FlowCellect™ Human iPS Cell Characterization Kit (100 tests)

Catalog No. FCSC100107

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Not for use in diagnostic procedures.

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Introduction

Human induced pluripotent stem cells (iPS cells) are somatic cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells. Because of their ability to mimic human embryonic stem cells (hESCs) with pluripotent capabilities and the ability to differentiate into any cell types, it is generally believed that iPS cells have the potential for alleviating incurable diseases and aiding organ transplantation [1]. Human iPS cells are useful tools for modeling diseases and for drug development, and by having the ability to develop "custom made" adult cells derived from iPS cells this may ultimately allow the treatment of patients with debilitating degenerative disorders [2]. In fact, some iPSC-based therapies are already being examined in animal models and showing signs of therapeutic utility [2,3].

In 2012, the Nobel Prize for Medicine was awarded to Sir John B. Gurden and Shinya Yamanaka for their work on iPS cells. In Takahashi and Yamanaka (2006), it examined if genetic reprogramming using specific transcription factors could induce pluripotency in somatic cells. And based on their findings, they were able to demonstrate that pluripotent stem cells can be directly generated from fibroblast cultures by the addition of only a few defined factors, a technique which has revolutionized the stem cell market [4]. The reprogramming of human cells allow scientists new opportunities to study diseases and develop methods for diagnosis without the need of embryonic stem cells. The use of embryonic stem cells (ESCs) have been controversial due to its origin (e.g. blastocytes) since it requires the destruction of the fertilized human embryo. This in itself has raised many ethical issues, hence, providing the need to find an alternative source of pluripotent stem cells with the same range of functions as ES cells [1,4]. Like human embryonic stem cells, iPS cells have the capacity to generate cells from each of the three germ layers (endoderm, mesoderm, and ectoderm), but also can be propagated in culture indefinitely [4,5,9].

Millipore's FlowCellect™ Human iPS Cell Characterization Kit provides a quantitative solution for clearly identifying human iPSC cultures using flow cytometry. The kit contains four directly conjugated antibodies to phenotypically characterize human iPSCs, with three positive expressing markers (Tra-1-60, SSEA4, and human Oct4), as well as one negative marker, SSEA1. These antibodies have been carefully evaluated and optimized on human induced pluripotent stem cells and human ES derived oligodendrocyte progenitor cells (negative control), ensuring that the kit can clearly monitor and characterize human iPSC cultures.

All kit antibodies are optimized for guava® bench top flow cytometers and provide a complete solution for human induced pluripotent stem cell analysis. However, this kit can be used on any flow cytometer following the same protocol, offering researchers a reliable, quantitative, and fully validated solution to study their iPSC cultures. All four directly conjugated antibodies provided in the kit have been carefully evaluated to ensure optimal performance for flow cytometry, alleviating the need for any additional validation of the kit reagents. Also, plate-based stability testing and quality control was performed to confirm the stability of the antibodies over time. After 120 minutes (2 hours) post sample preparation, kit antibodies did not lose activity (< 5-10% of signal strength). So many tests can be performed in a single experiment without the risk of losing antibody activity or performance over time.

Test Principle

Millipore's FlowCellect™ Human iPS Cell Characterization Kit includes three positive expressing markers, Tra-1-60/FITC, SSEA4/PE, and human Oct4/Alexa Fluor 647, and one negative marker, SSEA1/PECy5, all used for multi parametric analysis to phenotypically identify the state of their induced pluripotent stem cell cultures. Detailed assay instructions are included below to assist in cell preparation and analysis for flow cytometry, on guava easyCyte flow cytometers or on traditional sheath-fluid based flow cytometers. All four directly conjugated antibodies have been optimized together to ensure the ability to characterize human iPSC cultures accurately and confidently. Sufficient reagents are provided to perform 100 tests. Users can use the supplied antibodies and reagents with recommended isotype controls listed in the protocol.

In vitro, iPSCs are normally maintained and propagated on mouse fibroblast feeders for extended periods in media containing basic fibroblast growth factor (bFGF) [6]. However, spontaneous differentiation may occur in subpopulations of cells. Several pluripotent markers are commonly used to distinguish pluripotent iPSCs from differentiatied cells.

