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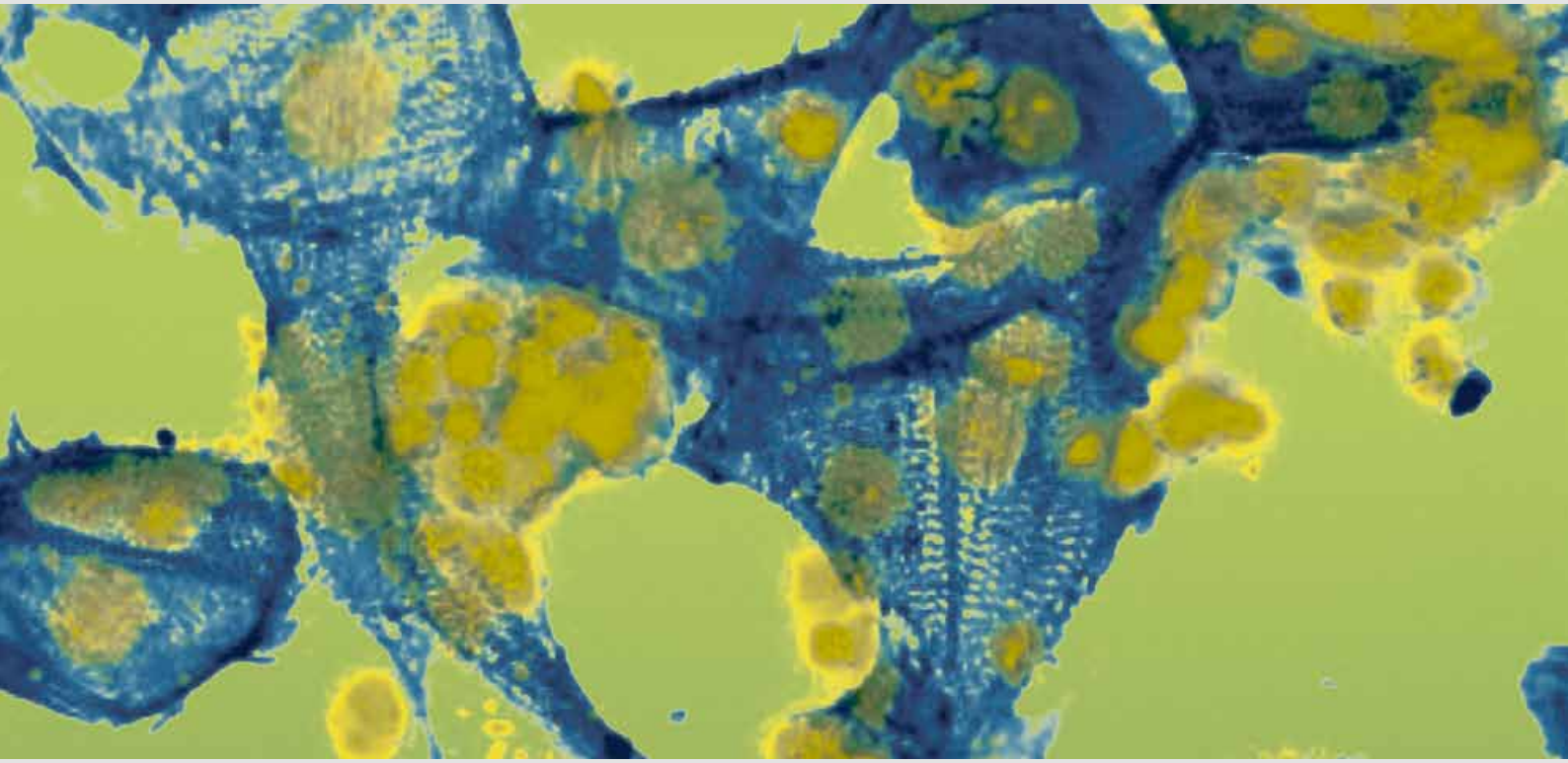
Emily Dick, Elena Matsa, Lorraine Young, and Chris Denning

Wolfson Centre for Stem Cells, Tissue Engineering & Modelling (STEM), Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK.



