TaqMan® Assays for rapid assessment of genome, epigenome, and quality of integration-free induced Pluripotent Stem Cells

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ABSTRACT

Pluripotent stem cells such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) are commonly identified and characterized based on biomarker expression. Current methods rely on a combination of *in vitro* and *in vivo* cellular methods to confirm pluripotency and tri-lineage differentiation potential. As the bottleneck in efficiency of reprogramming is alleviated with faster and better reprogramming systems, there is a need for high throughput characterization methods that allow for rapid confirmation of the quality of the resulting iPSC.

Molecular analysis platforms offer a quantitative, accurate and fast alternative to current methods and have recently been utilized to qualify pluripotent stem cells. Several platforms are available for gene expression analysis varying in content and complexity. To determine the optimal method and minimal set of genes required for definitive characterization of pluripotency, we have utilized high density array, medium and low density TaqMan® qPCR arrays to compare expression pattern of partially reprogrammed clones and fully reprogrammed iPSC in comparison to parental fibroblast and control embryonic stem cells. Results indicate that a focused set of genes in low and medium density arrays can recapitulate the information obtained with large scale arrays with distinct clustering of samples based on their pluripotency that correlated with cellular data. Further, this method was used to identify unique genes that were expressed differentially between partially reprogrammed cells and true iPSC clones as well as pluripotent cells and cells randomly differentiated via embryoid body formation.

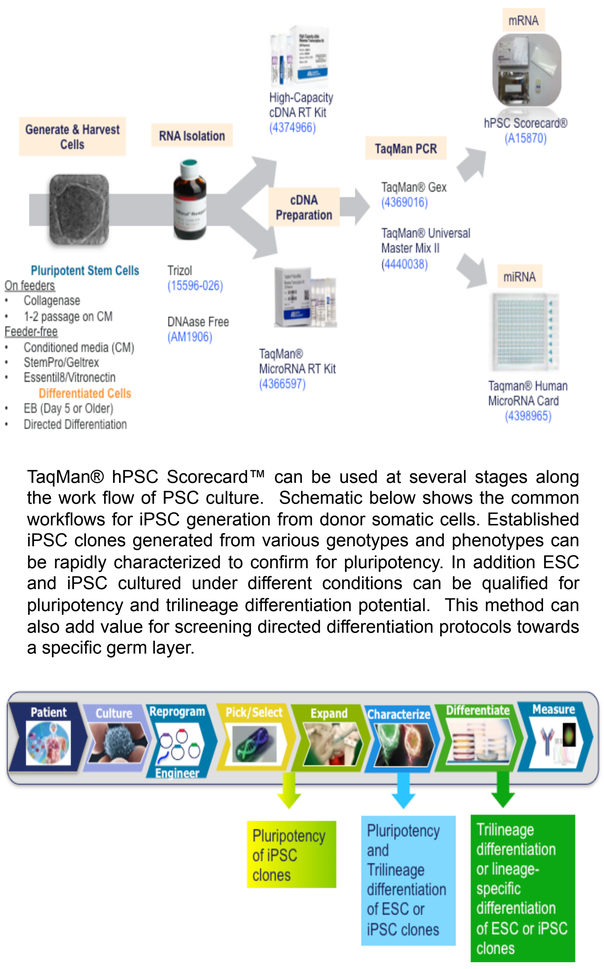
Additional assays were carried in parallel to assess epigenome signature using TaqMan® Array Human MicroRNA Card and TaqMan® assays for copy number variation. Comprehensive analysis of the resulting data indicates similarities between the pluripotent clones but also detects subtle differences that can be further evaluated for their impact on functionality and long-term stability.

BACKGROUND

Rapid advances in reprogramming technologies has led to the creation of iPSCs from diverse somatic cells using different methods and conditions. In order the confirm and standardize the quality of iPSCs, thorough characterization has become a necessity. Current methods that rely on a combination of *in vitro* and *in vivo* cellular staining, functional behavior, and differentiation potential are now being complemented with thorough molecular characterization (1,2). Such transcriptome and methylation analyses have been used to not only confirm pluripotency but also to ensure complete reprogramming (2) and determine differentiation bias of specific clones (1) and may soon replace traditional methods since they offer a high throughput solution.

