EXPANSION, HARVEST AND CRYOPRESERVATION OF INDUCED PLURIPOTENT STEM CELLS WITH CGMPS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application includes a claim of priority under 35 U.S.C. §119(e) to U.S. provisional patent application No. 63/551,650, filed February 9, 2024, the entirety of which is hereby incorporated by reference.

FIELD OF INVENTION

[0002] This invention relates to expansion, harvest and cryopreservation of induced pluripotent stem cells cell banks with current Good Manufacturing Practices (cGMPs).

BACKGROUND

[0003] All publications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

[0004] Cord blood and other cells such as fibroblasts, keratinocytes, peripheral blood cells, and mesenchymal stem cells are sources for cells that can be utilized for reprogramming into induced pluripotent stem cells (iPSCs) among other things. However, development of successful and reliable processes for clonal isolation post reprogramming, early passage stabilization, expansion, harvest and cryopreservation of induced pluripotent stem cells cell banks, and at the same time conform to current good manufacturing practices (cGMP) are difficult. Thus, there remains a need in the art for these processes.

SUMMARY OF THE INVENTION

[0005] The following embodiments and aspects thereof are described and illustrated in conjunction with compositions and methods which are meant to be exemplary and illustrative, not limiting in scope.

[0006] Various embodiments provide for a method for early passage and expansion of iPSCs, comprising: removing media from a plate or flask comprising iPSCs; adding PBS to each well in the plate or to the flask; adding ReLeSR and incubate at about 10-30°C for about 15-45 seconds; removing the ReLeSR; incubating the plate or flask at about 34-40°C for about 5-10 minutes adding Complete mTeSR Plus to the wall of each well of the plate or to each flask; immediately after adding the Complete mTeSR Plus, tapping the plate or flask to detach the cells from the plate or flask in detached cell aggregates; transferring the detached cell aggregates from each well or flask into a tube; adding a quantity of Complete

mTeSR Plus to the tube forming a cell suspension; triturating the cell suspension at least 3 times to break the detached cell aggregates into cell clumps; plating the cell clumps onto a Laminin CT521 coated plate, wherein the Laminin CT521 coated plate was Laminin CT521 coated and the Laminin CT521 was removed and replaced with mTeSR Plus media; rocking the Laminin CT521 coated plate in an incubator at about 34-40°C and about 3-7% CO_2 to distribute the cells.

[0007] In various embodiments, early passage and expansion of iPSCs can comprise removing media from the platesor flask comprising iPSCs; adding about 1 mL of PBS to each well in the plate, or about 10-50 mL/flask of mTeSR Plus for a T75-T225 flask; adding about 1 mL of ReLeSR and incubate at about about 20°C for about about 30 seconds; removing the ReLeSR; incubating the plates at about 37°C for about 7 minutes; adding about 1mL of Complete mTeSR Plus to the wall of each well or flask; immediately after adding the Complete mTeSR Plus, tapping the plate or flask to detach the cells from the plate in detached cell aggregates; transferring the detached cell aggregates from each well into a conical tube; adding a quantity of Complete mTeSR Plus to the conical tube to bring the total volume to 3, mL, 6 mL or 12 mL, forming a cell suspension; triturating the cell suspension at least 5 times to break the detached cell aggregates into cell clumps; plating the cell clumps onto a Laminin CT521 coated plate, wherein the plate was Laminin CT521 coated and the Laminin CT521 was removed and replaced with mTeSR Plus media; rocking the Laminin CT521 coated plate in an incubator at about 37°C and about 5% CO_2 to distribute the cells.

[0008] In various embodiments, tapping the plate or flask can comprise tapping the plate or flask against a BSC sash, or holding the plate or flask with one hand and use the other hand to firm tap the side of the plate or flask for about 30-60 seconds.

[0009] Various embodiments provide for a method of passaging iPSCs, comprising: removing media from a plate or flask comprising iPSCs; cutting an iPSC colony into multiple clumps; nudging the cut colony pieces off the plate or flask and onto a new plate or new flask, wherein the new plate or new flask comprises a quantity of mTeSR Plus; adding mTeSR Plus media to pool the clumps to the bottom of each well of the new plate, or the new flask; transferring the floating clumps to the new plate or the new flask having mTeSR Plus; rocking the new plate or the new flask comprising the floating clumps in an incubator at 34-40°C and 3-7% CO_2.

[0010] In various embodiments, passaging iPSCs can comprise: removing media from the plate or flask comprising iPSCs; cutting an iPSC colony into multiple clumps; using a P1000 micropipette tip inside a P20 tip to nudge the cut colony pieces off the plate or flask onto a new plate or new flask, wherein the new plate comprises a about 0.5 mL/well of mTeSR Plus for a 12 well plate, or 1 mL/well of mTeSR Plus for a 6 well plate, or 10-50 mL/flask of mTeSR Plus for a T75-T225 flask; adding mTeSR Plus media to pool the clumps to the bottom of each well of the new plate or to the new flask; transferring the colony

pieces to the new plate or the new flask; rocking the new plate or new flask comprising the floating clumps in an incubator at about 37°C and about 5% CO_2.

[0011] In various embodiments, the method can further comprise feeding the cells on day 1 post-passaging. In various embodiments, the method can further comprise pelleting the cells. In various embodiments, the method can further comprise cryopreserving the cells.

[0012] Various embodiments provide for a method for induced pluripotent stem cell (iPSC) harvesting, comprising: (a) obtaining iPSCs and measure percent confluency, wherein the iPSCs are in a plate or a flask; (b) removing conditioned media from the plates or flask; (c) incubating the iPSCs for 8-12 minutes to detach the iPSCs from the plate or flasks, and optionally for an additional 2-4 minutes if iPSCs are still adhered to bottom of the plate or flask; (d) rinsing the plate or flask with 6-10 mL of DPBS-/-; repeat step (d); (e) adding 6-10 mL of TrypLE to the plate or flask; (f) incubating the iPSCs for 8-12 minutes to detach the iPSCs from the plate or flask, and optionally for an additional 2-4 minutes if iPSCs are still adhered to bottom of the plate or flask; (f) adding 6-10 mL of mTeSR Plus media to the plate or flask and pipetting 1-4 times to assist in detaching the remaining iPSCs resulting in an iPSC suspension; (g) transferring the iPSC suspension into a tube; (h) optionally, add an additional 6-10 mL of mTeSR Plus media to the plate or flask and pipetting 1-4 times to assist in detaching the remaining iPSCs and transferring the remaining iPSCs into the tube.

[0013] In various embodiments, the method can further comprise preparing the iPSCs for cryopreservation, the method comprising: (i) spinning down the cells at 400-800 x g for 3-7 minutes at 10-30°C; (j) removing supernatant from the tube using a serological pipette leaving a pellet; (k) dislodging the pellet; (l) adding Complete mTeSR Plus media to the tube and pipetting up and down 2-4 times to resuspend the pellet; (m) adding 4-6 mL of Complete mTeSR Plus media to the tube; (n) optionally, combining the iPSC suspension with another iPSC suspension prepared by steps (f)-(h); (o) spinning down the tube at 400-800 x g for 3-8 minutes at 10-30°C; (p) removing supernatant and leaving a pellet; (q) adding cold CS10 and pipetting up and down 1-10 times to break up the pellet resulting in an iPSC cell suspension; (r) adding an additional 2-6 mL of cold CS10, and optionally adding additional CS10 to bring volume of the iPSC suspension to a desired volume; and (s) spinning down the tube at 200-400 x g for 3-7 minutes at 10-30°C;

[0014] In various embodiments, harvesting the iPSCs can comprise: (a) obtaining iPSCs and measure percent confluency, wherein the iPSCs are in the plate or flask; (b) removing conditioned media from the plate or flask; (c) incubating the iPSCs for 10 minutes to detach the iPSCs from the plate or flask, and optionally for an additional 3 minutes if iPSCs are still adhered to bottom of the plate or flask; (d) rinsing the flask with 8 mL of DPBS-/-; repeat step (d); (e) adding 8 mL of TrypLE to the plate or flask; (f) incubating the iPSCs for 10 minutes to detach the iPSCs from the plate or flask, and optionally for an additional 3 minutes if iPSCs are still adhered to bottom of the flask; (f) adding 8 mL of mTeSR Plus media to the plate or flask and pipetting 2-3 times to assist in detaching the remaining iPSCs resulting in an iPSC

suspension; (g) transferring the iPSC suspension into a 50 ml conical tube; (h) optionally, add an additional 8 mL of mTeSR Plus media to the plate or flask and pipetting 2-3 times to assist in detaching the remaining iPSCs and transferring the remaining iPSCs into the 50 ml conical tube.

[0015] In various embodiments, the method can further comprise preparing the iPSCs for cryopreservation, the method comprising: (i) spinning down the cells at about 300 x g for about 5 minutes at about 20°C; (j) removing supernatant from the tube using a serological pipette; (k) dislodging the pellet by flicking the 50 ml conical tube; (l) adding 1 mL of Complete mTeSR Plus media to the 50 ml conical tube and pipetting up and down about 3 times to resuspend the pellet using a P1000 micropipette; (m) adding about 4 mL of Complete mTeSR Plus media to the 50 ml conical tube; (n) optionally, combining the iPSC suspension with another iPSC suspension prepared by steps (f)-(h); (o) spinning down the 50 ml conical tube at about 300 x g for about 5 minutes at about 20°C; (p) removing supernatant from pellet using a serological pipette; (q) adding about 1 mL of cold CS10 and pipetting up and down 2-6 times to break up the pellet resulting in an iPSC cell suspension; (r) adding an additional about 4 mL of cold CS10, and optionally adding additional CS10 to bring volume of the iPSC suspension to a desired volume.

[0016] In various embodiments, the method can further comprise spinning down the 50 ml conical tube at 300 x g for 5 minutes at about 20°C.

[0017] In various embodiments, the method can further comprise cryopreserving the iPSCs. In various embodiments, cryopreserving the iPSC can comprise utilizing automated filling of cryovials.

[0018] Various embodiments provide for a method of cryopreserving iPSCs as single cells, comprising: placing a cryovial on a pre-cooled rack; pipetting up and down to resuspend iPSCs in a tube; adding about 0.5-2.0 mL of cell suspension to the cryovial; and transferring the filled cryovial to the controlled rate freezer (CRF).

[0019] In various embodiments, cryopreserving iPSCs as single cells can comprise placing a cryovial on a pre-cooled rack; pipetting up and down to resuspend iPSCs in a tube; adding about 1 mL of cell suspension to the cryovial; and transferring the filled cryovial to the controlled rate freezer (CRF).

[0020] In various embodiments, the method can further comprise allowing a sample probe to reach about 2-6°C; once at about 2-6°C, running the CRF; upon completion of CRF, transferring the cryovial to an LN2 tank.

[0021] In various embodiments, the method can further comprise allowing a sample probe to reach about 4°C; once at about 4°C, running the CRF; upon completion of CRF, transferring the cryovial to an LN2 tank.

[0022] In various embodiments, transferring the cryovial to an LN2 tank, can comprise first transferring the vials to a container with dry ice and then transferring the vial to an LN2 tank.

[0023] Various embodiments provide for a method of automated filling and cryopreserving iPSCs as single cells, comprising: (a) obtaining harvested induced pluripotent stem cell (iPSC), wherein the

harvested iPSCs have been spun down in a tube; (b) removing supernatant and leaving a pellet; (c) resuspending the pellet; (d) adding CS10 to the tube and resuspending the pellet into an iPSC suspension; (e) optionally, combing the iPSC suspension with one or more iPSC suspensions; (f) adding additional CS10 to bring the volume of the iPSC suspension to 15-35 mL; (g) transferring the iPSC suspension to a 125-375 mL bottle; (h) adding additional CS10 to bring the volume of the iPSC suspension to 100-200 mL; (i) inserting a tube into the bottle comprising the iPSC suspension and priming the tubing until some iPSC suspension is dispensed into a reservoir; (j) placing a multi-vial rack on a rack nest; (k) selecting a desired dispensing volume on an automated filling machine; (l) operating the automated filling machine to fill the vials in the multi-vial rack; and (m) transferring the filled cryovial to the controlled rate freezer (CRF).

[0024] In various embodiments, automated filling and cryopreserving iPSCs as single cells, can comprise: (a) obtaining harvested induced pluripotent stem cell (iPSC), wherein the harvested iPSCs have been spun down in a tube; (b) removing supernatant from pellet using a serological pipette; (c) resuspending the pellet by flicking the tube; (d) adding about 1mL of CS10 to the tube and pipette up and down no more than 3 times to break up the pellet resulting in an iPSC suspension; (e) optionally, combing the iPSC suspension with one or more iPSC suspensions; (f) adding additional CS10 to bring the volume of the iPSC suspension to about 25 mL; (g) transferring the iPSC suspension to a 250 mL sterile bottle; (h) adding additional CS10 to bring the volume of the iPSC suspension to about 157 mL; (i) inserting a tube into the bottle comprising the iPSC suspension and priming the tubing until some iPSC suspension is dispensed into a reservoir; (j) placing a 48-vial rack on a rack nest; and (k) selecting a desired dispensing volume of about 1000uL on the automated filling machine; (l) operating the automated filling machine to fill the vials in the multi-vial rack; and (m) transferring the filled cryovial to the controlled rate freezer (CRF).