Isolation of human induced pluripotent stem cell lines



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*not shown

Introduction

Human induced pluripotent stem cells (hiPSCs) can be maintained in an undifferentiated state and, in addition, be differentiated into a variety of cell types as required. Therefore, hiPSC lines derived from patients hold great potential for disease modeling and drug discovery. The generation of hiPSCs is most commonly achieved through transduction of fibroblasts with either retroviruses or adenovirus encoding the pluripotency genes for OCT4 and SOX2, with either LIN28 and NANOG or KLF4 and cMYC. For the establishment of sustainable hiPSC lines, it is necessary to identify and isolate truly reprogrammed iPSCs from the derivation culture. This is a challenging task, since the culture contains a mixture of mouse embryonic fibroblast (mEF) feeder cells, somatic cells, and partially reprogrammed cells, whose morphology is similar to hiPSCs. The manual selection process can be highly subjective, with mechanical dissection and passage being both technically difficult and laborious. To overcome these drawbacks we have developed a positive selection method for the magnetic isolation of true human iPS cells based on the surface expression of the pluripotency marker TRA-1-81. Fully reprogrammed cells are labeled with an anti-TRA-1-81, PE-conjugated antibody. Subsequently, hiPSCs bound with the antibody are magnetically labeled using Anti-PE-MicroBeads for fast and easy isolation by MACS® Technology.

Materials and methods

Culture and transduction of human dermal cell lines

Human dermal fibroblasts from patients with muscular dystrophy (DMD) or Long QT Syndrome type 2 (LQT2) were prepared as described[1,2]. As a control we used an embryonic fibroblast line that was derived from the human embryonic stem cell line HUES7. Transduction was performed as described[1,2].

Positive selection of true hiPSCs

After transduction with reprogramming factors and subsequent culture for 25–30 days, the derivation cell culture was treated with 0.5% trypsin/EDTA, and the entire culture was transferred to a 50 mL tube in approximately 20 mL of culture medium. Cells were pipetted up and down using a 10 mL pipette in order to detach iPS cells from the mEF layer. The cell suspension was then filtered through a 40 µm cell strainer to remove large mEF aggregates. The supernatant was centrifuged at 300×g for 5 minutes and the resulting pellet washed in ice-cold column buffer (Hanks balanced salt solution (HBSS) without calcium or magnesium, containing 2% fetal calf serum (FCS), pH 7.4). Subsequently, the cell pellet was resuspended in 200 µL ice-cold column buffer, containing a PE-conjugated TRA-1-81 antibody (1:200 dilution), and incubated in the dark at 4 °C for 15 minutes. Following the incubation, the cell suspension was washed by adding 5 mL of ice-cold column buffer and centrifuged at 300×g for 5 minutes. The cell pellet was resuspended in 80 µL ice-cold column buffer and 20 µL of superparamagnetic Anti-PE MicroBeads (Miltenyi Biotec), and incubated in the dark at 4 °C for 15 minutes. The cells were washed and centrifuged as described above. An MS Column (Miltenyi Biotec) was placed in the magnetic field of a MiniMACS™ Separator (Miltenyi Biotec) and equilibrated with 500 µL ice-cold column buffer. The cell pellet was suspended in 500 µL ice-cold column buffer and transferred to the column. The magnetically labeled hiPSCs were retained in the column, whereas the non-labeled cells were collected in the flow-through. The column was washed three times with column buffer. The column was then removed from the MiniMACS Separator and the hiPSCs were eluted by inserting the supplied plunger. The hiPSCs were centrifuged at 300×g for 2 minutes, resuspended in mEF-conditioned medium, and transferred to Matrigel™-coated dishes or flasks under feeder-free conditions[3]. An overview of the magnetic cell separation procedure is depicted in figure 1. Flow cytometric analysis of separated cells was performed as described.[1]

