# Characterization of pluripotent stem cells

Mercè Martí[1], Lola Mulero[1], Cristina Pardo[1], Cristina Morera[1], Meritxell Carrió[1], Leopoldo Laricchia-Robbio[1], Concepcion Rodriguez Esteban[1,2] & Juan Carlos Izpisua Belmonte[1,2]

[1]Center of Regenerative Medicine in Barcelona, Barcelona, Spain. [2]Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, California, USA. Correspondence should be addressed to J.C.I.B. (belmonte@salk.edu or izpisua@cmrb.eu).

Published online 10 January 2013; doi:10.1038/nprot.2012.154

**Characterization of pluripotent stem cells is required for the registration of stem cell lines and allows for an impartial and objective comparison of the results obtained when generating multiple lines. It is therefore crucial to establish specific, fast and reliable protocols to detect the hallmarks of pluripotency. Such protocols should include immunocytochemistry (takes 2 d), identification of the three germ layers in *in vitro*–derived embryoid bodies by immunocytochemistry (immunodetection takes 3 d) and detection of differentiation markers in *in vivo*–generated teratomas by immunohistochemistry (differentiation marker detection takes 4 d). Standardization of the immunodetection protocols used ensures minimum variations owing to the source, the animal species, the endogenous fluorescence or the inability to collect large amounts of cells, thereby yielding results as fast as possible without loss of quality. This protocol provides a description of all the immunodetection procedures necessary to characterize mouse and human stem cell lines in different circumstances.**



## INTRODUCTION

Since the first generation of embryonic stem cells (ESCs)[1,2] and induced pluripotent stem cells (iPSCs)[3,4], numerous cell culture techniques focusing on the culture of these cells have been presented in detail[5,6]. However, characterization protocols that use immunodetection to test the pluripotency and differentiation capacity of these newly generated lines have not always been described thoroughly. Although the fixation and antibodies used are usually described, technical pitfalls and suggested tips are frequently omitted.

When you are developing new ESC or iPSC lines, it is extremely important to carry out an exhaustive characterization of the new lines in order to unequivocally define the hESC or iPSC populations and to demonstrate their pluripotency. As recommended by the International Stem Cell Banking Initiative (ISCBI)[7], several tests need to be performed before banking a new hESC or iPSC line. These tests include the following: (i) pluripotency tests; (ii) differentiation tests both *in vitro* and *in vivo*; (iii) karyotype analysis to show that the new lines generated have maintained genetic stability, as it is already known that prolonged culture of pluripotent cell lines can result in genetic abnormalities, commonly causing aneuploidy; (iv) determination of cell identity, usually performed by DNA fingerprinting and HLA analysis; (v) gene expression profiling via a stem cell array, which detects the expression of a common set of genes expressed in undifferentiated cells downregulated upon differentiation; and (vi) a microbiological test to ensure that the cultures are free of any contaminants[8].

### Tests to characterize ESC and iPSC lines

**Alkaline phosphatase (AP) expression.** AP expression can be easily assayed because of the capacity of this enzyme to change the conformation of a colorimetric reagent from a soluble to a precipitated state. Although in humans there are different types of this membrane enzyme in different tissues, the placental alkaline phosphatase (hPLAP) is related to pluripotency. In addition to being an ESC marker, hPLAP resists high temperatures (68 °C)[9], which is why it has been used as a reporter of transfected cells, as endogenous AP can be inactivated with heat[10]. Thus, a high-temperature step for inactivating the other AP isoenzymes is added to the AP staining protocol to avoid false positives and any background from feeder cells. In mice, the PLAP is not resistant to high temperatures[11], and for this reason the heat step must be avoided. When you are using an AP detection kit according to the manufacturer's protocol, it is important to note that the instructions of these kits usually do not mention the need to perform heat inactivation.

**ESC marker expression.** Once AP-positive colonies are determined, the next step is to confirm their pluripotency by immunostaining. A panel of biochemical and molecular markers has been identified that are specific to ESC physiology and fundamental to maintaining the undifferentiated state[5–8]. In humans, in addition to the expression of AP, the markers include the nuclear transcription factors Oct4, Nanog and Sox2; the keratan sulfate antigens Tra-1-60 and Tra-1-81; and the glycolipid antigens SSEA3 and SSEA4. As SSEA1 is negative in pluripotent human cells, this marker can be used as a negative control. In mice, the main pluripotent markers are Oct4, Nanog, Sox2 and SSEA1. Maintenance of pluripotency or initiation of differentiation depends on a highly regulated equilibrium between Oct4, Nanog and Sox2. Nanog homodimerization is necessary for its interaction with these other crucial factors in the pluripotency network[12], and there are also different splice variants[13]. Thus, only the presence of all the markers and the demonstration that the cell line is able to differentiate to all germ layers *in vitro* and *in vivo* allow us to define it as a pluripotent stem cell line.

The antibody combinations we use include nuclear and surface markers. We have combined antibodies to require minimal numbers of cells while avoiding cross-reaction between the secondary antibodies. The first primary antibody combination is Oct4 (mouse IgG) with SSEA3 (rat IgM), using secondary antibodies that have been preabsorbed with the serum of the other species (the mouse-specific IgG antibody preabsorbed with rat serum and vice versa) to avoid cross-reaction between these highly related animal species. As these two secondary antibodies are made in goat, Nanog (goat IgG) cannot be added. The second combination consists of Sox2

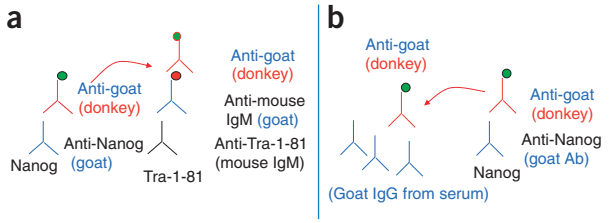


**Figure 1** | Possible cross-links. (**a**) Graphic showing the cross-link (red arrow) between the secondary antibody anti–goat-Cy2 (red), which should recognize the anti-Nanog antibody made in goat (in blue), and the secondary antibody anti-mouse IgM-Cy3 (blue, against the Tra-1-81 primary antibody), if it is made in goat. As a result, we will obtain a red signal in the cell surface, and a green signal in the surface (false) and in the nuclei of the cells. (**b**) Graphic showing the cross-link (red arrow) between the secondary antibody anti-goat IgG-Cy2 (red), which should recognize the primary antibody made in goat (blue), and the goat immunoglobulin present in the goat serum (blue). As a result, we will see a high background and a clear reduction of the real secondary antibody concentration.

