User Manual

# Fluorescent Human ES/iPS Cell Characterization Kit

**SCR078**

FOR RESEARCH USE ONLY

**Not for use in diagnostic procedures. Not for Human or Animal Consumption.**

## Product Overview

Human embryonic stem (hES) cells are pluripotent cells derived from the inner cell mass of pre-implantation blastocysts.[1] Human induced pluripotent (hiPS) cells are pluripotent cells generated by reprogramming human somatic cells using four transcription factors, Oct-4, Klf-4, Sox-2, and c-Myc, or their variants.[2] Both hESC and hiPSC can self-renew and have the ability to generate all three germ layers: ectoderm, mesoderm, and endoderm. *In vitro*, hESC/iPSC are normally maintained and propagated on mouse fibroblast feeders for extended periods in media containing basic fibroblast growth factor (bFGF).[3] However, spontaneous differentiation may occur in subpopulations of cells. Several pluripotent markers are commonly used to distinguish pluripotent hESC/iPSC from differentiated cells.

- **Alkaline phosphatase (AP)** is an enzyme that hydrolyzes the phosphate group from many types of molecules, including nucleotides, proteins and alkaloids. Although AP is primarily found in liver and bone, pluripotent stem cells have also been found to have elevated expression of AP.[5] Both human and mouse ESC/iPSC are characterized by high expression levels of AP.
- **Oct4, Sox-2, and Nanog** are three transcription factors that are highly expressed in pluripotent cells. They share a significant proportion of their target genes and form the core transcriptional regulatory circuitry that contributes to pluripotency and self-renewal of hESC/iPSC.[6] The successful reprogramming of somatic cells with Oct-4, Sox-2 together with Klf-4 and c-Myc genes further confirms the essential role of these transcription factors in maintaining pluripotency.[2,4]
- **TRA-1-60 and TRA-1-81** are cell surface antigens that are expressed in pluripotent human ES/iPS cells and not on mouse ES/iPS cells. Both antibodies recognize different proteoglycan epitopes on the same protein, podocalyxin.[7]
- **Dapi or 4',6-diamidino-2-phenylindole** is a fluorescent dye that binds strongly to A-T rich regions in DNA and is thus frequently used to label the cell nucleus.

Our fluorescent Human ES/iPS Cell Characterization Kit contains a range of sensitive tools for the phenotypic assessment of the pluripotent status of human ES/iPS cells. Included in the kit is an enzymatic assay to measure alkaline phosphatase activity in the cells along with validated directly conjugated antibodies to pluripotent transcription factors, Oct-4, Sox-2 and Nanog and cell surface epitopes TRA-1-60 and TRA-1-81 to enable rapid immunocytochemical marker analysis. The Dapi nuclear dye is conveniently included to aid in cell quantification. While the expression levels of pluripotent markers are expected to be diminished upon differentiation, each possess specific expression kinetics. For example, it has been noted that upon differentiation, Oct-4 and TRA-1-60 expressions are the first to be down-regulated while Nanog and alkaline phosphatase down-regulate at a much slower timeframe.[7]

## Materials Provided

- Fast Red Violet solution (90239): One 15 mL bottle.
- Naphthol AS-BI phosphate solution (2mg/mL) in a buffer, pH 8.5 (CS235583). One 15 mL bottle.
- Mouse anti-Oct-4 (POU5F1), clone 7F9.2, Alexa Fluor® 488 conjugate (MAB4419A4-50UL). One vial containing 50 mL of 0.5 mg/mL conjugated monoclonal antibody.
- Mouse anti-Sox-2, clone 10H9.1, Cy3 conjugate (MAB4423C3-50UL). One vial containing 50 mL of 0.5 mg/mL conjugated monoclonal antibody.
- Mouse anti-Nanog, clone 7F7.1, Alexa Fluor® 488 conjugate (MABD24A4-50UL). One vial containing 50 mL of 0.5 mg/mL conjugated monoclonal antibody.
- Mouse anti-TRA-1-60, clone TRA-1-60, Cy3 conjugate (MAB4360C3-50UL). One vial containing 50 mL of 0.5 mg/mL conjugated monoclonal antibody.
- Mouse anti-TRA-1-81, clone TRA-1-81, Cy3 conjugate (MAB4381C3-50UL). One vial containing 50 mL of 0.5 mg/mL conjugated monoclonal antibody.
- DAPI, 100 mL (90229). One vial containing 100 mL volume.