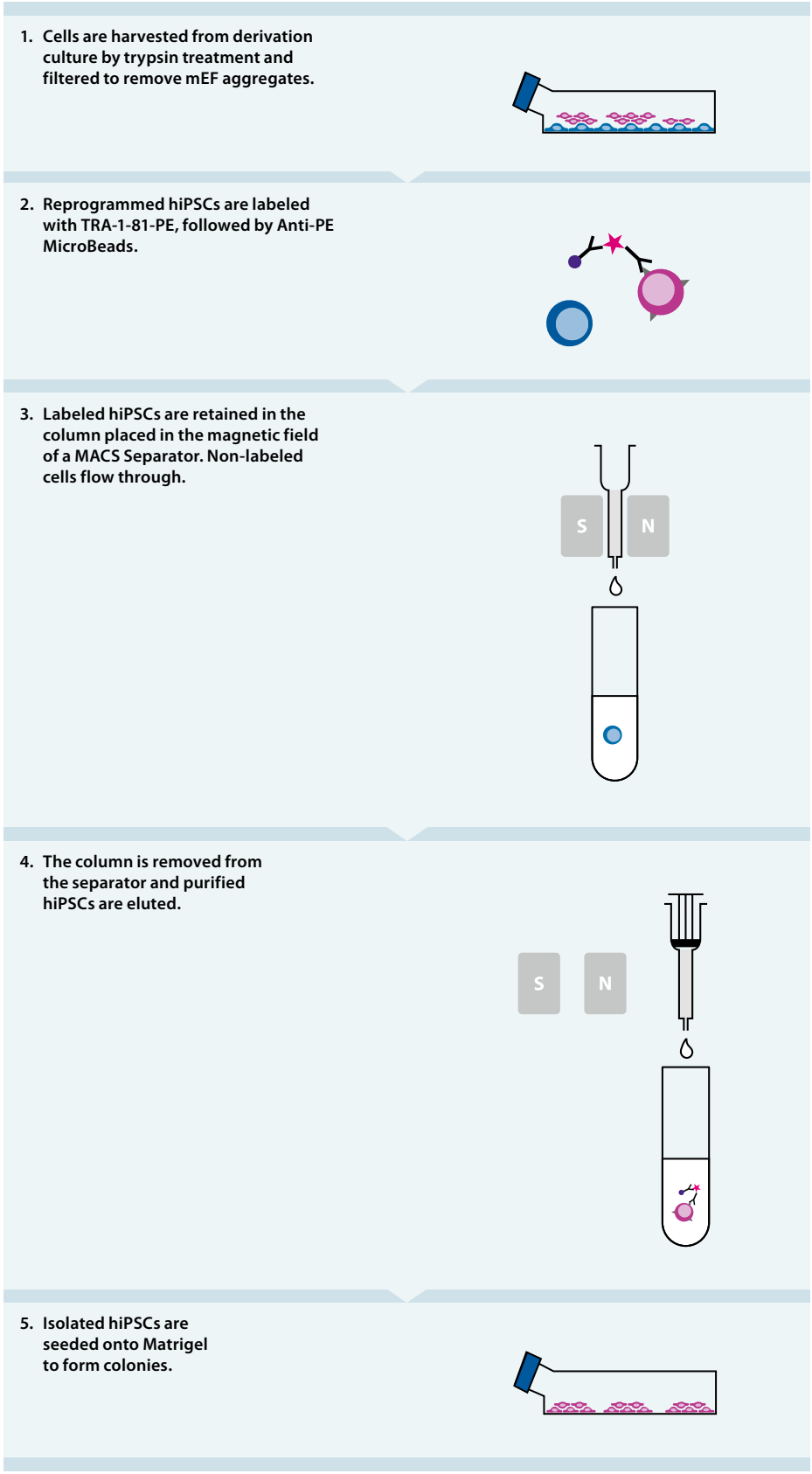

Figure 1 Principle of magnetic iPS cell isolation by MACS Technology.

Miltenyi Biotec now also offers a complete kit (Anti-TRA-1-60 MicroBead Kit, human) for the isolation of hiPSCs based on the expression of TRA-1-60, which is equivalent to TRA-1-81.

Detection of pluripotency marker expression.

