# Novel markers to distinguish pluripotent and non-pluripotent cells.

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## ABSTRACT

A steep challenge in stem cell research is the identification and characterization of cells. This issue is amplified during the process of somatic reprogramming, a long and complex process that lasts for several weeks. It is important to identify and pick the right colonies for further expansion and characterization. Commonly, surface antibodies against pluripotent specific markers such as SSEA4, Tra-1-60 and Tra-1-81 are used but this method is expensive and sterility is a concern. Differential expression of Alkaline Phosphatase (AP) in pluripotent cells is a useful tool but most available AP substrates are toxic to the cells and once stained cannot be propagated further.

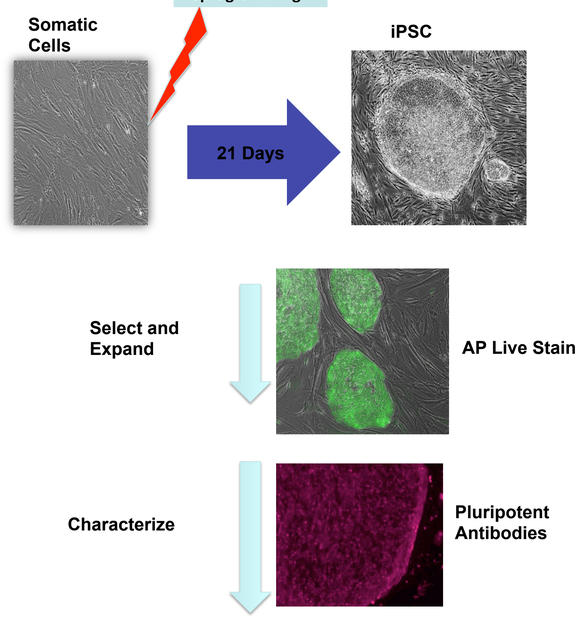
We have earlier reported the development of a novel Alkaline Phosphatase Live Stain (AP Live Stain) that specifically stains pluripotent stem cells while preserving cell integrity. More recently, using differentially expressed markers identified from transcriptome comparison of parental fibroblasts, partially and fully reprogrammed cells, we have identified several differential surface markers. One surface marker was highly expressed in parental fibroblast cells and partially reprogrammed cells but absent in embryonic stem cells and fully reprogrammed induced pluripotent stem cells. This marker was successfully used in combination with pluripotent markers to show distinct expression patterns between fully reprogrammed pluripotent colonies and partially or non-reprogrammed colonies during reprogramming. Antibody against the surface marker was utilized for rapid enrichment of pluripotent stem cells from parental fibroblast during reprogramming and from feeder murine fibroblasts in feeder-dependent culture systems. Gene expression pattern of a focused set of pluripotent and differentiation genes indicate a signature comparable to pluripotent stem cells harvested using traditional enzymatic and feeder-free culture methods. Identification of a combination of positive and negative markers will enable easier detection and early identification of true iPSC colonies with extended utility in isolation and enrichment.

## BACKGROUND

As the field of reprogramming somatic cells into Induced Pluripotent Stem Cells (iPSC) evolves and becomes more accessible and more accepted in the various scientific fields, there still remains the need for cellular characterization tools to assist in identifying and qualifying the emerging iPSC. To overcome the bottle neck in identifying truly pluripotent cells by morphology, scientists require a very well trained eye and very long and labor intensive cell culture and qualification of these cells prior to downstream applications. We previously reported the development of a novel Alkaline Phosphotase (AP) Live Stain which was engineered for the need to identify emerging iPSC in a non-destructive, non-invasive, and transient manner utilizing the highly elevated levels of AP activity of reprogrammed cells in comparison to the somatic parental cells and any feeder cells upon which they may be grown on.