(rabbit IgG), SSEA4 (mouse IgG) and Tra-1-60 (mouse IgM) primary antibodies. In this case, secondary antibodies specific to the μ- or γ-chain of the mouse immunoglobulin must be used because the general ones recognize both. As these secondary antibodies are made in goat, the Nanog-specific antibody (goat IgG) cannot be used in this combination. Finally, the third combination is Nanog (goat IgG) and Tra-1-81 (mouse IgM), in which secondary antibodies not made in goat (such as donkey or rabbit antibodies) must be used to avoid unspecific cross-reaction (**Fig. 1a**).

We prefer to prepare only one buffer for all the immunodetections, always without goat serum. Although theoretically goat serum should be added when secondary antibodies made in goat are used, we only use donkey serum. The rationale is that usually one primary antibody is made in goat and therefore we are using a secondary goat-specific IgG. This avoids the unspecific binding that these secondary antibodies would have had against the serum goat IgG if we had added it (**Fig. 1b**). Moreover, diluting the second antibodies with goat serum results in binding of the goat-specific IgG antibody, preventing its interaction with the first antibody. For the same reason, it is not recommended to use BSA, as the goat-specific antibodies can recognize the bovine ones.

One background that could lead us to analyze a false positive in pluripotency detection is produced by the use of mouse primary antibody on mouse samples. We noticed that the usual mouse IgG -specific secondary antibody, used in mouse cell immunodetections, gave a high background particularly in the nucleus where some markers, such as Oct4, were expected to appear. This background can be decreased by using the specific secondary antibody for the immunoglobulin isotype of the primary antibody (a mouse IgG2b-specific antibody, in the case of the Oct4-specific antibody 1.1). We have also tried the component M.O.M. (mouse-on-mouse) kit. Although this gave a clear decrease of the background, there was also a decrease in the positive signal. In view of this, we recommend using the mouse IgG2b-specific secondary antibody (**Fig. 2**).

If the cells express GFP, it can be necessary to detect whether the vector has been silenced. We use a chicken GFP-specific antibody to avoid interference with the mouse, rabbit or goat antibodies. In the case of DsRed colonies, we use an RFP (red fluorescent protein)-specific antibody that also recognizes the cherry and orange proteins. Unfortunately, we only found a rabbit and not a chicken antibody. This restricts the use of some primary antibodies.

An important consideration is that not all antibodies recognize the active form of the pluripotent antigen or the isoform of the protein related to pluripotency. In the case of Oct4, there are two splice variants[14,15]: Oct4A and Oct4B. Only Oct4A is related to pluripotency, and thus antibodies that recognize both isoforms can give false positives. The Oct4-specific antibody that we use, 1.1, recognizes only the C terminus of POU5F1-iB, and therefore only recognizes the variant Oct4A, which is present in the inner cell