[0025] In various embodiments, the method can further comprise allowing a sample probe to reach about 2-6°C; once at about 2-6°C, running the CRF; upon completion of CRF, transferring the cryovials to an LN2 tank.

[0026] In various embodiments, transferring the cryovial to an LN2 tank, can comprise first transferring the vials to a container with dry ice and then transferring the vial to an LN2 tank.

[0027] Various embodiments provide for induced pluripotent stem cells (iPSCs) reprogrammed from isolated cell fraction (ICF) from cord blood units (CBUs), and expanded and optionally passaged wherein (a) the iPSCs express one or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326, or (b)the iPSCs express one or more genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL, or (c) the iPSCs express one or more genes selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin), or (d) any combinations of (a), (b), or (c).

[0028] In various embodiments, the iPSCs are expanded and optionally passaged by any one of the methods of the present invention.

[0029] A composition comprising iPSCs of the present invention; and cell media.

[0030] Other features and advantages of the invention will become apparent from the following detailed description, taken in conjunction with the accompanying drawings, which illustrate, by way of example, various features of embodiments of the invention.

BRIEF DESCRIPTION OF THE FIGURES

[0031] Exemplary embodiments are illustrated in referenced figures. It is intended that the embodiments and figures disclosed herein are to be considered illustrative rather than restrictive.

[0032] Figure 1 shows an arrow and circle identifying an individual iPSC colony.

[0033] Figure 2 shows an arrow identifying an iPSC colony and the circle marks spontaneous differentiation.

[0034] Figures 3A-3E shows representative images for ranking reference. For Ranks 5-3, indicators are used to identify iPSC cultures with good morphology with none or some areas of spontaneous differentiation in the images. For Ranks 2 and 1 indicator correspond to cultures of very low quality and high percentages of differentiation, to identify good iPSC colonies or morphology; things not highlighted in the oval are spontaneous differentiation. 3A) Rank 5; 3B) Rank 4, 3C) Rank 3, bottom right - circle is highlighting an area of a colony that looks bumpy on the surface. The morphology of remaining good iPSC colonies amidst a lot of differentiation; 3D) Rank 2; 3E) Rank 1

[0035] Figure 4A-4B show UMAP clustering of single cell RNA-sequencing data separates distinct sample sets into defined clusters.

[0036] Figure 5 shows distinct subclusters and separation across all samples.

[0037] Figure 6 shows dot plot of specific marker genes across a matrix of clusters as defined in Figure 5 and categorizing them as specific cell type.

[0038] Figures 7-15 show unique CD genes, or cluster of differentiation genes, encode proteins that are cell surface markers expressed in iPSCs. These marker genes trace origin from cord blood isolated cell fraction and demonstrate a novel composition of iPSCs generated by our process.

[0039] Figures 16-25 show novel genes expressed uniquely in cord blood derived iPSC sample set.

[0040] Figures 26-30 shows that known pluripotency genes are also expressed in the cord blood derived iPSC sample set confirming that these are *bona fide* pluripotent stem cells

[0041] Figure 31 shows Reprogrammed Mix (Partially reprogrammed) Violin Plot and iPSCs Violin Plot.

DESCRIPTION OF THE INVENTION

[0042] All references cited herein are incorporated by reference in their entirety as though fully set forth. Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0043] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

[0044] As used herein the term "about" when used in connection with a referenced numeric indication means the referenced numeric indication plus or minus up to 5% of that referenced numeric indication, unless otherwise specifically provided for herein. In various embodiments, the term "about" when used in connection with a referenced numeric indication can mean the referenced numeric indication plus or minus up to 4%, 3%, 2%, 1%, 0.5%, or 0.25% of that referenced numeric indication, *if* specifically provided for in the claims.

[0045] "Room temperature" as used herein refers to a temperature between 15°C to 25°C. In various embodiments, "room temperature" refers to a temperature between 18°C to 22°C, or between 19°C to 21°C, or about 20°C, if specifically provided for in the claims.

[0046] StemSpan AOF referenced herein is an animal origin free media. It is a cGMP medium, for culture and expansion of human hematopoietic cells. It contains only recombinant proteins and synthetic components, and does not contain serum or other human- or animal-derived components.

[0047] StemSpan CD34+ expansion supplement is a serum-free culture supplement for expansion of human CD34+ hematopoietic cells. It contains a combination of recombinant human cytokines and other additives formulated to selectively promote the expansion of CD34+ cells isolated from human cord blood (CB) or bone marrow (BM) samples.

[0048] Table below provides the definitions of various acronyms used in the present application.

Acronym/Term	**Definition**
BSC	Biosafety Cabinet
BPR	Batch Production Record
CBC	Cedars-Sinai Biomanufacturing Center
cGMPs	current Good Manufacturing Practices
CRF	Controlled Rate Freezer
DIV	Days in vitro
DPBS -/-	DPBS, 1X (without Ca++/Mg++); Unless otherwise mentioned, DPBS refers to DPBS -/-
EM	Environmental Monitoring
ID	Identification
IPA	Isopropyl Alcohol

iPSCs	Induced Pluripotent Stem Cells
MFG	Manufacturing
MPR	Master Production Record
mTeSR Plus	This refers to Complete mTeSR Plus (Basal and Supplement mixed)
PCS	Primary Cell Stock
PCR	Polymerase Chain Reaction
PD	Process Development
PTK (Pick to Keep)	Passaging method of manually cutting iPSC colonies and transferring them to a new plate for expansion
Primary Cell Stock	First stock of iPSCs to be cryopreserved following reprograming and clone expansion to facilitate optimal iPSC clone screening. Generally, on the order of 10 vials. Generally, less than passage 10.
Seed Bank	Seed Bank is a bank of cells, approximately 25 Vials at 2 E+06 cells per vial.
PTK	Pick to Keep
QA	Quality Assurance
RCF	Relative Centrifugal Force
RT	Room Temperature
QC	Quality Control
R&D	Research and Development
RPM	Revolutions per Minute
SOP	Standard Operating Procedure
Trituration	Repeated pipetting to break clumps into smaller clumps
LN2	Liquid Nitrogen Tank. LN2 storage implies storing in a Liquid Nitrogen Tank

[0049] The procedure described herein applies to expanding early passage iPSCs with ReLeSR or the Pick-to-Keep method for Primary Cell Stock or Direct Seed Bank Generation. The procedure described herein applies to any number of clones (e.g., from 1 to 12) per parent line. Embodiments of procedure includes but are not limited to feeding and passaging iPSCs with ReLeSR or the Pick to Keep (PTK) method under current Good Manufacturing Practice (cGMP). The procedure described herein also applies to the generation of iPSC Seed Banks under Good Manufacturing Practices (cGMPs) Manufacturing facility.

[0050] CT521 laminin is a cell therapy grade laminin and it is well known in the field that it is challenging to successfully use it in reprogramming methods. CT521 has never been used for reprogramming cord blood derived iPSCs from previous literature. Described herein, the inventors have determined the process for successfully and consistently cultivating iPSCs using CT521 laminin.

[0051] Our innovative method for expansion of cord blood-derived iPSCs using cell therapy grade laminin 521 (CT521) per cGMPs has several unexpected aspects and benefits:

[0052] Enhanced efficiency: CT521 supports significantly increased proliferation compared to fragmented laminins, yielding 5-20 times more pluripotent stem cells from cord blood cells as a starting population compared to PBMCs and other starting cell types. CT521 is critical to achieving superior results

during reprogramming iPSCs like higher efficiency, faster colony formation, better cell viability. This leads to a more efficient reprogramming process for cord blood-derived iPSCs.

[0053] Our data shows that cord blood–derived iPSCs reprogram more quickly and yield higher-quality colonies this CT521-based method compared to standard fibroblast or peripheral blood methods. Given that cord blood cells are considered superior for reprogramming due to their young age and high proliferation rate, our method specifically optimized for cord blood-derived cells using CT521 yields higher efficiency and quality iPSCs compared to other cell sources.

[0054] Improved cell signaling: Unlike fragmented laminin products, full-length laminin CT521 can bind with all relevant cell surface receptors, including integrins, dystroglycans, and syndecans. This results in more authentic cell signaling during the reprogramming process, potentially improving the quality and stability of the resulting iPSCs.

[0055] Clinically relevant conditions: CT521 is designed for clinical research and complies with USP Chapter 1043, making it suitable for potential therapeutic applications. This allows for a seamless transition from research to clinical development.

[0056] Animal component-free culture: CT521 is animal component-free to the secondary level, reducing the risk of xenogenic contamination and improving the safety profile of the reprogramming process.

[0057] Biologically relevant microenvironment: CT521 recreates a more authentic culture environment, mimicking the natural stem cell niche leading to more stable and functionally superior iPSCs.

[0058] Compatibility with single-cell approaches: CT521 supports reliable single-cell expansion of human pluripotent stem cells, which is advantageous for developing more controlled and scalable reprogramming protocols.

[0059] Enhanced maturation and organization: CT521 has been shown to support efficient differentiation, maturation of differentiated cell types. This can be beneficial for downstream applications of the reprogrammed iPSCs.

[0060] Synergy with episomal reprogramming and expansion to create a fully integration-free and a cGMP clinically relevant reprogramming and expansion system.

[0061] Various embodiments of the present invention provide for a method for early passage and expansion of iPSCs, comprising:

removing media from a plate or flask comprising iPSCs;

adding PBS to each well in the plate or flask;

adding ReLeSR and incubate at about 10°C-30°C for about 15-45 seconds;

removing the ReLeSR;

incubating the plate or flask at about 34°C-40°C for about 5-9 minutes;

adding Complete mTeSR Plus to the wall of each well;

immediately after adding the Complete mTeSR Plus, tapping the plate or flask to detach the cells from the plate or flask in detached cell aggregates;

transferring the detached cell aggregates from each well into one tube;

adding a quantity of Complete mTeSR Plus to the one tube;

triturating the cell suspension at least 3 times to break the detached cell aggregates into cell clumps;

plating the cell clumps onto a plate, wherein the plate was Laminin CT521 coated and the Laminin CT521 was removed and replaced with mTeSR Plus media;

rocking the Laminin CT521 coated plate in an incubator at about 34°C-40°C and about 3-7% CO_2 to distribute the cells.

[0062] Various embodiments of the present invention provide for a method for early passage and expansion of iPSCs, comprising:

removing media from a plate or flask comprising iPSCs;

adding PBS to each well in the plate or flask;

adding ReLeSR and incubate at about 15°C-25°C for about 20-40 seconds;

removing the ReLeSR;

incubating the plate or flask at about 35°C-39°C for about 6-8 minutes;

adding Complete mTeSR Plus to the wall of each well;

immediately after adding the Complete mTeSR Plus, tapping the plate or flask to detach the cells from the plate or flask in detached cell aggregates;

transferring the detached cell aggregates from each well into one tube;

adding a quantity of Complete mTeSR Plus to the one tube;

triturating the cell suspension at least 3 times to break the detached cell aggregates into cell clumps;

plating the cell clumps onto a plate, wherein the plate was Laminin CT521 coated and the Laminin CT521 was removed and replaced with mTeSR Plus media;

rocking the Laminin CT521 coated plate in an incubator at about 35°C-39°C and about 4-6% CO_2 to distribute the cells.

[0063] In various embodiments, early passage and expansion of iPSCs comprises

removing media from a plate or flask comprising iPSCs;

adding about 1 mL of PBS to each well in the plate or flask;

adding about 1 mL of ReLeSR and incubate at about 20°C for about 30 seconds;

removing the ReLeSR;

incubating the plate or flask at about 37°C for about 7 minutes;

adding about 1mL of Complete mTeSR Plus to the wall of each well;

immediately after adding the Complete mTeSR Plus, tapping the plate or flask to detach the cells from the plate or flask in detached cell aggregates;

transferring the detached cell aggregates from each well into one conical tube;

adding a quantity of Complete mTeSR Plus to the conical tube to bring the total volume to about 3, mL, about 6 mL or about 12 mL;

triturating the cell suspension at least 5 times to break the detached cell aggregates into cell clumps;

plating the cell clumps onto a plate, wherein the plate was Laminin CT521 coated and the Laminin CT521 was removed and replaced with mTeSR Plus media;

rocking the Laminin CT521 coated plate in an incubator at about 37°C and about 5% CO_2 to distribute the cells.

