}$, 0.2 $\mu\text{g/mL}$, 0.3 $\mu\text{g/mL}$, 0.4 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, 0.6 $\mu\text{g/mL}$, 0.7 $\mu\text{g/mL}$, 0.8 $\mu\text{g/mL}$, 0.9 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, 3 $\mu\text{g/mL}$, 4 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 6 $\mu\text{g/mL}$, 7 $\mu\text{g/mL}$, 8 $\mu\text{g/mL}$, 9 $\mu\text{g/mL}$, or 10 $\mu\text{g/mL}$ and/or about 0.01 $\mu\text{g/cm}^2$, 0.05 $\mu\text{g/cm}^2$, 0.1 $\mu\text{g/cm}^2$, 0.2 $\mu\text{g/cm}^2$, 0.25 $\mu\text{g/cm}^2$, 0.3 $\mu\text{g/cm}^2$, 0.4 $\mu\text{g/cm}^2$, 0.5 $\mu\text{g/cm}^2$, 0.6 $\mu\text{g/cm}^2$, 0.7 $\mu\text{g/cm}^2$, 0.8 $\mu\text{g/cm}^2$, 0.9 $\mu\text{g/cm}^2$, 1 $\mu\text{g/cm}^2$, 2 $\mu\text{g/cm}^2$, 3 $\mu\text{g/cm}^2$, 4 $\mu\text{g/cm}^2$, 5 $\mu\text{g/cm}^2$, 6 $\mu\text{g/cm}^2$, 7 $\mu\text{g/cm}^2$, 8 $\mu\text{g/cm}^2$, 9 $\mu\text{g/cm}^2$, or 10 $\mu\text{g/cm}^2$.

In a preferred embodiment, the substrate is coated with laminin-521 using 10 µg/mL and 2 µg/cm² and with E-cadherin using 1.1 µg/mL and 0.22 µg/cm².

Rho-associated, coiled-coil containing protein kinase (ROCK) is involved mainly in regulating the shape and movement of cells by acting on the cytoskeleton. Thus, a "ROCK inhibitor" inhibits this function, and for present purposes a "ROCK inhibitor" enhances survival of iPSCs.

In one embodiment the ROCK inhibitor is Y27632 (CAS No: 129830-38-2). Other examples of ROCK inhibitors that may be used in the present method are AS 1892802 (CAS No: 928320-12-1), Fasudil hydrochloride (CAS No: 105628-07-7), GSK 269962 (CAS No: 850664-21-0), GSK 429286 (CAS No: 864082-47-3), H 1152 dihydrochloride (CAS No: 871543-07-6), Glycyl-H 1152 dihydrochloride (CAS No: 913844-45-8), HA 1100 hydrochloride (CAS No: 155558-32-0), OXA 06 dihydrochloride, RKI 1447 dihydrochloride, SB 772077B dihydrochloride (CAS No: 607373-46-6), SR 3677 dihydrochloride (CAS No: 1072959-67-1), and TC-S 7001 (CAS No: 867017-68-3).

The concentration of the ROCK inhibitor in which the cells are cultured may be about 0.1 µM, 0.5 µM, 1 µM, 2 µM, 3 µM, 4 µM, 5 µM, 6 µM, 7 µM, 8 µM, 9 µM, 10 µM, 11 µM, 12 µM, 13 µM, 14 µM, 15 µM, 16 µM, 17 µM, 18 µM, 19 µM, 20 µM, 25 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM, 80 µM, 90 µM, or 100 µM, for example.

Preferably, the ROCK inhibitor is Y27632 and the concentration of Y27632 in which the cells are cultured is about 10 µM.

According to the present method, for culturing the cells on a substrate coated with laminin-521 and E-cadherin in a medium comprising a Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor, the cells must be seeded on the substrate. The seeding density of the cells on the substrate may be about 1x10² cells/cm², 2x10² cells/cm², 3x10² cells/cm², 4x10² cells/cm², 5x10² cells/cm², 6x10² cells/cm², 7x10² cells/cm², 8x10² cells/cm², 9x10² cells/cm², 1x10³ cells/cm², 2x10³ cells/cm², 3x10³ cells/cm², 4x10³ cells/cm², 5x10³ cells/cm², 6x10³ cells/cm², 7x10³ cells/cm², 8x10³ cells/cm², 9x10³ cells/cm², 1x10⁴ cells/cm², 2x10⁴ cells/cm², 3x10⁴ cells/cm², 4x10⁴ cells/cm², 5x10⁴ cells/cm², 6x10⁴ cells/cm²,

7×10^4 cells/cm², 8×10^4 cells/cm², 9×10^4 cells/cm², 1×10^5 cells/cm², 2×10^5 cells/cm², 3×10^5 cells/cm², 4×10^5 cells/cm², 5×10^5 cells/cm², 6×10^5 cells/cm², 7×10^5 cells/cm², 8×10^5 cells/cm², 9×10^5 cells/cm², 1×10^6 cells/cm², 2×10^6 cells/cm², 3×10^6 cells/cm², 4×10^6 cells/cm², 5×10^6 cells/cm², 6×10^6 cells/cm², 7×10^6 cells/cm², 8×10^6 cells/cm², 9×10^6 cells/cm². In some embodiments, the seeding density may be about 1.1×10^4 cells/cm², 1.2×10^4 cells/cm², 1.3×10^4 cells/cm², 1.4×10^4 cells/cm², 1.5×10^4 cells/cm², 1.6×10^4 cells/cm², 1.7×10^4 cells/cm², 1.8×10^4 cells/cm², or 1.9×10^4 cells/cm². In a preferred embodiment, the seeding density is about 1.3×10^4 cells/cm².

As part of the present method, the cells may be cultured for about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or more before quantitating marker expression in the cultured cells. Preferably, the cells are cultured for about 5 days before quantitating marker expression in the cultured cells.

As part of the present method, the cells may be cultured in the presence of the ROCK inhibitor for about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or more before quantitating marker expression in the cultured cells.

After the cells have been cultured in the presence of the ROCK inhibitor, the cells may be cultured further in the absence of the ROCK inhibitor before quantitating marker expression in the cultured cells. In one embodiment, the cells are cultured further in the absence of the ROCK inhibitor for about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or more before quantitating marker expression in the cultured cells.

In a preferred embodiment, the cells are cultured in the presence of the ROCK inhibitor for about 3 days, then cultured further in the absence of the ROCK inhibitor for about 2 days before quantitating marker expression in the cultured cells.

In summary, expanding PSC-derived cells, such as MSCs, on a substrate, such as plastic culture ware, coated with laminin-521 and E-cadherin has been demonstrated to support the expansion of singularised, undifferentiated PSCs that reside within the population of PSC-derived cells, while simultaneously, a ROCK inhibitor enhances survival of the PSCs.

As used herein, "marker expression" refers to a gene that is expressed in residual, undifferentiated PSCs, but is not expressed, or only expressed at lower levels, in PSC-derived cells. Such a gene may be coding or non-coding, for example a mRNA, microRNA, or non-coding RNA.

Markers whose expression may be quantitated according to the present method include LIN28 (LIN28A), OCT4 (POU5F1), SOX2, FOXD3, NANOG, PODXL (protein isoforms of which are detected by antibodies TRA-1-60 and TRA-1-81), REX1 (ZFP42), SSEA1 (FUT4), SSEA4, DPPA2 and DPPA3. Preferably, marker expression is LIN28 expression. The LIN28 gene encodes the LIN28 protein that is an RNA-binding protein that promotes pluripotency and is highly expressed in PSCs, but is down-regulated in response to differentiation.

"Marker expression" may be quantitated as protein or mRNA expression. Preferably, marker expression is quantitated as mRNA expression.

Marker protein may be quantitated by processes involving gel electrophoresis (e.g. Western blot or two-dimensional gel electrophoresis), densitometry, fluorescence, luminescence, radioactivity, arrays, and/or mass spectrometry.

Marker mRNA may be quantitated by processes involving gel electrophoresis (e.g. Northern blot), densitometry, fluorescence, luminescence, radioactivity, arrays, and/or polymerase chain reaction (PCR). PCR generally relies on reverse transcription to generate marker cDNA from marker mRNA.

Preferably, marker expression is quantitated using PCR. Preferably, marker expression is quantitated using quantitative reverse transcription PCR (qRT-PCR). Preferably, qRT-PCR is real-time qRT-PCR in which a mRNA is reverse transcribed to a cDNA template and the cDNA template is amplified exponentially and quantitated by fluorescence in real-time.

Quantitating marker expression may be relative or absolute.

In one embodiment, quantitating marker expression is relative and relies on measuring the quantification cycle (Cq), which is the cycle where the fluorescent signal crosses the threshold for qRT-PCR assays. The Cq is inversely proportional to mRNA expression.

Relative quantitation of marker expression relies on comparison of marker expression in a test cell culture to marker expression in one or more reference cultures (i.e. controls).