## Materials Required (Not supplied)

- Tissue culture-wares and supplies
- Fixative (e.g., 4% Paraformaldehyde in 1X PBS)
- Millicell® EZ SLIDE 8-well glass, sterile (PEZGS0896)
- Phosphate-Buffered Saline (1X PBS) (BSS-1005-B)
- 1X Rinse Buffer (e.g., TBST: 20 mM Tris-HCL, pH 7.4, 0.15M NaCl, 0.05% Tween® 20)
- Blocking Solution (3% normal goat or donkey serum, 0.2% Trtion™ X-100, 0.05% $NaN_3$ in 1X PBS)
- Non-Permeable Blocking Solution (3% normal goat or donkey serum in 1X PBS)
- Anti-fading mounting solution (DABCO®/PVA)
- Microscope

## Storage and Stability

The Fluorescent Human ES/iPS Cell Characterization Kit contains two components used for alkaline phosphatase activity determination as well as 5 ES cell-specific antibodies and a nuclear staining dye. When stored at 2 to 8 °C, the kit components are good for 4 months from date of receipt. Do not freeze or expose to elevated temperatures.

## Protocol

### Preparation of Reagents

**Naphthol/Fast Red Violet Solution:** Mix Fast Red Violet (FRV) with Naphthol AS-BI phosphate solution (2 mg/mL) in a buffer, pH 8.5 in a 1:1 ratio (FRV:Naphthol) fresh before each staining assay.

## Staining Protocol

**Alkaline Phosphatase Staining Procedure**

1. Culture human ES/iPS cells for three to five days prior to analyzing AP activity.

   **Note:** This time-period is critical to be able to observe good levels of AP activity.
2. Aspirate the media and fix the human ES/iPS cells with a fixative (e.g., 4% paraformaldehyde in 1X PBS) for 1-2 minutes.

   **Note:** Do not over fix. Fixing cells longer than 2 minutes will result in the inactivation of alkaline phosphatase.
3. Aspirate the fixative and rinse with 1X Rinse Buffer. DO NOT allow the cells to dry.
4. Prepare reagents for Alkaline Phosphatase staining as described in "Preparation of Reagents" section.
5. Add enough stain solution to cover each well (e.g., 2 mL for a well of a 6-well plate). Incubate in the dark at room temperature for 15 minutes.
6. Aspirate the staining solution and rinse the wells with 1X Rinse Buffer. Cover the cells with 1X PBS to prevent drying and then count the number of colonies expressing AP (red stem cell colonies), versus the number of differentiated colonies (colorless).

   **AP staining criteria:** Greater than 90% of colonies should remain undifferentiated and express alkaline phosphatase.

**Immunofluorescent Staining Procedure**

For optimal results, cell staining should be performed on cell colonies that have been in culture for approximately 3-5 days after passaging. TRA-1-60 Cy3 and TRA-1-81 Cy3 conjugates can be used to stain live human ES/hiPS cells. In the case of live staining, skip steps 4-6 and go directly to step 7.

**Note:** Do not add $NaN_3$ to the blocking solution for live cell staining.

1. Culture human ES or iPS cells in a 6-well plate in human ESC expansion media of choice. The staining protocol will work similarly using feeder or feeder-free media systems so please follow the manufacturer's instructions for specific media.
2. Remove the media from the wells. Be careful to not aspirate the cells.
3. Rinse once with 1X PBS then aspirate.
4. Add 4% Paraformaldehyde (PFA, diluted in 1X PBS) to each well. Incubate for 15-30 minutes at room temperature.
5. Carefully aspirate the PFA from the wells. Be careful to not aspirate the cells.
6. Wash three times with 1X PBS (~2-3 minutes per wash). At this point the fixed cells can be stored in 1X PBS at 4 °C for a couple of weeks if necessary.
7. Aspirate the 1X PBS. Apply a blocking solution for 30-60 minutes at room temperature or overnight at 4 °C.

