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AN INTEGRATED APPROACH FOR GENERATION OF VALIDATED HUMAN IPSC CELL BANKS

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ABSTRACT

The reprogramming of somatic cells into induced Pluripotent Stem Cells (iPSC) has emerged as a powerful tool for dissecting basic biology with the added potential of applications in drug screening and cell therapy. With advances in reprogramming technologies, use of diverse media systems and somatic cell sources, there is a compelling need for comprehensive characterizations methods that facilitate the generation of high quality standardized iPSC cell bank.

In this study, we utilize an integrated approach to track somatic cell reprogramming, qualify resulting iPSCs to generate validated cell banks, and employ routine quality control measures to confirm high quality of the cells prior to use in downstream applications. Progression in reprogramming is monitored using a combination of positive and negative self-renewal markers via real-time monitoring and flow cytometry methods. Selected iPSC colonies are expanded and comprehensively characterized utilizing antibody staining and Taqman™ hPSC ScoreCard™ qPCR assays to confirm self-renewal marker expression and tri-lineage differentiation potential. Functionally pluripotent iPSC with normal karyotype, confirmed cell line identity, and free of mycoplasma and pathogens are banked to generate master frozen cell stocks. Viability and post thaw recovery of the frozen cell stocks are confirmed and a routine 2 marker flow cytometry analysis is carried out as a quality control measure prior to downstream applications such as gene editing and directed differentiation to specific lineages. A streamlined method of iPSC qualification and banking is critical for traceability to ensure the identity and quality of cells chosen for drug screening. In addition it serves as a foundational tool for the generation of clinical-grade banks that have potential use in extensive trails.

INTRODUCTION

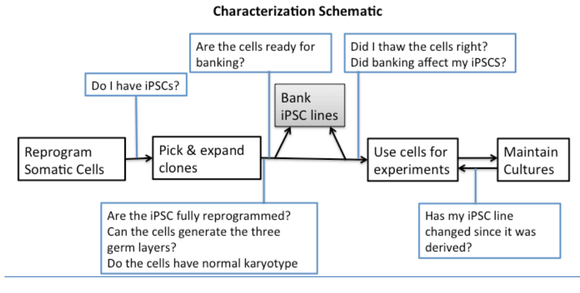
As the field of induced Pluripotent Stem Cells progresses to clinically relevant applications, it becomes very important to validated the reprogramming process and verify that the created cell line banks are truly pluripotent. We previously demonstrated the utility of using novel self renewal markers like Alkaline Phosphatase Live Stain[1] and negative somatic markers like CD44[2] to selected reprogrammed colonies for further clonal expansion for cell line generation. In this study we report an integrated approach in creating validated iPSC cell line banks by confirming functional pluripotency by confirming continued expression of markers of self renewal and tri-lineage differentiation potential, by not only using traditional immuno-staining, but also by looking at the gene expression levels using the Taqman™ hPSC ScoreCard™ to verify the functional capability of each cell line[3]. Once putative iPSC cell lines have been characterized, seed banks can be generated and further tested for chromosomal stability and identified via STR profiling. Validation of iPSC banks creates a traceable and verifiable way of ensuring cell line quality prior to downstream applications.

MATERIALS AND METHODS

All materials obtained from Thermo Fisher Scientific unless noted.

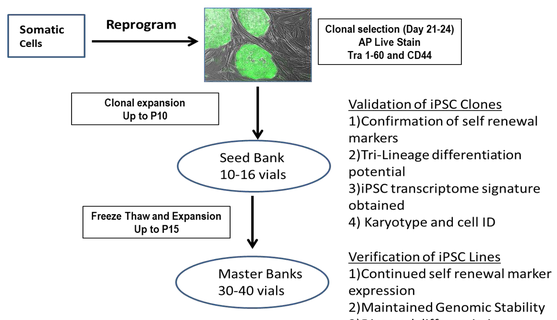
Human fibroblasts: BJ (ATCC), HDFn (C0045C), and HDFa (C0135C) were transduced with CytoTune™-iPSC and CytoTune™-iPS 2.0 Sendai Reprogramming Kits (A16517). iPSC colonies were generated in either feeder dependent conditions on iMEFs (A24903) and KnockOut™ Serum Replacement (10828010) or in feeder free conditions using Essential 8™ Medium (A1517001) on either Geltrex™ (A1413302) or Vitronectin (A14700). At day 21 post transduction, iPSC colonies were individually scored and passaged clonally in feeder dependent or feeder free conditions. Clones were passaged every 3-4 days.