- **Human Oct4** is a transcription factor that is highly expressed in pluripotent cells. It is expressed in a significant portion of their target genes and form the core transcriptional regulatory circuitry that contributes to pluripotency and self-renewal of iPSCs [7].
- **Tra-1-60 and SSEA4** are cell surface antigens that are expressed in pluripotent human iPS cells and not on mouse iPS cells. The antibodies recognize different proteoglycan epitopes on the same protein, podocalyxin [8].
- **SSEA1** is a cell surface antigen that is expressed on the surface of murine embryonic stem cells (ES) and murine and human germ cells (EG). No immunoreactivity is evident with undifferentiated human ES and iPS cells. In contrast to SSEA4 and Tra-1-60, the differentiation of human ES and iPS cells is characterized by an increase in SSEA-1 expression, making this a suitable negative marker.

While the expression levels of pluripotent markers are expected to be diminished (or in the case of SSEA1 activated) upon differentiation, each possess specific expression kinetics. For example, it has been noted that upon differentiation Oct4 and Tra-1-60 expressions are the first to be down regulated relative to other markers such as Nanog which down regulates at a much slower rate [8].

Human induced pluripotent stem cells have been highly utilized as a promising model with which to study disease mechanisms, discover new therapies, and develop truly personalized treatments. For example, Parkinson's disease (PD) is a common neurodegenerative disease in humans. It is characterized by severe motor symptoms of tremor, rigidity, bradykinsia, and postural instability, all a result from the loss of dopamine neurons in the substantia nigra [10]. Induced pluripotent stem cells derived from somatic cells can be a powerful tool for studying the pathogenesis of PD and provide a source for replacement therapies for this neurodegenerative disease. Cell replacement therapy such as transplantation of dopamine neurons or neural stem cells has shed new light as an alternative treatment strategy for PD [2,10].

In all, human iPS cells have attracted the attention of many researchers in the biomedical and research institutions given its potential to help alleviate many diseases. Due to their potential to mimic embryonic stem cells and act as potentially suitable alternatives for regenerative medicine techniques, as well as be utilized to study the mechanisms of disease for drug and toxicology testing, iPS cells are an attractive target for clinical studies as therapeutic agents.

Kit Components

- 20X Anti-Tra-1-60/FITC, clone TRA-1-60: (Part No. CS211422). One vial containing 500 µL
- 20X Anti-SSEA4/PE, clone MC-813-70: (Part No. CS211423). One vial containing 500 µL
- 20X Anti-SSEA1/PECy5, clone MC-480: (Part No. CS210082). One vial containing 500 µL
- 20X Anti-human Oct4/Alexa Fluor®647, clone 10H11.2: (Part No. CS211425). One vial containing 500 µL
- 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL
- Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL
- Permeabilizaton Buffer: (Part No. CS202125) One bottle containing 13 mL

Materials Not Supplied

1. Test tubes for sample preparation and storage
2. Tissue culture reagents, i.e. HBSS, PBS w/o Ca^{2+} or Mg^{2+}, cell dislodging buffers, etc.
3. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 µL
4. Tabletop centrifuge capable of achieving 300 x g
5. Mechanical vortex
6. Flow Cytometer
7. Deionized Water (for buffer dilution)
8. Cells of Interest (e.g. iPS cell cultures, embryonic stem cells, etc.)
9. Isotype Controls: Mouse IgM-FITC, Mouse IgG3-PE, Mouse IgG1-Alexa Fluor 647, or Mouse IgM-PECy5 (based on user preference)

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. Please refer to the MSDS sheet for specific information on hazardous materials (MSDS forms can be found on the web page or by contacting Millipore technical services).
- During storage and shipment, the directly conjugated antibodies may condense within the vial. For maximum recovery of the product, centrifuge original vial prior to removing cap.
- The conjugated antibody is light sensitive and must be stored in the dark at 2 - 8℃.
- Do not use reagents beyond the expiration date of the kit.

Storage

All reagents must be stored at 2 - 8ºC.