A "reference culture" may be a positive control or a negative control. When the reference culture is a positive control, the reference culture of cells comprises PSCs. Preferably, such a reference culture comprises a known proportion of PSCs. That is, the reference culture is "spiked" with a known proportion of undifferentiated PSCs, thereby enabling comparison between the test culture and the reference culture. In this embodiment, quantitation may be both relative (e.g. use of Cq as a unit of quantitation) and absolute (e.g. application of Cq to a known quantity). This may also be referred to as semi-quantitative. Thus, lower marker expression in the culture of cells being tested than marker expression in the reference culture of cells indicates absence of residual, undifferentiated PSCs in the cultured cells. Alternatively, lower marker expression in the culture of cells being tested than marker expression in the reference culture of cells indicates presence of residual, undifferentiated PSCs in the cultured cells, but presence at a proportion lower than the known proportion of PSCs in the reference culture of cells.

The known proportion of PSCs in the reference culture may be 0.000001%, 0.000005%, 0.00001%, 0.00005%, 0.0001%, 0.0005%, 0.0006%, 0.0007%, 0.0008%, 0.0009%, 0.001%, 0.002%, 0.003%, 0.004%, 0.005%, 0.006%, 0.007%, 0.008%, 0.009%, or 0.01% of the total number of cells. In a preferred embodiment, the known proportion of PSCs in the reference culture is 0.001% of the total number of cells.

In some embodiments, marker expression is "normalised", which compensates for intra- and inter-kinetic variations (sample-to-sample and run-to-run variations). Normalised data are particularly useful when quantitating gene expression using qRT-PCR. Different methods of normalisation will be known to the person skilled in the art, including normalisation to one or more unregulated or constitutive "housekeeping" genes, e.g. GAPDH, ACTB, LDHA, NONO, PGK1, or PPIH, and normalisation to total RNA. In one embodiment, marker expression is normalised to GAPDH expression.

PCR requires nucleotide primers. The person skilled in the art will understand how to design primers for PCR and qRT-PCR, and primer/probe combinations for qRT-PCR. Primers for some genes are available commercially. Primer/probe combinations for some genes also are available commercially. For example, the following TaqMan® Gene Expression assays are available commercially, all with Catalog No. 4331182: human LIN28 (LIN28A) assay Hs00702808_s1; human OCT4 (POU5F1) assay Hs04260367_gH; human SOX2 Hs01053049_s1; human FOXD3 assay Hs00255287_s1; human NANOG assay Hs04399610_g1; human PODXL (protein isoforms of which are detected by antibodies TRA-1-60 and TRA-1-81) assay Hs01574644; human REX1 (ZFP42) assay Hs01938187_s1; human SSEA1 (FUT4) assay Hs01106466_s1; human DPPA2 assay Hs00414515_m1 and human DPPA3 assay Hs01931905_g1.

Primers or primer/ probe combinations may be designed readily by the person skilled in the art using an online tool, for example, primer/ probe combinations may be custom designed using a tool such as the Custom TaqMan® Assay Design Tool or the GenScript Real-time PCR (TaqMan®) Primer Design tool. Primers may be designed using tools such as Primer3Plus or PrimerQuest Tool. These tools are examples only, and many more are readily available to the person skilled in the art.

Alternatively or additionally, primers or primer/ probe combinations may be designed readily from first principles, which include the following considerations.

PCR involves a cycle of: denaturing double stranded target DNA; annealing primers to the complementary regions of the single stranded DNA; extending the DNA by the action of DNA polymerase; and repeating, often for around 50 cycles. These steps are temperature sensitive and are commonly performed at 94°C, 60°C and 70°C respectively. Good primer design is essential for successful reactions. Important design considerations include:

1. primer length, often 18-22 bp;
2. primer melting temperature (T_m), often in the range of 52-58°C;
3. primer annealing temperature (T_a);
4. GC content, often 40-60%;

5. GC clamp, i.e. G or C bases located within the last five bases from the 3' end of primers;

6. primer and template secondary structures, i.e. intermolecular or intramolecular interactions, e.g. hairpins, self dimers, or cross dimers;

7. di-nucleotide repeats;

8. long runs of a single base;

9. 3' end stability;

10. cross homology/ specificity;

11. amplicon length, often around 100 bp for qRT-PCR and around 500 bp for standard PCR;

12. T_m of product;

13. primer pair T_m, often <5°C.

Primer T_m may be calculated as follows:

$T_m(K) = \{\Delta H / \Delta S + R \ln(C)\}$, or $T_m(^{\circ}C) = \{\Delta H / \Delta S + R \ln(C)\} - 273.15$, where

ΔH (kcal/mole): H is enthalpy and ΔH is the change in enthalpy

ΔS (kcal/mole): S is entropy and ΔS is the change in entropy.

ΔS (salt correction) = ΔS (1M NaCl) + $0.368 \times N \times \ln([Na^+])$,

where

N is the number of nucleotide pairs in the primer (primer length -1)

$[Na^+]$ is salt equivalent in mM

$[Na^+]$ calculation:

$[Na^+] = \text{Monovalent ion concentration} + 4 \times \text{free } Mg^{2+}$.

Primer T_a may be calculated as follows:

$T_a = 0.3 \times T_m (\text{primer}) + 0.7 T_m (\text{product}) - 14.9$

Example coding nucleotide sequences for human LIN28 (LIN28A), human OCT4 (POU5F1), human SOX2, human FOXD3, human NANOG, human PODXL, human REX1 (ZFP42), human SSEA1 (FUT4), human DPPA2 and human DPPA3 are provided in Figures 5 to 14 and SEQ ID NOs: 5 to 14, respectively. These sequences may be used by the person skilled in the art to design primers and primer/ probe combinations.

qRT-PCR relies on fluorescence to quantitate gene expression, e.g. marker expression. Such fluorescence may comprise (1) a non-specific fluorescent dye that intercalates with any double-stranded DNA, i.e. the amplified PCR product, or (2) a sequence-specific oligonucleotide probe labelled with a fluorescent reporter that fluoresces only after hybridisation of the probe with its complementary sequence. One example of the first type of fluorescence is SYBR® Green (CAS No. 163795-75-3). Commonly, the second type comprises a quencher covalently attached to the 3'-end of the probe that quenches the fluorescent reporter until the quencher is released from the probe during amplification.

In one embodiment, the method for detecting residual, undifferentiated PSCs in a culture of cells differentiated from PSCs disclosed herein may further comprise generating a report reporting the proportion of residual, undifferentiated PSCs detected in the culture of PSC-derived cells.

In one embodiment, the method for detecting residual, undifferentiated PSCs in a culture of cells differentiated from PSCs disclosed herein may further comprise formulating the culture of PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion into a therapeutic composition for administering to a subject. The method may further comprise treating or preventing a condition in a subject by administering the culture of PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion, or a therapeutic composition comprising such PSC-derived cells, to the subject. The condition may be bone cysts, bone neoplasms, fractures, cartilage defects, osteoarthritis, ligament injury, osteogenesis imperfecta, osteonecrosis, osteoporosis, aplastic anaemia, graft versus host disease (GvHD), myelodysplastic syndrome, Type 1 diabetes, Type 2 diabetes, autoimmune hepatitis, liver cirrhosis, liver failure, dilated cardiomyopathy, heart failure, myocardial infarction, myocardial ischemia, Crohn's disease, ulcerative colitis, burns, epidermolysis bullosa, lupus erythematosus, rheumatoid arthritis, Sjogren's disease, systemic sclerosis, bronchopulmonary dysplasia, chronic obstructive airways disease, emphysema, pulmonary fibrosis, amyotrophic lateral

sclerosis (ALS), Alzheimer's disease, brain injury, ataxia, degenerative disc disease, multiple system atrophy, multiple sclerosis, Parkinson's disease, retinitis pigmentosa, Romberg's disease, spinal cord injury, stroke, muscular dystrophy, limb ischaemia, kidney injury, lupus nephritis, endometriosis and complications of bone marrow or solid organ transplantation.

As used herein, the term "condition" includes a condition, disease or disorder, and symptoms thereof.

As used herein, the term "therapeutic composition" refers to PSC-derived cells as described herein that have been formulated for administration to a subject. Preferably, the therapeutic composition is sterile. This is readily accomplished by filtration through sterile filtration membranes. Preferably, the therapeutic composition is pyrogen-free.

As used herein, "formulating" refers to mixing a culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion with optional pharmaceutically acceptable carriers, excipients or stabilisers, and maintaining cell viability. Acceptable carriers, excipients, or stabilisers are nontoxic to recipient subjects at the dosages and concentrations employed, and may include: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulated pharmaceutical composition may also comprise other active compounds as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. The composition may comprise a cytotoxic agent, cytokine, and/or growth inhibitory agent. Such

molecules are suitably present in combination in amounts that are effective for the purpose intended.

PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion, or a therapeutic composition comprising such PSC-derived cells, may be administered before, during or after a condition presents. In one embodiment, PSC-derived cells or a therapeutic composition are administered during inflammation. PSC-derived cells may be administered (a) as a preventative measure, (b) as soon as the condition has been diagnosed, (c) when other treatments fail, and/or (d) when a condition advances to a pre-defined degree of severity.

In one embodiment, PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion are, or a therapeutic composition comprising such PSC-derived cells is, pre-treated prior to administration. Pre-treatment may be with a growth factor or by gene editing, for example, where a growth factor may prime the PSC-derived cells and gene editing may confer a new therapeutic property on the PSC-derived cells.