TaqMan® assays are comprehensive real-time PCR assays that can be utilized for generation of quantitative transcriptome and epigenome analysis. Using data generated earlier with whole transcriptome and medium density TaqMan® OpenArray® Human Stem Cell Panel, a focused set of genes that are commonly expressed across pluripotent stem cells and differentially expressed between pluripotent and differentiating cells was identified. Using a scorecard approach as previously described (1), a focused set of genes in the TaqMan® hPSC Scorecard™ was used to confirm pluripotency and differentiation potential of several pluripotent clones. A similar approach was also utilized to identify key miRNAs that identify common and differential signatures between pluripotent state and in differentiated cells. A combination of molecular signatures provides a useful tool to define and standardize pluripotent cells and their derivatives.

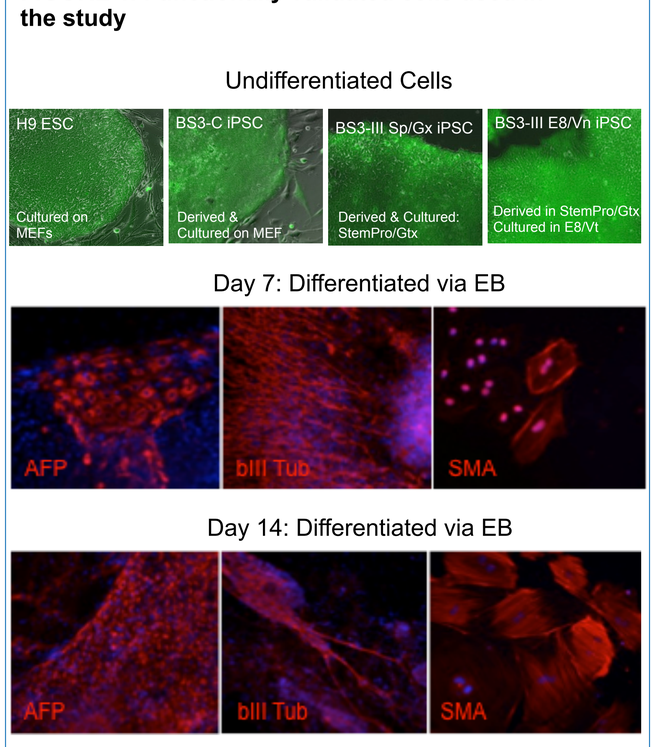
Methods outlined below was used to generate and characterize samples.



TaqMan® hPSC Scorecard™ can be used at several stages along the work flow of PSC culture. Schematic below shows the common workflows for iPSC generation from donor somatic cells. Established iPSC clones generated from various genotypes and phenotypes can be rapidly characterized to confirm for pluripotency. In addition ESC and iPSC cultured under different conditions can be qualified for pluripotency and trilineage differentiation potential. This method can also add value for screening directed differentiation protocols towards a specific germ layer.