Characterization was performed on live cultures for self renewal surface markers and on fixed cells for intracellular self renewal transcription factors, using the appropriate antibodies against each target In vitro tri-lineage potential was tested by generating embryoid bodies and permitting spontaneous differentiation for 21 day, followed by intracellular immuno-staining for markers indicative of cells from the 3 germ lineages. Genomic Analysis of undifferentiated iPSC and 7 day embryoid bodies was conducted using the Taqman™ hPSC Scorecard™ Array (A15872). iPSC samples were sent out for G-banding karyotyping and STR profiling (Wicell or Cell line Genetics). Directed differentiation was performed using PSC Neural Induction Medium (A1647801), PSC Cardiomyocyte Differentiation Kit (A2921201), and PSC Definitive Endoderm Induction Kit (A27654SA). All flow cytometry was performed using the Attune™ NxT Flow Cytometer (A24858) and analyzed using FlowJo v10 (FloJo, LLC).



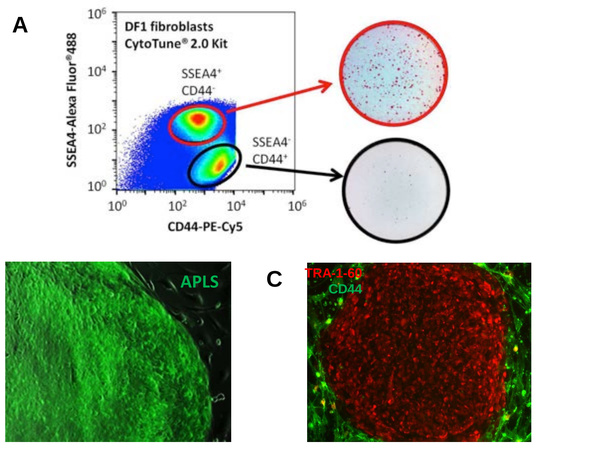
RESULTS

Figure 1. iPSC Cell Line generation timeline



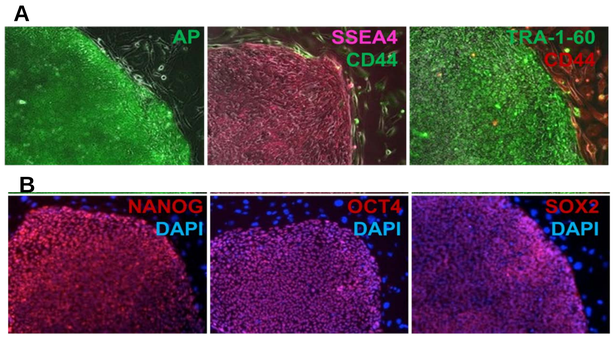
iPSC colonies at 21 days post transduction are selected and clonally expanded until passage 10 (~5 weeks). At P10, the clones characterized and karyotyped, and seed banks are created. Following validation of genome stability, and functional pluripotency via self renewal marker and tri-lineage confirmation, master banks are created prior to downstream application.

Figure 2. Monitoring Reprogramming kinetics and identification of true iPSC colonies



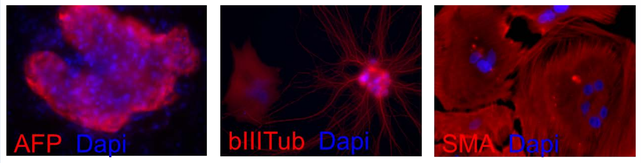
(A) Reprogrammed cells, can be sorted at day 7 based on expression of SEEA4 (self renewal marker) and down regulation of CD44 (fibroblast marker). SSEA4+/CD44- popluations yielded true iPSC colonies, while SSEA4-/CD44+ populations failed to yield viable colonies at day 21. iPSCs at day 21 post transfection can be positively selected for clonal isolation and expansion using either **(B)** Alkaline Phosphatase Live Stain, or the positive/negative expression of **(C)** Tra1-60 (self renewal) and CD44 (fibroblast marker), to ensure selection of fully reprogrammed colonies.