All kit components are stable up to four (4) months from date of receipt if stored and handled correctly. **Please avoid repeated changes in temperature as this will affect the integrity of the product.**

Preparation of Reagents

1. **Assay Buffer**
   Assay Buffer is supplied at 5X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Assay Buffer is stable up to one year. Store at 2 - 8ºC

2. **Antibody Working Cocktail Solution**
   The kit contains four (4) antibodies which can be performed in multiplex. Prior to antibody staining of cells, prepare an antibody working cocktail solution by addition of the following: Add 5 µL of anti-Tra-1-60/FITC, 5 µL of anti-SSEA4/PE, 5 µL of anti-human Oct4/Alexa Fluor 647, and 5 µL of anti-SSEA1/PECy5 conjugated antibodies into a centrifuge tube for a final volume of 20 µL total. This amount is good for one (1) test.
   **Based on the number of tests being performed, it is up to the end user to adjust antibody volume amounts at similar ratios (e.g. for 10 tests, the working cocktail solution will contain 50 µL of Tra-1-60, 50 µL of SSEA4, 50 µL of human Oct4, and 50 µL of SSEA1 for a total of 200 µL).*

Assay Instructions

It is recommended that the end user provide either a negative control cell line (e.g. human ES derived oligodendrocyte progenitor cells, or OPC cells) or secure the appropriate isotype controls for cell staining. These can be suitable controls to validate the iPS cells being investigated.

I. Compensation Controls

It is highly recommended that the end user set up a series of compensation controls for every experiment performed. Compensation controls can be achieved by following the quick instructions below:

- **Well or Tube #1:** 5 µL of IgM/FITC, 5 µL of IgG3/PE, 5 µL of IgM/PECy5, and 5 µL of IgG1/Alexa Fluor 647 in 80 µL of 1x Assay Buffer.
- **Well or Tube #2:** 5 µL of Anti-Tra-1-60/FITC, 5 µL of IgG3/PE, 5 µL of IgM/PECy5, and 5 µL of IgG1/Alexa Fluor 647 in 80 µL of 1x Assay Buffer.
- **Well or Tube #3:** 5 µL of Anti-SSEA4/PE, 5 µL of IgM/FITC, 5 µL of IgM/PECy5, and 5 µL of IgG1/Alexa Fluor 647 in 80 µL of 1x Assay Buffer.
- **Well or Tube #4:** 5 µL of Anti-SSEA1/PECy5, 5 µL of IgM/FITC, 5 µL of IgG3/PE, and 5 µL of IgG1/Alexa Fluor 647 in 80 µL of 1x Assay Buffer.
- **Well or Tube #5:** 5 µL of Anti-human Oct4/Alexa Fluor 647, 5 µL of IgM/FITC, 5 µL of IgG3/PE, and 5 µL of IgM/PECy5 in 80 µL of 1x Assay Buffer.

II. General Assay Protocol

1. Prepare cells of interest into a tissue culture flask and follow the proper guidelines for cell line propagation and maintenance.

2. On the day of the assay, collect cell culture(s) and place into a centrifuge tube(s). Depending on the number of assays to be performed, determine the proper amount of cells required for experimentation. Each test is defined as 100,000 cells.

3. In the centrifuge tube, spin at 300 x g for 5 minutes. Aspirate off media and resuspend cell pellet with 1X Assay Buffer. Using 100 µL per 100,000 cells should be sufficient. The intent of this step is to wash the cells and remove any residual growth media and/or cell treatments prior to cell staining.

4. Aspirate media and resuspend cells in 50 µL 1X Assay Buffer per 100,000 cells in culture (per test). For example, if preparing to perform 10 tests, resuspend the cell pellet containing approximately one million cells with 500 µL 1X Assay Buffer.

5. For each test, add 50 µL of Fixation Buffer for a final volume of 100 µL for each 100,000 cells in suspension (for 10 tests, add 500 µL Fixation Buffer for a final volume of one mL and one million cells). Incubate for 20 minutes on ice.

6. After 20 minutes, add 100 µL Permeabilization Buffer per test (for a total of 200 µL per test). So if performing 10 tests, add one mL Permeabilization Buffer to the centrifuge tube for two mLs total volume in one million cells (Adjust buffer ratios according to the number of tests performed). Allow to incubate on ice for 10 minutes.

7. After Fixation/Permeabilization step, spin cell sample at 300 x g for 5 minutes. Aspirate off media and resuspend cells in 80 µL per 100,000 cells in culture (per test). Again, if performing multiple tests, please adjust the resuspension volumes accordingly, i.e.—for 10 tests, resuspend cell pellet in 800 µL 1X Assay Buffer.