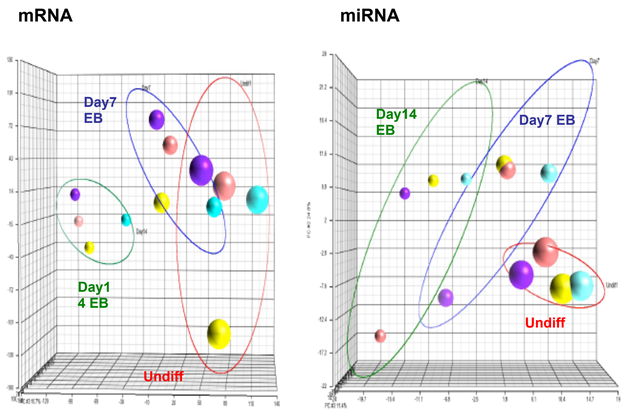
RESULTS

FIGURE 1. Functionally validated cells used in the study



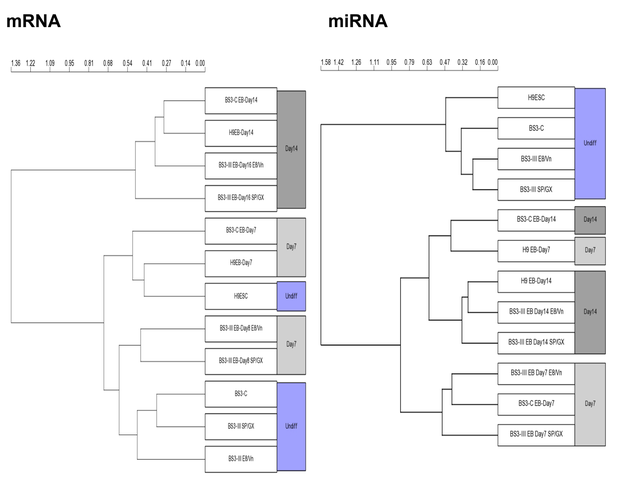
H9 ESC and iPSC derived using CytoTune® iPSC reprogramming kits on feeders (BS3-C) or under feeder free conditions using StemPro® hESC media and Geltrex® coated dishes (BS3-III Sp/Gx) or Essential 8™ media on Vitronectin coated dishes (BS3-III E8/Vn) were used for the study. Undifferentiated cells were confirmed to be homogenous via staining with AP Live Stain and upon randomly differentiation for 1 or 2 weeks via embryoid body (EB) formation. All the pluripotent lines cells stained positive for cells representing the three germ lineages: Endoderm (AFP), Ectoderm (βIII-Tubulin), and Mesoderm (SMA).

FIGURE 2. Principal Component Analysis



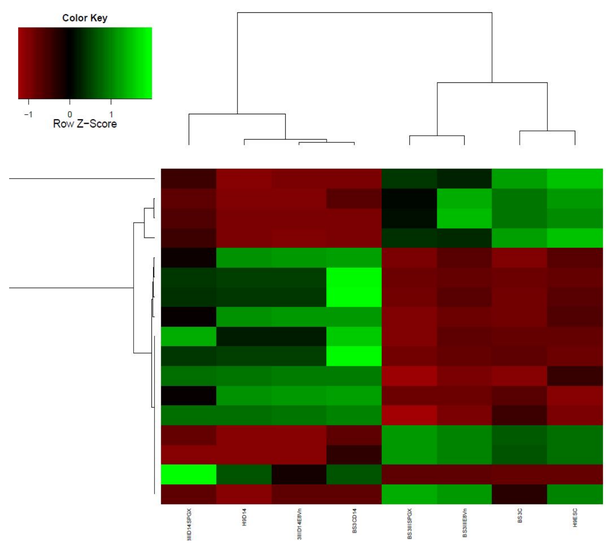
TaqMan® hPSC Scorecard™ Gene Expression analysis of 93 focused assays comprising of control, pluripotent and differentiation markers and Taqman® Array Human MicroRNA analysis of miRNAs were used to analyze H9 ESC (purple), and iPSCs generated on feeders (orange), feeder-free in StemPro® hESC media (Blue) and Essential 8™ (Yellow) media. PCA analysis of both mRNA and miRNA expression data shows distinct clustering of undifferentiated cells and day 7 and day 14 differentiated cells.

FIGURE 3. Hierarchical Clustering



Hierarchical clustering of mRNA and miRNA TaqMan® expression data shows relatedness of the samples based on their pluripotent or differentiation state. In both cases, the undifferentiated samples clustered together irrespective of media conditions. iPSCs showed closer relatedness compared to embryonic stem cells suggesting subtle differences. Nevertheless, undifferentiated cells clearly clustered away from week 1 and week 2 differentiated cells indicating that changes in mRNA and miRNA expression signatures was accurately captured by the focused list of assays.

Figure 4. Differential expressed miRNA that correlate with differentially expressed genes



Heat map of differentially expressed miRNAs between undifferentiated and day14 EBs, that are up-regulated (Green) and down-regulated (Red). Partek® software analysis using ANOVA model was used to identify 10 uniquely upregulated miRNAs and 7 unique downregulated miRNAs. These 17 differentially expressed miRNA also correlated to differential expressed mRNA.

