Application Note

Multiplex pluripotency analysis of STEMCCA™ vector-reprogrammed iPS cells using MILLIPLEX® MAP magnetic bead assays

Introduction

Due to their potential to differentiate into a wide range of specialized cells, induced pluripotent stem cells (iPSC), as well as human embryonic stem cells (hESC), are of great interest for regenerative medicine and drug discovery. For the application of both of these types of stem cells in research, it is crucial to characterize the identity and potency status of pluripotent stem cells by measuring molecular markers during cultivation. Despite the many phenotypic, morphological, functional and molecular similarities between hESC and iPSC, it has become clear that the differences may be equally numerous[1]. These differences (in epigenetic markers, expression of somatic genes, specific responses to cell fate modulators and more) can be understood only by profiling large numbers of stem cell biomarkers to gain more accurate, "higher resolution" snapshots of stem cells[1].

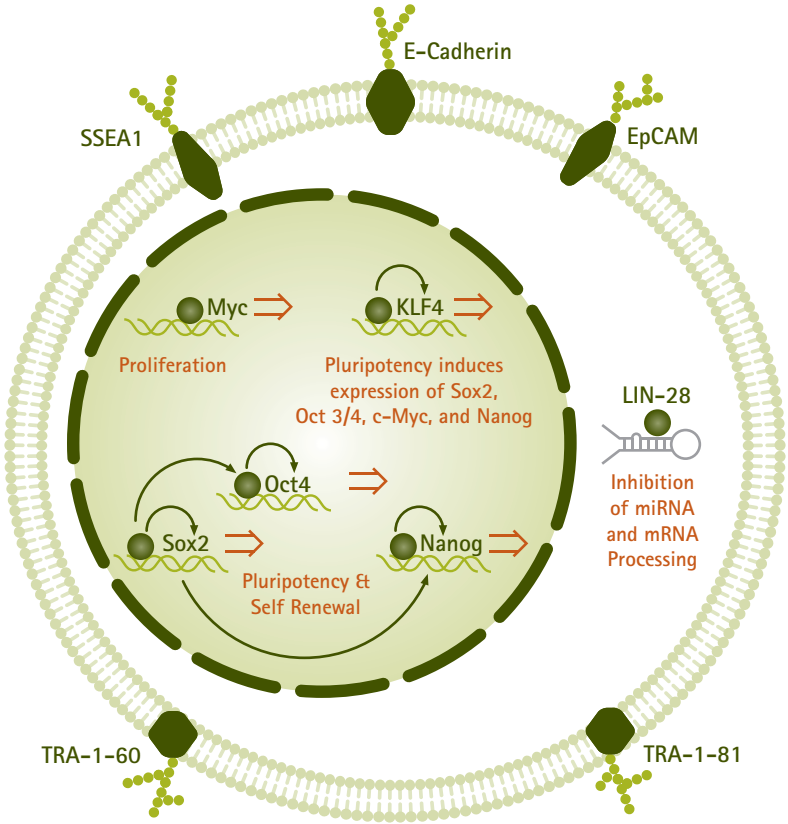
Commonly, flow cytometry and PCR techniques are used to observe the expression of pluripotency markers, and several fluorescently labeled antibodies are commercially available for immunofluorescence, immunocytochemistry and flow cytometry studies. In this study, we describe novel MILLIPLEX® MAP multiplexed bead-based immunoassays, which use Luminex® xMAP® technology to provide a complementary assay to flow cytometry for quickly assessing the potency status of a cell population. This method is a quick, simple and accurate way to detect a large number of pluripotency markers in a single measurement, providing fast feedback on the potency status of a cell culture by measuring protein expression levels in cell lysates. Quantitative RT-PCR has proven to be a robust method for quantitating RNA levels corresponding to pluripotency genes, and multiplex RT-PCR kits (such as Merck Millipore's STEMCCA™ Gene Detection Kits) are available for assessing the expression of multiple pluripotency genes per sample. However, given that mRNA levels do not necessarily correlate with protein levels, a complete picture of pluripotency can only be obtained by measuring numerous protein, as well as nucleic acid, stem cell markers.