8. If using a guava 8HT system for cell acquisition transfer 80 µL aliquots per well into a “V” bottomed 96-well plate (Costar: 3897). If using another flow cytometer, cells can be distributed into individual cell tubes for acquisition, each containing 80 µL of 100,000 cells each.

9. For each experimental sample, add 20 µL of the antibody working cocktail solution (**See “Preparation of Reagents” section for more detailed instructions).* Allow each experimental sample to stain for 30 minutes on ice in the dark.

10. Following cell staining, add 100 µL of 1X Assay Buffer to each sample (for a total of approximately 200 µL per test) into each well and/or sample tube, and place into a centrifuge. Spin samples at 300 x g for 5 minutes.

11. Aspirate supernatant from each sample, and resuspend each cell pellet in 200 µL 1X Assay Buffer once more. Spin at 300 x g for 5 minutes. The intent of this step is to wash away any unbound, residual antibody from experimental samples.

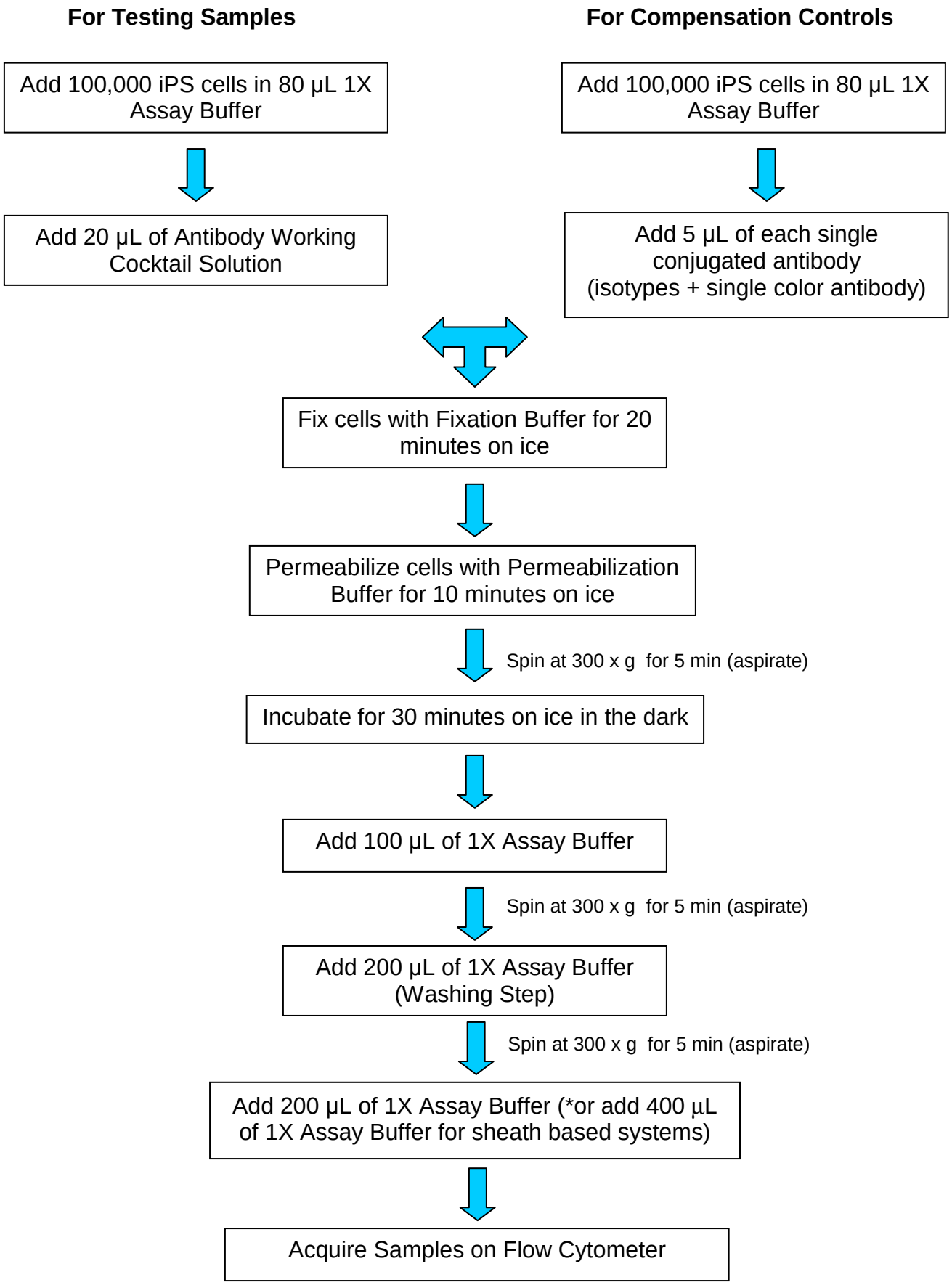
12. Aspirate supernatant from each sample, and resuspend each cell pellet in 200 µL 1X Assay Buffer (*NOTE: If using a standard sheath fluid based system, resuspend the final cell pellet into 400 µL 1X Assay Buffer).