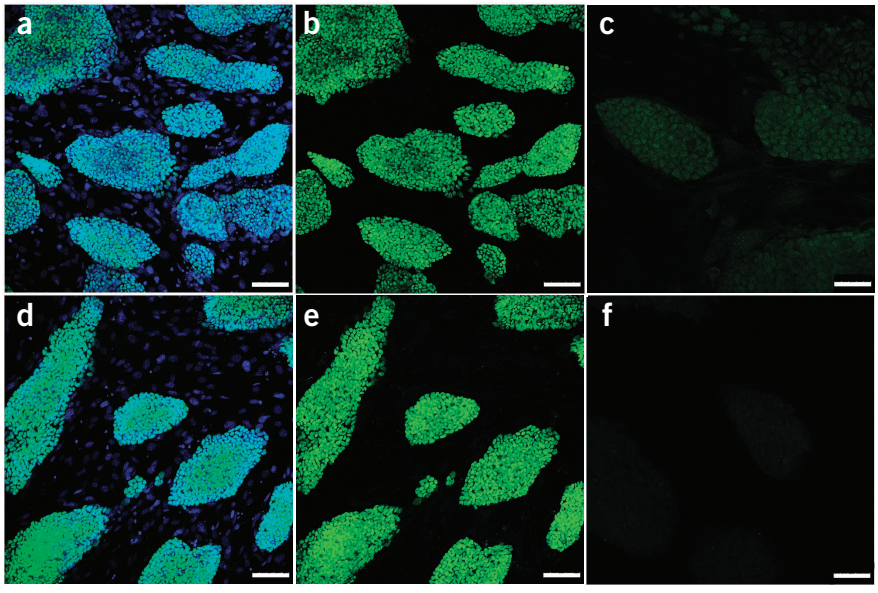


**Figure 2** | Background as a result of using mouse antibodies in mouse samples. (**a**–**c**) Oct4 detection in G4 mouse cells with a general anti-mouse IgG-Cy2 (**a**); Oct4 signal in the green channel (**b**); nuclear background in the negative control, taking the images with the same settings (**c**). (**d**–**f**) Oct4 detection with a specific anti-mouse IgG2b-Alexa Fluor 488. Merged image of Oct4 and DAPI signal (**d**); Oct4 signal in the green channel (**e**); background in the negative control (**f**). Note that with the general anti-mouse IgG antibody a nuclear background is visible, which can lead to a false positive if the detection settings of the confocal are increased. With the specific subgroup antibody, only a general and weak background is visible. (**a**,**b**,**d**,**e**) *Image information*: confocal microscope (Leica TCS SP5); HCX PL APO ×20.0/0.70 IMM UV 11506191 objective; sequential mode; 405-nm diode and 488-nm argon lasers; AOBS detection system; emission bandwidths of 415–529 nm/501–546 nm; 1,024 × 1,024 image pixels; 8 bits resolution; maximum projection of a *xy/z* series; scale bars, 100 μm. (**c**,**f**) *Image information*: Confocal microscope (Leica TCS SP5); HCX PL APO CS ×40.0/1.25 OIL UV 11506251 objective; sequential mode; 488-nm argon laser; AOBS detection system; 501- to 546-nm emission bandwidth; 1,024 × 1,024 image pixels; 8-bit resolution; *xy* plane; scale bars, 50 μm.

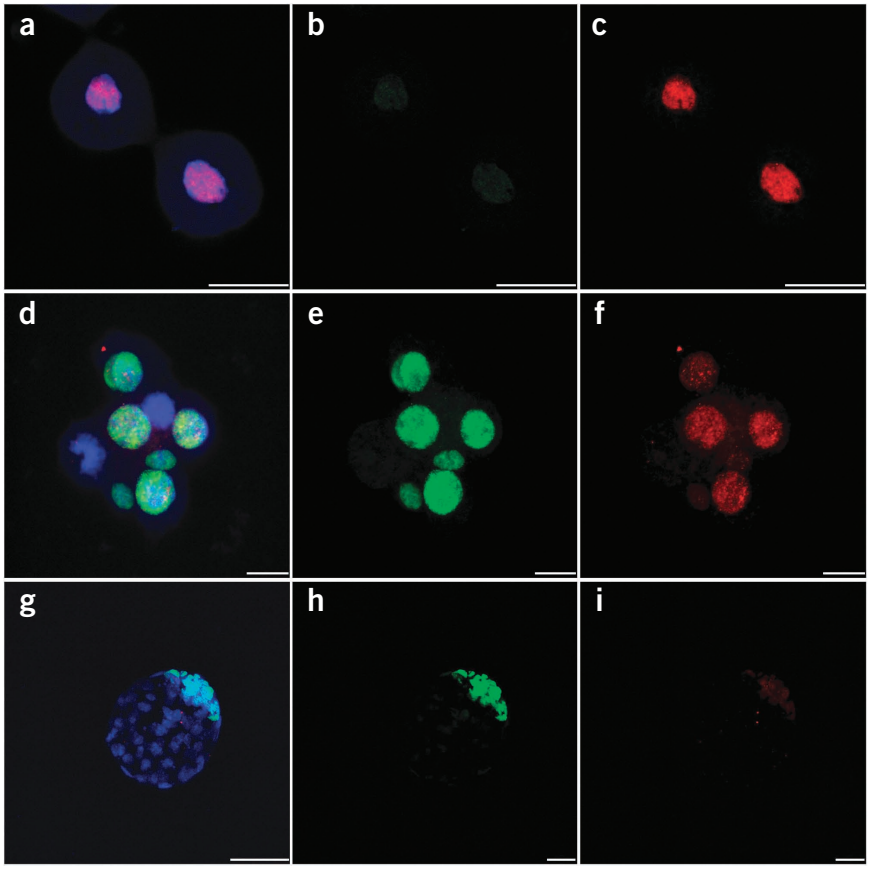


**Figure 3** | Isoforms of Oct4. (**a**–**i**) Isoform Oct4A (green; **b**,**e**,**h**) and isoform Oct4B (red; **c**,**f**,**i**) detection in mouse embryo with DAPI in blue (merge; **a**,**d**,**g**). (**a**–**c**) Two-cell mouse embryo blastomeres, positive for the isoform Oct4B. (**d**–**f**) Eight-cell mouse embryo blastomeres positive for isoform Oct4A and Oct4B. (**g**–**i**) Mouse embryo blastocysts positive for isoform Oct4A in the inner cell mass cells. *Image information*: confocal microscope: SP5 AOBS. Objective: HCX PL APO lambda blue ×63.0/1.40 OIL ultraviolet. Sequential mode. Laser: 405-nm diode and 488-nm argon. Emission bandwidth: 415–529 nm/515–565 nm/582–689 nm. Image pixels: 1,024 × 1,024. Resolution: 8 bits. Image: *xyz* series, maximum projection. Scale bars, (**a**–**c**,**h**,**i**) 25 µm, (**d**–**f**) 10 µm, (**g**) 50 µm. All animal experiments were conducted in accordance with experimental protocols previously approved by the Institutional Ethics Committee on Experimental Animals of the Barcelona Biomedical Research Park, in full compliance with Spanish and European laws and regulations.

mass of blastocysts and pluripotent stem cell colonies. Antibody 1.36, which maps near the N terminus of POU5F1-iB and recognizes only the variant Oct4B, stains the nuclei of precompaction blastomeres. We have only detected both variants simultaneously in the precompacting eight-cell embryo (**Fig. 3**).

**Differentiation tests.** If pluripotency markers are expressed by the cells, the next stage in characterization is to demonstrate that ESCs or iPSCs can differentiate to form tissues derived from the three primordial germ layers of the embryo. This can be shown via an *in vitro* or *in vivo* differentiation test. Nowadays, the necessity of performing an *in vivo* test is highly debated, and some groups argue that *in vitro* test is enough. On the other hand, other labs argue that the behavior of cells in culture can be totally different from that seen *in vivo*, and therefore their capacity to differentiate and stay alive in a tissue must be demonstrated. Moreover, it has been proposed that a better understanding of the spontaneous teratoma formation could help in the studies related to potential hazards of stem cell therapeutics[16,17]. Currently, the *in vivo* test is necessary to register a cell line, and thus this protocol includes the procedure we use to analyze the teratomas.