[0064] In various embodiments, tapping the plate or flask comprises tapping the plate or flask against a BSC sash, or holding the plate or flask with one hand and use the other hand to firm tap the side of the plate or flask for about 30-60 seconds. In various embodiments, Laminin CT521 was removed means that excess Laminin CT521 was removed, but a coating to Laminin CT521 is still on the plate.

[0065] In various embodiments, the iPSCs express one or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express five or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express 10 or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express 15 or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express all genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, and CD326.

[0066] In various embodiments, the iPSCs express one or more genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 5 or more genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 10 or more genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 15 or more genes selected from TCFP2L1,

CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express all genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, and COBL.

[0067] In various embodiments, the iPSCs express one or more genes selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express 5 or more genes selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express 8 or more genes selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express all genes selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, and PODXL (CD34 sialomucin).

[0068] Various embodiments provide for a method of passaging iPSCs, comprising:

removing media from a plate or flask comprising iPSCs;

cutting an iPSC colony into multiple clumps;

nudging the cut colony pieces off the plate or the flask and onto a new plate or new flask, wherein the new plate or new flask comprises a quantity of mTeSR Plus;

adding mTeSR Plus media to the plate or the flask to rinse the plate or the flask and pooling the clumps to the bottom of each well of the plate or the flask;

transferring the floating clumps from the plate or the flask to the new plate or new flask having mTeSR Plus;

rocking the new plate or new flask comprising the floating clumps in an incubator at 34-40°C and 3-7% CO_2.

[0069] In various embodiments, passaging iPSCs, comprises:

removing media from a plate or flask comprising iPSCs;

cutting an iPSC colony into multiple clumps;

using a micropipette tip inside a pipette tip to nudge the cut colony pieces off the plate or flask onto a new plate or new flask, wherein the new plate or new flask comprises about 0.25-0.75 mL/well of mTeSR Plus for a 12 well plate, or about 0.5-1.5 mL/well of mTeSR Plus for a 6 well plate, or 5-75 mL/flask of mTeSR Plus for a T75-T225 flask;

adding mTeSR Plus media to the plate or the flask to rinse the plate or the flask and pooling the clumps to the bottom of each well of the plate or the flask;

transferring the clumps from the plate or the flask to the new plate or the new flask having mTeSR Plus; and

rocking the new plate or the new flask comprising the floating clumps in an incubator at about 35-38°C and about 4-6% CO_2.

[0070] In various embodiments, passaging iPSCs, comprises:

removing media from a plate or a flask comprising iPSCs;

cutting an iPSC colony into multiple clumps;

using a P1000 micropipette tip inside a P20 tip to nudge the cut colony pieces off the plate or flask onto a new plate or new flask, wherein the new plate or new flask comprises about 0.5 mL/well of mTeSR Plus for a 12 well plate, or about 1 mL/well of mTeSR Plus for a 6 well plate, or about 10-50 mL/flask of mTeSR Plus for a T75-T225 flask;

adding mTeSR Plus media to the plate or the flask to rinse the plate or the flask and pooling the clumps to the bottom of each well of plate or the flask;

transferring the clumps from the plate or the flask to the new plate the new flask having mTeSR Plus; and

rocking the new plate comprising the floating clumps in an incubator at about 37°C and about 5% CO_2.

[0071] In various embodiments, the mTeSR Plus media added to the plate or the flask to rinse the plate or the flask is from the new plate or new flask. While the plate and flask are termed as "new", this does require a never used plate or flask. Rather, the "new" plate for "new" flask is a clean and/or sterile plate or flask that is receiving the pieces of the iPSC colony.

[0072] In various embodiments, the method further comprises feeding the cells on day 1 post-passaging. The feeding schedule can be as listed in Table 1. In various embodiments, the method further comprises pelleting the cells. In various embodiments, the method further comprises cryopreserving the cells.

[0073] In various embodiments, the iPSCs express one or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express five or more of markers selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express 10 or more of markers selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express 15 or more of markers selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express all markers

selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, and CD326.

[0074] In various embodiments, the iPSCs express one or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 5 or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 10 or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 15 or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express all markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, and COBL.

[0075] In various embodiments, the iPSCs express one or more of markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express 5 or more of markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express 8 or more of markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express all markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, and PODXL (CD34 sialomucin).

[0076] Various embodiments provide for a method for induced pluripotent stem cell (iPSC) harvesting, comprising:

(a) obtaining iPSCs and measure percent confluency, wherein the iPSCs are in a plate or a flask;

(b) removing conditioned media from the plate or the flask;

(c) incubating the iPSCs for 8-12 minutes to detach the iPSCs from the plate or flask, and optionally for an additional 2-4 minutes if iPSCs are still adhered to bottom of the plate or flask;

(d) rinsing the plate or the flask with 6-10 mL of DPBS-/-;

repeat step (d);

(e) adding 6-10 mL of TrypLE to the plate or the flask;

(f) incubating the iPSCs for 8-12 minutes to detach the iPSCs from the plate or flask, and optionally for an additional 2-4 minutes if iPSCs are still adhered to bottom of the plate or flask;

(f) adding 6-10 mL of mTeSR Plus media to the plate or the flask and pipetting 1-4 times to assist in detaching the remaining iPSCs resulting in an iPSC suspension;

(g) transferring the iPSC suspension into a tube;

(h) optionally, add an additional 6-10 mL of mTeSR Plus media to the plate or flask and pipetting 1-4 times to assist in detaching the remaining iPSCs and transferring the remaining iPSCs into the tube.

[0077] In various embodiments, harvesting the iPSCs comprises:

(a) obtaining iPSCs and measure percent confluency, wherein the iPSCs are in a plate or a flask;

(b) removing conditioned media from the plate or the flask;

(c) incubating the iPSCs for 9-11 minutes to detach the iPSCs from the plate or the flask, and optionally for an additional 2.5-3.5 minutes if iPSCs are still adhered to bottom of the plate or the flask;

(d) rinsing the plate or the flask with 6-10 mL of DPBS-/-;

repeat step (d);

(e) adding 7-9 mL of TrypLE to the plate or the flask;

(f) incubating the iPSCs for 9-11 minutes to detach the iPSCs from the plate or the flask, and optionally for an additional 1.5-3.5 minutes if iPSCs are still adhered to bottom of the plate or flask;

(f) adding 7-9 mL of mTeSR Plus media to the plate or the flask and pipetting 1-4 times to assist in detaching the remaining iPSCs resulting in an iPSC suspension;

(g) transferring the iPSC suspension into a tube;

(h) optionally, add an additional 7-9 mL of mTeSR Plus media to the plate or the flask and pipetting 1-4 times to assist in detaching the remaining iPSCs and transferring the remaining iPSCs into the tube.

[0078] In various embodiments, harvesting the iPSCs comprises:

(a) obtaining iPSCs and measure percent confluency, wherein the iPSCs are in a plate or flask;

(b) removing conditioned media from the plate or flask;

(c) incubating the iPSCs for about 10 minutes to detach the iPSCs from the plate or flask, and optionally for about an additional 3 minutes if iPSCs are still adhered to bottom of the plate or flask;

(d) rinsing the plate or flask with about 8 mL of DPBS-/-;

repeat step (d);

(e) adding about 8 mL of TrypLE to the plate or flask;

(f) incubating the iPSCs for about 10 minutes to detach the iPSCs from the plate or flask, and optionally for about an additional 3 minutes if iPSCs are still adhered to bottom of the plate or flask;

(f) adding about 8 mL of mTeSR Plus media to the plate or flask and pipetting about 2-3 times to assist in detaching the remaining iPSCs resulting in an iPSC suspension;

(g) transferring the iPSC suspension into a 50 ml conical tube;

(h) optionally, add about an additional 8 mL of mTeSR Plus media to the plate or flask and pipetting about 2-3 times to assist in detaching the remaining iPSCs and transferring the remaining iPSCs into the 50 ml conical tube.

[0079] In various embodiments, the method further comprises(s) spinning down the 50 ml conical tube at about 150-450 x g for about 3-7 minutes at about 18-22°C. In various embodiments, the method further comprises(s) spinning down the 50 ml conical tube at about 200-400 x g for about 4-6 minutes at about 19-21°C. In various embodiments, the method further comprises (s) spinning down the 50 ml conical tube at about 300 x g for about 5 minutes at about 20°C.

[0080] In various embodiments, the iPSCs express one or more of markers selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express five or more of markers selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express 10 or more of markers selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express 15 or more of markers selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express all markers selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, and CD326.

[0081] In various embodiments, the iPSCs express one or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 5 or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 10 or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 15 or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express all markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, and COBL.

[0082] In various embodiments, the iPSCs express one or more of markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express 5 or more of markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express 8 or more of markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express all markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, and PODXL (CD34 sialomucin).

[0083] In various embodiments, the method further comprises preparing the iPSCs for cryopreservation. In various embodiments, the method of preparing the iPSCs for cryopreservation comprises:

(i) spinning down the cells at about 400-800 x g for about 3-7 minutes at about 10-30°C;

(j) removing supernatant from the tube using a serological pipette;

(k) dislodging the pellet;

(l) adding Complete mTeSR Plus media to the tube and pipetting up and down about 2-4 times to resuspend the pellet;

(m) adding about 4-6 mL of Complete mTeSR Plus media to the tube;

(n) optionally, combining the iPSC suspension with another iPSC suspension prepared by steps (f)-(h);

(o) spinning down the tube at about 400-800 x g for about 3-8 minutes at about 10-30°C;

(p) removing supernatant from pellet;

(q) adding cold CS10 and pipetting up and down about 1-10 times to break up the pellet resulting in an iPSC cell suspension;

(r) adding an additional about 2-6 mL of cold CS10, and optionally adding additional CS10 to bring volume of the iPSC suspension to a desired volume; and

(s) spinning down the tube at about 200-400 x g for about 3-7 minutes at about 10-30°C.

[0084] CS10 is an animal component-free, defined cryopreservation medium with 10% DMSO. In various embodiments, cold CS10 can be about 2-8°C. In various embodiments, cold CS10 can be about 9-12°C. In various embodiments, cold CS10 can be up to 15 °C.

[0085] In various embodiments, In various embodiments, the method of preparing the iPSCs for cryopreservation comprises:

(i) spinning down the cells at 350-700 x g for 4-6 minutes at 15-25°C;

(j) removing supernatant from the tube using a serological pipette;

(k) dislodging the pellet;

(l) adding Complete mTeSR Plus media to the tube and pipetting up and down 2-4 times to resuspend the pellet;

(m) adding 4-5 mL of Complete mTeSR Plus media to the tube;

(n) optionally, combining the iPSC suspension with another iPSC suspension prepared by steps (f)-(h);

(o) spinning down the tube at 350-700 x g for 4-7 minutes at 15-25°C;

(p) removing supernatant from pellet;

(q) adding cold CS10 and pipetting up and down 2-6 times to break up the pellet resulting in an iPSC cell suspension;

(r) adding an additional 3-5 mL of cold CS10, and optionally adding additional CS10 to bring volume of the iPSC suspension to a desired volume; and

(s) spinning down the tube at 150-350 x g for 3-7 minutes at 10-30°C.

[0086] In particular embodiments, the method of preparing the iPSCs for cryopreservation comprises:

(i) spinning down the cells at about 300 x g for about 5 minutes at about 20°C;

(j) removing supernatant from the tube using a serological pipette;

(k) dislodging the pellet by flicking the 50 ml conical tube;

(l) adding about 1 mL of Complete mTeSR Plus media to the 50 ml conical tube and pipetting up and down about 3 times to resuspend the pellet using a P1000 micropipette;

(m) adding about 4 mL of Complete mTeSR Plus media to the 50 ml conical tube;

(n) optionally, combining the iPSC suspension with another iPSC suspension prepared by steps (f)-(h);

(o) spinning down the 50 ml conical tube at about 300 x g for about 5 minutes at about 20°C;

(p) removing supernatant from pellet using a serological pipette;

(q) adding about 1 mL of cold CS10 and pipetting up and down about 2-6 times to break up the pellet resulting in an iPSC cell suspension;

(r) adding an additional about 4 mL of cold CS10, and optionally adding additional CS10 to bring volume of the iPSC suspension to a desired volume.

[0087] Various embodiments provide for a method of cryopreserving iPSCs as single cells, comprising placing a cryovial on a pre-cooled rack (e.g., 2-6°C); pipetting up and down to resuspend iPSCs in a tube; adding 0.5-2.0 mL of cell suspension to the cryovial; and transferring the filled cryovial to the controlled rate freezer (CRF). In various embodiments, the method further comprises allowing a sample

probe to reach about 2-6°C; once at about 2-6°C, running the CRF; upon completion of CRF, transferring the cryovial to an LN2 tank.