Pluripotency marker expression was determined by immunocytochemistry. Cells were grown to confluence in chamber slides (NUNC) and then fixed in 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton®-X-100, and blocked with 8% goat or rabbit serum in PBS, as appropriate. The cells were then incubated with primary antibodies overnight at 4 °C, washed and then incubated with secondary antibodies at room temperature for 1 hour in the dark. Cells were imaged using a Nikon™ Eclipse™ 90i fluorescence microscope.

In vitro differentiation into three germ layers

Pluripotency potential of hiPSCs was determined by differentiation into the three germ layers following embryoid body (EB) formation.[4] At day 30, EBs were fixed in 4% PFA, then subjected to the immunocytochemistry protocol described above using primary antibodies specific for markers representing the three germ layers: β-III-tubulin for ectoderm, α-fetoprotein for endoderm, and α-actinin for mesoderm.

Teratoma formation in NOD/SCID mice

The teratoma forming potential of human iPS cell lines was tested[5]. Briefly, iPS cells were injected into the kidney capsule of NOD/SCID mice. Tumors were collected after 50–60 days, fixed in 4% PFA, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. The three different germ layers were identified using a light microscope.

Results and discussion

Isolation of hiPSCs by MACS® Technology

The magnetic cell separation method is quick and simple: purified cells can be obtained in one hour and many cell lines can be purified in parallel. Magnetic isolation of hiPSCs resulted in a purity of up to 95% TRA-1-81–positive cells (fig. 2C).

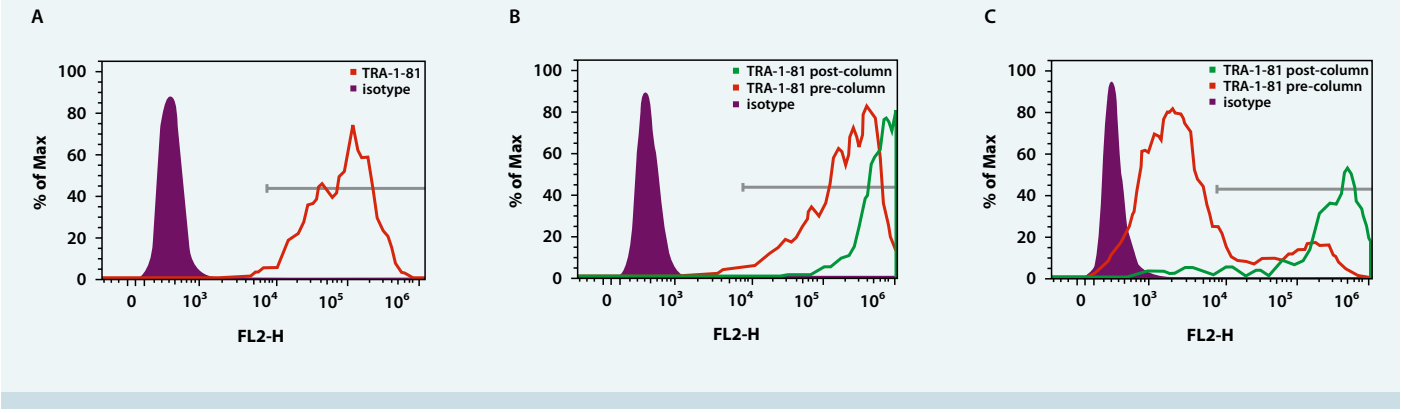

Figure 2 Purification of human iPS cells. Representative flow cytometric analyses of TRA-1-81 expression in (A) the hESC line, HUES7; (B) TRA-1-81^{high} cells (green) enriched from an HUES-iPS cell line (red); (C) TRA-1-81–positive cells (green) enriched from a patient iPS cell line (red) at passage 3 after initial column. Filled histograms denote the isotype control.