To perform the *in vitro* differentiation test, colonies (or regions of colonies) are first cultured in suspension so that they form large aggregates called embryoid bodies (EBs). If the first stages of differentiation are being studied, these aggregates can be analyzed via *in toto* immunodetection. Alternatively, the EBs are transferred to six-well plates or SlideFlasks, where they grow adhered to the surface[18]. Pluripotent cells growing as EBs should differentiate spontaneously to different cells types derived from the three germ layers (spontaneous differentiation), or they can be cultured in different substrates with different media, to favor differentiation toward a specific cell lineage (guided differentiation)[19,20].

Manual generation of EBs is amenable to the formation of large quantities of aggregates, but this does not allow accurate control of their size. Size can be more precisely controlled by the use of the hanging drop protocol[21,22]. The rounded bottom of the hanging drop allows the aggregation of ESCs, providing a good environment for forming EBs. The number of cells aggregated in a hanging drop can be controlled by varying the number of cells in the initial cell suspension to be hung as a drop from the lid of a Petri dish. By using this method, we have reproducibly formed homogenous EBs. Independently of the chosen method to generate EBs, after their

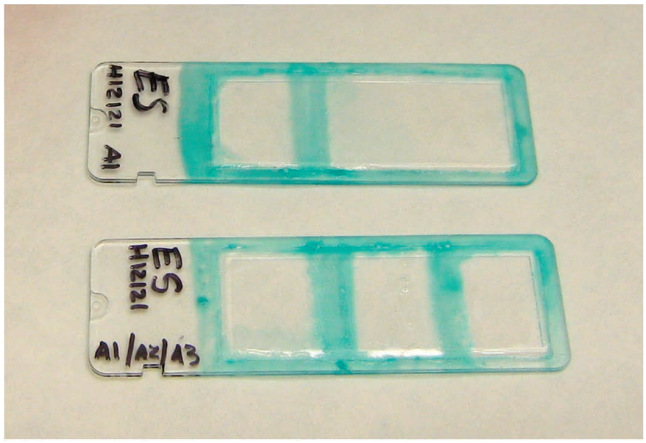


**Figure 4** | The use of SlideFlasks in immunodetection. SlideFlasks with colonies. In the upper one, a small area has been created with a PAP pen for a negative control. The other SlideFlask has been divided into three areas to perform the three antibody combinations in the same slide.

## Box 1 | AP live staining protocol ● TIMING 45 min

This is based on the manufacturer's guidelines:
1. Remove the growth medium from the culture plate.
2. Wash it with prewarmed (37 °C) DMEM/F-12 culture medium for 2–3 min. Aspirate the medium and repeat this step.
3. Add 1× AP live stain working solution by following the data sheet recommendations.
4. Incubate the mixture for 20–30 min.
5. Aspirate the AP live stain and wash twice for 5 min with DMEM/F-12.
6. Add fresh DMEM/F-12 and take images within 30–90 min of staining.
7. Replace DMEM/F-12 with fresh growth medium and return the cells to normal culture conditions.

formation differentiation should be confirmed by immunocytochemistry for differentiated markers.

The *in vivo* differentiation test consists of injecting ESCs or iPSCs into severe combined immunodeficient (SCID) mice. If the cells are pluripotent, they will proliferate and differentiate in the tissue where they are injected, and ultimately they will form a teratoma that contains multiple tissues differentiated from the three primordial germinal layers. Different methodologies have been described to generate teratomas, with changes in the culture cell conditions (with or without Matrigel), the amount of injected cells and the region of injection (intratesticular, subcutaneous or intramuscular)[17,23,24]. *In vivo* differentiation tests must be approved by the regional legal authorities, and they must cause minimal pain and stress to the animals. Once the teratoma has formed, differentiation should be confirmed by immunohistochemistry for differentiation markers.

**Karyotype analysis.** The most commonly used technique to evaluate genomic integrity is the traditional karyotype analysis. This technique is based on arresting the cells in the metaphase stage of cell division; this is followed by staining (usually Giemsa staining or 'G-banding'). On the basis of the cytogenetic characteristics of the individual chromosomes at metaphase, chromosomal abnormalities can be observed under a microscope. The first part of the methodology, the generation of the nuclear preparation, can be easily performed in the laboratory[25]. Thereafter, the samples can be analyzed by a cytogeneticist using an automated karyotyping system such as the CytoVision (Olympus). The karyotype of 30 metaphases is usually analyzed. It is very important that hESCs and newly created iPSC lines be routinely karyotyped.

Alternatively, spectral karyotyping can be performed. This is a molecular cytogenetic technique that improves the resolution of the traditional karyotype[26]. Chromosome-specific DNA sequences are labeled with different fluorophores, generating unique fluorescent probes for each pair of chromosomes. The individual colored chromosomes are then visualized, and genomic changes can be detected.

**DNA fingerprinting and HLA analysis.** There are different techniques available for cell identification. One of the most common methods for DNA fingerprinting is based on PCR and uses short tandem repeats (STRs). This method uses highly polymorphic regions that have short repeated sequences of DNA. These repeated sequences are unique to each cell line. Thus, analysis of STR will allow cell identification. Briefly, we extract genomic DNA using the QIAamp DNA mini kit. STR profile sites (D13S317, D7S820, D16S539, vWA, TH01, TPOX, CSF1PO, D8S1179, D21811, D19S433, D18851, D3S1358, D2S1338, D5S818, FGA and amelogenin for sex determination) are obtained using the AmpFlSTR identifier PCR amplification kit, and the STR analysis is performed by a genomic technological platform (usually available as a core facility in the university or research center) or by American Type Culture Collection (ATCC).