13. Acquire samples using the flow cytometer. Compare signals (-) isotype control and/or negative control cell line versus (+) antibody staining against the iPSC cultures of interest.

14. Analyze data

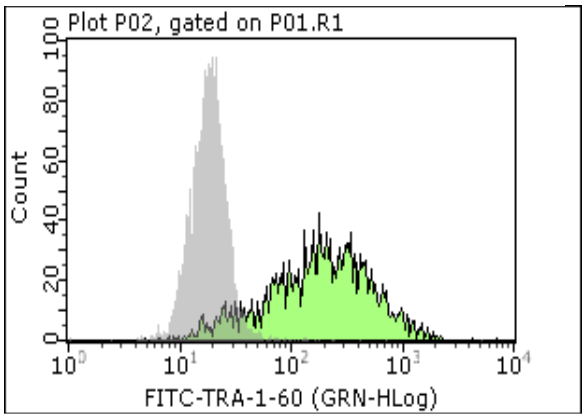
Note: Results are tested using Guava easyCyte systems. However, the kit can be used on any flow cytometer with the correct laser and filter configurations. Also, human iPSCs expression can vary depending on the cell line being evaluated. Different iPSC cultures can produce different levels of surface expression for each given marker.

***Quick Assay Workflow (This protocol can be applied to any flow cytometer):**

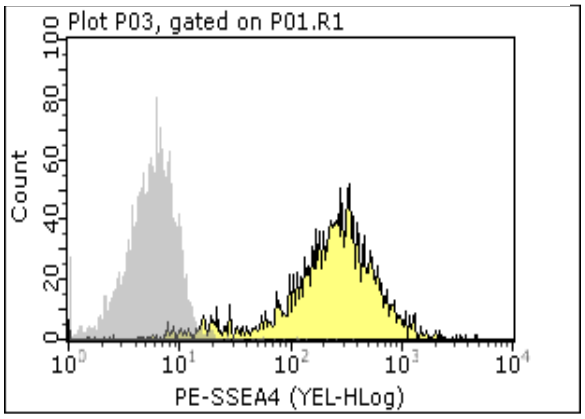


Sample Data

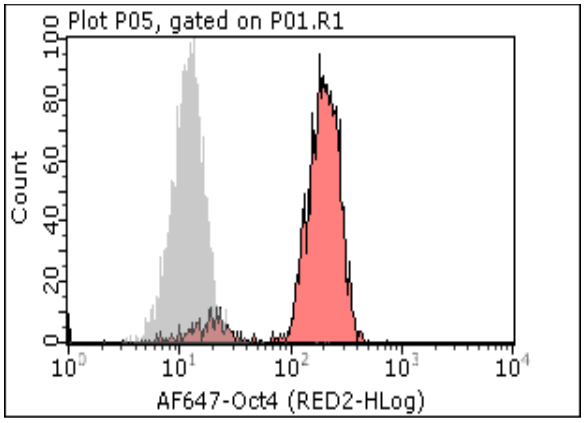
A. Anti-Tra-1-60/FITC



B. Anti-SSEA4/PE



C. Anti-human Oct4/Alexa Fluor 647



D. Anti-SSEA1/PECy5

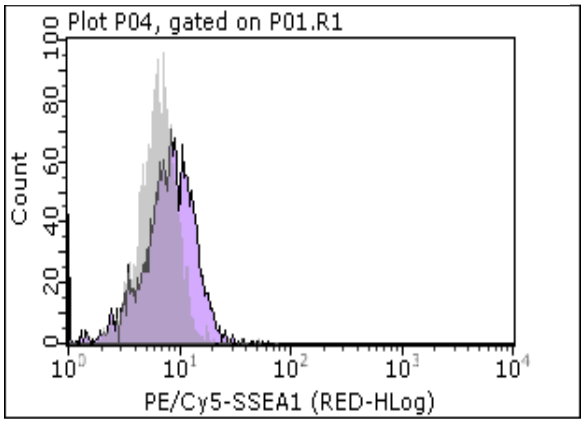
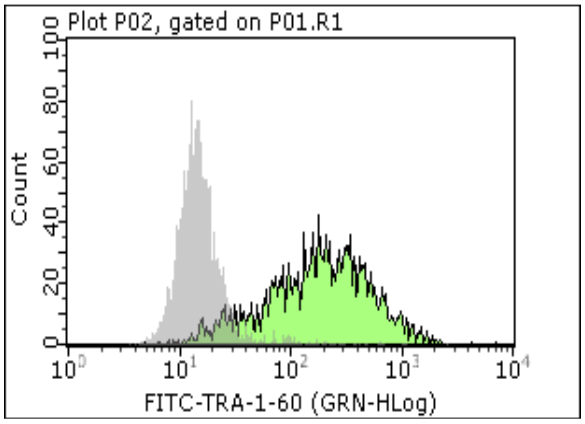
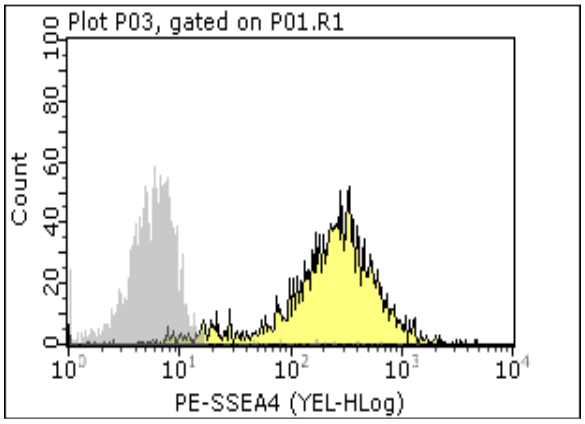


Figure 1. Validation of surface and intracellular marker Oct 4 for characterization of human induced pluripotent stem cells (iPSCs) by flow cytometry. Tra-1-60, SSEA4, human Oct 4, and SSEA1 were performed in multiplex and analyzed by histogram individually. Human iPS cells were stained and compared versus the corresponding isotype control. As indicated by the histograms for Tra-1-60, SSEA4, and human Oct4, in all cases positive expression of each is indicated by the shift in fluorescence relative to the negative control (A, B, and C). Conversely, SSEA1 does not show any expression on human iPSCs, serving as a suitable negative control.