It will be appreciated by the person skilled in the art that the exact manner of administering to a subject a therapeutically effective amount of PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion, or a therapeutic composition comprising such PSC-derived cells, will be at the discretion of the medical practitioner with reference to the condition to be treated or prevented. The mode of administration, including dosage, combination with other agents, timing and frequency of administration, and the like, may be affected by the diagnosis of a subject's likely responsiveness to treatment with the PSC-derived cells or therapeutic composition, as well as the subject's condition and history.

The PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular condition being treated or prevented, the particular subject being treated, the clinical status of the subject, the site of administration, the method of administration, the scheduling of

administration, possible side-effects and other factors known to medical practitioners. The therapeutically effective amount of the PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion, or therapeutic composition comprising such PSC-derived cells, to be administered will be governed by such considerations.

PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion, or a therapeutic composition comprising such PSC-derived cells, may be administered systemically or peripherally, for example by routes including intravenous (IV), intra-arterial, intramuscular, intraperitoneal, intracerebrospinal, subcutaneous (SC), intra-articular, intrasynovial, intrathecal, intracoronary, transendocardial, surgical implantation, topical and inhalation (e.g. intrapulmonary). Most preferably, the PSC-derived cells are, or therapeutic composition is, administered IV. PSC-derived cells or a therapeutic composition may be administered in combination with a scaffold of biocompatible material.

The term "therapeutically effective amount" refers to an amount of PSC-derived cells in which residual, undifferentiated PSCs are absent or lower than a known proportion, or a therapeutic composition comprising such PSC-derived cells, effective to treat a condition in a subject.

The terms "treat", "treating" or "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the aim is to prevent or ameliorate a condition, disease or disorder in a subject or slow down (lessen) progression of a condition, disease or disorder in a subject. Subjects in need of treatment include those already with the condition, disease or disorder as well as those in which the condition, disease or disorder is to be prevented.

The terms "preventing", "prevention", "preventative" or "prophylactic" refers to keeping from occurring, or to hinder, defend from, or protect from the occurrence of a condition, disease or disorder, including an abnormality or symptom. A subject in need of prevention may be prone to develop the condition, disease or disorder.

The term "ameliorate" or "amelioration" refers to a decrease, reduction or elimination of a condition, disease or disorder, including an abnormality or symptom. A subject in need of treatment may already have the condition, disease or disorder, or may be prone to have the condition, disease or disorder, or may be in whom the condition, disease or disorder is to be prevented.

As used herein, the term "subject" refers to a mammal. The mammal may be a primate, particularly a human, or may be a domestic, zoo, or companion animal. Although it is particularly contemplated that the method and its resulting MSC or population of MSCs disclosed herein are suitable for medical treatment of humans, they are also applicable to veterinary treatment, including treatment of domestic animals such as horses, cattle and sheep, companion animals such as dogs and cats, or zoo animals such as felids, canids, bovids and ungulates.

Also disclosed herein is a culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion when detected by the method of the first aspect.

Also disclosed herein is therapeutic composition when manufactured by the method of the second aspect.

Also disclosed herein is a kit for detecting residual, undifferentiated pluripotent stem cells (PSCs) in a culture of cells differentiated from PSCs, the kit comprising:

- laminin-521; and
- E-cadherin; and
- a ROCK inhibitor.

In one embodiment, the ROCK inhibitor is a ROCK inhibitor disclosed herein.

In one embodiment, the kit further comprises PCR primers and optionally a PCR probe for quantitating in the cultured cells expression of a marker of residual, undifferentiated PSCs. The PCR primers and probe are specific for the marker residual, undifferentiated PSCs. In one embodiment, the PCR primers and/or probe are PCR primers and/or probe disclosed herein.

In one embodiment, the kit comprises a ready-to-use composition for PCR, optionally qRT-PCR, referred to as a "master mix" comprising the components for PCR except for DNA template and

primers, and optionally a probe. The components may include buffer, deoxynucleotide triphosphates (dNTPs), Mg^{2+} , and a polymerase. Master mixes are available commercially, for example 2X TaqMan® GE Master Mix.

In another embodiment, the kit further comprises a medium. In one embodiment, the medium is a medium disclosed herein. In one embodiment, the medium comprises the ROCK inhibitor.

In another embodiment, the kit further comprises a substrate. Such a substrate is suitable for culturing cells differentiated from PSCs in which residual, undifferentiated PSCs are to be detected. Preferably, the substrate is plastic culture ware such as a dish, multi-well plate, or flask. In one embodiment, the substrate is coated with the laminin-521 and E-cadherin.

In one embodiment, the kit comprises instructions for using the kit in a method comprising:

- culturing the cells on a substrate coated with laminin-521 and E-cadherin in a medium comprising a ROCK inhibitor;

- quantitating marker expression in the cultured cells; and

- comparing marker expression in the cultured cells with marker expression in a reference culture of cells comprising a known proportion of PSCs,

wherein lower marker expression in the culture of cells than marker expression in the reference culture of cells indicates absence of residual, undifferentiated PSCs in the cultured cells or presence of residual, undifferentiated PSCs in the cultured cells at a proportion lower than the known proportion of PSCs in the reference culture of cells.

In one embodiment, the kit comprises instructions for using the kit according to the method of the first aspect.

In another embodiment, the kit is used according to the method of the first aspect. This may be indicated by the term "when used".

In a preferred embodiment, iPSC-MSCs are cultured for 3 days in E8 Medium comprising 10 μ M Y27632, a ROCK inhibitor, then cultured for 2 days in the absence of a ROCK inhibitor, and LIN28 expression is quantitated by qRT-PCR and normalised to GAPDH

expression, allowing detection of 0.001% residual, undifferentiated iPSCs.

Other definitions that may be of assistance in understanding the present disclosure, particularly the examples, include the following.

a) TaqMan® Gene Expression (GE) Assay (20X): A type of real-time qRT-PCR assay available from Life Technologies that is designed using proprietary Applied Biosystems® software and reagents. These assays are used to quantitate specific mRNAs in a sample, and are available as pre-designed or custom assays. The TaqMan® GE Assays are provided as a 20X mix of gene specific primers and probe.

i) FAM™/MGB-NFQ Probe: A fluorescent reporter molecule (FAM: 6-carboxyfluorescein), covalently attached to the 5'-end of a TaqMan® probe. MGB-NFQ is a Minor Groove Binder-Non-Fluorescent Quencher molecule, covalently attached to the 3'-end of the TaqMan® probe. During amplification, the probe is cleaved, releasing the reporter from the quencher. The resulting fluorescent signal is proportional to the mRNA concentration in the sample. Other fluorescent dyes are available in place of FAM™. VIC® is commonly used for Endogenous Control Genes used for normalisation, such as GAPDH.

b) 2X TaqMan® GE Master Mix: contains AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure) for hot start activation, a blend of dNTPs with dTTP/dUTP and Uracil-DNA Glycosylase (UDG) to minimise carry-over PCR contamination, and a passive internal reference based on proprietary ROX™ dye.

c) cDNA: Complementary DNA, which is reverse transcribed from mRNA using Reverse Transcriptase (RT).

d) mRNA: messenger RNA.

e) gDNA: genomic DNA.

f) Template: a nucleic acid target (e.g., cDNA) intended to be amplified by PCR.

g) Total RNA: a complex mixture of RNA species, including mRNA (~1-5%), ribosomal RNA (rRNA) (~80%), transfer RNA (tRNA) and micro RNAs (miRNAs). The exact composition varies, depending on the cell type.

h) RNase: Ribonuclease, a class of enzymes that degrade RNA and are very difficult to inactivate.

i) Endogenous Control Gene: a positive control gene that is expressed in all sample types. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase is a common endogenous control gene used for normalisation.

j) Target Gene: a gene of interest that is differentially expressed in stem cells versus differentiated cells (e.g., iPSC vs. iPSC-MS), such as LIN28.

k) +RT and -RT Master Mixes: contain the reagents needed for reverse transcription of Total RNA samples. -RT is a negative control containing Total RNA, but lacking Reverse Transcriptase. The -RT samples test for gDNA contamination of Total RNA samples.

l) +/-RT Reaction Mixes: A combination of +/-RT Master Mix with each Total RNA sample or control for reverse transcription.

m) cDNA Amplification Mix: a combination of 2X TaqMan® GE Master Mix with each +/-RT Reaction Mix.

n) GE Assay Mix (e.g., LIN28 or GAPDH Assay Mix): A combination of a 20X TaqMan® GE Assay with each cDNA Amplification Mix for real-time qRT-PCR.

o) No Template Control (NTC): A type of Negative Control (NEG) in which Total RNA is replaced by water in the +/-RT cDNA reaction. Used to detect contamination of reagents by nucleic acid templates.

p) SNP: Single Nucleotide Polymorphism.

q) Cq: Quantification cycle (e.g., Cq(X)) is the cycle where the fluorescent signal crosses the threshold for real-time qPCR and qRT-PCR assays. The Cq is inversely related to the mRNA concentration. As the Cq increases, the mRNA concentration decreases, and vice versa. "Cq(50)" indicates that 50 cycles were run. "No Cq(50)" indicates that no Cq value was generated in 50 cycles.