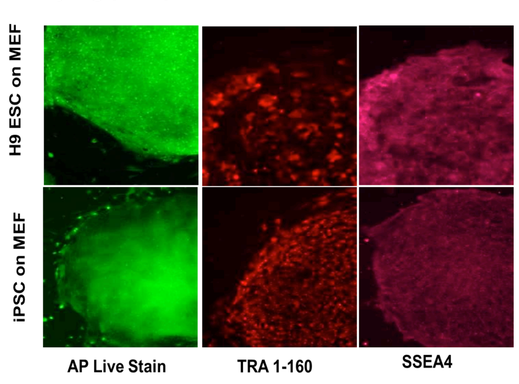
AP activity is used to monitor and select cells that are further down the reprogramming pathway. Further analysis is required to confirm pluripotency and thus the need to utilize other pluripotent surface markers, such as SSEA4, Tra-1-60, and Tra-1-81, for positive selection. In this study we utilized these cellular techniques to qualify iPSC lines derived from BJ human fibroblasts (ATCC®, CRL-2522™) which were re-programmed using CytoTune® iPS Sendai Reprogramming Kits (Cat #A1378001) and selected solely using AP Live stain. Large and mid density transcriptome analysis was performed on these iPSC , along with H9 hESC and the parental fibroblasts. The analysis revealed novel target surface proteins that were up-regulated in the parental fibroblast, over the established iPSC and hESC. This study explores the cellular expression of one of these markers, CD44, which may be utilized as negative selection marker for identification of pluripotent cells.

The subsequent study utilizes AP Live Stain (Cat #A14353) and antibodies against SSEA4, directly conjugated to Alexa Fluor® 647 (Cat #SSEA421), CD44 (Cat# RM5700), and TRA-1-60 (Cat #411000) which were then visualized with Alexa Fluor® 488 secondary (Cat #A11006) and Alexa Fluor® 594 secondary (Cat #A11005) respectfully for the later antibodies. FACS analyses were performed and analyzed using the Attune® Acoustic Focusing Cytometer (Cat# 4469120).



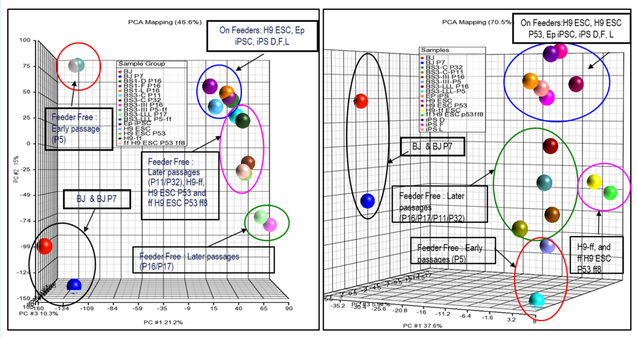

## RESULTS

**Figure 1. AP Live Stain is a useful tool for identifying pluripotent cells**




H9 hESC and CytoTune® derived iPSC can be identified as pluripotent using AP Live Stain and further characterized using pluripotent surface antibodies such as Tra-1-60 and SSEA4.

**Figure 2. Transcriptome analysis of pluripotent and non pluripotent cells**

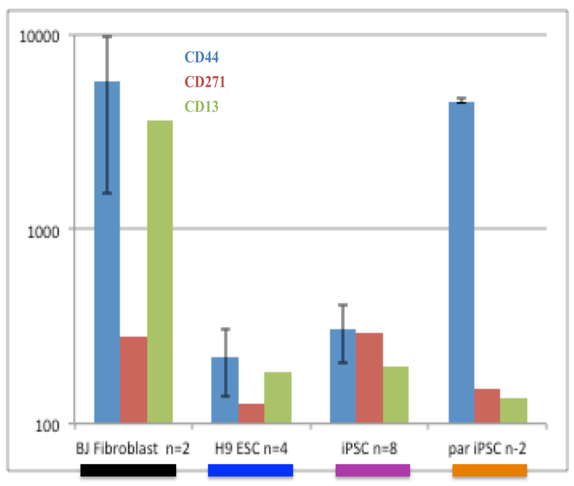



Human HT-12 Illumina® Bead array

TaqMan® OpenArray® Human Stem Cell Panel

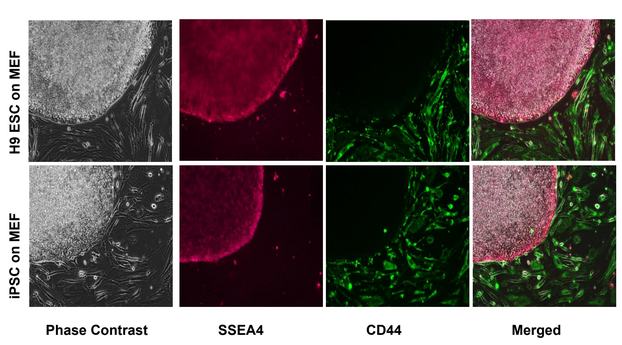
High density arrays and medium density OpenArrays® show distinct clustering of parental fibroblast, early passage iPSC (partially reprogrammed) and late passage iPSC and pluripotent cells. Molecular signatures were analyzed to determine cell states and their respective gene expression.