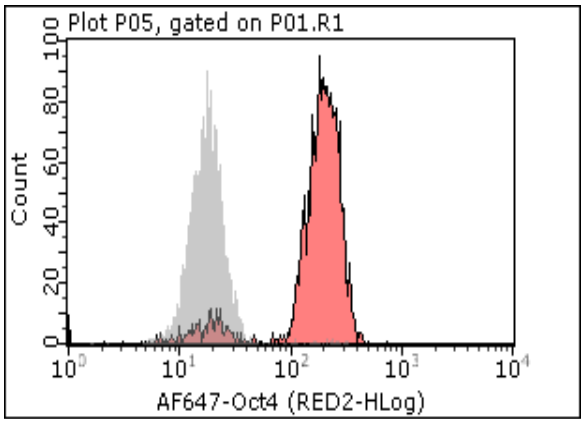
A. Anti-Tra-1-60/FITC



B. Anti-SSEA4/PE



C. Anti-human Oct4/Alexa Fluor 647



D. Anti-SSEA1/PECy5

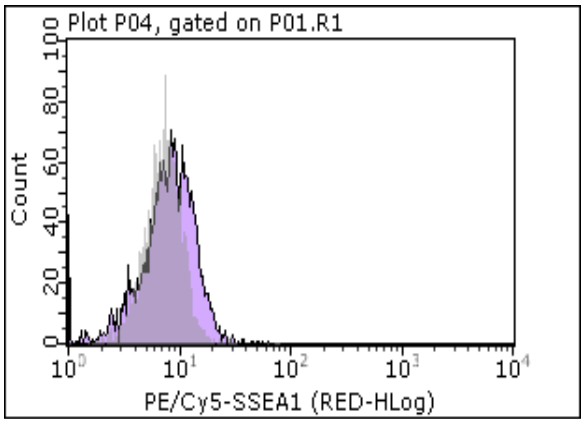


Figure 2. Human induced pluripotent stem cells are phenotypically identified by Tra-1-60, SSEA4, and human Oct4 conjugated antibodies. Using a negative control cell line (e.g. human ES derived oligodendrocyte progenitor cells, or OPC cells), when compared to human iPSCs both cultures are individually stained with positive markers, Tra-1-60, SSEA4, and human Oct4. As illustrated, positive expression is evident for all three cultures when compared to the negative control cell line. And as expected, when SSEA1 is examined in a similar fashion there is no expression, further validating that these four antibodies performed in multiplex are a legitimate way to characterize human iPSCs.