EXAMPLES**Example 1 - Production of iPSC-MSCs****Materials***Table 1. Reagents*

Description	Vendor / Cat # or Ref #
DMEM/F12 Base Medium	Invitrogen / A1516901
E8 supplement	Invitrogen / A1517101
vitronectin	Life Technologies / A14700
collagen IV	Sigma / C5533
H-1152 ROCK Inhibitor	EMD Millipore / 555550
Y27632 dihydrochloride ROCK Inhibitor	Tocris / 1254
FGF2	Waisman Biomanufacturing / WC-FGF2-FP
human endothelial-SFM	Life Technologies / 11111-044
stemline II hematopoietic stem cell expansion medium	Sigma / S0192
GLUTAMAX	Invitrogen / 35050-061
insulin	Sigma / I9278
lithium chloride (LiCl)	Sigma / L4408
collagen I solution	Sigma / C2249
fibronectin	Life Technologies / 33016-015
DMEM/F12	Invitrogen / 11330032
recombinant human BMP4	Peprtech / 120-05ET
activin A	Peprtech / 120-14E
Iscove's modified Dulbecco's medium (IMDM)	Invitrogen / 12200036
Ham's F12 nutrient mix	Invitrogen / 21700075
sodium bicarbonate	Sigma / S5761
L-ascorbic acid 2-phosphate Mg ²⁺	Sigma / A8960
1-thioglycerol	Sigma / M6145
sodium selenite	Sigma / S5261
non essential amino acids	HyClone / SH30853.01
chemically defined lipid concentrate	Invitrogen / 11905031
embryo transfer grade water	Sigma / W1503
polyvinyl alcohol (PVA)	MP Bio / 151-941-83
holo-transferrin	Sigma / T0665
ES-CULT M3120	Stem Cell Technologies / 03120
STEMSPAN serum-free expansion medium (SFEM)	Stem Cell Technologies / 09650
L-ascorbic acid	Sigma / A4544
PDGF-BB	Peprtech / 110-14B

The reagents listed in Table 1 are known to the person skilled in the art and have accepted compositions, for example IMDM and Ham's F12. GLUTAMAX comprises L-alanyl-L-glutamine dipeptide, usually supplied at 200 mM in 0.85% NaCl. GLUTAMAX releases L-glutamine upon cleavage of the dipeptide bond by the cells being cultured. Chemically defined lipid concentrate comprises arachidonic acid 2 mg/L, cholesterol 220 mg/L, DL-alpha-tocopherol acetate 70 mg/L, linoleic acid 10 mg/L, linolenic acid 10 mg/L, myristic acid 10 mg/L, oleic acid 10 mg/L, palmitic acid 10 mg/L, palmitoleic acid 10 mg/L, pluronic F-68 90 g/L, stearic acid 10 mg/L, TWEEN 80® 2.2 g/L, and ethyl alcohol. H-1152 and Y27632 are highly potent, cell-permeable, selective ROCK (Rho-associated coiled coil forming protein serine/threonine kinase) inhibitors.

Table 2. IF6S medium (10X concentration)

10X IF6S	Quantity	Final Concentration
IMDM	1 package, powder for 1 L	5X
Ham's F12 nutrient mix	1 package, powder for 1 L	5X
sodium bicarbonate	4.2 g	21 mg/mL
L-ascorbic acid 2-phosphate Mg ²⁺	128 mg	640 µg/mL
1-thioglycerol	80 µL	4.6 mM
sodium selenite (0.7 mg/mL)	24 µL	84 ng/mL
GLUTAMAX	20 mL	10X
non essential amino acids	20 mL	10X
chemically defined lipid concentrate	4 mL	10X
embryo transfer grade water	To 200 mL	NA

Table 3. IF9S medium (1X concentration; based on IF6S)

IF9S	Quantity	Final Concentration
IF6S	5 mL	1X
polyvinyl alcohol (PVA; 20 mg/mL)	25 mL	10 mg/mL
holo-transferrin (10.6 mg/mL)	50 µL	10.6 µg/mL
insulin	100 µL	20 µg/mL
embryo transfer grade water	To 50 mL	NA

Table 4. Differentiation medium (1X concentration; based on IF9S)

Differentiation Medium	Quantity	Final Concentration
IF9S	36 mL	1X
FGF2	1.8 µg	50 ng/mL
LiCl (2M)	36 µL	2mM
BMP4 (100 µg/mL)	18 µL	50 ng/mL
Activin A (10 mg/mL)	5.4 µL	1.5 ng/mL

Table 5. Mesenchymal colony forming medium (1X concentration)

M-CFM	Quantity	Final Concentration
ES-CULT M3120	40 mL	40%
STEMSPAN SFEM	30 mL	30%
human endothelial-SFM	30 mL	30%
GLUTAMAX	1 mL	1X
L-ascorbic acid (250 mM)	100 µL	250 µM
LiCl (2M)	50 µL	1 mM
1-thioglycerol (100 mM)	100 µL	100 µM
FGF2	600 ng	20 ng/mL

Table 6. Mesenchymal serum-free expansion medium (1X concentration)

M-SFEM	Quantity	Final Concentration
human endothelial-SFM	5 L	50%
STEMLINE II HSFM	5 L	50%
GLUTAMAX	100 mL	1X
1-thioglycerol	87 µL	100 µM
FGF2	100 µg	10 ng/mL

Method

1. Thawed iPSCs in E8 Complete Medium (DMEM/F12 Base Medium + E8 Supplement) + 1 µM H1152 on Vitronectin coated (0.5 µg/cm²) plastic ware. Incubated plated iPSCs at 37°C, 5% CO₂, 20% O₂ (normoxic).
2. Expanded iPSCs three passages in E8 Complete Medium (without ROCK inhibitor) on Vitronectin coated (0.5 µg/cm²) plastic ware and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) prior to initiating differentiation process.
3. Harvested and seeded iPSCs as single cells/small colonies at 5x10³ cells/cm² on Collagen IV coated (0.5 µg/cm²) plastic ware

- in E8 Complete Medium + 10 μ M Y27632 and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) for 24 h.
4. Replaced E8 Complete Medium + 10 μ M Y27632 with Differentiation Medium and incubated at 37°C, 5% CO₂, 5% O₂ (hypoxic) for 48 h.
 5. Harvested colony forming cells from Differentiation Medium adherent culture as a single cell suspension, transferred to M-CFM suspension culture and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) for 12 days.
 6. Harvested and seeded colonies (Passage 0) on Fibronectin/Collagen I coated (0.67 μ g/cm² Fibronectin, 1.2 μ g/cm² Collagen I) plastic ware in M-SFEM and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) for 3 days.
 7. Harvested colonies and seeded as single cells (Passage 1) at 1.3x10⁴ cells/cm² on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) for 3 days.
 8. Harvested and seeded as single cells (Passage 2) at 1.3x10⁴ cells/cm² on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) for 3 days.
 9. Harvested and seeded as single cells (Passage 3) at 1.3x10⁴ cells/cm² on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) for 3 days.
 10. Harvested and seeded as single cells (Passage 4) at 1.3x10⁴ cells/cm² on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) for 3 days.
 11. Harvested and seeded as single cells (Passage 5) at 1.3x10⁴ cells/cm² on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO₂, 20% O₂ (normoxic) for 3 days.
 12. Harvested passage 5 (P5) iPSC-MSCs as single cells and froze final product.

Example 2 - Quantitation of residual undifferentiated stem cells by qRT-PCR

1) PURPOSE

This example protocol describes quantitation of residual, undifferentiated iPSCs by TaqMan® Gene Expression Assays (qRT-PCR). Although detection of undifferentiated iPSCs amongst iPSC-MSCs using LIN28 expression is exemplified, the protocol is applicable generally to PSC-derived cell culture using any PSC marker differentially expressed in PSCs, but not expressed in PSC-derived cells. The protocol is intended for Quality Control of PSC-derived cells destined for formulation into a therapeutic composition.