**Figure 3: Gene expression of known fibroblast markers**




CD 44 gene expression , compared to other known fibroblast markers, shows that it is highly expressed in BJ parental fibroblasts and partially reprogrammed iPSC, and very lowly expressed in hESC and fully reprogrammed iPSC

**Figure 4. CD44 can be used as surface marker for distinguishing pluripotent and non-pluripotent cells**

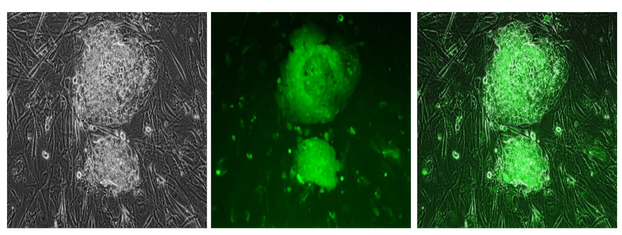



H9 ESC and established iPSC , grown on MEFs demonstrate typical staining patterns , where the pluripotent colonies are positive for SSEA4 and negative for CD44 while the MEFs are positive for CD44 and negative for SSEA4.

**Figure 5. Characterization of differential CD44 expression in emerging iPSC**

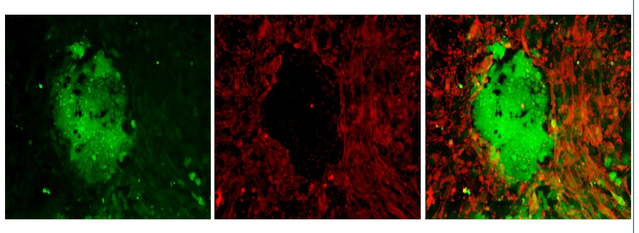

BJ fibroblasts, transduced with the CytoTune® iPS Sendai reprogramming kits for 7 days followed by re-seeding onto MEFs for an additional 14 days demonstrate down regulation of CD44 by 21 days post transduction. Prior to picking and expanding iPSC clones. Colonies are positive for the early pluripotent marker, SSEA4, and are negative for CD44, while the MEFs show a definitive expression of CD44, as gauged by non-invasive immuno-fluorescence.

**Figure 6. AP Live Stain can be used as a transient measure of early pluripotency during reprogramming.**



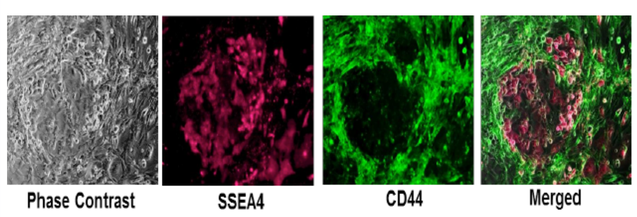

BJ Fibroblasts, transduced with CytoTune® iPSC Sendai reprogramming kits and seeded on MEFS can be probed with AP Live stain as early as 14 days post transduction. Increased AP expression in emerging iPSC colonies serves to identify clones that are reprogrammed much earlier than traditional antibody selection, without altering the normal growth conditions and growth kinetics of the colonies to be selected.