[0088] Various embodiments provide for a method of cryopreserving iPSCs as single cells, comprising placing a cryovial on a pre-cooled rack; pipetting up and down to resuspend iPSCs in a tube; adding 0.5-1.5 mL of cell suspension to the cryovial; and transferring the filled cryovial to the controlled rate freezer (CRF). In various embodiments, the method further comprises allowing a sample probe to reach about 3-5°C; once at about 3-5°C, running the CRF; upon completion of CRF, transferring the cryovial to an LN2 tank.

[0089] In various embodiments, the method of cryopreserving iPSCs as single cells, comprises placing a cryovial on a pre-cooled rack; pipetting up and down to resuspend iPSCs in a tube; adding about 1 mL of cell suspension to the cryovial; and transferring the filled cryovial to the controlled rate freezer (CRF). In various embodiments, the method further comprises allowing a sample probe to reach about 4°C; once at about 4°C, running the CRF; upon completion of CRF, transferring the cryovial to an LN2 tank.

[0090] In various embodiments, transferring the cryovial to an LN2 tank, comprises first transferring the vials to a container with dry ice and then transferring the vial to an LN2 tank.

[0091] Various embodiments provide for a method of automated filling and cryopreserving iPSCs as single cells, comprising:

(a) obtaining harvested induced pluripotent stem cell (iPSC), wherein the harvested iPSCs have been spun down in a tube;

(b) removing supernatant and leaving a pellet;

(c) resuspending the pellet;

(d) adding CS10 to the tube and resuspending the pellet into an iPSC suspension;

(e) optionally, combining the iPSC suspension with one or more iPSC suspensions;

(f) adding additional CS10 to bring the volume of the iPSC suspension to 15-35 mL;

(g) transferring the iPSC suspension to a 125-375 mL sterile bottle;

(h) adding additional CS10 to bring the volume of the iPSC suspension to 100-200 mL;

(i) inserting a tube into the bottle comprising the iPSC suspension and priming the tubing until some iPSC suspension is dispensed into a reservoir;

(j) placing a multi-vial rack on a rack nest; and

(k) selecting a desired dispensing volume on an automated filling machine;

(l) operating the automated filling machine to fill the vials in the multi-vial rack; and

(m) transferring the filled cryovial to the controlled rate freezer (CRF).

[0092] In various embodiments, the method of automated filling and cryopreserving iPSCs as single cells, comprises:

(a) obtaining harvested induced pluripotent stem cell (iPSC), wherein the harvested iPSCs have been spun down in a tube;

(b) removing supernatant and leaving a pellet;

(c) resuspending the pellet;

(d) adding CS10 to the tube and resuspending the pellet into an iPSC suspension;

(e) optionally, combining the iPSC suspension with one or more iPSC suspensions;

(f) adding additional CS10 to bring the volume of the iPSC suspension to 20-30 mL;

(g) transferring the iPSC suspension to a 150-350 mL sterile bottle;

(h) adding additional CS10 to bring the volume of the iPSC suspension to 125-175 mL;

(i) inserting a tube into the bottle comprising the iPSC suspension and priming the tubing until some iPSC suspension is dispensed into a reservoir;

(j) placing a multi-vial rack on a rack nest; and

(k) selecting a desired dispensing volume on an automated filling machine;

(l) operating the automated filling machine to fill the vials in the multi-vial rack; and

(m) transferring the filled cryovial to the controlled rate freezer (CRF).

[0093] In various embodiments, automated filling and cryopreserving iPSCs as single cells, comprises:

(a) obtaining harvested induced pluripotent stem cell (iPSC), wherein the harvested iPSCs have been spun down in a tube;

(b) removing supernatant and leaving a pellet using a serological pipette;

(c) resuspending the pellet by flicking the tube;

(d) adding 1mL of CS10 to the tube and pipette up and down no more than 3 times to break up the pellet resulting in an iPSC suspension ;

(e) optionally, combing the iPSC suspension with one or more iPSC suspensions;

(f) adding additional CS10 to bring the volume of the iPSC suspension to 25 mL;

(g) transferring the iPSC suspension to a 250 mL sterile bottle;

(h) adding additional CS10 to bring the volume of the iPSC suspension to 157 mL;

(i) inserting a tube into the bottle comprising the iPSC suspension and priming the tubing until some iPSC suspension is dispensed into a reservoir;

(j) placing a 48-vial rack on a rack nest; and

(k) selecting a desired dispensing volume of 1000μL on the automated filling machine;

(l) operating the automated filling machine to fill the vials in the multi-vial rack; and

(m) transferring the filled cryovial to the controlled rate freezer (CRF).

[0094] In various embodiments, the method further comprises allowing a sample probe to reach about 2-6°C; once at about 2-6°C, running the CRF; upon completion of CRF, transferring the cryovials to

an LN2 tank. In various embodiments, the method further comprises allowing a sample probe to reach about 3-5°C; once at about 3-5°C, running the CRF; upon completion of CRF, transferring the cryovials to an LN2 tank. In various embodiments, the method further comprises allowing a sample probe to reach about 4°C; once at about 4°C, running the CRF; upon completion of CRF, transferring the cryovials to an LN2 tank.

[0095] In various embodiments, transferring the cryovial to an LN2 tank, comprises first transferring the vials to a container with dry ice and then transferring the vial to an LN2 tank.

[0096]

[0097] Various embodiments of the present invention provide for iPSCs that have been expanded, passaged, and/or harvested in accordance with the methods of the present invention.

[0098] Various embodiments provide for iPSC.

[0099] Various embodiments provide for a composition comprising iPSC and cell media.

[0100] In various embodiments, the iPSCs express one or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express five or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express 10 or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express 15 or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326. In various embodiments, the iPSCs express all genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, and CD326.

[0101] In various embodiments, the iPSCs express one or more genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 5 or more genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 10 or more genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL. In various embodiments, the iPSCs express 15 or more of markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL.

In various embodiments, the iPSCs express all markers selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, and COBL.

[0102] In various embodiments, the iPSCs express one or more of markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express 5 or more genes selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express 8 or more of markers selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin). In various embodiments, the iPSCs express all genes selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, and PODXL (CD34 sialomucin).

KITS

[0103] The present invention is also directed to a kit for expansion, harvest and/or cryopreservation. The kit is useful for practicing the inventive method of expansion, harvest and/or cryopreservation. The kit is an assemblage of materials or components. Thus, in some embodiments the kit contains a composition including the components of the buffers as described herein.

[0104] The exact nature of the components configured in the inventive kit depends on its intended purpose. For example, some embodiments are configured for the purpose of expansion. In one embodiment, the kit is configured particularly for the purpose of harvest. In another embodiment, the kit is configured particularly for the purpose of cell cryopreservation.

[0105] Instructions for use may be included in the kit. "Instructions for use" typically include a tangible expression describing the technique to be employed in using the components of the kit to effectuate a desired outcome. Optionally, the kit also contains other useful components, such as, diluents, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, or other useful paraphernalia as will be readily recognized by those of skill in the art.

[0106] The materials or components assembled in the kit can be provided to the practitioner stored in any convenient and suitable ways that preserve their operability and utility. For example, the components can be in dissolved, dehydrated, or lyophilized form; they can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging material(s). As employed herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit, such as inventive compositions and the like. The packaging material is constructed by well-known methods, preferably to provide a sterile, contaminant-free environment. As used herein, the term "package" refers to a suitable solid matrix or material such as glass, plastic, paper, foil, and the like, capable

of holding the individual kit components. The packaging material generally has an external label which indicates the contents and/or purpose of the kit and/or its components.

EXAMPLES

[0107] The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art may develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

Example 1

Early Passage and Expansion of iPSC for Primary Cell Stock or Direct Seed Bank Generation

PRIMARY CELL STOCK OR SEED BANK GENERATION

[0108] Cells having undergone reprogramming will be isolated, fed and expanded following this procedure.

[0109] iPSCs clones will be expanded to cryopreserve and generate a Primary Cell Stock (PCS) or Seed Bank (SB). The PCS or SB will be generated based on the following criteria:

- The clone has reached at least passage 5.
- The clone has a rank of 3 or higher for two weeks in a PCS: The clone has been expanded into at least 1 - 6 well plate or T75 flask.
- SB: The clone can be expanded into as many plates/flasks to reach the desired number of cells.
- The clone has reached at least 80% confluency.

[0110] When cryopreserving cells as clumps (generally for PCS), 6-well plates that are >80% yield 2 vials per well.

[0111] When cryopreserving cells as single cells (generally for SB), refer to the following table for approximate cell counts per vessel

Vessel	Expected Number of Cells per Vessel
6 well plate	1.4E+07
T-75 flask	1.8E+07

[0112] Feeding will be performed as per below until cell confluency is at least 80% or 8 days in culture.

[0113] Post Passage Imaging and Ranking:

[0114] DIV 1: cells will be viewed and imaged to verify cell attachment DIV 4 – 8: cells will be imaged and assessed for quality and confluency.

[0115] Cells will be expanded from 12 well plates, into 6 Well Plates and into T75 flasks where applicable.

[0116] The PCS or SB will be harvested and frozen per project specific cryopreservation. Before generating a PCS or SB, freeze at least vials between passage 2 and 4 following the steps described below.

FEEDING

[0117] Feeding iPSCs will begin on Day 1 post-passaging and will continue based on the schedule listed in Table 1 below.

[0118] Table 1. iPSC Feeding Schedule

Vessel	**Volume of mTeSR Plus to Add**	**Type of Feed**
12 well plate	1 mL/well	Standard
6 well plate	2.5 mL/well	Standard
T-75 flask	20 mL	Standard
12 well plate	2 mL/well	Weekend (Double)
6 well plate	4 mL/well	Weekend (Double)
T-75 flask	40 mL	Weekend (Double)

[0119] Disinfect BSC.

[0120] Obtain required Complete mTeSR Plus media aliquots. mTeSR Plus can be used cold, directly from 4°C, it is not required to equilibrate to room temperature before using.

[0121] Image cells as per the schedule described in herein. Transfer the cells to the microscope. Take a picture and store the image.

[0122] **NOTE:** If confluency is below 80%, proceed to feed the cells as described below. If confluency is above 80% proceed to passage the cells as described below or harvest cells using relevant project-specific Harvest and Cryopreservation Procedure.

[0123] Transfer iPSCs to the BSC. When a single clone is contained in more than one plate/flask, only handle a maximum of 4 plates or 4 T-75 flasks at a time. Remove all the media from the plates/flasks and discard. Add the appropriate volume of fresh mTeSR Plus using a serological pipette according to Table 2. Record volume of media added to each vessel.

[0124] Transfer vessel(s) to incubator and record incubator information. Proceed to feed each clone repeating these steps.

CELL PASSAGING INITIAL STEPS

[0125] Disinfect BSC.

[0126] Obtain cells to be passaged from incubator and check cell confluency under microscope.

[0127] Record the cell rank, percent (%) confluency, harvesting methodology and anticipated passing dilution, if using ReLeSR. For cells with a ranking of 3 or higher, passage using ReLeSR method. For cells that have a ranking of 2, passage using PTK.

[0128] Take a picture store the picture.

[0129] Transfer the cells back to the incubator.

[0130] Ensure enough plate(s)/flask(s) have been coated with Laminin CT521 and record coating and expiration date. Record the number of plates or flasks to be used. Transfer reagents into BSC. **NOTE:** mTeSR Plus can be used cold, directly from 4°C, it is not required to equilibrate to room temperature before using.

[0131] Transfer Laminin CT521 coated plate(s)/flask(s) into the BSC.

[0132] Remove Laminin CT521 coating and add the appropriate volume of mTeSR Plus as described in the table 2 below.

Vessel	**Volume of mTeSR Plus to Add**
12 well plate	0.5 mL/well
6 well plate	2 mL/well
T-75 flask	15 mL

7.5. ReLeSR CELL PASSAGING

[0133] Transfer plates/flasks with cells to be harvested into the BSC. Note, when harvesting multiple plates/flasks, only process 4 plates/flasks at a time.

[0134] Remove media and discard. Add 1 mL of PBS to each well to be passaged. Remove PBS and discard. Add 1 mL of ReLeSR and incubate at room temperature for approximately 30 seconds. Remove ReLeSR and discard. Move plates/flasks to the incubator and incubate at 37°C for 7 minutes. Following the incubation, move plates/flasks back to the BSC. Gently add 1 mL of Complete mTeSR Plus to the wall of the well.

[0135] Immediately after adding mTeSR Plus, tap the plate against the inside of the BSC sash. Alternatively, hold the plate with one hand and use the other hand to firmly tap the side of the plate for approximately 30 - 60 seconds. If very few cells lift, use serological pipette to rinse well with medium already there.

[0136] Using a serological pipette, transfer the detached cell aggregates to an appropriately sized conical tube labelled as “iPSC”. Multiple wells should be pooled into one conical.