2) MATERIALS

a) Materials for iPSC-MSC Laminin-521/E-Cadherin Expansion Culture Protocol:

- i) LN521™ Human rLaminin-521, Biolamina, Catalog No. LN521-03
- ii) E-Cadherin, Human Recombinant, Advanced BioMatrix, Catalog No. 5085-0.1MG
- iii) Dulbecco's Phosphate-Buffered Saline (1X) with Mg^{2+} and Ca^{2+} (DPBS++) (6 x 500 mL), Corning/Mediatech, Catalog No. 21-030-CV or equivalent
- iv) HyClone™ Dulbecco's Phosphate Buffered Saline without Mg^{2+} and Ca^{2+} , solution, (DPBS--), GE Healthcare Life Sciences, Catalog No. SH30028 or equivalent
- v) ROCK Inhibitor Y27632, Sigma-Aldrich, Catalog No. Y0503-1MG or Y0503-5MG
- vi) Essential 8™ Medium (Kit), Thermo Fisher Scientific, Catalog No. A1517001
 - (1) Essential 8™ Basal Medium (500 mL)
 - (2) Essential 8™ Supplement (Vitronectin) (10 mL)
- vii) TrypLE™ Select Enzyme (1X), no phenol red (100 mL), Thermo Fisher Scientific, Catalog No. 12563011
- viii) Dimethyl sulfoxide (DMSO), Sigma-Aldrich, Catalog No. D2650
- ix) Falcon® 75cm² Rectangular Canted Neck Cell Culture Flask with Vented Cap, Corning Life Sciences, Catalog No. 353136 or equivalent

x) Falcon® Cell Scraper with 25cm Handle and 1.8cm Blade, Sterile, Corning Life Sciences, Catalog No. 353086 or equivalent

xi) HyClone™ 0.4% Trypan Blue, Thermo Fisher Scientific, Catalog No. SV30084 or equivalent

xii) Hemocytometer with cover slip, Reichert Right Line or equivalent

b) RNeasy® Protect Cell Mini Kit (50): QIAGEN® Catalog No. 74624. Consists of two QIAGEN® products:

i) RNaProtect® Cell Reagent (Box 1 of 2): Designed for cultured or sorted cells. Can be added directly to cells in culture medium. Arrests gene expression patterns, providing immediate stabilization of Total RNA. Samples can be stored at 4°C, -20°C or archived at -80°C.

ii) RNeasy® **Plus** Mini Kit (Box 2 of 2): Designed to isolate total RNA from a variety of sample types. "Plus" indicates that the kit contains genomic DNA (gDNA) Eliminator Columns and related reagents.

c) QIAshredder (50): QIAGEN® Catalog No. 79654. Required to homogenise cell lysates for RNA isolation.

d) RNase-Free DNase Set (50 preps), QIAGEN®, Catalog No. 79254. For on-membrane DNase treatment using QIAGEN® RNA purification kits.

e) Ethanol (96-100%): Fisher BioReagents, Molecular Biology Grade, Absolute (200 Proof), 100 mL, Fisher Scientific Catalog No. BP2818100 or equivalent. Required for the QIAGEN® RNeasy® Plus Mini Kit.

f) UltraPure™ DNase/RNase-Free Distilled Water, Life Technologies, Catalog No. 10977-015 or equivalent.

g) β-Mercaptoethanol (β-ME), 14.3 M, Thermo Fisher Scientific, Catalog No. BP176-100 or equivalent. Required for the QIAGEN® RNeasy® Plus Mini Kit to inactivate RNases.

h) Safetec Green-Z™ Biohazard Fluid Control Powder, Thermo Fisher Scientific, Catalog No. 19-023-901. For disposal of the RNaProtect® Cell Reagent, which is an environmental hazard.

i) RNase AWAY™ Decontamination Reagent (250 mL), Life Technologies, Catalog No. 10328-011 or equivalent. Apply the ready-to-use solution to surfaces, such as lab benches, pipets, glassware

or plastic ware. Rinse with Milli-Q water to eliminate RNase and DNA contamination.

j) High-Capacity RNA-to-cDNA™ Kit (50): Thermo Fisher Scientific, Catalog No. 4387406

k) SUPERase·In™ RNase Inhibitor: Thermo Fisher Scientific, Catalog No. AM2694

l) TaqMan® Gene Expression Master Mix (2X): Thermo Fisher Scientific, Catalog No. 4369016 (1-Pack, 5 mL: 200 x 50 µL Rxns)

m) Ambion® RT-PCR Grade Water: Thermo Fisher Scientific Catalog No. AM9935 (10 x 1.5 ml) (preferred) or equivalent. Note: AM9935 is autoclaved, membrane-filtered and is not DEPC-treated. AM9935 is tested for prokaryotic (16S rRNA) and eukaryotic (18S rRNA) genomic DNA contamination by real-time PCR. It is certified RNase-free, DNase-free and genomic DNA-free.

n) TaqMan® Gene Expression Assay(s), which contain a 20X, ready-to-use mix of primers and FAM/MGB-NFQ probe in TE. FAM is the most common fluorescent reporter. (1X = 250 nM TaqMan Probe + 900 nM of each PCR primer (forward & reverse)). The catalog numbers below refer to custom assay sizes. Light sensitive. Preferably store in one-time use aliquots at -20°C in 1.5 ml Ambion® Non-Stick Tubes.

i) Custom TaqMan® Gene Expression Assays (20X, Single Tube), Thermo Fisher Scientific, made to order. (Note that custom assays can only be ordered with FAM-MGB probes.)

(1) Catalog No. 4331348 (Small = 360 rxn @ 20 µL qRT-PCR)

(2) Catalog No. 4332078 (Medium = 750 rxns @ 20 µL qRT-PCR)

ii) LIN28 Custom TaqMan® Gene Expression Assay, Thermo Fisher Scientific; Catalog No: 4331348; Assay ID: AIVI48S; Assay Name: LIN28.QRT-PCR; Scale: S: 360 rxns; Made to Order. This is a custom designed assay with known primer and probe sequences as disclosed in Kuroda, et al. (PLoS ONE 7, 1-9 (2012) *Highly Sensitive In Vitro Methods for Detection of Residual Undifferentiated Cells in Retinal Pigment Epithelial Cells Derived from Human iPS Cells*). LIN28 Probe sequence (5'→3') CGCATGGGGTTCGGCTTCCTGTCC (SEQ ID NO: 14); LIN28 forward primer sequence (5'→3') CACGGTGCGGGCATCTG (SEQ ID NO: 15); LIN28 reverse primer sequence (5'→3') CCTTCCATGTGCAGCTTACTC (SEQ ID NO: 16).

iii) TaqMan® Endogenous Control Assays: These assays have been predesigned and may be inventoried. Endogenous Controls are available with FAM-MGB or VIC-MGB probes. Preferably select the VIC-MGB probe to distinguish the control assay from the distinguishing assay.

(1) GAPDH TaqMan® Gene Expression Assay (Gene Symbol: GAPDH, hCG2005673), Life Technologies; Catalog No: 4448489; Assay ID: Hs02758991_g1 (VIC-MGB Probe); Made to order. The "Best Coverage" assay was chosen from multiple predesigned GAPDH assays. This assay spans an intron and should therefore not detect genomic DNA.

o) Ambion® Non-Stick RNase-free Microfuge Tubes (1.5 ml): Life Technologies Catalog No. AM12450. Note: AM12450 1.5 ml tubes have a low binding surface and are certified RNase-free and DNase-free. Preferably used to store aliquots of the Custom TaqMan® GE Assay (20X) and prepare cDNA.

p) 1.5mL Screw Cap Microtube (Sarstedt 72.692.005): Fisher Scientific Catalog No. 50809238 or equivalent; Conical Bottom, Sterile With Assembled O-Ring Cap. Preferably used to prepare cDNA Amplification Mixes. The exterior-threaded screw cap prevents the generation of aerosols, which could otherwise cause PCR contamination.

q) White Hard-Shell Low-Profile 96-Well Skirted PCR Plates: Bio-Rad Catalog No. HSP-9655. A white plate will reduce background noise and increase the fluorescent signal over clear 96-well plates and tubes. Strip tubes should not be used for this procedure.

r) Microseal 'B' Adhesive Seals (100): Bio-Rad Catalog No. MSB-1001. This is the recommended, optically clear, plate sealer for the Hard-Shell 96-well plates.

s) Sealing Roller: Bio-Rad Catalog No. MSR-0001. Used to seal a Hard-Shell 96-well plate with a Microseal 'B' Adhesive Seal.

t) Bio-Rad CFX96 Real-Time PCR Detection System with Bio-Rad CFX Software (ID 0394) or equivalent.

u) Eppendorf MiniSpin Plus Microcentrifuge (ID 0569) or equivalent.

v) Nanodrop 2000 UV-Visible Spectrophotometer (ID 0504) or equivalent.

3) PROCEDURE

a) Preparation of P5 iPSC-MSCs final product (1 vial) for qRT-PCR analysis using selective expansion of residual undifferentiated iPSCs.

i) This procedure selectively expands undifferentiated iPSCs in a background of iPSC-MSCs, in order to increase the sensitivity of qRT-PCR for residual undifferentiated iPSCs.

ii) Preparation of Cell Culture Media:

(1) Preparation of E8 Complete Medium (E8CM):

(a) Thaw E8 Supplement overnight at 2-8°C.

(b) Remove 10 ml of E8 Basal Medium from 500 ml bottle.

(c) Add 10 ml of E8 supplement to 490 ml of E8 Basal Medium.

Mix well and store at 2-8°C. Expires 2 weeks after preparation.

(2) Preparation of E8CM + 10µM Y27632 Medium:

(a) Prepare 10 mM Y27632 by adding 312 µl DPBS-- (without Ca²⁺ & Mg²⁺) to 1 mg of Y27632. Aliquot and freeze at -20°C.

(b) Add 1 µl of 10 mM Y27632 to each ml of E8CM for a final concentration of 10 µM Y27632 (e.g. 75 µl of 10 mM Y27632 to 75 ml E8CM). Store at 4°C. Expires 2 weeks after preparation.

iii) Preparation of Freeze Medium (E8CM + 20% DMSO):

(1) Prepare 10 ml of Freeze Medium as follows: Combine 8 ml E8CM with 2 ml DMSO. Transfer to 4°C to chill prior to use.