Figure 3. Self-renewal Marker Expression



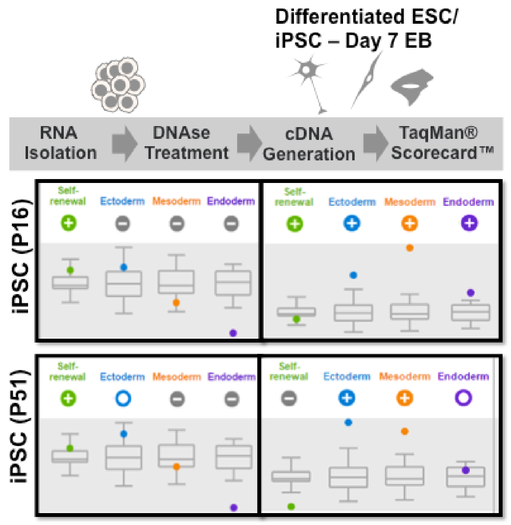
At passage 10, putative iPSC lines must continue to express self renewal self renewal markers **(A)** AP Live Stain, SSEA4, and Tra 1-60 (left to right) using immuno-detection in live cell cultures**. (B)** Nanog, Oct4, and SOX2 (left to right) via intracellular immuno-cytochemistry on fixed cells

Figure 4. Trilineage Differentiation potential



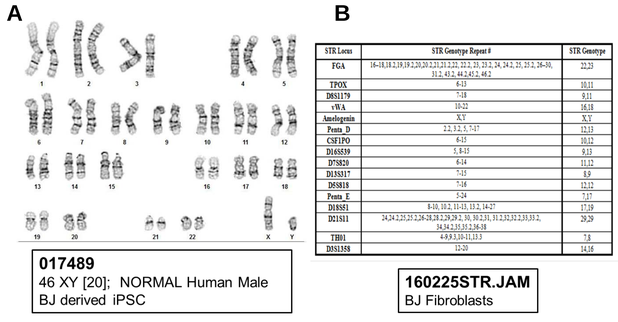
In vitro embryoid body spontaneous differentiation (21 days) confirms the potential of iPSC lines to generate cells indicative of the 3 germ layers as measured by ICC: Alpha-fetoprotein (AFP) for endoderm, , and beta-III Tubulin (bII Tub) for ectoderm and Smooth Muscle Actin (SMA) for mesoderm.

Figure 5. Gene expression analysis to confirm functional pluripotency of iPSC lines



Taqman™ hPSC Scorecard™ Analyses of iPSC seed banks are used to confirm functional pluripotency using gene expression patterns, where undifferentiated cells demonstrate positive scores for self renewal while having all parental somatic gene expression down regulated. iPSCs allowed to spontaneously differentiate as EBs for 7 days show that the markers of self renewal are down regulated and the tri-lineage scores are up regulated to confirm differential potential. This genomic analysis can be routinely used to monitor functional pluripotency through passage.

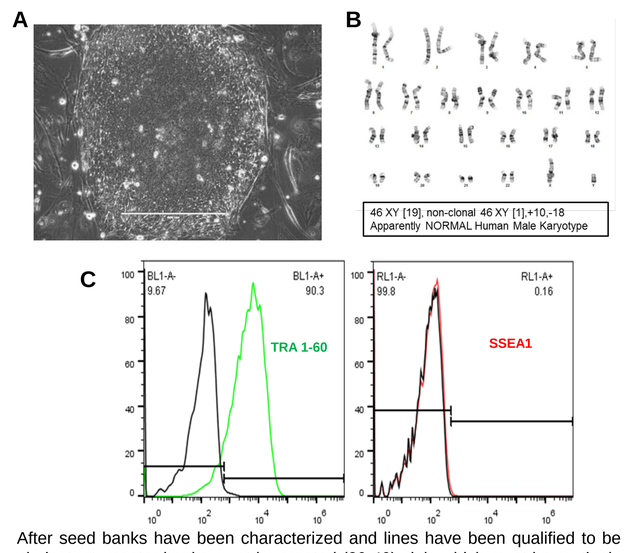
Figure 6. iPSC Seed banks must have stable karyotype and be traceable to parental donor cells



