Human Embryonic Stem Cell Starter Panel

Catalog Number SC007

Reagents for the expansion of human embryonic stem cells.

This package insert must be read in its entirety before using this product.

FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
THE SAFETY AND EFFICACY OF THIS PRODUCT IN DIAGNOSTIC
OR OTHER CLINICAL USES HAS NOT BEEN ESTABLISHED.

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INTRODUCTION

Embryonic stem (ES) cells, derived from the inner cell mass of pre-implantation embryos, have been recognized as the most pluripotent stem cell population (1, 2). Human ES cells can be maintained and propagated on mouse fibroblast feeders for extended periods in media containing **fibroblast growth factor basic** (FGF-basic) (3). While the undifferentiated/ pluripotent state of ES cells can be best defined functionally, a good number of molecular markers have been used to characterize it.

Oct-3/4 (also termed Oct-3 or Oct-4), a member of the POU transcription factor family, was originally identified as a DNA-binding protein that activates gene transcription via a cis-element containing octamer motif (4). It is expressed in totipotent embryonic stem and germ cells (5, 6). A critical amount of Oct-3/4 expression is required to sustain stem cell self-renewal and pluripotency (7). When embryonic stem cells are induced to differentiate, Oct-3/4 is downregulated, which is essential for a proper and divergent developmental program (8). Therefore, the Oct-3/4 molecule is not only a master regulator of pluripotency that controls lineage commitment, but also is the first, most recognized marker used for the identification of totipotent embryonic stem cells.

SSEAs (Stage Specific Embryonic Antigens) were originally identified by three monoclonal antibodies (Abs) recognizing defined carbohydrate epitopes associated with lacto- and globo-series glycolipids that constitute the SSEA-1, -3 and -4 (9). SSEA-1 is expressed on the surface of preimplantation stage murine embryos at the eight cell stage and has been found on the surface of teratocarcinoma stem cells but not on their differentiated derivatives (10, 11). SSEA-3 and -4 are synthesized during oogenesis and are present in the membranes of oocytes, zygotes and early cleavage stage embryos (12, 13). Biological roles of these carbohydrate-associated molecules have been suggested in controlling cell surface interactions during development (9). Undifferentiated primate ES cells, human ES cells and human embryonic carcinoma (EC) cells express SSEA-3 and SSEA-4, but not SSEA-1. Undifferentiated mouse ES cells, however, do express SSEA-1 but do not express SSEA-3 or SSEA-4 (3, 14).

Alkaline Phosphatase is an enzyme in the blood, intestines, liver, and bone cells and exists as membrane-bound isoforms of glycoproteins sharing a common protein structure but differing in carbohydrate content. These enzymes are most active at alkaline pH - hence the name (15). Undifferentiated human EC, ES and embryonic germ (EG) cells have been shown to express a very high level of the liver/bone/kidney isozyme of alkaline phosphatase (16 - 18). Expression levels of alkaline phosphatase decrease following stem cell differentiation.

DESIGN OF THE PANEL

The Human Embryonic Stem Cell Starter Panel is designed for users who are interested in *in vitro* expansion of human ES cells. The panel contains human FGF-basic, a growth factor used in human ES cell culture and a group of antibodies; anti-Oct-3/4, anti-SSEA-4 and anti-alkaline phosphatase for monitoring the differentiation status of human ES cells by analyzing ES cell marker expressions.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- The safety and efficacy of this product in diagnostic or other clinical uses have not been established.

REAGENTS

Human FGF basic (Part # 962646) - 10 μg of lyophilized recombinant human fibroblast growth factor (FGF) basic, enough to make 2 liters of human ES cell culture media at the concentration of 5 ng/mL.

Goat anti-Oct-3/4 (Part # 962649) - 25 μg of lyophilized goat anti-Oct-3/4 polyclonal antibody, enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 μg/100 μL.

Mouse anti-SSEA-4 (clone MC-813-70, Isotype mouse IgG_3, Part # 962648) - 25 μg of lyophilized mouse anti-SSEA-4 monoclonal antibody, enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 μg/100 μL.

Mouse anti-liver/bone/kidney alkaline phosphatase (clone B4-78, isotype mouse IgG_1, Part # 962647) - 25 μg of lyophilized mouse anti-alkaline phosphatase monoclonal antibody, enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 μg/100 μL.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Use within 1 year of receipt.</td></tr>
<tr><td rowspan="4">Opened Reagents</td><td>Human FGF basic</td><td rowspan="4">Aliquot and store at ≤ -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Goat anti-Oct-3/4</td></tr>
<tr><td>Mouse anti-SSEA-4</td></tr>
<tr><td>Mouse anti-alkaline phosphatase</td></tr>
</table>

OTHER SUPPLIES REQUIRED

- FACS Buffer - 2% fetal bovine serum, 0.1% sodium azide in Hank's buffer
- SAP Buffer - 2% fetal bovine serum, 0.5% saponin, 0.1% sodium azide in PBS
- 4% paraformaldehyde in PBS
- 8% paraformaldehyde in PBS
- 1% BSA in PBS
- 0.1% Triton X-100, 1% BSA, 10% normal donkey serum in PBS
- 1% BSA, 10% normal donkey serum in PBS
- Secondary developing reagents
- Fluorescence microscope
- Sterile PBS

REAGENT AND MATERIAL PREPARATION

Use serological pipettes to transfer and remove solutions.