HLA analysis (variation in human leukocyte antigen gene) is another option for cell identification and is required to register a cell line. We extract DNA using the MagNA Pure LC machine (Roche Applies Sciences). HLA-A, HLA-B, HLA-DRB1 and HLA-DQB1 loci are typed at low resolution using the Lipa HLA kit. The PCR sequence-specific primers method is used to perform high-resolution typing of the HLA-DRB1 and HLA-DQB1 alleles according to the manufacturer's instructions. The HLA analysis can usually be performed by a genomic technological platform of a university core facility or research center.

**GeneChip expression analysis.** An important characterization step is to test whether the global transcriptional profile of the generated iPSCs is close to that of human ESCs. We perform a global comparison of the transcriptomes of the original cells

## Box 2 | Surface marker live detection ● TIMING 30 min

1. Add the antibody directly to 1 ml of HES medium in a six-well culture plate. If the fluorochromes used are different, you can use them together. If their fluorescence emissions are in the same bandwidth, then add them separately in different wells. For Tra-1-60 PE (1.34), use a 1:20 dilution (50 µl in 1 ml of medium). For Tra-1-81 FITC (1.35), use 1:50 dilution (20 µl in 1 ml of medium).
2. Incubate the mixture for 20 min at 37 °C.
3. Wash it twice with HES medium.
4. Observe the cell staining with an inverted microscope and take images if the result is positive.
5. Return the cells to incubator and grow them under normal culture conditions.

before being reprogrammed, such as fibroblasts or keratinocytes and the generated iPSC lines, with hESCs.

First, RNA is isolated from the different cell lines. We use the miRNeasy mini kit from Qiagen, which allows purification of total RNA and microRNA because of their important roles in pluripotency. RNA integrity is then assessed and RNA samples with high integrity are subsequently used in microarray experiments. Our GeneChip microarray processing is performed by a core facility. We plot results as 'dendrograms', representing hierarchical clustering of genome-wide transcriptional profiles of keratinocytes,