[0137] Add the appropriate volume of Complete mTeSR Plus to the tube to bring the total volume to 3 mL, 6 mL or 12mL (as per below). Use the table below as guide for Total Cell Suspension Volume.

Dilution	Total Cell Suspension Volume	Cell Suspension Volume to Plate
1:2	3 mL	1.5 mL
1:3	3 mL	1 mL
1:6	3 mL	0.5 mL
1:9	6 mL	667 µL
1:12	6 mL	500 µL
1:18	6 mL	333 µL
1:24	6 mL	250 µL
1:30	6 mL	200 µL
1:50	6 mL	120 µL
1:60	12 mL	200 µL
1:75	12 mL	160 µL
1:90	12 mL	134 µL
1:120	12 mL	100 µL

[0138] If moving from a 6 well plate to a T-75 flask, there is an expansion factor of 8. Expansion Factor = 75 cm^2/ 9.6 cm^2 = 8

[0139] The volume of cell suspension required to be added from a 6-well plate to a T-75 is calculated as: Volume (µL) = [Cell Suspension Volume to plate] x [Expansion Factor]

[0140] Using a serological pipette, triturate the cell suspension at least 5 times to break up the clumps to be more evenly distributed. Plate the cell clumps at the desired density using the table in above a guide. Record the dilution, volume plated and the number of wells plated. **NOTE:** If multiple dilutions are plated, record the dilutions, volume plated per well and number of wells plated.

[0141] Transfer the plate/flask(s) with cells outside the BSC and label it, for example, as follows: iPSC Line ID + clone #, BPR #, if applicable, Passage Number, ReLeSR, Dilution, Date, Initials

[0142] Transfer the plate to a 37°C 5% CO2 incubator and rock plate/flask back and forth and side to side to distribute cells evenly. Record incubator RB# and shelf.

[0143] If additional iPSC clones are going to be passaged, ensure appropriate clone-to-clone separation is maintained .

[0144] If additional clones are to be harvested using ReLeSR repeat steps these steps.

[0145] Ensure to record the data for each clone on its respective BPR.

[0146] If additional iPSC clones are going to be passaged using the PTK method, utilize the PTK method as described below.

[0147] Begin feeding on day 1 post-passaging.

PTK CELL PASSAGING

[0148] Transfer plate(s) with cells to be harvested into the BSC. Remove media and discard. Add the volume of mTeSR Plus defined in the table below depending on the plate type:

Plate	Volume of mTeSR Plus to Add
12 well plate	0.5 mL/well
6 well plate	1 mL/well

[0149] Working under microscope inside the BSC at 4X objective, use an insulin syringe needle to cut iPSC colonies into small clumps. If 3 or more colonies are available, pick and transfer into 1 well of a 6 well plate. Whenever possible, pick at least 8 colonies. No more than 16 colonies should be transferred into 1 well of a 6 well plate. If fewer than 3 colonies are available to be picked, pick and transfer colonies to 1 well of a 12 well plate.

[0150] Place a P1000 micropipette tip inside a P20 tip. Use P1000+P20 tip to nudge cut colony pieces off the plate. From the new plate(s) prepared in above pull up 0.5 – 1 mL of mTeSR Plus to rinse the PTK plate. Tilt PTK plate and add media from previous step to pool all the clumps to the bottom of the well. Transfer floating clumps to new plate.

[0151] Transfer the plate(s) outside the BSC and label them with information, for example: Batch #, if applicable, iPSC Line ID + clone #, Passage Number, PTK, Date, Initials

[0152] Transfer plate with cells to a 37°C 5% CO_2 incubator and rock plate back and forth and side to side to distribute cells evenly. Record incubator RB# and shelf.

[0153] If additional iPSC clones are going to be passaged, ensure appropriate clone-to-clone separation is maintained.

[0154] If additional iPSC clones are going to be passaged using PTK method repeat steps above.

[0155] Ensure to record the data for each clone on its respective BPR.

[0156] If additional iPSC clones are going to be passaged using ReLeSR, utilize ReLeSR method described above.

[0157] Begin feeding on day 1 post-passaging.

CELL PELLETING

[0158] Pellet cells at desired passages based on project requirements (generally above P5). Obtain plate designated for cell pelleting from the incubator.

[0159] Check and record confluency. Assign a ranking. Transfer the cells back to the incubator Record the number of plates and wells to be collected. Determine the expected number of cell pellets to be made (each well of a 6-well plate will generate 2 cell pellets). Transfer cells to be pelleted into the BSC. Remove spent media and discard. Add 1 mL of DPBS -/- to each well to be plated. Using a cell scraper, gently lift the cells from the plate.

[0160] **NOTE:** It is important not to exert too much pressure when using the cell scraper. Too much pressure can cause the cell scraper to destroy or smear the colonies, rendering them unusable.

[0161] Using a P1000, triturate the cell suspension 3-4 times to ensure evenly sized aggregates are formed. Using a P1000, transfer the cell suspension to the appropriate number of microcentrifuge tubes (1 well into 2 microcentrifuge tubes) Spin the cells down at 300 x g for 2 mins at room temperature. NOTE: Be sure to install the appropriate centrifuge rotor/adaptors for this step. Transfer the tubes back into the

BSC. Using a P1000, remove DPBS -/- from each tube and discard. Be careful not to disturb the cell pellet. Close the tubes and transfer them to personnel out of the BSC for labelling.

[0162] Outside the BSC, inspect the labels, ensure they contain for example, the following information: Project, iPSC Line ID, Passage, Primary Cell Stock or Seed Bank, BPR #, if applicable, Store at -80°C, Initials, Date

[0163] Take a picture of the label and attach to the BPR. Affix labels to microcentrifuge tubes containing the cell pellets. Submit all the pellets to the QC laboratory .

CRYOPRESERVATION OF iPSC AT EARLY PASSAGES

[0164] Early Passage Cryopreservation will be performed between passages 2 to 5 (following clone isolation).

[0165] Additional wells will be plated during prior passage to be cryopreserved. For example, if planning to cryopreserve an iPSC clone at Passage 3, 1-2 additional wells will be plated during passaging.

[0166] Expanded iPSCs with a confluency greater than 80% will be harvested to be cryopreserved. Transfer plate(s) with cells to be harvested into the BSC. \

[0167] Remove media and discard. Add 1 mL of DPBS-/- to each well to be cryopreserved from that clone. Remove DPBS-/- and discard. Add 1 mL of ReLeSR and incubate at room temperature for about 30 seconds. Remove ReLeSR and discard.

[0168] Move plate to the incubator and incubate at 37°C for 7 minutes. Record incubation time.

[0169] Move plate back to the BSC after the incubation step. Gently add 1 mL of mTeSR Plus Media to the wall of the well. Immediately after adding mTeSR Plus Media, tap the plate against the inside of the BSC sash.

[0170] Alternatively, hold the plate with one hand and use the other hand to firmly tap the side of the plate for approximately 30 - 60 seconds.

[0171] If very few cells lift, use serological pipette to rinse well with medium already there.

[0172] Using a serological pipette, transfer the detached cell aggregates to an appropriately sized conical tube labelled as “iPSC”. Pool multiple wells into a single conical tube. Add 6 mL of mTeSR Plus to the tube for adequate trituration volume. Using a serological pipette, triturate the cell suspension at least 5 times to break up the clumps to be more evenly distributed. Spin the cells down at 300xg for 5 minutes at room temperature. Record speed, time, and temperature on BPR. Transfer the tube(s) into the BSC. Remove supernatant using a serological pipette and discard. Do not disturb the cell pellet. Close tube and flick pellet gently. Based on the number of wells harvested for cryopreservation, record number of vials to be cryopreserved. 1 well of a 6 well plate will be split into 2 cryovials (each containing 1 mL).

[0173] Record volume of CS10 to be used based on number of vials defined above. Each vial will contain 1 mL of cells suspended in CS10. Using a P1000 add 1 mL of cold CS10 and pipette no more than 3 times to break up the cell pellet. Using a serological add remaining volume of CS10 to reach the total

volume calculated (2mL of CS10 per well of a 6 well plate) and mix. Record volume added on BPR. Use a serological pipette to mix the cell suspension by pipetting up and down twice. Fill cryovials with 1 mL of cell suspension using a serological pipette. Record number of vials filled. Transfer vials outside the BSC and label them with, for example: iPSCs Line ID + clone #, Passage number (do not add one passage to the cells being cryopreserved), BPR#, Date, Initials

[0174] Transfer the vials to a Mr. Frosty at room temperature and transfer to a -80°C freezer as soon as possible. Record freezer RB# and location on BPR. At least 72 hours after placing Mr. Frosty in the -80°C freezer, transfer the vials to the LN2 tank. Record RB# and location on BPR.

Example 2

Induced Pluripotent Stem Cells (iPSCs) Single Cell Harvesting and Cryopreservation for Seed Bank Generation

CLONE EXPANSION

[0175] In order to proceed with Seed Bank Generation, clones should receive a rank of 3 for 2 consecutive passages and receive an EBNA copies/cells value below LoQ .

[0176] Seed Banks can be generated from clones that have not passed the above criteria with approval.

[0177] Once it has been decided to generate a Seed Bank from a specific clone, cells must be passaged into:

- 1 x 6 Well plate containing at least 2 wells for Cell Pelleting
- 1 x T75 Flask for Karyotype Analysis
- 2 x T75 Flasks for Seed Banking

CONTROLLED RATE FREEZER SET UP

[0178] Identify and log CRF use.

[0179] Connect laptop to the CRF and log into CRF software.

[0180] Click open profile and select iPSCs Single Cell Cryopreservation Profile.

[0181] Ensure profile settings are as follows:

- Wait at 4.0 °C
- 1.0 °C/m S to -4.0 °C
- 20.0 °C/m C to -45.0 °C
- 10.0 °C/m C to -10 °C
- 0.5 °C/m C to -20 °C
- 1.0 °C/m S to -80.0 °C
- Hold at -80.0 °C

[0182] Under Run File name, write iPSCs cryopreservation.

[0183] Press Start to pre-cool chamber to 4°C and ensure program is running.

KARYOTYPE ANALYSIS

[0184] On the day of harvesting, transfer the T75 flask designated for Karyotyping to the QC Laboratory for testing.

CELL PELLETING

[0185] Obtain 6 well plates designated for cell pelleting from the incubator. Check and record confluency.

[0186] Assign a ranking. Transfer the cells back to the incubator. Record the number of wells to be pelleted. Pellet at least 3 wells. Each well will generate 2 cell pellets. When ready to collect cells, transfer cells to be pelleted into the BSC.

[0187] For one well at a time:

- Remove spent media and discard.
- Add 1 mL of DPBS -/- to each well.
- Remove the DPBS -/- from each well and add 1 mL of
- fresh DPBS -/- to each well.
- Using a cell scraper, gently lift the cells from the plate.
- It is important not to exert too much pressure when using the cell scraper. Too much pressure can cause the cell scraper to destroy or smear the colonies, rendering them unusable.

[0188] Using a P1000, triturate the cell suspension 3-4 times to ensure evenly sized aggregates are formed. Using a P1000, transfer the cell suspension to 2 microcentrifuge tubes (1 well into 2 x 1.5mL microcentrifuge tubes). Spin the cells down at 300 x g for 2 mins at room temperature. Be sure to install the appropriate centrifuge rotor/adaptors for this step.

[0189] Transfer the tubes back into the BSC. Using a P1000, remove DPBS from each tube and discard. Be careful not to disturb the cell pellet.

Close the tubes and transfer them to personnel out of the BSC for labelling. Affix the Cell Pellet label to the tubes containing the cell pellets. Submit all the pellets to the QC laboratory.

CELL HARVESTING

[0190] Expanded iPSCs at 80-100% confluency will be harvested to be cryopreserved. The target is to generate a 25 vial Seed Bank at 2.0E+06 cells/mL at 1 mL/vial. Obtain cells to be cryopreserved from incubator, place the vessel under microscope and record the confluency and rank. Record the cell rank and percent (%) confluency.

[0191] Take a picture and store the picture: CBC Manufacturing>*Project*>iPSC line ID>Clone>Pictures. Identify it with, for example, Cell Line ID, passage, DIV, initials and date.

[0192] **NOTE:** Verify the confluency and rank from all the flasks and provide an average value.

[0193] Obtain mTeSR Plus medium, record total volume and expiration date. Approximately 60 mL of mTeSR Plus will be required for processing.

[0194] Label 2 x 50 mL conical tubes, one with FLASK-1 and the other with FLASK-2. Transfer T75 flasks into the BSC. Using a serological pipette remove the conditioned media and discard. Rinse each flask with 8 mL of DPBS-/-. Repeat step once. Add 8 mL of TrypLE to each flask. Transfer cells to incubator and incubate for 10 minutes. After 10 minutes check cells under microscope to see if they have detached.