(2) Add 1 µl of 10 mM Y27632 to each ml of E8CM for a final concentration of 10 µM Y27632 (e.g. 75 µl of 10 mM Y27632 to 75 ml E8CM). Store at 4°C. Expires 2 weeks after preparation.

iv) Preparation of Laminin-521/E-Cadherin Coating (in DPBS++):

(1) Thaw Laminin-521 and E-Cadherin overnight at 2-8°C.

(2) Prepare the coating material (30 ml required for 2 x T75 flasks):

Materia	Conc.	Volume	T75 -
DPBS++	1X	180 µl	13.5 ml
Laminin	100	20 µl	1.5 ml
E-	500	0.444	33.3 µl

(3) Coat culture ware (2 x T75 flasks) and store at 2-8°C for at least overnight prior to use (1 month expiration). Make sure that the coating solution covers the entire culture area.

v) Preparation of Expansion Culture

(1) Minimum 1 hour before seed, remove coating material from flasks and add 15 ml E8CM + 10 μ M Y27632/T75. Let equilibrate at 37°C.

(2) Thaw P5 iPSC-MSK in a 37°C water bath.

(3) Transfer the contents of the vial into a 15 ml tube containing 9 ml of E8CM + 10 μ M Y27632.

(4) Centrifuge at 200 x g for 5 minutes.

(5) Aspirate the supernatant and resuspend the pellet in 5 ml E8CM + 10 μ M Y27632.

(6) Count the cells using a hemocytometer. Estimated concentration 5×10^6 cells/5 ml = 1×10^6 cells/ml. Suggested dilution in trypan blue is 1:2. Calculate the percent viability.

(7) Calculate the number of cells required to seed each T75 flask at 1.3×10^4 cells/cm² or 1×10^6 cells/T75).

(8) Seed the Laminin-521/E-Cadherin coated flasks with the calculated number of cells.

(9) Feed the cells on the following schedule:

(a) Day 1 - No feed.

(b) Day 2 - Feed each T75 flask with 15 ml E8CM + 10 μ M Y27632.

(c) Day 3 - No feed.

(d) Day 4 & 5 - Feed each T75 flask with 15 ml E8CM (without Y27632).

(10) Check the cultures daily for signs of microbial contamination (e.g., turbidity). Discard any contaminated cultures and start over at Step 3)a).

vi) Harvest (Day 6 post-seed)

(1) Notes: Cell are difficult to detach from Laminin-521/ E-cadherin coated culture ware. Use of cell scrapers is recommended to get all the cells to detach. The average harvest density is 4×10^4 cells/cm² or 3×10^6 cells/T75 flask. If an insufficient number of cells is harvested from a single T75 flask ($< 2 \times 10^6$ cells) harvest of the second T75 may be required.

(2) Aspirate the growth medium and rinse the flask with 10 ml DPBS--.

(3) Add 8 ml TrypLE to the T75 flask. Incubate at 37°C for 15 minutes.

(4) Rap flask firmly to dislodge the cells and transfer to a 50 ml conical tube (approximately 8 ml).

(5) Add 8 ml E8CM + 10 μ M Y27632 to the flask, pipet up and down and combine with cell suspension in the 50 ml tube (16 ml total).

(6) Add 8 ml E8CM + 10 μ M Y27632 to the flask and use a sterile disposable cell scraper to gently scrape off the cells. Collect the cells by rinsing the scraped surface with medium and combine with the cell suspension (24 ml total).

(7) Repeat scraping after adding an additional 8 ml E8CM + 10 μ M Y27632 to the flask. Collect cell suspension as above (32 ml total).

(8) Centrifuge cells at 200 x g for 10 minutes. Aspirate the supernatant.

(9) Resuspend the cell pellet in E8CM + 10 μ M Y27632 and count cells. Suggested resuspension volume is 2 ml with estimated concentration 3×10^6 cells/2 ml = 1.5×10^6 cells/ml. Suggested dilution in trypan blue is 1:2. In order to accurately count, cells may require dissociation into single cells using a 1 ml pipet tip.

(a) Count the cells using a hemocytometer.

(b) The required percent viability is $\geq 70\%$. The required yield is $\geq 2 \times 10^6$ cells.

(10) Spin cells at 200 x g for 10 minutes. Aspirate supernatant.

(11) Resuspend cell pellet in E8CM at 2×10^6 cells/ml.

vii) Freeze the cells prior to analysis.

(1) Add an equal volume of COLD Freeze Medium (E8CM with 20% DMSO) for a final concentration of 1×10^6 cells/ml.

(2) Add 1 ml of cell suspension to each cryovial.

(3) Immediately transfer cryovials to -80°C freezer.

(4) Transfer to liquid Nitrogen tank the next day.

b) Preparation of Reagents for the RNeasy® Plus Mini Kit:

i) Preparation of QIAGEN® Buffer RPE wash solution:

(1) Add 100% ethanol, as indicated on the bottle, to Buffer RPE concentrate (i.e., 44 mL for a 50 prep RNeasy® Protect Cell Mini Kit).

ii) Preparation of 70% Ethanol:

(1) Combine 7 mL of 100% Ethanol with 3 mL of Nuclease-Free Water. Expiration is one month after preparation.

iii) Preparation of Lysis Buffer for Total RNA isolation:

(1) Add 10 μ L β -mercaptoethanol (β -ME) per 1 mL of Buffer RLT Plus before use, using a chemical fume hood.

iv) Preparation of RNase-free DNase for the QIAGEN® RNase-Free DNase Set:

(1) Add the indicated amount of RNase-Free Water (i.e., 550 μ L) to the lyophilised DNase, using a sterile needle and syringe. Gently resuspend the DNase by inversion. Do not vortex. Store the reconstituted DNase at -20°C in 20 μ L aliquots, preferably in Ambion® Non-Stick RNase-free Microfuge Tubes. The reconstituted RNase expires nine months after preparation. Store the remaining kit components at $2-8^{\circ}\text{C}$.

c) Preparation of cultured cells for Total RNA isolation, using RNAProtect® Cell Reagent.

i) RNAProtect® Cell Reagent immediately stabilises Total RNA in treated cells, preserving the gene expression profile. The reagent is added directly to cells, with or without medium present. Frozen cells in DMSO can also be prepared, using a modified protocol.

(1) The recommended cell number is $1-2 \times 10^6$ cells per Total RNA miniprep. This can be increased to a maximum of 5×10^6 cells per miniprep, without changing the reagent volumes. For larger cell numbers, process multiple minipreps or use an RNA midiprep procedure, following the manufacturer's protocol.

(2) Use a ratio of 1 mL of sample to 5 mL of RNAProtect® Cell Reagent.

ii) Prepare an appropriate number of 15 mL tubes containing 5 mL of RNAProtect® Cell Reagent.

iii) Frozen Cells: Place vials of frozen cells on dry ice to prevent thawing. Do not thaw the cells prior to reagent addition. Process one vial at a time to maximise protection of the RNA.

(1) Transfer ~750 µL of RNAp Protect® Cell Reagent from one 15 mL tube to a cryovial of frozen cells containing ~1 mL of freezing medium. Adjust the volumes as necessary for other scenarios. Do not overfill.

(a) Use high quality, low-retention pipet tips to prevent pipetting losses.

(2) Immediately vortex the mixture for ~10 seconds. Quickly transfer the partially thawed mixture back to the same 15 mL tube containing RNAp Protect® Cell Reagent.

(3) Repeat steps 3)c)iii)(1) and 3)c)iii)(2) until the mixture is completely thawed. This will take 3-4 iterations.

(4) Mix well by vortexing, until the sample is homogenous.

iv) Proceed immediately with Total RNA isolation or store the prepared samples in RNAp Protect® Cell Reagent. Store samples at 2-8°C for up to 4 weeks or archive at -20°C or -80°C.

d) Preparation of Cell Lysates for Total RNA isolation.

i) If frozen, thaw the RNAp Protect® cell mixture at room temperature without mixing.

ii) Centrifuge the RNAp Protect® cell mixture for 5 minutes at 4000 rpm (Sorvall ID 0018 or equivalent) to collect the cells and any precipitate that may have formed. A visible white pellet should form.

iii) Remove as much supernatant as possible to a waste container. Flick the tube or vortex to loosen the pellet. This is very important for complete solubilization in the next step.

(1) Add 350 µL of freshly prepared Buffer RLT Plus + β-ME to the pellet. Vortex vigorously for 1-2 minutes, until the pellet is completely dissolved. If needed, the lysate can be stored at -80°C. Thaw at room temperature and vortex before use.

e) Isolation of Total RNA using the QIAGEN® RNeasy® Plus Mini Kit. All steps are performed at room temperature. Isolate Total RNA from the following cryopreserved samples. Elute in 30 µL Nuclease-Free Water.