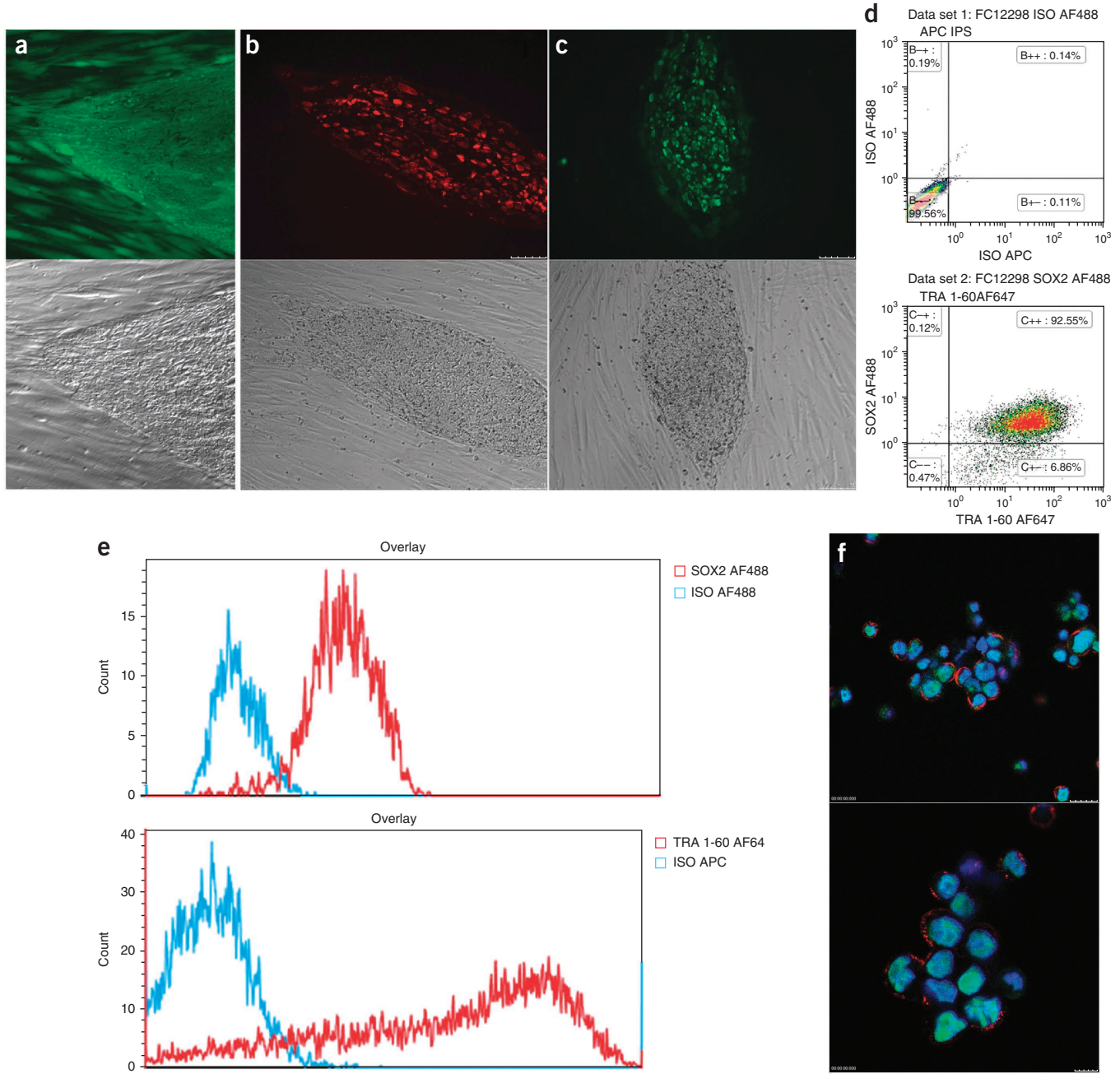


**Figure 5** | Quick pluripotency assays. (**a**) AP live stain test in an hESC line showing a positive colony, but also a high background in the feeders. Fluorescence and Nomarsky images of the same colony. (**b**,**c**) *In vivo* detection of Tra-1-60 PE (red) (**b**) and Tra-1-60 FITC (green) (**c**) in hESCs. In this case, no background is detected in the feeders. Fluorescent images and Nomarsky image of the same colonies. (**d**) Flow cytometry analysis. Representative dot plots showing the distribution of hESCs along the signal obtained with the isotype-specific control antibodies (top) or with the Sox2 and Tra-1-60 direct conjugated antibodies (bottom). (**e**) Flow cytometry analysis. Representative overlay histograms showing the profile of Sox2 and Tra-1-60 reactivity on hESCs (red) matched with the reactivity of the isotype-specific control antibodies (blue). (**f**) Confocal image of the positive cells sorted after an analysis with Sox2 (green) and Tra-1-60 (red) direct conjugated antibodies. DAPI signal is shown in blue. *Image information*: Inverted microscope: Leica DMI4000. Objectives: (**a**) N PLAN ×10/0.25 pH 1; (**b**,**c**) HCX PL FLUOTAR L ×20/0.40/C pH1. Image pixels: 1,024 × 1,024. Resolution: 8 bits. Image: *xy* plane. Scale bars in **b** and **c**, 75 µm. Cell sorter: MoFlo Dako Cytomation. Confocal microscope: Leica SP5 AOBS, Sequential mode. Laser: 405-nm Diode/488-nm Argon/561-nm DPSS. Emission bandwidth: 415–529 nm/515–565 nm/582–689 nm. Image pixels: 1,024 × 1,024. Resolution: 8 bits. Image: *xy* plane. Scale bars in **f**, 25 µm (top); 10 µm (bottom).

## Box 3 | Flow cytometry analysis of pluripotency ● TIMING 3–4 h

1. Trypsinize the cell culture with 0.05% (wt/vol) trypsin for 1–2 min at 37 °C.
2. Add 1 ml of cell culture medium to block the effect of the trypsin.
3. Adjust the concentration of the cell suspension to ~$10^4$ cells by adding culture medium.
4. Put 100 µl of this cell suspension into a flow cytometry tube.
5. Add the recommended amount of direct conjugated antibody (see the product data sheet). In our example, add 5 µl of Tra-1-60 antibody (1.38) into the tube.
6. Incubate the mixture for 15 min at RT in the dark.
7. Wash the cells with 1× PBS. Centrifuge the mixture at 500*g* for 5 min at RT.
8. Discard the supernatant.
9. Resuspend the cell pellet and add 0.5 ml of 4% (wt/vol) PFA for 30 min.
10. Wash it with 1× TBS. Centrifuge the mixture at 500*g* for 5 min at RT. Repeat this step.
11. Discard the supernatant.
12. Resuspend the cell pellet and add 0.5 ml of TBS++ for 30 min.
13. Centrifuge the mixture at 500*g* for 5 min at RT.
14. Discard the supernatant.
15. Resuspend the cell pellet and add 100 µl of TBS++ and 5 µl of Sox2 antibody (1.37).
16. Incubate the mixture for 30 min at RT in the dark.
17. Wash with 1× TBS. Centrifuge the mixture at 500*g* for 5 min at RT.
18. Discard the supernatant. Resuspend the cell pellet with 0.5 ml of 1× TBS.
19. Analyze the tube by flow cytometry.
20. Sort the positive population.
21. Centrifuge the mixture at 500*g* for 5 min at RT. Discard the supernatant and resuspend the cell pellet in 20 µl of 1× TBS.
22. Take one drop of the cell solution and put it in a slide. Cover it with a coverslip.
23. Capture images under the microscope.



fibroblasts, ESCs and iPSCs. By using a hierarchical clustering, usually the iPSC and hESC lines cluster together, with fibroblasts and keratinocytes as outgroups. The overall transcriptional profiles of iPSCs and hESCs should appear indistinguishable in the sense that the differences between the two lines are in the same range as the differences between different hES cell lines.

**Microbiological test.** In addition to mycoplasma testing, which should be routinely performed in the laboratory, in order to register an hESC line, a complete microbiological test is required to ensure that the cell line is free of any possible contaminant. This test can be performed by an external microbiology laboratory. In our case, we send supernatant samples from our cultures to General Lab (Barcelona, Spain) and they test the cultures for the presence of Gram-positive and Gram-negative bacteria, mycoplasma, yeast and fungi.

### Comparison with other methods

Characterization is often performed on cells that have been grown in six-well culture plates, with each well dedicated to a specific antibody. To be sure that the full area of the plate is covered by the diluted antibody solution, at least 1 ml is needed. Moreover, some papers describe different reagents needed for each antibody, for example, with or without Triton X-100, depending on whether their location is intracellular or over the plasma membrane[4–6].

To decrease the quantity of cells and antibody needed, we use SlideFlasks and test more than one antibody together. In this way, expression of nuclear and surface pluripotency markers can be seen concurrently in the same colony. Moreover, we have observed that with the same reagent (Tris-buffered saline (TBS) with Triton X-100 and donkey serum) we can detect all antigens, thereby simplifying the protocol. We either use three SlideFlasks for each human line to be characterized using seven antibodies or two SlideFlasks for four antibodies in mouse cell lines, making a vertical line with a PAP pen to create a small area for a negative control on each slide. PAP pens are special marker pens that provide a thin hydrophobic barrier on the slide to reduce the volume of antibody solution and to avoid any spills. If necessary, only one SlideFlask per cell line is used and divided with a PAP pen into three different areas (or two areas for mouse cell lines), one for each combination of antibodies (**Fig. 4**). When the chamber is taken out, 200 µl of diluted antibody solution is enough to fill the surface of the SlideFlask.