   **Important:** Do not shake the cells. For optimal results, use the Blocking Solution (3% Normal Goat or Donkey Serum, 0.2% Triton X-100, and 0.05% $NaN_3$ in 1X PBS) with antibodies directed against intracellular gene targets, Oct-4, Sox-2, and Nanog. Use the Non-Permeable Blocking Solution (3% Normal Goat or Donkey Serum in 1X PBS) with antibodies directed against cell surface epitopes TRA-1-60 and TRA-1-81.
8. Before the end of the incubation time, prepare 1:100 dilutions of the conjugated antibodies in the appropriate blocking buffer (protected from light).
9. Aspirate the blocking buffer. Be careful to not aspirate the cells.
10. Add the 1:100 diluted antibodies to the designated well(s). Incubate for 1-2 hours at room temperature. Cover the plate(s) with tin foil to protect from the light.
11. Aspirate to remove the antibodies. Be careful to not aspirate the cells.
12. Wash three times with 1X PBS (3-4 minutes per wash).
13. Prepare the DAPI dye. Dilute the DAPI in 1X PBS at 1:1000 dilution.
14. Remove the last wash, add DAPI staining solution and incubate at room temperature for 5-10 minutes.

16. Remove the DAPI solution; wash three times with 1X PBS (3-4 minutes per wash).
17. If cell staining is on plates, cells should be covered with 1X PBS for visualization. However, if using glass coverslips, mount the coverslip onto glass slides using anti-fading mounting solution (e.g., DABCO®/PVA).
18. Visualize the cell staining using a fluorescence microscope.