#1: iPSC-MSC (non-expanded) (Reference culture)

#2: iPSC-MSC (expanded) (Reference culture)

#3: iPSC-MSC plus 0.001% iPSC Spike (non-expanded) (Reference culture)

#4: iPSC-MSC plus 0.001% iPSC Spike (expanded) (Reference culture)

#5: iPSC-MSC P5 Final Product (non-expanded)

#6: iPSC-MSC P5 Final Product (expanded)

i) Transfer the lysate to a QIAshredder. Centrifuge at full speed for 2 minutes to shear and homogenise the lysate. A thin round pellet may form in the bottom of the collection tube.

ii) Transfer the homogenised lysate to an assembled gDNA Eliminator spin column, avoiding any pelleted material. Centrifuge at full speed for 30 seconds. If necessary, repeat until all of the lysate has passed through the spin column.

iii) Add 350 µL of 70% ethanol to the flow-through and mix well by pipetting. Do not centrifuge. Immediately transfer the mixture to an assembled RNeasy® spin column, with any precipitate that may have formed.

iv) Gently close the cap and centrifuge at full speed for 15 seconds. Discard the flow-through from the collection tube. The RNA is now bound to the RNeasy® spin column.

v) On-Membrane DNase Treatment:

(1) For maximum removal of genomic DNA, the bound RNA is treated with RNase-Free DNase.

(a) Add 350 µL Buffer RW1 to the spin column. Gently close the cap and centrifuge at full speed for 15 seconds. Discard the flow-through from the collection tube.

(b) For each sample, combine 20 µL of reconstituted DNase, 140 µL Buffer RDD and 2 µL SUPERase. In™. Mix gently by pipetting and combine all into one tube. Do not vortex.

(c) Pipet 80 µL of the DNase mix directly onto the center of the RNeasy® membrane. Incubate at room temperature for 20 minutes.

(d) Add 350 µL Buffer RW1 to the spin column. Gently close the cap and centrifuge at full speed for 15 seconds. Discard the flow-through from the collection tube.

vi) Add 500 µL Buffer RPE to the spin column. Gently close the cap and centrifuge at full speed for 15 seconds. Discard the flow-through from the collection tube.

vii) Repeat the wash by adding 500 μ L Buffer RPE to the spin column. Gently close the cap and centrifuge at full speed for 2 minutes.

viii) Transfer the spin column to a fresh 2 mL collection tube. Centrifuge at full speed for 1 minute to remove any residual wash solution.

ix) Transfer the spin column to a 1.5 mL collection tube. Add 30 μ L RNase-free water directly to the membrane. Gently close the cap and centrifuge at full speed for 1 minute to elute the RNA.

x) Discard the spin column and cap the tube. Label the tube with the date and sample ID.

xi) Normalise the volume of each Total RNA sample to 30 μ L.

(1) Set a 10-100 μ L pipet to 30 μ L and measure the eluate volume.

(2) If the volume is less than 30 μ L, add Nuclease-Free Water from the RNeasy® kit to a final volume of 30 μ L.

(3) The sample is now normalised to the starting number of cells.

xii) Determine the Total RNA concentration for each sample using a Nanodrop 2000.

(1) Clean the sample pedestal with Milli-Q water before launching the software, to remove any potential contamination from previous use.

(2) Once the software initialises, uncheck the 340 nm Baseline Correction box.

(3) Blank the instrument with Milli-Q water.

(4) Test a Milli-Q water sample to ensure that the background is zero. If necessary, re-clean the sample pedestal with Milli-Q water.

(5) Determine the Total RNA concentration of each sample using a single determination.

(6) Calculate the Total RNA Yield and Yield per Cell.

(7) Calculate the volume of 1.0-2.0 μ g Total RNA for each sample. (A maximum of 2.0 μ g Total RNA can be Reverse Transcribed per cDNA reaction).

(8) The Total RNA concentration must be ≥ 100 ng/ μ L to meet the requirement of 1 μ g Total RNA per 10 μ L (maximum volume) for

Reverse Transcription. Re-isolate Total RNA from a fresh vial of cells if necessary.

The expected Total RNA yield per 1×10^6 cryopreserved iPSC-MSCs is ~5-10 μg . This is equivalent to ~5-10 pg Total RNA per cell.

xiii) Proceed to cDNA synthesis or store the Total RNA at -20°C for up to 1 year.

f) Preparation of cDNA using the High Capacity RNA-to-cDNA™ Kit.

i) For each Total RNA sample, prepare cDNA +/-RT using 1 μg Total RNA / 22 μL RT. Include H_2O (NTC) +/-RT Controls (N=14).

ii) Thaw the kit components on ice.

iii) Prepare one +RT and one -RT Master Mix on ice, depending on the number of Total RNA samples to be analyzed. The -RT samples serve as negative controls, lacking reverse transcriptase. Include H_2O (NTC) +/- RT Controls by replacing Total RNA with RT-PCR Grade Water.

iv) Prepare individual +/-RT Reaction Mixes on ice for each Total RNA sample.

(1) The maximum volume of Total RNA is 10 μL per 22 μL +/-RT. The volume of Total RNA will vary, depending on the Total RNA concentration.

(2) Calculate the volume of RT-PCR Grade Water for each tube by subtracting the Total RNA volume from 10 μL .

(3) Pipet the appropriate volume of RT-PCR Grade Water into each tube. Use 10 μL for the +/-RT H_2O Controls.

(4) Add the appropriate volume of Total RNA to each tube.

(5) Add 12 μL of +RT or -RT Master Mix to each tube and mix gently by pipetting.

v) Incubate at 37°C for 60 minutes.

vi) Heat inactivate at 95°C for 5 minutes.

vii) Briefly centrifuge the tubes to collect the cDNA.

viii) Measure the cDNA volume using a calibrated 10-100 μL pipet.

(1) Set the pipet to the cDNA reaction volume (20-22 μL) and measure the cDNA volume.

(2) Adjust the cDNA volume to 100 µL with Nuclease-Free Water. This will dilute inhibitors and provide enough cDNA for multiple qRT-PCR assays.

ix) Mix by vortexing and briefly spin down the samples.

x) Proceed to qRT-PCR or store at -20°C.

g) qRT-PCR (TaqMan® Gene Expression Assays)

i) For each cDNA (N=14), amplify 50 ng cDNA (Total RNA equivalents) per 20 µL qRT-PCR for LIN28 and GAPDH (N=3 each). Perform qRT-PCR using 50 cycles. (Protocol File: TaqMan qRT-PCR Assays Cq(50).prcl).

ii) Prepare a cDNA Amplification Mix for each +RT and -RT cDNA, depending on the number of replicates and number of TaqMan® GE Assays to be performed.

(1) The recommended maximum amount of cDNA per reaction is 100 ng (Total RNA equivalents).

iii) Prepare a GE Assay Mix for each cDNA, depending on the number of TaqMan® GE Assays and replicates. One GE Assay Mix will be prepared for each cDNA and each TaqMan® GE Assay to be performed.

iv) Transfer 20 µL of each GE Assay Mix into a white CFX96 plate and seal with an optical plate sealer.

v) Centrifuge the plate at 400 rpm for 1 minute in a centrifuge with a plate adapter (ID #0018) to collect the contents to the bottom of the wells.

vi) Carefully place the plate in the thermal cycler, being careful not to disturb the contents of the wells.

vii) Program the Bio-Rad CFX96 Real-Time PCR Detection System using the Bio-Rad CFX Manager™ software on the attached computer. with the following Thermal Cycling Parameters:

(1) 2 minutes @ 50.0°C (UNG incubation; required for optimal uracil-N-glycosylase activity; degrades previously amplified, dUTP-incorporated PCR products to reduce PCR contamination).

(2) 10 minutes @ 95°C (Hot Start: AmpliTaq Gold®, UP Polymerase activation).

(3) [15 seconds @ 95°C (denaturation) + 1 minute @ 60°C (annealing/extension) + Plate Read] - repeat 49 times for 50 cycles (amplification and real-time detection).

viii) Set the Sample Volume to 20 µL.

- ix) The Estimated Run Time should be 01:59:00 h.
- x) Read all wells and all channels during the run to make sure that all data is collected during the run.
 - (1) The Preview settings, located above the plate layout, should be:
 - (a) Fluorophores: FAM, HEX, Texas Red, Cy5, Quasar 705 (VIC can be added later)
 - (b) Plate Type: BR White
 - (c) Scan Mode: All Channels
- xi) All wells should be labeled "Unk," indicating that the software considers all wells to be Unknown during the run. The actual plate layout will be set up after the run is complete.
- xii) Click on Next to move to the Start Run Tab. Enter identifying information about the run in the Notes section.
- xiii) Click on the Start Run Button to begin qRT-PCR.

h) Data Analysis

- i) The Data Analysis window will open at the end of the run.
 - (1) qRT-PCR data is reported as Cq values (i.e. Cq(50)).
- ii) Click on the Plate Setup button on the top, right side of the window.
 - (1) Select View/Edit Plate. Follow the Plate Loading Guide to set up the plate with sample information.
 - (a) Clear any empty wells.
 - (b) Do not check, "Exclude Wells in Analysis." All data must be analyzed.
 - (2) Click on OK and save the file.
- iii) Click on the Quantification tab near the top of the window.
- iv) Check the following Settings:
 - (1) Cq Determination Mode: Single Threshold
 - (2) Baseline Setting: Baseline Subtracted Curve Fit
 - (3) Analysis Mode: Fluorophore
- v) Generate a report from the Tools menu with the following sections and associated subsections: Header, Run Setup, Quantification and QC Parameters.
 - (1) Deselect Gene Expression options, Allelic Discrimination and End Point, as these sections are not relevant.

vi) Export data for analysis by selecting Export > "Export All Data Sheets to Excel." This will provide the data in spreadsheet format.

vii) Analyze the data using a spreadsheet.