FGF basic Stock (1000X) - Reconstitute human FGF basic with 2 mL of sterile PBS containing 0.1% BSA. Mix gently. Aliquot and store at ≤ -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

Goat anti-Oct-3/4 (10X) - Reconstitute the goat anti-Oct-3/4 with 250 μL of sterile PBS. Mix gently. Aliquot and store at ≤ -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

Mouse anti-SSEA-4 (10X) - Reconstitute the mouse anti-SSEA-4 with 250 μL of sterile PBS. Mix gently. Aliquot and store at ≤ -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

Mouse anti-alkaline phosphatase (10X) - Reconstitute the mouse anti-alkaline phosphatase with 250 μL of sterile PBS. Mix gently. Aliquot and store at ≤ -20° C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

PROCEDURE

Use serological pipettes to transfer and remove solutions.

Usage of FGF-basic for human ES cell expansion in culture

Embryonic stem cells are pluripotent cell lines that are derived from the blastocyst-stage early mammalian embryo. These unique cells are characterized by their capacity for prolonged undifferentiated proliferation in culture while maintaining the potential to differentiate into derivatives of all three germ layers. FGF-basic is required for undifferentiated proliferation of human ES cells in culture and is suggested to be used at the concentration of 5 μg/mL.

Please see the following references for the methods described in detail:

Thomson, J.A. *et al*. (1998) Science. **282**:1145.
Amit, M. *et al.* (2000) Dev. Biol. **227**:271.
Laslett, A.L. *et al.* (2003) Trends Cardiovasc. Med.**13**:295
Xu, C. *et al.* (2001) Nat. Biotechnol. **19**:971.

Surface Marker Analysis of SSEA-4 and Alkaline Phosphatase by Flow Cytometry

1. Resuspend the cells in FACS buffer at the concentration of 1 x 10^6 cells/mL.
2. Transfer 90 μL of cell suspension into a 5 mL tube. Add 10 μL of either anti-SSEA-4 or anti-alkaline phosphatase.
3. Incubate for 30 minutes at 2 - 8° C.
4. Following incubation, wash the sample twice in 3 mL of FACS buffer.
5. Resuspend the cells in 200 μL of FACS buffer and add stock solution of a secondary developing reagent such as goat anti-mouse IgG conjugated to a fluorochrome [*e.g.* PE-conjugated goat anti-mouse IgG secondary antibody (Caltaq, Catalog # M35004-3)] according to the manufacturer's suggestion.
6. Incubate for 30 minutes at 2 - 8° C **in the dark.**
7. Following incubation, wash the sample twice in 3 mL of FACS buffer.
8. Resuspend the cells in 400 μL of FACS buffer for flow cytometric analysis.

   Note: *As a control for analysis, cells in a separate tube should be treated with mouse IgG_3 control for anti-SSEA-4 or mouse IgG_1 isotype control for anti-alkaline phosphatase antibody. Negative control using secondary developing reagent only is an alternative when isotype control gives high background.*

Intracellular Marker Analysis of Oct-3/4 by Flow Cytometry

A. Fixation of cells prior to intracellular staining

1. Wash the cells twice in PBS. Resuspend in PBS at the concentration of 1 x 10^6 cells/mL.
2. For each staining, transfer 100 μL of cell suspension to a 5 mL tube and add 400 μL of sterile PBS.
3. Add 500 μL of 8% paraformaldehyde (prepared in PBS) to the cell sample. Incubate for 10 minutes at room temperature.
4. Wash the cells twice with sterile PBS.

B. Intracellular staining

1. Wash the cells once with SAP buffer.
2. Resuspend the cells in 90 μL of SAP buffer and add 10 μL of goat anti-Oct-3/4.
3. Incubate the staining sample for 30 minutes at 2 - 8° C.
4. Following incubation, remove the unreacted antibody by washing the sample twice in 3 mL of SAP buffer.
5. Resuspend the cells in 200 μL of SAP buffer and add stock solution of a secondary developing reagent such as PE-conjugated swine anti-goat (Caltaq, Catalog # G50004).
6. Incubate the sample for 30 minutes at 2 - 8° C **in the dark.**
7. Following incubation, wash the sample twice in 3 mL of FACS buffer.
8. Resuspend the cells in 400 μL of FACS buffer for flow cytometric analysis.