Table 1. Taqman® hPSC ScoreCard™ data analysis

	Expected Category	Pluripotent	Endoderm	Mesoderm	Ectoderm
H9 ESC	Pluri	-1.39	-0.26	0.71	0.62
H9 Day7 EB	Endo,Ecto,Meso	-2.89	1.77	4.22	0.87
H9 Day14 EB	Endo,Ecto,Meso	-7.27	1.98	4.78	1.03
BS3C iPSC	Pluri	-0.54	0.2	0.52	0.25
BS3C Day7 EB	Endo,Ecto,Meso	-2.23	1.62	3.55	1.98
BS3C Day14 EB	Endo,Ecto,Meso	-3.59	2.67	5.13	2.2
BS3III Sp/Gx iPSC	Pluri	-0.75	-1.03	-0.99	-1.58
BS3III Sp/Gx Day7 EB	Endo,Ecto,Meso	-1.73	0.14	1.37	1.7
BS3III Sp/Gx Day14 EB	Endo,Ecto,Meso	-3.26	2.03	4.34	2.68
BS3III E8/Vn iPSC	Pluri	-0.03	-1.4	-1.43	-0.61
BS3III E8/Vn Day7 EB	Endo,Ecto,Meso	-1.86	1.16	2.67	2.24
BS3III E8/Vn Day14 EB	Endo,Ecto,Meso	-3.31	2.79	4.22	2.47
DF4 iPSC	Pluri	-0.25	-0.65	0.13	0.83
DF4 Day7 EB	Endo,Ecto,Meso	-2.53	1.56	3.93	2.26
DF4 Day14 EB	Endo,Ecto,Meso	-5.63	2.46	5.24	2.14

Comparable Higher Lower

TaqMan® hPSC ScoreCard™ data was analyzed using T-statistics as reported earlier (1). T- and P- values was computed from the normalized CT values between the test sample and distribution defined by a reference standard that comprised of 6 Biological replicates of H9 ESC and BS3-C iPSC line. Each input sample mean of t-statistic over gene categories of pluri and differentiation lineages is represented as a number with values -1 to 1 indicating comparable, <1 lower expression and >1 higher expression relative to the reference standard. Table 1 indicates that for 5 different pluripotent lines, undifferentiated cells shows comparable expression of pluripotent genes and comparable or lower expression of differentiation. This pattern is reversed upon differentiation of all the pluripotent lines with decrease in pluripotent category of genes and increase in the endoderm, ectoderm and mesoderm set of genes.

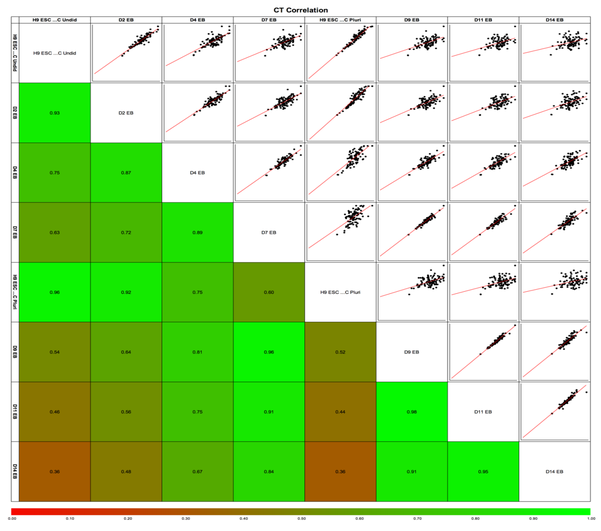
Table 2. TaqMan® hPSC ScoreCard™ analysis of pluripotent, nonpluripotent and lineage skewed samples

	Expected Category	Pluripotent	Endoderm	Mesoderm	Ectoderm	
H9 ESC	Pluri	-0.32	0.97	-0.10	0.20	100% SSEA4+
H9 Day4 EB monolayer	Endo.Ecto, Meso	-2.70	1.89	2.05	3.54	77% SSEA4+
H9 Day7 EB monolayer	Endo.Ecto, Meso	-3.74	2.44	3.24	3.95	57% SSEA4+
H9 Day4 EB suspension	Endo.Ecto, Meso	-2.65	3.22	2.85	3.38	40% SSEA4+
H9 Day7 EB suspension	Endo.Ecto, Meso	-3.28	3.29	3.73	3.61	12% SSEA4+
hNSC	Ecto	-6.18	-0.01	-0.40	2.04	
hNSC -Day5 Diff	Ecto	-4.69	0.07	-1.54	1.56	
BJ Fibroblast	Non Pluri	-7.53	1.04	2.74	0.96	
HDFfetal	Non Pluri	-12.73	-1.91	0.37	-3.00	