Cells isolated by MACS Technology could be directly seeded onto Matrigel™ under feeder-free conditions. The cells could be cryopreserved at high viability rates (approx. 85%) using standard techniques, avoiding labor-intensive and highly technical vitrification methods. Depending on the quantity of hiPSC colonies in the derivation culture, it was possible to yield approximately 3×10^6 iPS cells after just one week in culture, allowing large-scale cryopreservation using DMSO as a protectant. In contrast, production of such a high amount of cells by mechanical passaging takes several weeks and 5–10 passages in multiple dishes. If required, monoclonal hiPSC lines can be established at any time by single-cell cloning. It is important to note that many lines required a second MACS Separation after 2–3 passages to eliminate mEFs and differentiated cells, which appeared after the initial isolation[2] (fig. 2C).

Characterization of human iPS cell lines

In order to analyze pluripotency of the iPS cell lines isolated by magnetic separation, cells were grown to passage 15 (p15) on Matrigel under feeder-free conditions and used for characterization between p15 and p25. The hESC line, HUES7, was used as a control between p17 and p30. Cells were analyzed for expression of various markers by immunofluorescence and flow cytometry (fig. 3). All cell lines expressed the pluripotency markers OCT4, LIN28, TRA-1-81, and SSEA4 (one representative example is shown in figs. 3A and B), as well as SOX2, NANOG, KLF4, DNMT3B, REX1, and cMYC (data not shown). The cell lines were negative for the differentiation marker SSEA1

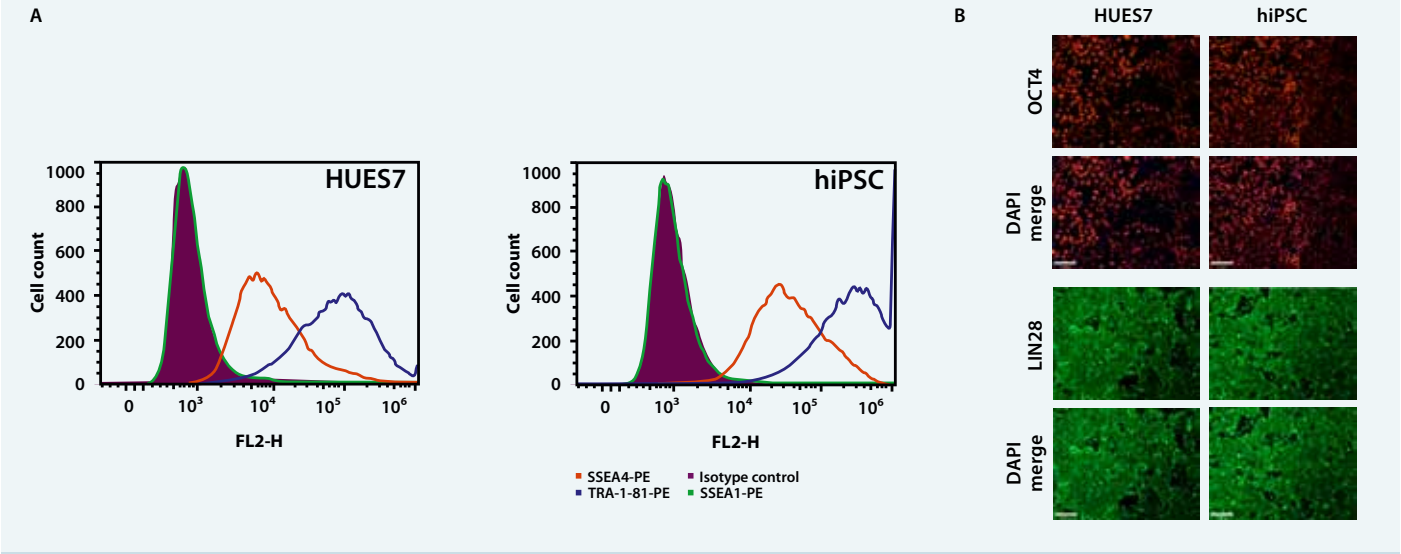

Figure 3 Magnetically isolated human iPS cells express pluripotency markers. The analysis of a representative cell line is shown. The hESC line, HUES7, was used as control. (A) Flow cytometric analysis of SSEA4, TRA-1-81, and SSEA1 expression. Filled histograms denote the isotype control. (B) Expression of OCT4 and LIN28 is demonstrated by immunofluorescence. Scale bars represent 100 µm.