Note: *As a negative control for analysis, cells in a separate tube should be treated with swine anti-goat conjugated to a fluorochrome only.*

Immunocytochemistry of Oct-3/4

Note: *This protocol of immunocytochemistry is for cells grown in a 24-well tissue culture plate.*

1. Wash the cells twice with 1 mL of PBS.
2. Fix the cells with 0.5 mL of 4% paraformaldehyde (prepared in PBS) for 20 minutes at room temperature.
3. Wash the cells twice with 1 mL of PBS for 5 minutes.
4. Permeabilize and block the cells with 0.5 mL of 0.1% Triton X-100, 1% BSA, 10% normal donkey serum in PBS at room temperature for 45 minutes.
5. During the blocking, prepare the goat anti-Oct-3/4 antibody working solution by diluting the 10X stock with PBS containing 1% BSA, 10% normal donkey serum to a final concentration of 10 μg/mL.

   Note: *For staining 10 sample wells at 300 μL per well, add 30 μL of goat anti-Oct-3/4 antibody (10X) into 2.7 mL of PBS containing 1% BSA, 10% normal donkey serum.*
6. After blocking, incubate the cells with 300 μL/well of goat anti-Oct-3/4 antibody working solution overnight at 2 - 8° C.
7. Wash the cells three times with 1 mL of PBS containing 1% BSA for 5 minutes.
8. Dilute the secondary antibody [*e.g.* Rhodamine Red-conjugated donkey anti-goat secondary antibody (Jackson Immunoresearch, Catalog # 705-026-147)] according to the manufacturer's suggestion in PBS containing 1% BSA. Incubate the cells with secondary antibody at 300 μL per well for 60 minutes at room temperature **in the dark.**
9. Wash the cells three times with 1 mL of PBS containing 1% BSA for 5 minutes.
10. Cover the cells with 1 mL of PBS and visualize with a fluorescence microscope.

Immunocytochemistry of SSEA-4 and Alkaline Phosphatase

Note: *This protocol of immunocytochemistry is for cells grown in a 24-well tissue culture plate.*

1. Wash the cells twice with 1 mL of PBS.
2. Fix the cells with 0.5 mL of 4% paraformaldehyde (prepared in PBS) for 20 minutes at room temperature.
3. Wash the cells twice with 1 mL of PBS for 5 minutes.
4. Block the cells with 0.5 mL of 0.1% BSA, 10% normal donkey serum in PBS at room temperature for 45 minutes.
5. During the blocking, prepare the primary antibody working solution by diluting the 10X stock with PBS containing 1% BSA and 10% normal donkey serum to a final concentration of 10 μg/mL.

   Note: *For staining 10 sample wells at 300 μL per well, add 30 μL of anti-SSEA-4 (10X) or anti-alkaline phosphatase antibody (10X) into 2.7 mL of PBS containing 1% BSA and 10% normal donkey serum.*
6. After blocking, incubate the cells with 300 μL/well of primary antibody working solution overnight at 2 - 8° C.
7. Wash the cells three times with 1 mL of PBS containing 1% BSA for 5 minutes.
8. Dilute the secondary antibody [*e.g.* Rhodamine Red-conjugated donkey anti-mouse IgG secondary antibody (Jackson Immunoresearch, Catalog # 715-295-150)] according to the manufacturer's suggestion in PBS containing 1% BSA. Incubate the cells with secondary antibody at 300 μL per well for 60 minutes at room temperature **in the dark.**
9. Wash the cells three times with 1 mL of PBS containing 1% BSA for 5 minutes.
10. Cover the cells with 1 mL of PBS and visualize with a fluorescence microscope.

FIGURES AND IMAGES OF EMBRYONIC STEM CELL STAINING

Courtesy of Dr. Jong-Hoon Kim and Dr. Ron McKay from the National Institute of Neurological Disorders and Stroke & Stem Cell Unit at NIH.

Immunocytochemistry

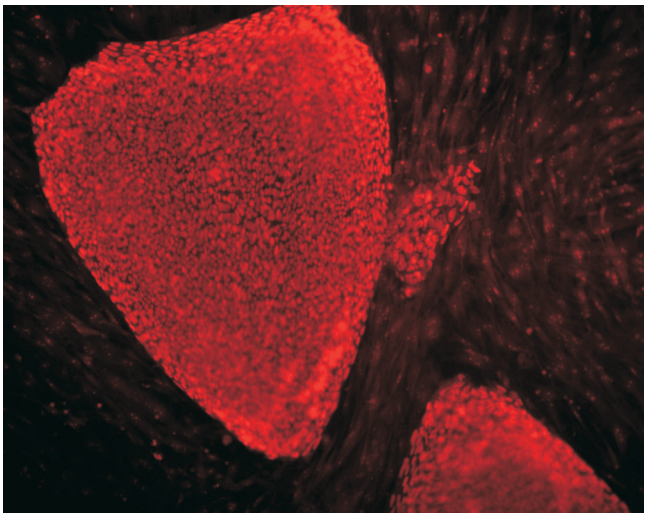

Figure 1A: Detection of Oct-3/4 in human embryonic stem cells using R&D Systems' goat anti-Oct-3/4 affinity-purified polyclonal antibody (Catalog # AF1759). Cells were stained using a Rhodamine Red-conjugated donkey anti-goat IgG secondary antibody.