Comparable Higher Lower

TaqMan® hPSC ScoreCard™ analysis method was tested using samples randomly differentiated for 7 days either as suspension or as monolayer cultures and part of the sample analyzed by FACS to determine percent of pluripotent samples. A little over 20% differentiation was sufficient to see a measurable change in the pluri and differentiation markers. To determine the ability to detect lineage differentiation bias, human ESC-derived NSC and NSC differentiated for 5 days was analyzed using TaqMan® hPSC ScoreCard™ and the results showed increased expression of only the Ectoderm category of differentiation genes. Nonpluripotent samples commonly used as somatic cell source for reprogramming were negative for pluri and did not show a consistent lineage pattern suggesting heterogeneity of these cells.

Figure 5. TaqMan® *hPSC Scorecard™ Analysis Software*



D2 EB
Ecto Endo Meso
Limited general purpose pluripotent cell line. Not recommend...

D7 EB
Ecto Endo Meso
Good general purpose pluripotent cell line.

Sample Name	Expected Cell Type	Pluripotent	Ectoderm	Mesoderm	Endoderm
H9 ESC P50	Pluripotent	-0.09	-1.21	-0.97	-0.59
H9-ESC Day0	Pluripotent	-0.25	-0.91	-1.26	-0.27
H9-EB-Day2	Endo.Ecto, Meso?	-1.14	-0.37	-0.67	1.29
H9-EB-Day4	Endo.Ecto, Meso?	-2.32	0.45	1.18	3.78
H9-EB-Day7	Endo.Ecto, Meso	-3.58	1.34	2.30	4.19
H9-EB-Day9	Endo.Ecto, Meso	-3.83	1.90	3.54	4.17
H9-EB-Day11	Endo.Ecto, Meso	-3.97	2.23	4.57	4.52
H9-EB-Day14	Endo.Ecto, Meso	-5.43	2.31	4.78	4.30

Based on the statistical approach, the cloud-based hPSC ScoreCard™ Analysis software utilized a reference standard, which is comprised of comprising 9 different ESC and iPSC lines, to analyze T-value scores as depicted in Tables 1 and 2 as well as visual pass/fail results and routine analysis plots such as correlation graphs. A time course of H9 ESC differentiating randomly via EB formation shows the progressive deviation of cells from potency towards trilineage differentiation.

CONCLUSIONS

Transcriptome and miRNA analyses offer a fast and high-throughput alternative to cellular characterization methods for qualifying pluripotent stem cells.

TaqMan® hPSC ScoreCard™ and TaqMan® Array Human miRNA card were used to identify unique signatures of undifferentiated pluripotent stem cells and their randomly differentiated cells.

The focused gene expression array in TaqMan® hPSC ScoreCard™ was further utilized to assess pluripotency and trilineage differentiation potential of several pluripotent stem cell lines by analyzing undifferentiated and parallel week 1 and 2 differentiated cells.

Based on published results, the T-statistic method was used to compare data generated with samples against a functionally validated pluripotent reference standard. This analysis was used to analyze data from pluripotent cells, differentiating cells, lineage skewed samples and nonpluripotent cells.

This analysis method was the basis for a cloud-based TaqMan® hPSC ScoreCard™ Analysis software for sensitive, fast and accurate measure to determine pluripotency and differentiation into the three germ layers: endoderm, ectoderm and mesoderm.

ACKNOWLEDGMENTS

We thank Harrison Leong for building the algorithm and analysis tool. Special thanks to Deborah Tieberg, Robert Horton, Kevin Vedvik for their role in shaping this project. The gene content for the TaqMan® hPSC ScoreCard™ array and final reference data was a collaborative effort with team at Harvard lead by Alex Meissner.

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