[0195] **NOTE:** Tap the bottom of the flask to help cell detachment. If cells are still adhered to bottom return to incubator for up to an additional 3 minutes.

[0196] Add 8 mL of mTeSR Plus media to the first T75 flask and use a serological pipette to flush 2-3 times to help detach remaining cells. Transfer the cell suspension into the 50mL conical labeled FLASK-1. Add an additional 8mL of mTeSR Plus media to the first T75 flask and use a serological pipette to flush 2-3 times to help detach remaining cells.

[0197] Repeat steps with the second flask and transfer the cells into the tube labeled FLASK-2.

[0198] Spin down cells at 300 x g for 5 min at room temperature. Record speed, time, and temperature. Transfer the tubes into the BSC.

[0199] Remove supernatant from each tube using a serological pipette and discard. Do not disturb the cell pellet. Flick the tube to dislodge the pellet. Add 1 mL of Complete mTeSR Plus media to each 50 mL conical tube and pipette up and down 3 times to resuspend the pellet using a P1000 micropipette. Record volume added. Add 4 mL of Complete mTeSR Plus media to each 50 mL conical tube. Record total volume of cell suspension.

[0200] Transfer the cell suspension from the conical tube labeled FLASK-2 into the conical tube labeled FLASK-1. There should be approximately 10 mL of cell suspension in the tube labeled FLASK-1.

[0201] Using a serological pipette, add 10 mL of mTeSR plus to the tube labeled FLASK-2 to wash remaining cells. Collect media and transfer to the cell suspension in the tube labeled FLASK-1. Use a serological pipette to mix cell suspension and measure the volume.

[0202] Label 2 x 1.5 mL microcentrifuge tubes. One with “CELLS” and one with “COUNT.”

[0203] Using a P1000, transfer 1 mL of well mixed cell suspension to the 1.5 mL microcentrifuge tube labeled CELLS. Transfer 20 µL of well-mixed cell suspension from the tube labeled CELLS to the tube labeled COUNT. Add 180 µL of Moxi Viability reagent to ‘cell count’ tube and mix. Record volume. Incubate in the dark for 5 minutes. Record time.

[0204] Measure cell viability using MoxiGO II. Include the average viability, average live cell concentration and the total live cell count. Confirm CVs for viability and live cell concentration are ≤25%. Attach MoxiGO II spectra to BPR following production.

[0205] Depending on the total number of live cells harvested, there will be 3 scenarios:

- If total live cell number is equal to or greater than 60E+06 cells, prepare 30 vials at 2E+06/vial.
- 7.7.32.2. If total live cell number is equal to or greater than 50E+06 cells, but less than 60E+06 prepare 25 vials at 2E+06/vial.
- If total live cell number is less than 50E+05, notify manufacturing supervisor for approval to prepare as many vials as possible at 2E+06/vial.

[0206] Label a 50 mL conical tube as "FINAL CELLS." Calculate the volume of cell suspension containing the desired number of cells using the formula - Volume Cell Suspension = [Number of Cells Required] / [Live Cell Concentration from Step 7.7.31.1]

[0207] Transfer the volume of cell suspension calculated in the previous step into the conical tube labeled FINAL CELLS. (In the event that there is less than 50E+06 cells, transfer the total volume into the new conical tube).

[0208] Prepare a balance using DPBS -/- containing the same volume as the conical tube labeled FINAL CELLS. Spin down cells at 300 x g for 5 min at room temperature. Record speed, time, and temperature. During centrifugation, transfer cool CS10 from refrigerator to be used for the final formulation and notify CRF team to ensure CRF is ready for processing. After centrifugation, transfer the tubes back into the BSC.

[0209] Remove supernatant from pellet using a serological pipette and discard. Do not disturb the cell pellet. Using a P1000 micropipette, add 1 mL of cold CS10 and pipette up/down several times to break up the pellet. Add an additional 4 mL of cold CS10 and use a serological pipette to measure the current volume of cell suspension. Add enough CS10 to bring volume of cell suspension to the desired volume (based on the conditions described in section 7.7.32).

[0210] Required Volume = [Target Volume (30 mL, 25 mL etc.)] – [Current Volume of Cell Suspension]

[0211] Transfer 1 mL of CS10 into a cryovial to be used a temperature probe for the CRF. Label cryovial with TEMP PROBE.

VIAL FILLING

[0212] Ensure CRF chamber is 4°C and cryovials are pre-labelled. Place the cryovials on a pre-cooled rack and uncap them. Using a serological pipette, pipette up and down to resuspend the cells, add 1 mL of cell suspension to each cryovial.

[0213] **NOTE:** Use a serological pipette to resuspend the cells in the conical tube after each 10 cryovials filled.

[0214] Once completed filling, record the total number of vials filled. Transfer filled cryovials to CRF as quickly as possible.

CRYOPRESERVATION USING CRF

[0215] Insert the sample probe into the cryovial labeled as TEMP PROBE. Wait for sample probe to reach approximately 4°C and then press button to continue run.

[0216] **NOTE:** Ensure the program properly moves to next step.

[0217] Upon completion of CRF, transfer the vials to a bucket with dry ice and quickly transfer vials to LN2 tank.

[0218] Separate the vials into two groups:

QC Tests and Retains	3 Vials
Main Bank	Remaining Vials (Target is ≥20 Vials)

[0219] Record number of vials placed in each group. Separate them in separate boxes. Record box location in LN2 tank for each group of vials. Make sure Seed Bank vials are in a separate box from the QC samples.

[0220] On CRF equipment save report and store the report and attach a copy of the cryopreservation report to the BPR.

REMAINING CELLS

[0221] If there are any remaining cells in the tube labeled "FLASK-1" proceed below.

[0222] Label 2 x 15mL conical tubes as "Remaining Cells." Using a serological pipette measure the volume of cell suspension in the conical tube labeled "FLASK-1" and record on the BPR.

[0223] Transfer the cell suspension into the conical tubes labeled "Remaining Cells" (half volume into each conical). Calculate the total number of cells transferred into each tube: Number of Cells = [Volume] x [Cell Concentration]

[0224] Centrifuge the two tubes at 300 x g for 5 minutes at room temperature. Record speed, time and temperature. Once centrifuge is complete, transfer tube into BSC and remove supernatant without disturbing pellet. Transfer tubes out of the BSC and affix the label. Manually write the total number cells in each tube on the conical tube. Submit all the QC samples, as well as the cell pellet from the remaining cells, to the QC laboratory.

Example 3

Automated Filling

EQUIPMENT SETUP AND CALIBRATION

[0225] Final formulation and automated filling will take place in dedicated suite.

[0226] The following steps must be performed and completed before the cells being harvested and placed in the centrifuge for their final spin.

[0227] Ensure CRF chamber is at 4 °C and cryovials in 48-vial racks are labelled. Disinfect BSC. Disinfect Fill-It unit. Transfer CryoStor CS10, tubing set and any other materials needed . Turn on Fill-It unit and push 'reset.' Set up the Fill-It unit with a V8 tube immersed in a bottle with CryoStor CS10 and a disposable reservoir placed on the rack nest for priming and calibration.

[0228] **NOTE:** This set up procedure needs to be performed before the cells are harvested or in parallel.

[0229] Select the "PRIME" function and prime the tubing by holding 'GO' until liquid is evenly dispensed, and no significant bubbles appear in the tubing.

[0230] Measure the weight of the disposable reservoir before dispensing 48 mL of CryoStor CS10. Record the weight of empty reservoir.

[0231] Using the "CALIBRATE INTO TUB" function, select the volume intended to fill per vial (1 mL). After selecting "GO" this will fill 48x that volume into the reservoir. Record weight of filled reservoir.

[0232] Calculate and record the volume dispensed per vial using the formula below:

$$Volume\ of\ liquid\ dispensed\ per\ via = \frac{(post\ dispense\ weight) - (pre\ dispense\ weight)}{(48\ vials)\ *\ 1.069}$$

[0233] **NOTE**: The volume is calculated based on the dispensed weight and CS10's associated density (1.069 g/mL).

[0234] Enter the volume of liquid dispensed into the Fill-It unit for calibration and press "Function" to save the value. If the "volume per vial" calculated is within 5% of the intended volume (for example 950-1050 µL for a 1000 µL intended vial fill) the calibration was successful and continue to the next step.

[0235] If the "volume per vial" calculated is more than 5% from the intended volume, discard the liquid in the reservoir and repeat the steps using a new reservoir until the calculated volume falls within the 5% range.

[0236] Once calibrated, use the PRIME function to remove the CS10 from the tubing set. Once the tubing is empty, keep the tubing inside a bottle.

[0237] Ensure CRF is at 4°C. At this point, the Filling Team will wait to receive the centrifuged cells from the harvesting team.

[0238] After receiving the centrifuged cells from the Harvest Team, remove supernatant from pellet using a serological pipette and discard. Do not disturb the cell pellet.

[0239] Close tube(s) and flick tube gently to resuspend the cell pellet.

[0240] Using a P1000 micropipette, add 1 mL of CryoStor CS10 and pipette up/down no more than 3 times to break up the pellet. If you have more than 1 tube, add 1 mL to each tube.

[0241] Use a serological pipette to combine the cell suspension from all tubes into 1 tube and measure volume. Record volume. Add enough CS10 to bring volume of cell suspension to 25 mL. Record volume added.

[0242] Transfer the cell suspension to a 250 mL sterile bottle labeled as "CELLS FINAL". Bring the cell suspension up to 157 mL.

[0243] Note: The volume of CryoStor CS10 is determined based on the total number of cells (3.15 E8) prepared at a concentration of 2 E+06 cells/ml. This yields a target solution of 157ml.

[0244] From this step on, ensure processing is performed on cool beads. Ensure CRF chamber is 4°C and cryovials are labelled. Ensure bottle with cell suspension remains on cool beads. Prepare one cryovial to be used as a control to introduce the sample temperature probe by adding 1 mL of CryoStor CS10 using a P1000 micropipette. Label it manually as 'Temp Probe.' Place a new reservoir on the rack nest. Swirl the bottle containing the cell suspension to keep the cells suspended.

[0245] Introduce the tubing into the container with cell suspension (CELLS FINAL), select the PRIME function, and select 'GO' to PRIME the tubing until some cell suspension gets dispensed into the reservoir to ensure the tube is filled with homogenous cell suspension. Remove the reservoir from the rack nest. Place a pre-labelled 48-vial rack on the rack nest (ensure correct orientation) and ensure that the tube is immersed in the source container with cell suspension

[0246] Select 'DECAP-DISPENSE-CAP' function, and select the desired dispensing volume (1000 μL). Record fill volume. Press 'GO' for the Fill-It unit to automatically start the process of DECAP-DISPENSE AND CAP for 48 vials.

[0247] Once the system finishes, a second person will move the rack to the CRF to keep it at ~4°C.

[0248] Manually swirl the bottle containing the cell suspension after filling each rack, and if filling more than 2 racks, manually resuspend the cells using a serological pipette between each 2 racks being filled.

[0249] Repeat until the cell suspension has been filled in all the vials. Note, the Fill-it machine can fill vials in multiples of 8. If filling less than a full rack of vials, be sure to remove the vials not being filled in multiples of 8. Record total number of vials filled.

[0250] Move to next step to start cell cryopreservation.

CRYOPRESERVATION USING CONTROLLED RATE FREEZER

[0251] Transfer cryovials in the rack to the CRF as quickly as possible.

[0252] Insert the sample probe into the cryovial designated as the "TEMP PROBE" vial.

[0253] Wait for sample to reach approximately 4°C and then press button to continue run.

[0254] **NOTE:** Ensure the program properly moves to next step.

[0255] Upon completion of CRF, transfer the vials to a bucket with dry ice and quickly transfer vials to LN2 tank.

[0256] Separate the vials into two groups:

QC Tests and Retains	11 Vials
Main Bank	At least 85 Vials

[0257] Record number of vials placed in each group. Separate them in separate boxes. Record box location in LN2 tank for each group of vials. Make sure Seed Bank vials are in a separate box from the QC samples. On CRF equipment save report as PDF, store the file and attach a copy of the cryopreservation report to the BPR. Record equipment and room use in electronic Quality Management System.

Example 4

Evaluation and Ranking of induced Pluripotent Stem Cells (iPSCs) in Culture

[0258] iPSCs are also characterized by their natural tendency to spontaneously differentiate. Therefore, it is common to see iPSC cultures in expansion with varying percentages of spontaneous differentiation (Figure 2).

[0259] The quality of iPSC cultures is generally defined by the percentage of differentiated versus un-differentiated cells.