We demonstrate the use of MILLIPLEX® MAP magnetic bead-based multianalyte assay panels to examine different sets of protein biomarkers that indicate the potency of stem cells to quickly and accurately distinguish pluripotent cells from multipotent or differentiating cells. We first generated iPSCs from human foreskin fibroblasts using a polycistronic, excisable lentiviral vector, and then monitored the resulting culture using the MILLIPLEX® MAP panels. Expression of Oct 3/4, Sox2 and Nanog as well as the presence of E-Cadherin, EpCam, KLF4, LIN-28, TRA-1-60 and TRA-1-81 could be observed after reprogramming, indicating that the examined cell culture was indeed pluripotent.

Materials

MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kit 1 (Catalogue No. 48-617MAG)

This 4-plex panel monitors the expression of the transcription factors Oct 3/4, Sox2, Nanog and c-Myc (Figure 1). These proteins play critical roles in the maintenance of pluripotency and self renewal in stem cells. Octamer 4 (Oct4), a member of the POU family of transcription factors, has been demonstrated to be vital for the formation of self-renewing pluripotent stem cells[2]. Sox2, also known as SRY-related HMG BOX gene 2, binds linear DNA in a sequence-specific manner and has been shown to help stem cells maintain pluripotency[3]. Nanog is a member of the Nanog homeobox family of DNA binding proteins. It is expressed in embryonic stem cells conferring pluripotency to these cells and is suppressed upon embryonic stem cell differentiation[4]. c-Myc (bHLHe39), a member of the helix-loop-helix-leucine zipper (bHLH-LZ) family of transcription factors, is involved in apoptosis, cell differentiation and cell proliferation[5]. In this kit, c-Myc serves as an internal control, since it is expressed in many cell types.



MILLIPLEX® MAP Human Stem Cell Pluripotency Kit 1
Oct 3/4
Sox2
Nanog
c-Myc

MILLIPLEX® MAP Human Stem Cell Pluripotency Kit 2
E-Cadherin
EpCam
KLF4
LIN-28
SSEA1
TRA-1-60
TRA-1-81

Figure 1.

Stem cell pluripotency markers. Analytes detected by the MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kits 1 and 2.

MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kit 2 (Catalogue No. 48-620MAG)

This 7-plex panel measures the concentrations of additional pluripotency-related markers (Figure 1), including a transcription factor, a translational regulator, transmembrane proteins and cell surface glycostructures. The zinc finger-containing transcription factor, KLF4, has regulatory functions in cell growth, proliferation, differentiation and embryogenesis. It is one of the factors for iPSC reprogramming used by Takahashi and Yamanaka in 2006[6]. The translational enhancer, LIN-28, contributes to stem cell pluripotency by possibly blocking miRNA-mediated differentiation[7]. The transmembrane proteins, E-Cadherin and EpCam, are adhesion molecules. E-Cadherin is a calcium-dependent transmembrane protein influencing pluripotency and self-renewal signaling pathways in ES and iPS cells[8]. EpCam is a hemophilic, calcium-independent adhesion molecule that is expressed in undifferentiated hESCs and is localized to Oct4-positive pluripotent cells[9,10]. TRA-1-60 and TRA-1-81 are glycan modifications of the membrane protein podocalyxin and are expressed on ES cells[11,12]. Finally, SSEA1 is the stage-specific embryonic antigen-1 that is expressed in mouse ES cells[13] but repressed in human ES cells[14], therefore serving as a negative control in the kit.

Cells and Reprogramming Reagents

IPS cells were generated from FibroGro™ Xeno-Free Human Foreskin Fibroblasts (passage 6 HFF, Catalogue No. SCC058) using the STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Catalogue No. SCR511). STEMCCA™ transgenes were excised from iPS cells using a cell-permeable TAT-Cre Recombinase (Catalogue No. SCR508).

Methods

Tissue Culture

MCF-7, Ntera-2cl.D1, HeLa and Jurkat cells were cultured according to ATCC® guidelines in recommended media. Cells were grown to approximately 85% confluence. HFF cells were cultured in FibroGRO™ LS Complete Medium (Catalogue No. SCMF002) and reprogrammed according to the STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Catalogue No. SCR511) protocol. iPS cells were cultured in HEScGRO® Medium for Human ES Cell Culture.