    **Note:** Be sure to use the correct filter when visualizing fluorescent-labeled cells.

## Product Performance

### Result

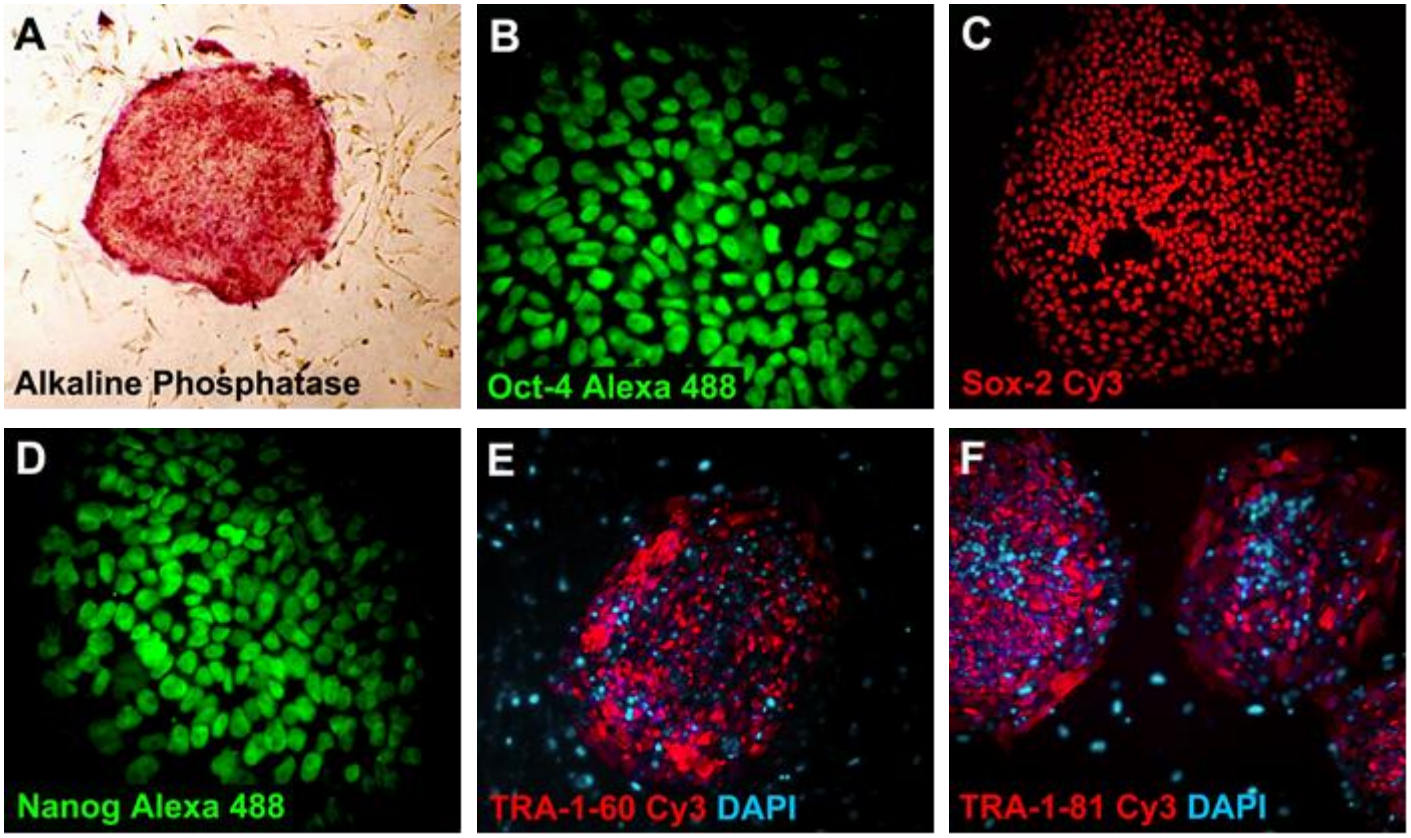


**Figure 1:** (**A**) Pluripotent hES/iPS cells express pluripotent markers, alkaline phosphatase (40x), (**B**) Oct-4 Alexa Fluor® 488 (400x), (**C**) Sox-2 Cy3 (100x), (**D**) Nanog Alexa Fluor® 488 (400x), (**E**) TRA-1-60 Cy3 (100x), and (**F**) TRA-1-81-Cy3 (100x). All conjugated antibodies were used at 1:100 dilutions. Nuclei were counterstained with DAPI (blue). Human foreskin fibroblasts were reprogrammed using the Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (SCR545).

## Related Products

The following related products are available on our website as separate items:

- ES Cell Characterization Kit (SCR001)
- ES Cell Marker Sample Kit (SCR002)
- Alkaline Phosphatase Detection Kit (SCR004).
- Quantitative Alkaline Phosphatase ES Characterization Kit (SCR066)
- Anti-OCT-4 [POU5F1], clone 7F9.2, Alexa Fluor® 488 conjugate, 100 mL (MAB4419A4)
- Anti-OCT-4 [POU5F1], clone 7F9.2, Cy3 conjugate, 100 mL (MAB4419C3)
- Anti-OCT-4, clone 10H11.2, Alexa Fluor® 488 conjugate, 100 µL (MAB4401A4)
- Anti-OCT-4, clone 10H11.2, Cy3 conjugate, 100 µL (MAB4401C3)
- Anti-NANOG, clone 7F7.1, Alexa Fluor® 488 conjugate, 100 mL (MABD24A4)
- Anti-NANOG, clone 7F7.1, Cy3 conjugate, 100 mL (MABD24C3)
- Anti-TRA-1-60, clone TRA-1-60, Cy3 conjugate, 100 mL (MAB4360C3)
- Anti-TRA-1-60, clone TRA-1-60, Alexa Fluor® 488 conjugate, 100 mL (MAB4360A4)
- Anti-TRA-1-81, clone TRA-1-81, Cy3 conjugate, 100 mL (MAB4381C3)
- Anti-TRA-1-81, clone TRA-1-81, Alexa Fluor® 488 conjugate, 100 mL (MAB4381A4)
- Anti-Sox-2, clone 10H9.1, Cy3 conjugate, 100 mL (MAB4423C3)
- Anti-Sox-2, clone 10H9.1, Alexa Fluor® 488 conjugate, 100 mL (MAB4423A4)

## References


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