(1) Report "N/A" as "No Cq(50)."

(2) Calculate the Av. Cq(50) and % relative standard deviation (RSD, or coefficient of variation (CV)) for each LIN28 and GAPDH +/- RT data set.

(3) Normalise the Av. Cq(50) values by subtracting Av. GAPDH Cq(50) (N=3) from Av. LIN28 Cq(50) (N=3).

(4) Calculate the Av. GAPDH Cq(50) and % RSD for all samples.

(5) Compare the Normalised Av. Cq(50) values of Samples #2 (iPSC-MSC (expanded)) and #4 (iPSC-MSC plus 0.001% iPSC Spike (expanded)). Compare the Normalised Av. Cq(50) values of Samples #4 (iPSC-MSC plus 0.001% iPSC Spike (expanded)) and #6 (iPSC-MSC P5 Final Product (expanded)).

4) ACCEPTANCE CRITERIA

a) The Normalised Av. Cq(50) value of Sample #2 (iPSC-MSC (expanded)) must be greater than the Normalised Av. Cq(50) value of Sample #4 (iPSC-MSC plus 0.001% iPSC Spike (expanded)). This demonstrates that the 0.001% iPSC Spike (expanded) can be detected above background.

Table 7. Expected Av. Norm. Cq(50) values for reference cultures for iPSC-MSC P5 final product.

Sample #	Spike	Expansion	Expected Av. Norm. Cq(50) *
1	No	No	15.16 ± 0.29
2	No	Yes	19.85 ± 0.17
3	0.001% iPSC	No	14.93 ± 0.06
4	0.001% iPSC	Yes	13.86 ± 0.07

*Norm. LIN28 Cq(50) = (LIN28 Cq(50) - GAPDH Cq(50)) +/- Std. Dev. (N=3x3)

b) The GAPDH Cq(50) % RSD for all samples must be ≤5%. The % RSD for all Average Cq(50) values and Normalised Cq(50) values must be ≤ 5%.

c) The H₂O (NTC) Controls (+/-RT) must not produce a detectable amplification signal within 50 amplification cycles for all replicates.

i) If any of the H₂O (NTC) Controls (+/-RT) are assigned a Cq(50) value, repeat the TaqMan® qRT-PCR assays from Step 3)g). This indicates contamination of the reagents by template DNA.

Example 3 - Results of protocol performed according to example 2

Table 8. Av. Norm. Cq(50) values of protocol performed according to example 2 for reference cultures and iPSC-MS C P5 final product.

Sample #	Description	Av. Norm. Cq(50): iPSC-MS C-P5		
		Culture 1	Culture 2	Culture 3
1	iPSC-MS C (non-expanded) (Reference culture)	15.23	15.33	14.73
2	iPSC-MS C (expanded) (Reference culture)	19.97	21.51	20.53
3	iPSC-MS C plus 0.001% iPSC Spike (non-expanded) (Reference culture)	14.90	15.02	14.69
4	iPSC-MS C plus 0.001% iPSC Spike (expanded) (Reference culture)	14.30	14.20	13.99
5	iPSC-MS C P5 Final Product (non-expanded)	15.97	15.42	14.09
6	iPSC-MS C P5 Final Product (expanded)	20.46	21.06	20.72

Example 4 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by OCT4 (POU5F1). The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 5 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by SOX2. The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 6 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by FOXD3. The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 7 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by NANOG. The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 8 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by PODXL. The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 9 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by REX1 (ZFP42). The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 10 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by SSEA1 (FUT4). The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 11 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by SSEA4. The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 12 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by DPPA2. The anticipated results are in line with example 2, table 7 and example 3, table 8.

Example 13 - Quantitation of residual undifferentiated stem cells by qRT-PCR

This example describes quantitation of residual, undifferentiated iPSCs essentially by the protocol of example 2, but LIN28 is replaced by DPPA3. The anticipated results are in line with example 2, table 7 and example 3, table 8.

CLAIMS

1. A method for detecting residual, undifferentiated pluripotent stem cells (PSCs) in a culture of cells differentiated from PSCs, the method comprising:

culturing the cells on a substrate coated with laminin-521 and E-cadherin in a medium comprising a Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor;

quantitating in the cultured cells expression of a marker of residual, undifferentiated PSCs; and

comparing the marker expression in the cultured cells with the marker expression in a reference culture of cells comprising a known proportion of PSCs,

wherein lower marker expression in the culture of cells than marker expression in the reference culture of cells indicates absence of residual, undifferentiated PSCs in the cultured cells or presence of residual, undifferentiated PSCs in the cultured cells at a proportion lower than the known proportion of PSCs in the reference culture of cells.

2. The method of claim 1, wherein the marker expression is LIN28, OCT4, SOX2, FOXD3, NANOG, PODXL, REX1, SSEA1, SSEA4, DPPA2 or DPPA3 expression.

3. The method of claim 1 or claim 2, wherein the ROCK inhibitor is Y27632.

4. The method of claim 3, wherein the cells are cultured in about 10 μ M Y27632.

5. The method of any one of claims 1 to 4, comprising culturing the cells in the medium comprising the ROCK inhibitor for about 3 days.

6. The method of claim 5, further comprising culturing the cells in a medium not comprising a ROCK inhibitor for about 2 days after culturing in the medium comprising the ROCK inhibitor.

7. The method of any one of claims 1 to 6, wherein the marker expression is quantitated by polymerase chain reaction (PCR).

8. The method of claim 7, wherein the PCR is quantitative real time PCR (qRT-PCR).

9. The method of claim 8, wherein the qRT-PCR comprises a probe consisting of 5'→3' sequence CGCATGGGGTTCGGCTTCCTGTCC (SEQ ID NO: 14), a primer consisting of 5'→3' sequence CACGGTGCGGGCATCTG (SEQ ID NO: 15), and a primer consisting of 5'→3' sequence CCTTCCATGTGCAGCTTACTC (SEQ ID NO: 16)

10. The method of any one of claims 1 to 9, wherein the marker expression is normalised.

11. The method of claim 10, wherein the marker expression is normalised to GAPDH expression.

12. The method of any one of claims 1 to 11, wherein the known proportion is 0.001%.

13. The method of any one of claims 1 to 12, wherein the PSCs are iPSCs.

14. The method of any one of claims 1 to 13, wherein the cells are MSCs.

15. The method of claim 14, wherein the MSCs are cultured in E8 Complete Medium.

16. The method according to any one of claims 1 to 15 further comprising formulating into a therapeutic composition the culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion.

17. A method for manufacturing a therapeutic composition, the method comprising formulating into a composition for therapeutic administration to a subject a culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion when detected by the method of any one of claims 1 to 15.

18. Use of a culture of cells in which residual, undifferentiated PSCs are absent or lower than a known proportion in the manufacture of a medicament for treating or preventing a condition in a subject, wherein residual, undifferentiated PSCs in the culture of cells differentiated from PSCs are detected by the method of any one of claims 1 to 15.

19. The use of claim 18, wherein the condition is selected from bone cysts, bone neoplasms, fractures, cartilage defects, osteoarthritis, ligament injury, osteogenesis imperfecta, osteonecrosis, osteoporosis, aplastic anaemia, graft versus host disease (GvHD), myelodysplastic syndrome, Type 1 diabetes, Type 2 diabetes, autoimmune hepatitis, liver cirrhosis, liver failure,

dilated cardiomyopathy, heart failure, myocardial infarction, myocardial ischemia, Crohn's disease, ulcerative colitis, burns, epidermolysis bullosa, lupus erythematosus, rheumatoid arthritis, Sjogren's disease, systemic sclerosis, bronchopulmonary dysplasia, chronic obstructive airways disease, emphysema, pulmonary fibrosis, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, brain injury, ataxia, degenerative disc disease, multiple system atrophy, multiple sclerosis, Parkinson's disease, retinitis pigmentosa, Romberg's disease, spinal cord injury, stroke, muscular dystrophy, limb ischaemia, kidney injury, lupus nephritis, endometriosis and complications of bone marrow or solid organ transplantation.

20. A kit for detecting residual, undifferentiated PSCs in a culture of cells differentiated from PSCs, the kit comprising:

laminin-521; and
E-cadherin; and
a ROCK inhibitor.

21. The kit of claim 20, further comprising PCR primers and optionally a PCR probe for quantitating in the cultured cells expression of a marker of residual, undifferentiated PSCs.

22. The kit of claim 20 or claim 21, further comprising a medium, optionally comprising the ROCK inhibitor.

23. The kit of any one of claims 20 to 22, further comprising a substrate, optionally coated with the laminin-521 and E-cadherin.