This protocol is designed for line registration, which requires immunocytochemistry images of all the pluripotency markers, both surface and nuclear. However, if you are just confirming whether a previously characterized cell line is still pluripotent, and whether to proceed with the experiment, an *in vivo* detection of only the surface antigens via an AP live stain test (**Box 1**) or with direct conjugated antibodies (**Box 2**) can suffice (**Fig. 5a–c**). However, we find that these tests do not produce optimal results and that the reagents required are more expensive. Undoubtedly, they could be alternative methods if time is a priority, or if it is not possible to obtain replicas of the cell line, as the stained cells can be grown further after detection. After washing, the cell line can follow *in vitro* growth without any alterations (**Supplementary Videos 1** and **2**).

However, we see a high background in the feeders when we perform AP live staining. In addition, the high-temperature step is not allowed; thus, it is not clear whether hPLAP or another AP enzyme is detected (**Fig. 5a**). A further disadvantage of *in vivo* detection is that the staining decreases quickly, and thus cells must be analyzed promptly.

## Box 4 | The use of antigen retrieval in immunodetection of stem cells

● TIMING 1 h 30 min

To perform antigen retrieval on colonies in a SlideFlask, use the same reagent as that used for paraffin sections (1× citrate buffer, pH 9). The retrieval will be weaker. Antigen retrieval is optional and should be performed between Steps 12 and 13 of the protocol. The antigen retrieval can also be performed in immunodetections of EBs in suspension (PROCEDURE Step 38A(vi–vii)), or in immunodetections of EBs over SlideFlask (PROCEDURE Step 38B(ii,iii)).

1. Remove the 1× TBS.
2. Add 2 ml of the 1× citrate buffer (prewarmed to 60 °C).
3. Place the SlideFlask into the oven for 1 h at 60 °C.
4. Take the SlideFlask out of the oven and let it cool down at RT in the same buffer (wait for 10 min).
5. Wash with 1× TBS three times for 5 min each.
6. Proceed with Step 13.

Flow cytometric analysis of pluripotency markers using direct conjugated antibodies (**Box 3**) is a quick alternative method to determine whether the cell line is pluripotent (**Fig. 5d**,**e**). Images of the stained and sorted cells can be taken under a microscope, although morphology is not well preserved and staining can be less bright (**Fig. 5f**).

Although the described protocol gave us good results in the characterization of all the lines, some other studies require the optimization of other markers also present in the stem cells. For example, we were interested in detecting the changes in E-cadherin and connexin43 expression while the differentiation process was in progress. Immunodetection with E-cadherin–specific (1.32) or connexin43-specific (1.33) antibodies was clearly improved by performing a weak antigen retrieval (**Box 4**). Pluripotency markers are not affected by this process, and therefore antibodies to detect their expression can be added if desired (**Fig. 6**). However, the antigen retrieval process will alter the actin structure of the cytoskeleton, and thus it must be avoided when phalloidin staining is necessary. In addition, it will decrease or eliminate the endogenous fluorescence of GFP and DsRed proteins. The expression of GFP and DsRed can be monitored, if required, after antigen retrieval by using antibodies against the fluorescent proteins.

### Experimental design

In our laboratory, several ESC and iPSC lines from different sources (for example, fibroblast, keratinocytes, cord blood cells), species (zebrafish, pig, mouse and human) and with up to two endogenous fluorescent markers (GFP or DsRed fluorescence) have been fully characterized to be registered[17,27–31] (**Fig. 7**). The different protocols for immunodetection of pluripotency (**Supplementary Figs. 1** and **2**) and differentiation tests we use (**Supplementary Figs. 3–5**), with the various optimizations and improvements we have added, are detailed below.

To maintain consistency, we standardized protocols that we could use for all samples, taking into account the amount of time required, economic costs and reproducibility of the results. However, some time points can be changed if required. For example, the primary antibody incubations (24 h at 4 °C) can be carried out at 4 °C for the entire week or reduced to 2 h at 37 °C. However, incubations at 37 °C will increase the background.

Usually, we analyze the EBs growing adhered to the SlideFlask surface. In addition to the advantages discussed earlier to using a SlideFlask, it can be covered by a coverslip after the immunodetection, and confocal microscopy can be used to recover the 3D structure of the EBs.

Even though the easiest way to study the differentiation in EBs is to perform immunodetection in SlideFlasks, the immunodetection should be done when the EBs are still cultured in suspension if study of the earliest markers of differentiation is required. In these cases, the immunodetection can be performed *in toto* in 96-well plates under the stereomicroscope, moving the EBs with a Pasteur pipette from one well to successive wells containing the reagents