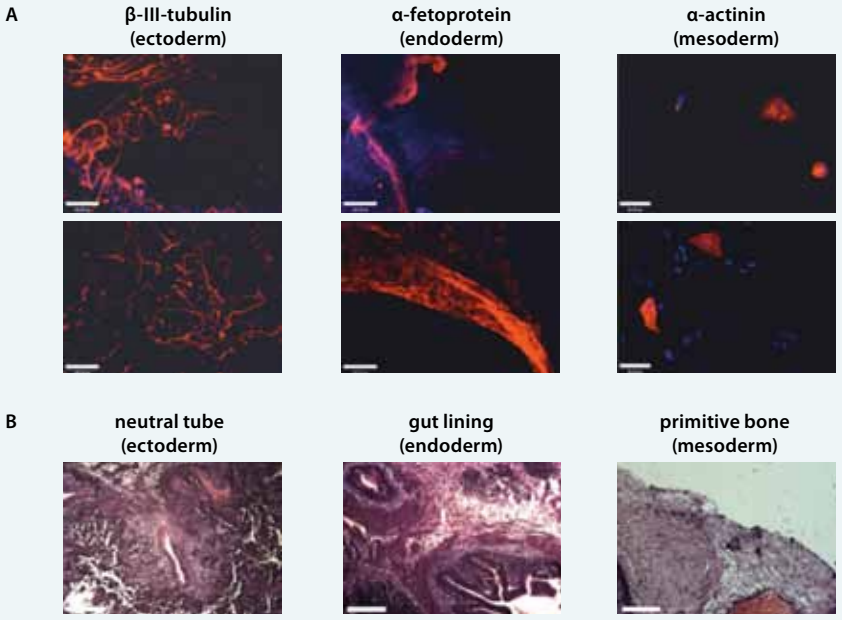

Figure 4 ***In vitro*** **and** ***in vivo*** **formation of the three germ layers.** (A) Cells were differentiated *in vitro* to form EBs. After 30 days cells were fixed and stained for expression of β-III-tubulin (ectoderm), α-fetoprotein (endoderm), and α-actinin (mesoderm). (B) *In vivo* formation of the three germ layers 50–60 days after injection of human iPS cells into the kidney capsule of NOD/SCID mice is shown by hematoxylin/eosin staining of teratoma sections. Scale bars represent 100 µm.

(fig. 3A) and the fibroblast marker P4HB (data not shown). Metaphase spreads at p15 and p25 showed that the iPS cell lines have a normal karyotype (data not shown).

To further demonstrate pluripotency of the magnetically isolated human iPS cell lines, we performed both *in vitro* and *in vivo* differentiation experiments. The iPS cells were differentiated *in vitro* into EBs[4], and fixed after 30 days. All three germ layers developed within the EBs as indicated by expression of β-III-tubulin (ectoderm), α-fetoprotein (endoderm), and formation of α-actinin–positive beating clusters (mesoderm) (fig. 4A). *In vivo* differentiation was monitored by the formation of teratomas. Fixed teratoma sections were stained with hematoxylin and eosin and showed formation of all three germ layers, i.e., early neuroectoderm, primitive gut endothelium (endoderm), and premature cartilage (mesoderm) (fig. 4B).

Conclusion

Magnetic cell separation by MACS Technology offers a significant advantage over labor-intensive mechanical passaging for the production of human iPS cell lines. It allows simultaneous processing of multiple cell lines and generation of large amounts of cells in a short period of time. The cell lines phenotypically fulfill the accepted criteria for the classification of true human iPS cells[1,6,7]. The method can be adapted for the purification of any cell type based on surface marker expression, e.g., the isolation of hESCs from cultures containing differentiated cells, or the purification of specific cell lineages in differentiation protocols.

Relevant products	Order no.
Stemgent Phycoerythrin (PE) anti-Human TRA-1-81 Antibody	130-095-627
Anti-PE MicroBeads	130-048-801
Anti-TRA-1-60 MicroBead Kit, human	130-095-816

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