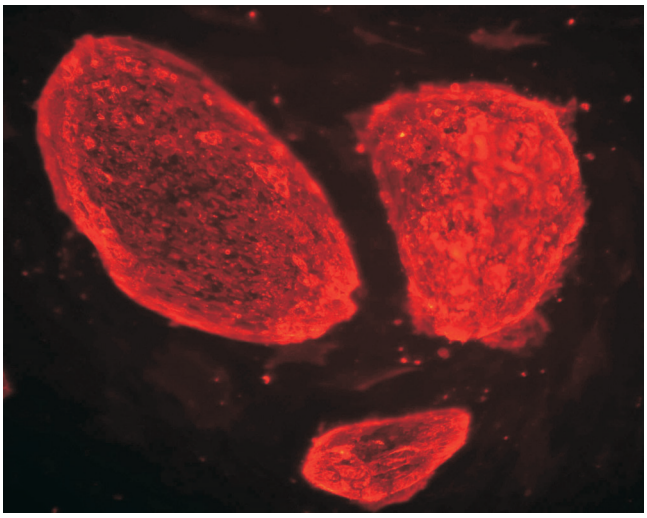

Figure 1B: Detection of SSEA-4 in human embryonic stem cells using R&D Systems' mouse anti-SSEA-4 monoclonal antibody (Catalog # MAB1435). Cells were stained using a Rhodamine Red-conjugated donkey anti-mouse IgG secondary antibody.

Flow Cytometry

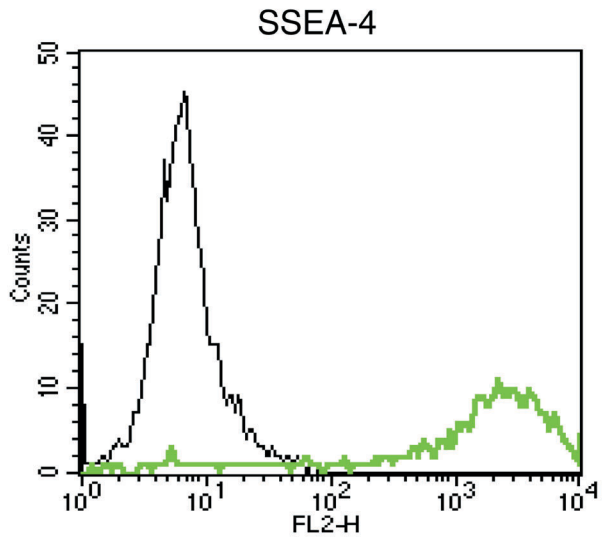

Figure 2A: Reactivity of human embryonic stem cells stained with mouse anti-SSEA-4 monoclonal antibody (Catalog # MAB1435) (right) or secondary negative only control (left). Cells were stained using a PE-conjugated goat anti-mouse IgG secondary antibody.

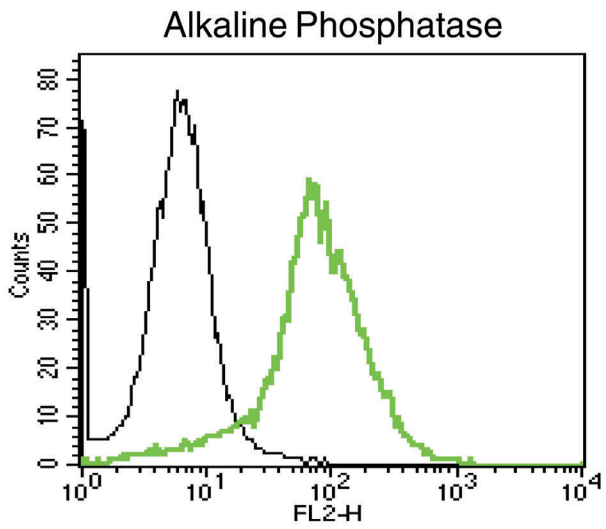

Figure 2B: Reactivity of human embryonic stem cells stained with mouse anti-Alkaline Phosphatase monoclonal antibody (Catalog # MAB1448) (right) or isotype control (left). Cells were stained using a goat anti-mouse IgG secondary antibody.

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NOTES