Sample Preparation

Cells were lysed and samples collected according to the protocols for the MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kits 1 and 2.

Microspheres

We developed the MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kits 1 and 2 by conjugating specific capture antibodies to MagPlex® microsphere beads purchased from Luminex Corporation. Each set of beads is distinguished by different ratios of two internal dyes, yielding a unique fluorescent signature to each bead set.

MILLIPLEX® MAP Assay Protocol

The multiplex assay was performed in a 96-well plate according to product instructions supplied for the MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kits 1 and 2 (Catalogue Nos. 48-617MAG and 48-620MAG). Each plate was first rinsed with 50 µL assay buffer. Controls and samples were diluted 1:1 in Assay Buffer and 10 µg of total protein (25 µL) and 25 µL beads were added to each well. Plates were incubated overnight at 4 °C (alternatively, plates can be incubated 2 hours at room temperature). Beads were washed twice with 100 µL assay buffer, and then incubated for 1 hour at room temperature (RT) with biotinylated detection antibody cocktail. The detection antibody cocktail was replaced with 25 µL streptavidin-phycoerythrin (SAPE) and incubated for 15 minutes at RT. 25 µL of amplification buffer was added and incubated for another 15 minutes at RT. Finally, the SAPE/amplification buffer was removed and beads were resuspended in 150 µL assay buffer. The assay plate was read and analyzed in a Luminex 200™ system (Catalogue No. 40-013). This is a compact unit consisting of an analyzer, a computer and software.

Immunoprecipitation and Western Blotting

MILLIPLEX® MAP capture beads were incubated with 100 µg total protein lysate diluted 1:3 with assay buffer at 4 °C overnight on an orbital shaker. Beads were separated from the lysate sample and washed 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween® 20 using the PureProteome™ magnetic stand (Catalogue No. LSKMAGS08). The beads were then resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Samples were resolved by SDS-PAGE, and the gels were transferred to Immobilon®-FL PVDF membranes (Catalogue No. IPFL00010). These membranes were subsequently blocked and probed with individual biotinylated MILLIPLEX® MAP detection antibodies. Finally, the membrane was incubated with fluorescently labeled streptavidin and the blots were imaged.

Flow Cytometry

Flow cytometry analysis was performed according to the product instructions provided in the FlowCellect® Human iPS Cell Characterization Kit (Catalogue No. FCSC100107). Human iPS stem cell and human foreskin fibroblast cultures were dislodged from a tissue culture plate using a mild enzyme (Accutase™, Catalogue No. SCR005) and placed in suspension in a 96-well plate (100,000 cells per well). Cells were fixed for 20 minutes, followed by a 10 minute permeabilization step. Using a working antibody cocktail solution containing fluorophore-conjugated antibodies to TRA-1-60, Oct4, SSEA1 and SSEA4, cells were stained for 30 minutes on ice in the dark. Following staining, cells were washed once in 1X Assay Buffer and resuspended in 200 mL 1X Assay Buffer in preparation for sample acquisition.

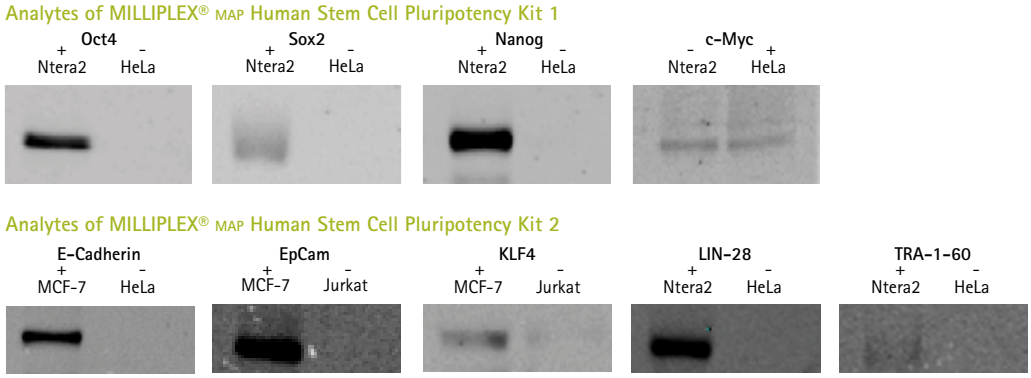
Samples were assayed on the guava easyCyte™ 8HT benchtop flow cytometer (Catalogue No. 0500-4008), and analysis was performed using the InCyte™ software module.