**Figure 7. Positive-negative selection of pluripotency during early Reprogramming**



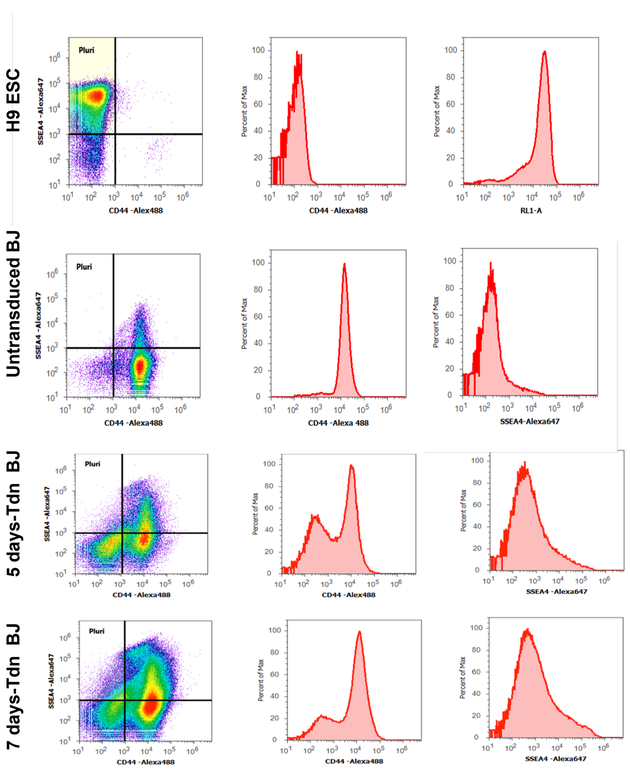

BJ Fibroblasts, transduced with CytoTune® iPSC Sendai reprogramming kits begin to demonstrate the up-regulation of pluripotent markers and down-regulation of fibroblast markers early in the reprogramming process. This image is of BJ fibroblasts at 8 day post transduction, growing in the original transduction wells. Clusters of reprogrammed cells begin to show robust AP activity while demonstrating a decrease expression of CD44.

**Figure 8. Marker expression for early iPSC generation**




BJ Fibroblasts, transduced with CytoTune® iPSC Sendai reprogramming kits for 8 days, prior to re-seeding on matrices or feeders, also begin to demonstrate up-regulation of SSEA4 and down regulation of CD44 which can utilized as means of identifying or selecting for individual pluripotent cells via a positive/negative profile.

**Figure 9. FACS Analyses of pluripotency profiles**




FACS analyses of H9 ESC, BJ Fibroblasts prior to transduction with CytoTune® iPS Sendai reprogramming kit, and at 5 and 7 days post transduction. Double antibody staining of cells with CD44 antibody and Alexa Fluor® 488 secondary, and SSEA4-Alexa Fuor® 647 dyes demonstrate the typical profile for pluripotent and non-pluripotent cells where pluripotent cells are CD44 negative and SSEA4 positive while non-pluripotent cells have the converse profile. The profiles of partially reprogrammed cells show the beginning of the transitions of pluripotency where populations of cells start to become positive for SSEA4, while losing their CD44 expression.

## CONCLUSIONS

- AP Live stain has been demonstrated to be an effective tool to demonstrate pluripotency during the reprogramming process and has been utilized in selecting fully reprogrammed iPSC.
- CytoTune® reprogrammed iPSC demonstrate molecular signatures similar to H9 hESC, and distinctive from the BJ parental fibroblasts. Transcriptome analysis revealed novel surface markers which were highly up-regulated in fibroblasts.
- CD44 was compared to other known fibroblast markers and selected for further analysis to confirm its ability to be used as a cellular marker to negatively qualify un-reprogrammed cells, partially reprogrammed cells, and murine embryonic fibroblasts which are typically used in iPSC culture conditions.
- CD44 can be used as a surface marker to identify non-pluripotent cells, and the lack of its expression in pluripotent cells. A combined approach of CD44 for non-pluripotent or partially re-programmed iPSC with a pluripotent positive marker like AP Live Stain, SSEA4, and Tra-1-60 can serve to show cellular characteristics of established pluripotent cells and emerging iPSC.
- CD44 and AP Live Stain, or SSEA4 can be used to identify reprogrammed cells very early in the reprogramming process. AP activity and decreased CD44 expression can be seen as early as 8 days post transduction.
- FACS analysis of pluripotent and non pluripotent cells demonstrate correlative cellular signatures which can be used to look at the cellular expression of CD44 and pluripotent markers such as SSEA4 during the reprogramming process. Cells undergoing reprogramming go through transitions stages which can be identified via surface marker expression and may be used to enrich for pluripotent populations.

## REFERENCES

1. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. (2009) Proc Jpn Acad Ser V Phys Biol Sci. 85(8): 348-362.
2. Singh U, Quintanilla RH, Grecian S, Gee KR, Rao MS, Lakshmipathy U. (2012) Stem Cell reviews and reports.

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