STR Locus	STR Genotype Repeat #	STR Genotype
FGA	16-18,18.2,19,19.2,20,20.2,21,21.2,22,.22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26-30, 31.2, 43.2, 44.2,45.2, 46.2	22,23
TPOX	6-13	10,11
D8S1179	7-18	9,11
vWA	10-22	16,18
Amelogenin	X,Y	X,Y
Penta_D	2.2, 3.2, 5, 7-17	12,13
CSF1PO	6-15	10,12
D16S539	5, 8-15	9,13
D7S820	6-14	11,12
D13S317	7-15	8,9
D5S818	7-16	12,12
Penta_E	5-24	7,17
D18S51	8-10, 10.2, 11-13, 13.2, 14-27	17,19
D21S11	24,24.2,25,25.2,26-28,28.2,29,29.2, 30, 30.2,31, 31.2,32,32.2,33,33.2, 34,34.2,35,35.2,36-38	29,29
TH01	4-9.9,3,10-11,13.3	7,8
D5S1358	12-20	14,16

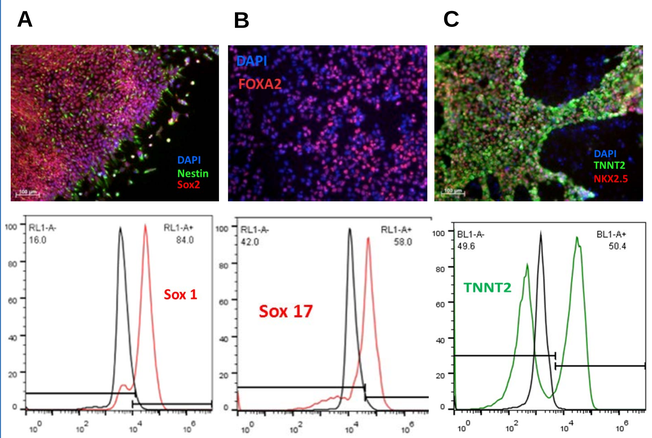
(A) G-Band Karyotype analysis of seed bank of iPSC lines are required to demonstrate stable karyotype to validate downstream usage. **(B)** STR profiling of the parental line will help identify clones of different donors apart from each other and is necessary for traceability of iPSC lines.

Figure 7. Master banks of derived iPSC lines must continue to demonstrate self renewal and genomic stability



After seed banks have been characterized and lines have been qualified to be pluripotent, master banks must be created (30-40) vials which must be routinely qualified to demonstrate continued proliferation and colony morphology **(A)**, genomic stability **(B)**, and the continued expression of self renewal markers as demonstrated by > 85% Tra 1-60 expression and less than 10% SSEA1 expression, as demonstrated via flow cytomery **(C)**.

Figure 8. Directed differentiation of iPSC banks confirms pluripotency of validated cell line



Master Banks of iPSC cell lines are further qualified via directed differentiation into the 3 germ lineages . Using Neural induction Kit **(A)** for confirmation of ectodermal commitment via ICC staining of Nestin and SOX2, and flow cytometry expression of SOX1. Definitive Endoderm differentiation **(B)** for confirmation of endodermal commitment via ICC staining of FOXA2, and flow cytometry expression of SOX17. Cardiomyocyte differentiation **(C)** was confirmed for mesodermal commitment measured via ICC staining of TNNT2 and NKX2.5 and flow cytometry expression of TNNT2.

CONCLUSIONS

•IPSC generation from different donor fibroblasts requires validation of functional pluripotency at passage 10 via expression of established markers of self renewal and the ability of the cells to spontaneously differentiate into cells indicative of the 3 germ lineages once seed banks are established. Gene expression analysis using the Taqman™ hPSC Scorecard™ Panel permits the comparison of undifferentiated and differentiated iPSC cell lines against a 94 gene panel that includes reference standards to confirm functional pluripotency.

•Karyotypic stability of the seed banks must be confirmed and the cell lines must be traceable to the parental donor tissues using STR profiling, especially when working with multiple donors. Confirmation of functional pluripotency of established iPSC lines can be further verified using established protocols and commercially available kits for each germ lineage.

•Once iPSC lines have been validated, subsequent master banks can be generated. Routine verification of continued self renewal and genomic stability are required to ensure iPSCs are of good quality prior to downstream applications.

•An integrated approach at iPSC cell line qualification and validation will ensure cell used for future applications are truly pluripotent without lineage bias. This approach to validating iPSC banks can be readily applied to iPSCs generated from multiple donor somatic cells, using any reprogramming technology, and various media and matrices.

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TRADEMARKS/LICENSING

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