Results and Discussion

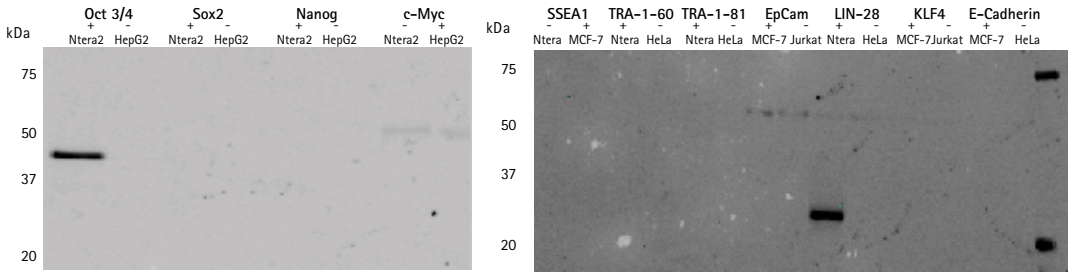
The MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kits 1 and 2 enabled the detection of all of the panel analytes with excellent specificity, sensitivity and precision (Figure 2). The assays provide high specificity, indicated by the detection of proteins at the expected molecular weights as shown by immunoprecipitation/Western blot (Figure 2A).

Specificity was also demonstrated by individual detection of the analytes in immunoprecipitation/Western blot using the capture beads in multiplex (Figure 2B) and individual detection antibodies. In addition, demonstrations of sample linearity (Figure 2C), high signal-to-noise ratios (Figure 2D) and precision lent support to the robustness of this kit.

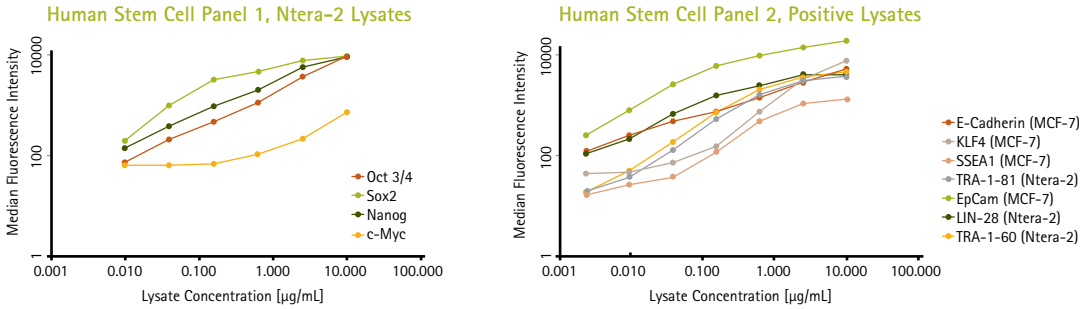
A. IP/Western Blots



B. Cross Reactivity by IP/Western Blots



C. Lysate Titrations



D. Statistics

MILLIPLEX® MAP Human Stem Cell Pluripotency Kit 1

	Intra-assay CV (%) n = 16	Inter-assay CV (%) n = 18	Signal-to-Noise Ratio n = 16
Oct 3/4	3.9	12.4	186
Sox2	2.5	7.4	262
Nanog	3.6	6.2	164
c-Myc	7.9	15.8	94