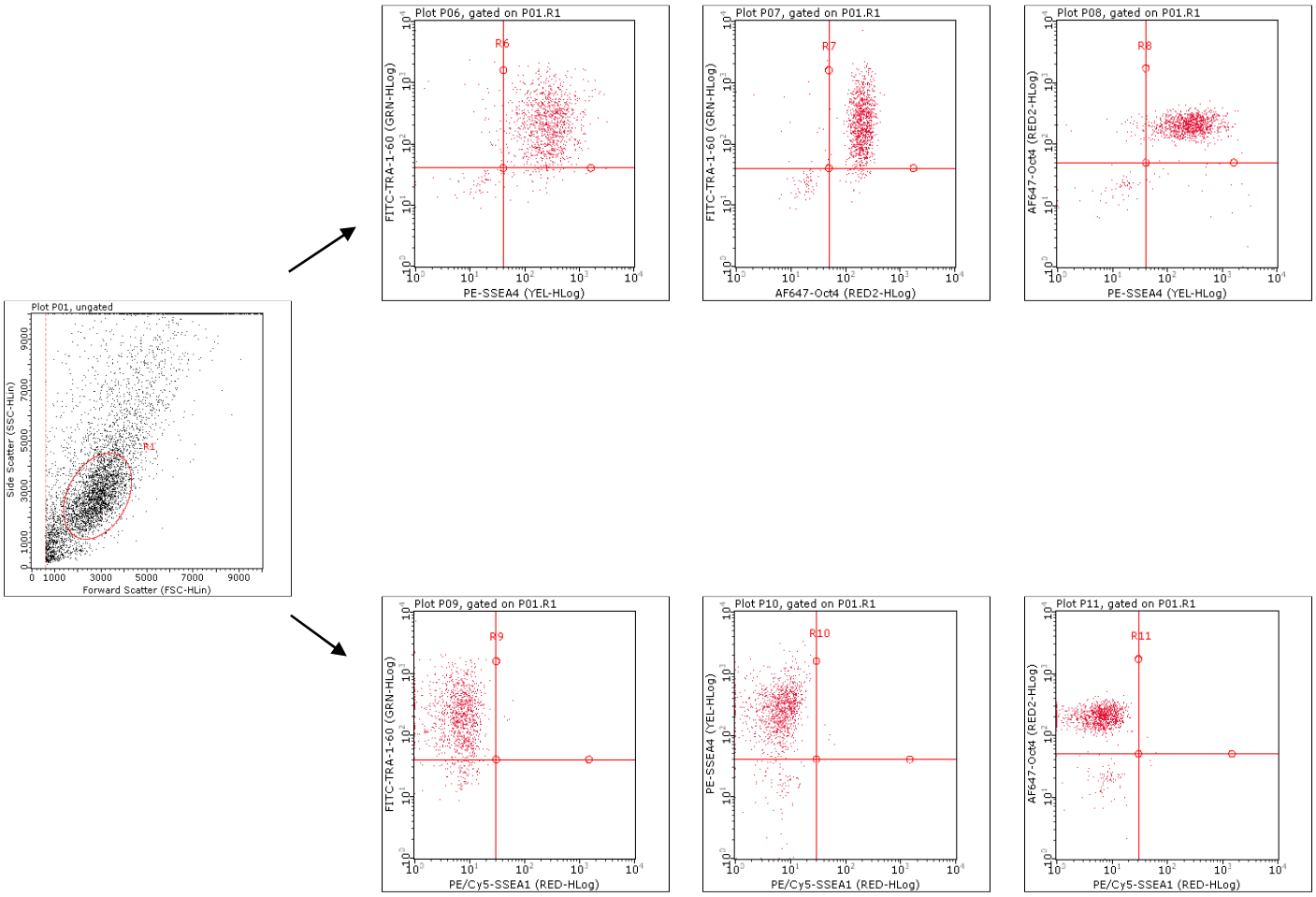


Figure 3. Human induced pluripotent stem cell markers performed in multiplex (dot plot representation). All four antibodies are performed in multiplex and represented above in bivariate plots. Human iPSC cultures were gated to exclude debris in the scatter plot and analyzed for staining as shown against **Tra-1-60, SSEA4, and human Oct4 conjugated antibodies, and for negative expression of SSEA1**. Positive markers for each is validated by plotting against SSEA1 (negative marker), confirming the specificity of each positive marker for iPSC cultures.

Technical Hints

- If minor precipitate is detected in the 5X Assay Buffer, place the bottle in a warm water bath for 15 minutes, followed by mixing the contents on a mechanical vortex.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- Do not mix or interchange reagents from various kit lots.

Troubleshooting

Assay Step	Potential Problem	Experimental Suggestions
Reagent Preparation	Precipitation found in 5X Wash buffer	• If storing at -20ºC, precipitate can form in the 5X Assay Buffer. Prior to use, place bottle in a 37ºC water bath, swirling the contents occasionally. If this does not remove the precipitate completely, allow 5X Assay Buffer to sit at room temperature overnight.
Acquisition	Acquisition rate decreases dramatically	This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following: • Decreasing number of cells for analysis. Guava flow cytometers have the capacity of analyzing a steady stream of 300 – 500 cells per microliter. Using cell concentrations in excess of the recommended level can block the normal flow, causing disruption during the assay. Decrease the number of cells being analyzed by diluting the sample to approximately 0.5 million cells per milliliter (5 x10^5 cells per mL). • Adherent cells can result in cellular clumping. Using a cell declumping agent such as the guava ViaCount CDR Cell Dispersal Reagent (Cat. No. 4700-0050) after cell harvesting should help keep cells in single suspension. Alternatively, using a cell strainer can help disrupt cell clumping if needed. • After many uses, it is possible that the fluid system on any standard flow cytometer will require cleaning. Run standard cleaning procedures to clean the fluid system during or after an assay. This will prevent any material from forming where the steady flow steam takes place.
Cellular Analysis	A loss or lack of signal	• Cell numbers may need to increase. Cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to a loss of signal. Make sure that cell density remains at approximately 0.5 million cells per milliliter during analysis. • Poor staining: Follow the assay instructions for proper cell staining to ensure that the staining of cells is done appropriately. Shorter incubation times can lead to inadequate surface staining.
Cellular Analysis	Variability in day to day experiments	• Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results. • When using any guava easyCyte™ instrument for flow analysis, make sure that a quality check on the instrument (e.g. calibration) is performed on a daily basis prior to use. (**See Analytical Sensitivity and Detection Limits Section for Guava Check standards*)

***For Technical Service, please visit www.millipore.com/techservice**

References

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Related Products

1. FlowCellect™ Human Mesenchymal Stem Cell Characterization Kit (Catalog No. FCSC100184).
2. FlowCellect™ Human ESC (HESCA-1) Surface Marker Characterization Kit (Catalog No. FCHEC25104)
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4. FlowCellect™ Human ESC Nucleus Marker Characterization Kit (Catalog No. FCHEC25102)
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6. FlowCellect™ Rodent NSC Characterization Kit (Neural) (Catalog No. FCRNC25112)
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