[0260] Maintenance of iPSCs cultures require keeping them with low percentages of differentiation. Which consists of passaging only un-differentiated iPSCs. This can be achieved by using ReLeSR when the percentage of differentiation is low or picking manually un-differentiated colonies when the percentage of differentiation is high. Therefore, passaging methodology will be determined based on iPSC ranking. See table 3 for additional details.

[0261] iPSCs are evaluated and ranked as part of the expansion process to generate an iPSC Primary Cell Stock (PCS), Seed Bank or Master Cell Bank. Specifically, iPSCs are always ranked prior to being passaged.

[0262] EVALUATION AND RANKING OF IPSCs DURING CULTURE

[0263] To evaluate the quality of iPSC cultures and rank them, transfer the iPSC plate to an inverted microscope for observation. Use a magnification that allows to see details about cell morphology as well as colony morphology and confluency.

[0264] Representative images of the cells in the wells are taken and saved.

[0265] Pictures are evaluated and notes are recorded on morphology of the cells and colonies, confluence, and percentage of differentiated versus un-differentiated iPSCs. Records will be captured on forms or BPRs.

[0266] Based on the evaluation, a rank will be assigned to the iPSC culture.

[0267] iPSC Ranking is defined in a scale from 1 to 5. Where Rank 1 corresponds to iPSC cultures of very low quality and high percentages of differentiation and Rank 5 representing very high-quality iPSC cultures with low percentages of differentiation. See table 3 below for ranking guidelines.

Table 3. Ranking Guidelines

Rank	Features	Rate of Spontaneous Differentiation	Recommended Passing Action
5	Very little to no spontaneous differentiation observed	<10%	ReLeSR 7-minute incubation
4	Some spontaneous differentiation observed	10-30%	ReLeSR 7-minute incubation
3	Spontaneous differentiation is moderate	30-60%	ReLeSR 7-minute incubation
2	A large amount of differentiation, very few good colonies.	60-90%	>50% confluent: ReLeSR 7-minute incubation <50% confluent: pick-to-keep (PTK)
1	Almost no good colonies visible, usually a partially reprogrammed iPSC line.	90-100%	Passage by pick-to-keep (PTK) or discard

[0268] Refer to representative images in Figure 3A-3E for ranking.

Example 5

Single cell RNA-sequencing

[0269] Single-cell RNA sequencing (scRNA-seq) is a powerful technology that enables the measurement of transcriptomes at the resolution of individual cells. It is a powerful technique that allows researchers to analyze gene expression profiles at the individual cell level. This method provides unprecedented resolution for understanding cellular heterogeneity, identifying rare cell populations, and tracking cellular differentiation processes.

[0270] We used the 10X Genomics Chromium system, which you used, employs a droplet-based approach for scRNA-seq. Here's a brief overview of the process:

- Single cells are encapsulated in Gel Beads in Emulsion (GEMs) along with barcoded oligonucleotides.
- Within each GEM, cell lysis occurs, and mRNA is captured and barcoded.
- The barcoded cDNA is amplified and used to prepare sequencing libraries.
- Libraries are sequenced using next-generation sequencing platforms.

[0271] Described herein is a brief overview of the process and how it is used to identify gene markers and cluster cells using methods like UMAP, particularly in the context of the 10x Genomics platform:

[0272] **Single-Cell Isolation and Library Preparation (10x Genomics):** Thousands of individual cells are encapsulated into microdroplets along with uniquely barcoded beads. Each bead carries a unique oligonucleotide barcode. Within each droplet, polyadenylated mRNA from a single cell is captured on the barcoded bead, followed by reverse transcription to generate cDNA. This barcode identifies all transcripts that originate from the same cell. After breaking the emulsion, the cDNA is amplified, and sequencing libraries are prepared. Each resulting library contains transcript information linked to an individual cell.

[0273] **Sequencing and Data Processing:** Libraries are sequenced, typically on Illumina platforms. Raw reads are demultiplexed using the 10x Genomics Cell Ranger pipeline, which aligns reads to a reference genome, quantifies gene expression, and outputs a cell-by-gene expression matrix.

[0274] **Identifying Marker Genes:** Expression data are normalized to account for differences in sequencing depth and other technical variations. Marker genes for clusters or specific cell types are identified by comparing the expression profiles of groups of cells (e.g., cluster 1 vs. all other cells). After sequencing, the data is processed to generate a gene expression matrix for each cell. This matrix is then used to identify marker genes that characterize different cell populations. High expression (and specificity) of a gene within a cluster suggests it may be a marker for that cell population. Known markers can help assign identities to clusters (e.g., T cells, neurons, epithelial cells, etc.), while novel markers may suggest new cell subtypes or states. Differential expression analysis is performed between clusters or cell types. Genes that are significantly upregulated in a specific cluster compared to others are considered potential markers. The FindAllMarkers() function in Seurat or Loupe Browser, that are popular packages for scRNA-seq analysis

[0275] **Clustering and UMAP Analysis**: Dimensionality reduction is used. Uniform Manifold Approximation and Projection (UMAP) is a dimensionality reduction technique widely used for visualizing and clustering scRNA-seq data. Given that the data can include thousands of genes and thousands of cells, techniques like principal component analysis (PCA) followed by Uniform Manifold Approximation and Projection (UMAP) are used to project the high-dimensional data into a lower-dimensional space. UMAP creates a two-dimensional (or sometimes three-dimensional) representation that preserves local and global similarities between cells. Cells with similar expression profiles cluster together in this reduced space. The UMAP plot is used to visualize how cells group based on their gene expression, and each cluster can be annotated based on known or newly This allows for visualization of cellular relationships and identification of distinct cell populations.

scRNA-Seq Sample Prepration, Library Preparation, Sequencing and Bioinformatics

[0276] Single cell suspensions were stained with ViaStain AOPI Staining Solution (Nexcelom Bioscience, Lawrence, MA) and imaged on an EVOS M7000 Imaging System (Thermo Fisher Scientific, Waltham, CA) to determine cell suspension concentration and cell viability. As per the Fixation of Cells

& Nuclei for Chromium Fixed RNA Profiling Demonstrated Protocol (10x Genomics, CG000478), $1x10^6$ cells per sample with a viability >80% were fixed at 4°C using the Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (10x Genomics, Pleasanton, California). Fixed cells were quenched of enzymatic activity, processed for long-term storage at –80°C, and stored for ~1 month.

[0277] Following the Chromium Fixed RNA Profiling Reagents Kit for Multiplexed Samples User Guide (10x Genomics; CG000527), stored samples were thawed and 300,000 cells were used for probe hybridization for 24 hours, using one probe barcode per sample using the Chromium Fixed RNA Kit, Human Transcriptome kit. Post-hybridization wash was performed on individual samples, which were then pooled using an equal number of cells for each sample for a target capture of 10,000 cells per sample. Gel beads-in-EMulsion (GEMs) were generated on the Chromium X Controller (10x Genomics). Cell barcoding, GEM recovery, pre-amplification PCR, and gene expression library construction were performed following the manufacturer's user guide. Libraries were quantified using a Qubit fluorometer (Thermo Fisher Scientific) and library size was measured via the 4200 TapeStation (Agilent Technologies, Santa Clara, CA). Libraries were sequenced on a 10B 200 cycle kit on the NovaSeq X Plus (Illumina, San Diego, CA) as per the Chromium Fixed RNA Profiling Reagents Kit for Multiplexed Samples User Guide, with a sequencing depth of 40,000 reads/cell.

[0278] Cell Ranger multi v8.0.0 (10X Genomics) was used for barcode identification, read alignment, and UMI quantification with default parameters and aligning to the human reference genome GRCh38. Count matrix from each sample were filtered to exclude low-quality cells and doublets, based on the following criteria: gene counts between 150 and 10,000, total UMI counts between 0 and 50,000, mitochondrial gene expression between 0% and 10%, and a doublet score of ≤ 0.5. UMAP plots were constructed based on gene expression counts, total UMI counts, and mitochondrial gene expression counts. The filtered cells from all samples were then integrated into a unified dataset and analyzed using python package Scanpy. The unified dataset was normalized and log transformed using normalized_total() and log1p() respectively. Dimension reduction and batch correction were done using ScVI. Neighbors was computed using scVI latent representation using neighbors(). Leiden clustering with latent representation from scVI and resolution of 0.3 was subsequently performed to identify cell populations.

[0279] To validate cell type identities, known marker genes from the literature were visualized using a dot plot. Differential gene expression analysis was conducted to compare each cluster against all other cells, highlighting genes that were significantly upregulated in each cluster. The cluster identities were further confirmed through enrichment analysis using Enrichr.

[0280] Single cell RNA-sequencing was performed on sample data sets from cord blood post wash, cord blood post enrichment, reprogramed mix (partially reprogrammed cells), and iPSCs (cord blood-derived).

[0281] iPSCs that have been expanded for 4 to 6 passages were characterized. These iPSCs are at about day 45 to day 65 post reprogramming; and 28 to 42 days pot clone isolation.

[0282] UMAP clustering of single cell data separates distinct sample sets and distinct subclusters are found across samples. Dot plot of markers across clusters and samples are shown in Figure 6.

[0283] CD genes expressed in the iPSCs include but are not limited to CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, and CD326. (Figures 7-15.)

[0284] Novel genes expressed uniquely in iPSCs include but are not limited to TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, and COBL. (Figures 16-25)

[0285] Known pluripotency genes expressed in the various data sets include but are not limited to GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, and PODXL (CD34 sialomucin). (Figures 26-30.)

[0286] Known blood and hematopoietic stem/ progenitor marker genes expressed include but are not limited to CD11a (ITGAL), CD11c (ITGAX), CD34, CD49d (ITGA4), RUNX1, GATA2, TAL1, CBFAT2T3, MMLT3, and PTPN22. (Data not shown.)

[0287] Various embodiments of the invention are described above in the Detailed Description. While these descriptions directly describe the above embodiments, it is understood that those skilled in the art may conceive modifications and/or variations to the specific embodiments shown and described herein. Any such modifications or variations that fall within the purview of this description are intended to be included therein as well. Unless specifically noted, it is the intention of the inventors that the words and phrases in the specification and claims be given the ordinary and accustomed meanings to those of ordinary skill in the applicable art(s).

[0288] The foregoing description of various embodiments of the invention known to the applicant at this time of filing the application has been presented and is intended for the purposes of illustration and description. The present description is not intended to be exhaustive nor limit the invention to the precise form disclosed and many modifications and variations are possible in the light of the above teachings. The embodiments described serve to explain the principles of the invention and its practical application and to enable others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. Therefore, it is intended that the invention not be limited to the particular embodiments disclosed for carrying out the invention.

[0289] While particular embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that, based upon the teachings herein, changes and modifications may be made without departing from this invention and its broader aspects and, therefore, the appended

claims are to encompass within their scope all such changes and modifications as are within the true spirit and scope of this invention. As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are useful to an embodiment, yet open to the inclusion of unspecified elements, whether useful or not. It will be understood by those within the art that, in general, terms used herein are generally intended as "open" terms (e.g., the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.). Although the open-ended term "comprising," as a synonym of terms such as including, containing, or having, is used herein to describe and claim the invention, the present invention, or embodiments thereof, may alternatively be described using alternative terms such as "consisting of" or "consisting essentially of."

[0290] Unless stated otherwise, the terms "a" and "an" and "the" and similar references used in the context of describing a particular embodiment of the application (especially in the context of claims) may be construed to cover both the singular and the plural. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein may be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (for example, "such as") provided with respect to certain embodiments herein is intended merely to better illuminate the application and does not pose a limitation on the scope of the application otherwise claimed. The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example." No language in the specification should be construed as indicating any non-claimed element essential to the practice of the application.

[0291] "Optional" or "optionally" means that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not.

[0292] Groupings of alternative elements or embodiments of the present disclosure disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. One or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for early passage and expansion of iPSCs, comprising:

removing media from a plate or flask comprising iPSCs;

adding PBS to each well in the plate or to the flask;

adding ReLeSR and incubate at about 10-30°C for about 15-45 seconds;

removing the ReLeSR;

incubating the plate or flask at about 34-40°C for about 5-10 minutes

adding Complete mTeSR Plus to the wall of each well of the plate or to each flask;

immediately after adding the Complete mTeSR Plus, tapping the plate or flask to detach the cells from the plate or flask in detached cell aggregates;

transferring the detached cell aggregates from each well or flask into a tube;

adding a quantity of Complete mTeSR Plus to the tube forming a cell suspension;

triturating the cell suspension at least 3 times to break the detached cell aggregates into cell clumps;

plating the cell clumps onto a Laminin CT521 coated plate, wherein the Laminin CT521 coated plate was Laminin CT521 coated and the Laminin CT521 was removed and replaced with mTeSR Plus media;

rocking the Laminin CT521 coated plate in an incubator at about 34-40°C and about 3-7% CO_2 to distribute the cells.