MILLIPLEX® MAP Human Stem Cell Pluripotency Kit 2

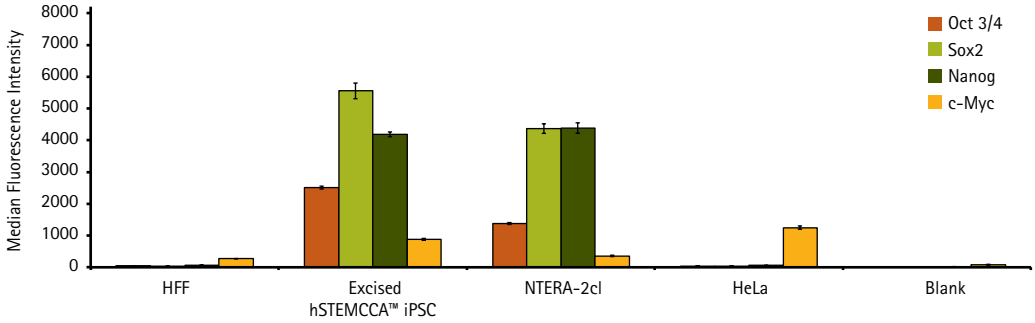
	Intra-assay CV (%) n = 16	Inter-assay CV (%) n = 20	Signal-to-Noise Ratio n = 16
E-Cadherin	4.0	6.8	176
EpCam	3.5	5.9	405
KLF4	4.5	10.7	133
LIN-28	6.5	7.3	194
SSEA1	4.7	9.5	25
TRA-1-60	6.6	7.6	110
TRA-1-81	16.6	15.3	71

Figure 2.

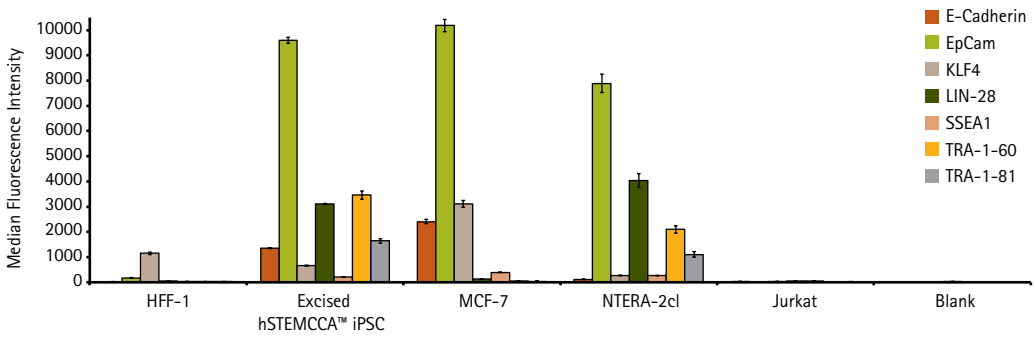
Specificity, sensitivity, and precision of the MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kits 1 and 2.
All analytes were simultaneously detected in multiple cell lines. (A) IP of analytes were performed with capture beads and detected by Western blotting with the biotinylated detection antibodies in control cell lines. (B) Cross-reactivity tests were performed using capture beads in multiplex by IP/Western blotting and individual detection antibodies. As an example, results for Oct 3/4 (45 kDa) and LIN-28 (30 kDa) are shown. (C) Lysate titrations were performed on lysates of Ntera-2cl.D1 and MCF-7 cells. (D) Signal-to-noise ratios were calculated for signals obtained from Ntera-2cl.D1 and MCF-7 cell lysates and background fluorescent signals from assay buffer. Intra- and inter-assay coefficients of variation (CVs) were calculated and reported as percentages.

All pluripotency markers in the MILLIPLEX® MAP Human Stem Cell Pluripotency Magnetic Bead Kits 1 and 2 were detected in iPS cells generated from human foreskin fibroblasts (HFF). Almost all pluripotency marker analytes showed a 57-177 fold increase in expression after reprogramming (Figure 3A, 3B). These data were consistent with the expression profiles of these markers in flow cytometry experiments (Figure 3C). Pluripotency-associated transcription factor KLF4 was expressed in HFF; on the other hand, the negative marker, SSEA1, was expressed in neither HFFs nor in iPSCs. The control marker c-Myc was expressed at detectable levels in all cells.