2. The method of claim 1, wherein early passage and expansion of iPSCs comprises

removing media from the platesor flask comprising iPSCs;

adding about 1 mL of PBS to each well in the plate, or about 10-50 mL/flask of mTeSR Plus for a T75-T225 flask;

adding about 1 mL of ReLeSR and incubate at about about 20°C for about about 30 seconds;

removing the ReLeSR;

incubating the plates at about 37°C for about 7 minutes;

adding about 1mL of Complete mTeSR Plus to the wall of each well or flask;

immediately after adding the Complete mTeSR Plus, tapping the plate or flask to detach the cells from the plate in detached cell aggregates;

transferring the detached cell aggregates from each well into a conical tube;

adding a quantity of Complete mTeSR Plus to the conical tube to bring the total volume to 3, mL, 6 mL or 12 mL, forming a cell suspension;

triturating the cell suspension at least 5 times to break the detached cell aggregates into cell clumps;

plating the cell clumps onto a Laminin CT521 coated plate, wherein the plate was Laminin CT521 coated and the Laminin CT521 was removed and replaced with mTeSR Plus media;

rocking the Laminin CT521 coated plate in an incubator at about 37°C and about 5% CO_2 to distribute the cells.

3. The method of claim 1 or claim 2, wherein tapping the plate or flask comprises tapping the plate or flask against a BSC sash, or holding the plate or flask with one hand and use the other hand to firm tap the side of the plate or flask for about 30-60 seconds.

4. A method of passaging iPSCs, comprising:

removing media from a plate or flask comprising iPSCs;

cutting an iPSC colony into multiple clumps;

nudging the cut colony pieces off the plate or flask and onto a new plate or new flask, wherein the new plate or new flask comprises a quantity of mTeSR Plus;

adding mTeSR Plus media to pool the clumps to the bottom of each well of the new plate, or the new flask;

transferring the floating clumps to the new plate or the new flask having mTeSR Plus;

rocking the new plate or the new flask comprising the floating clumps in an incubator at 34-40°C and 3-7% CO_2.

5. The method of claim 4, wherein passaging iPSCs, comprising:

removing media from the plate or flask comprising iPSCs;

cutting an iPSC colony into multiple clumps;

using a P1000 micropipette tip inside a P20 tip to nudge the cut colony pieces off the plate or flask onto a new plate or new flask, wherein the new plate comprises a about 0.5 mL/well of mTeSR Plus for a 12 well plate, or 1 mL/well of mTeSR Plus for a 6 well plate, or 10-50 mL/flask of mTeSR Plus for a T75-T225 flask;

adding mTeSR Plus media to pool the clumps to the bottom of each well of the new plate or to the new flask;

transferring the colony pieces to the new plate or the new flask;

rocking the new plate or new flask comprising the floating clumps in an incubator at about 37°C and about 5% CO_2.

6. The method of claim 4 or claim 5, further comprising feeding the cells on day 1 post-passaging.

7. The method of any one of claims 4-6, further comprising pelleting the cells.

8. The method of any one of claims 1-7, further comprising cryopreserving the cells.

9. A method for induced pluripotent stem cell (iPSC) harvesting, comprising:

(a) obtaining iPSCs and measure percent confluency, wherein the iPSCs are in a plate or a flask;

(b) removing conditioned media from the plates or flask;

(c) incubating the iPSCs for 8-12 minutes to detach the iPSCs from the plate or flasks, and optionally for an additional 2-4 minutes if iPSCs are still adhered to bottom of the plate or flask;

(d) rinsing the plate or flask with 6-10 mL of DPBS-/-;

repeat step (d);

(e) adding 6-10 mL of TrypLE to the plate or flask;

(f) incubating the iPSCs for 8-12 minutes to detach the iPSCs from the plate or flask, and optionally for an additional 2-4 minutes if iPSCs are still adhered to bottom of the plate or flask;

(f) adding 6-10 mL of mTeSR Plus media to the plate or flask and pipetting 1-4 times to assist in detaching the remaining iPSCs resulting in an iPSC suspension;

(g) transferring the iPSC suspension into a tube;

(h) optionally, add an additional 6-10 mL of mTeSR Plus media to the plate or flask and pipetting 1-4 times to assist in detaching the remaining iPSCs and transferring the remaining iPSCs into the tube.

10. The method of claim 9, further comprising preparing the iPSCs for cryopreservation, the method comprising:

(i) spinning down the cells at 400-800 x g for 3-7 minutes at 10-30°C;

(j) removing supernatant from the tube using a serological pipette leaving a pellet;

(k) dislodging the pellet;

(l) adding Complete mTeSR Plus media to the tube and pipetting up and down 2-4 times to resuspend the pellet;

(m) adding 4-6 mL of Complete mTeSR Plus media to the tube;

(n) optionally, combining the iPSC suspension with another iPSC suspension prepared by steps (f)-(h);

(o) spinning down the tube at 400-800 x g for 3-8 minutes at 10-30°C;

(p) removing supernatant and leaving a pellet;

(q) adding cold CS10 and pipetting up and down 1-10 times to break up the pellet resulting in an iPSC cell suspension;

(r) adding an additional 2-6 mL of cold CS10, and optionally adding additional CS10 to bring volume of the iPSC suspension to a desired volume; and

(s) spinning down the tube at 200-400 x g for 3-7 minutes at 10-30°C;

11. The method of claim 9, wherein harvesting the iPSCs comprises:

(a) obtaining iPSCs and measure percent confluency, wherein the iPSCs are in the plate or flask;

(b) removing conditioned media from the plate or flask;

(c) incubating the iPSCs for about 10 minutes to detach the iPSCs from the plate or flask, and optionally for an additional about 3 minutes if iPSCs are still adhered to bottom of the plate or flask;

(d) rinsing the flask with about 8 mL of DPBS-/-;

repeat step (d);

(e) adding about 8 mL of TrypLE to the plate or flask;

(f) incubating the iPSCs for about 10 minutes to detach the iPSCs from the plate or flask, and optionally for an additional about 3 minutes if iPSCs are still adhered to bottom of the flask;

(f) adding about 8 mL of mTeSR Plus media to the plate or flask and pipetting about 2-3 times to assist in detaching the remaining iPSCs resulting in an iPSC suspension;

(g) transferring the iPSC suspension into a 50 ml conical tube;

(h) optionally, add an additional about 8 mL of mTeSR Plus media to the plate or flask and pipetting about 2-3 times to assist in detaching the remaining iPSCs and transferring the remaining iPSCs into the 50 ml conical tube.

12. The method of claim 11, further comprising preparing the iPSCs for cryopreservation, the method comprising:

(i) spinning down the cells at about 300 x g for about 5 minutes at about 20°C;

(j) removing supernatant from the tube using a serological pipette;

(k) dislodging the pellet by flicking the 50 ml conical tube;

(l) adding about 1 mL of Complete mTeSR Plus media to the 50 ml conical tube and pipetting up and down about 3 times to resuspend the pellet using a P1000 micropipette;

(m) adding about 4 mL of Complete mTeSR Plus media to the 50 ml conical tube;

(n) optionally, combining the iPSC suspension with another iPSC suspension prepared by steps (f)-(h);

(o) spinning down the 50 ml conical tube at about 300 x g for about 5 minutes at about 20°C;

(p) removing supernatant from pellet using a serological pipette;

(q) adding about 1 mL of cold CS10 and pipetting up and down about 2-6 times to break up the pellet resulting in an iPSC cell suspension;

(r) adding an additional about 4 mL of cold CS10, and optionally adding additional CS10 to bring volume of the iPSC suspension to a desired volume.

13. The method of claims 9 or 11, further comprising spinning down the 50 ml conical tube at about 300 x g for about 5 minutes at about 20°C.
14. The method of claims 9 or 11, further comprising cryopreserving the iPSCs.
15. The method of claim 14, wherein cryopreserving the iPSC comprises utilizing automated filling of cryovials.
16. A method of cryopreserving iPSCs as single cells, comprising:

placing a cryovial on a pre-cooled rack;

pipetting up and down to resuspend iPSCs in a tube;

adding about 0.5-2.0 mL of cell suspension to the cryovial; and

transferring the filled cryovial to the controlled rate freezer (CRF).
17. The method of cryopreserving iPSCs as single cells of claim 16, comprising:

placing a cryovial on a pre-cooled rack;

pipetting up and down to resuspend iPSCs in a tube;

adding about 1 mL of cell suspension to the cryovial; and

transferring the filled cryovial to the controlled rate freezer (CRF).
18. The method of claim 16 or claim 17, further comprising

allowing a sample probe to reach about 2-6°C;

once at about 2-6°C, running the CRF;

upon completion of CRF, transferring the cryovial to an LN2 tank.
19. The method of claim 16 or claim 17, further comprising

allowing a sample probe to reach about 4°C;

once at about 4°C, running the CRF;

upon completion of CRF, transferring the cryovial to an LN2 tank.
20. The method of claim 18 or claim 19, wherein transferring the cryovial to an LN2 tank, comprises first transferring the vials to a container with dry ice and then transferring the vial to an LN2 tank.
21. A method of automated filling and cryopreserving iPSCs as single cells, comprising:

(a) obtaining harvested induced pluripotent stem cell (iPSC), wherein the harvested iPSCs have been spun down in a tube;

(b) removing supernatant and leaving a pellet;

(c) resuspending the pellet;

(d) adding CS10 to the tube and resuspending the pellet into an iPSC suspension;

(e) optionally, combing the iPSC suspension with one or more iPSC suspensions;

(f) adding additional CS10 to bring the volume of the iPSC suspension to 15-35 mL;

(g) transferring the iPSC suspension to a 125-375 mL bottle;

(h) adding additional CS10 to bring the volume of the iPSC suspension to 100-200 mL;

(i) inserting a tube into the bottle comprising the iPSC suspension and priming the tubing until some iPSC suspension is dispensed into a reservoir;

(j) placing a multi-vial rack on a rack nest;

(k) selecting a desired dispensing volume on an automated filling machine;

(l) operating the automated filling machine to fill the vials in the multi-vial rack; and

(m) transferring the filled cryovial to the controlled rate freezer (CRF).

22. The method of claim 21, wherein automated filling and cryopreserving iPSCs as single cells, comprises:

(a) obtaining harvested induced pluripotent stem cell (iPSC), wherein the harvested iPSCs have been spun down in a tube;

(b) removing supernatant from pellet using a serological pipette;

(c) resuspending the pellet by flicking the tube;

(d) adding about 1mL of CS10 to the tube and pipette up and down no more than 3 times to break up the pellet resulting in an iPSC suspension;

(e) optionally, combing the iPSC suspension with one or more iPSC suspensions;

(f) adding additional CS10 to bring the volume of the iPSC suspension to about 25 mL;

(g) transferring the iPSC suspension to a 250 mL sterile bottle;

(h) adding additional CS10 to bring the volume of the iPSC suspension to about 157 mL;

(i) inserting a tube into the bottle comprising the iPSC suspension and priming the tubing until some iPSC suspension is dispensed into a reservoir;

(j) placing a 48-vial rack on a rack nest; and

(k) selecting a desired dispensing volume of about 1000uL on the automated filling machine;

(l) operating the automated filling machine to fill the vials in the multi-vial rack; and

(m) transferring the filled cryovial to the controlled rate freezer (CRF).

23. The method of claim 22, further comprising

allowing a sample probe to reach about 2-6°C;

once at about 2-6°C, running the CRF;

upon completion of CRF, transferring the cryovials to an LN2 tank.

24. The method of claim 23, wherein transferring the cryovial to an LN2 tank, comprises first transferring the vials to a container with dry ice and then transferring the vial to an LN2 tank.

25. Induced pluripotent stem cells (iPSCs) reprogrammed from isolated cell fraction (ICF) from cord blood units (CBUs),), and expanded and optionally passaged, wherein

(a) the iPSCs express one or more genes selected from CD15, CD13, CD133, CD135, CD90, CD117, CD56, CD71, CD10, CD24, CD9, CD49d, CD44, CD71, CD138, KITLG (ligand for KIT/CD117), CD74, or CD326, or

(b) the iPSCs express one or more genes selected from TCFP2L1, CD10, FOXD3, MIR1915HG, IDO1, PRDM14, GRID2, HHLA1, TRDN, C9ORF135, CLDN7, RAB17, APELA, SERPINB9, FLT1 (VEGFR1), GRPR, CXCL5, CXCL12, CUZD1, or COBL, or

(c) the iPSCs express one or more genes selected from GDF3, POU5F1B, DNMT3B, ZIC3, SOX2, NANOGP8, DPPA2, DPPA4, ZFP42, or PODXL (CD34 sialomucin), or

(d) any combinations of (a), (b), or (c).

26. The iPSCs of claim 25, wherein the iPSCs are expanded and optionally passaged, and optionally cryopreserved by any one of the methods of claim 1-24.

27. A composition comprising iPSCs of claim 26; and cell media.