A. MILLIPLEX® MAP Human Stem Cell Pluripotency Kit 1



B. MILLIPLEX® MAP Human Stem Cell Pluripotency Kit 2



C. FlowCellect® Human iPS Cell Kit

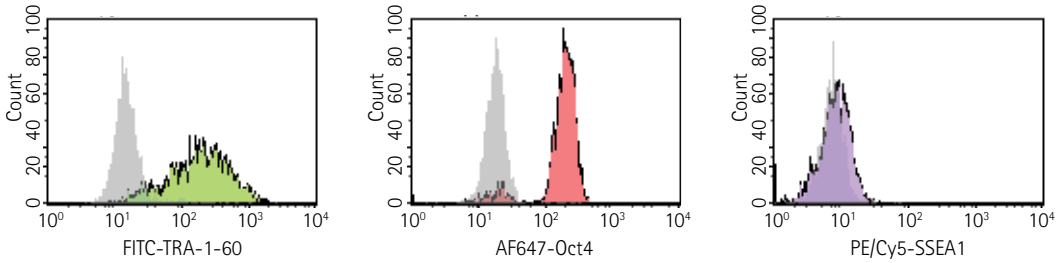


Figure 3.

Human foreskin fibroblasts (HFF P6, Catalogue No. SCC058) were reprogrammed by transduction with the STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Catalogue No. SCR511) followed by excision of viral transgenes using a TAT-Cre Recombinase, resulting in virus-excised hSTEMCCA™ iPSC. (A) HFF and iPS cells were assayed with the 4-plex MILLIPLEX® MAP Human Stem Cell Pluripotency Kit 1 using Ntera-2cl.D1 and HeLa as control cell lines. (B) HFF and iPS cells were also analyzed with the 7-plex MILLIPLEX® MAP Human Stem Cell Pluripotency Kit 2 using Jurkat, MCF-7 and Ntera-2cl.D1 as controls. (C) The markers Oct4, TRA-1-60 and SSEA1 were measured using the FlowCellect® Human iPS Cell Characterization Kit (Catalogue No. FCSC100107). The histograms of the stained HFF control cell lines (grey) were overlaid with the histograms of the stained iPSC generated thereof.

Conclusions and Features of Merck Millipore's MILLIPLEX® MAP Stem Cell Assays

As shown by our pluripotency marker data, the wide number of molecular markers that are needed to characterize the potency of a stem cell may be analyzed by bead-based multiplex assays successfully. Intracellular proteins, transmembrane proteins as well as glycostructures serving as pluripotency markers can be detected in the presented multiplex assays.

Merck Millipore's MILLIPLEX® MAP Stem Cell Assays enable the analysis of a great number of pluripotency markers in a single measurement, saving valuable time and resources, while serving as a fast and reliable complementary method for conventional flow cytometry analyses.

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FibroGRO™-LS Complete Media Kit for Culture of Human Fibroblasts	SCMF002
Accutase™	SCR005
STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	SCR511
TAT-Cre Recombinase	SCR508
FlowCellect® Human iPS Cell Characterization Kit	FCSC100107
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Fluorescent Mouse ES/iPS Cell Characterization Kit	SCR077
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Human STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	SCR545
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Related Products (continued)

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Anti-Oct-4 [POU5F1], clone 7F9.2, Cy3 conjugate	MAB4419C3
Anti-Nanog, clone 7F7.1, Alexa Fluor® 488 conjugate	MABD24A4
Anti-Nanog, clone 7F7.1, Cy3 conjugate	MABD24C3
Anti-Sox2, clone 10H9.1, Alexa Fluor® 488 conjugate	MAB4423A4
Anti-Sox2, clone 10H9.1, Cy3 conjugate	MAB4423C3
Anti-TRA-1-60, clone TRA-1-60, Alexa Fluor® 488 conjugate	MAB4360A4
Anti-TRA-1-60, clone TRA-1-60, Cy3 conjugate	MAB4360C3
Anti-TRA-1-81, clone TRA-1-81, Alexa Fluor® 488 conjugate	MAB4381A4
Anti-TRA-1-81, clone TRA-1-81, Cy3 conjugate	MAB4381C3
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Anti-Nuclei, clone 235-1, Alexa Fluor® 488 conjugate	MAB1281A4
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Anti-BCRP1, clone 5D3, Cy3 conjugate	MAB4155C3
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Anti-Nestin, clone rat-401, Cy3 conjugate	MAB353C3
Anti-Nestin, clone 10C2, Alexa Fluor® 488 conjugate	MAB5326A4
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