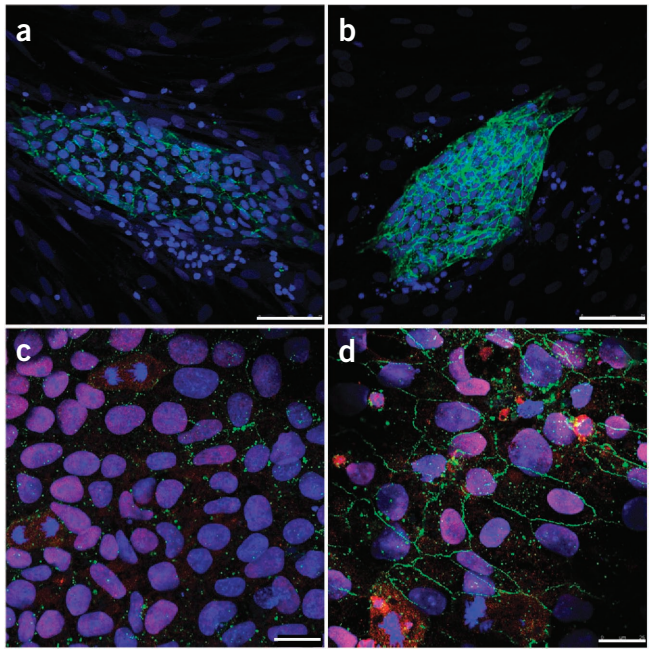


**Figure 6** | Antigen retrieval process over cultured stem cells. (**a**,**b**) E-cadherin detection (green) without antigen retrieval (**a**) and with antigen retrieval (**b**) in hESCs. Even though there is a weak staining without the antigen retrieval, it can be markedly improved with this method. DAPI is shown in blue (**c**,**d**). Similar improvement can be obtained in the Connexin43 immunodetection (green) in hESCs. Shown are results without antigen retrieval (**c**) or with antigen retrieval (**d**). Nanog is shown in red, DAPI in blue. *Image information*: Confocal microscope: Leica TCS SP5 AOBS. Objectives: (**a**,**b**) HCX PL APO ×20.0/0.70 IMM UV 11506191; (**c**,**d**) HCX PL APO lambda blue ×63.0/1.40 OIL UV. Sequential mode. Laser: 405-nm diode/488-nm argon/561-nm DPSS. Emission bandwidth: 415–529 nm/515–565 nm/582–689 nm. Image pixels: 1,024 × 1,024. Resolution: 8 bits. Image: *xy* plane. Scale bars, (**a**,**b**) 75 µm, (**c**,**d**) 25 µm.

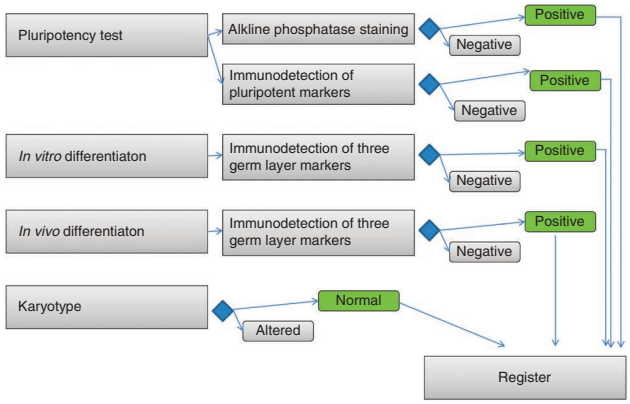


**Figure 7** | Characterization of a pluripotent stem cell line. Flowchart showing the different steps to characterize one cell line.

for immunodetection. If the EBs are too big to be transferred via a pipette, the reagent can be aspirated from the well and the next reagent can be added. If you are using EBs in 96-well plates, increase the time for antibody incubation, permeabilization and washes, and shake the plate continuously.

Teratomas must be embedded in paraffin to be analyzed, by following the classic protocol (dehydration with ethanol, clearing with xylene and paraffin embedding) and obtaining 5-µm sections by rotary microtome. The same antibodies can be used to detect mouse or human differentiation markers in EBs and teratomas.

If you are assaying cell proliferation in a teratoma (**Supplementary Fig. 6**), prepare specimens by cryostat (cryopreservation with 1× PBS plus 30% (wt/vol) sucrose, and optimum cutting temperature compound (OCT) embedding), avoiding paraffin embedding. The morphology may be affected, but some proliferation markers are not suitable for paraffin sections. Ki67 is a suitable marker, although it requires aggressive antigen retrieval and the signal can be weak. To resolve this, we suggest amplifying the obtained signal with a FITC/anti-FITC system. Other cell proliferation markers are PCNA (proliferating-cell nuclear antigen) or phosphohistone H3 (phH3). These two epitopes also require antigen retrieval, although it does not need to be as aggressive as is required for Ki67. If a comparison between the cell proliferation/cell death ratios among different lines in generated teratomas is needed (**Supplementary Fig. 7**), we suggest using PCNA and phH3 proliferative markers with the TUNEL kit. Proteinase K treatment is required for the TUNEL protocol and Ki67 detection requires boiling the sample, and thus these two processes together destroy the section.

In our opinion, previous protocols for the analysis of differentiation have omitted important details that must be considered when interpreting results. For example, Gata4 can be detected in two germ layers (endoderm and mesoderm), and is only a specific marker of cardiomyocytes when expressed with α-sarcomeric actinin. In teratomas, one must be sure that the structures that the antibodies are showing (for example, α-sarcomeric actin–positive cells) are not from the mouse (in an intramuscular injected teratoma). To avoid this, we explain in detail the morphology of the cells resulting from the different germ layers that are usually observed in EBs and teratomas, with the aim of helping non-expert researchers in the interpretation of the differentiation immunodetections.

## MATERIALS

### REAGENTS

- ESCs or iPSCs of interest
- Animals: SCID/beige mice (Charles River laboratories) for teratomas. Male mice aged 6–12 weeks to 18 months old. Two individual mice per line are required for characterization **! CAUTION** All animal experiments must conform to relevant institutional and governmental regulations. Our protocol was approved by the Institutional Ethics Committee on Experimental Animals of the Barcelona Biomedical Research Park, in full compliance with Spanish and European laws and regulations.
- Six-well plate for AP staining (Corning, cat. no. 153516)
- 96-well plate (Nunc, cat. no. 167008)
- SlideFlask (Nunc, cat. no. 734-2107)
- PBS without Ca, Mg (Gibco, cat. no. 10010)
- Paraformaldehyde, 4% (wt/vol; PFA), in 0.1 M PB (Sigma, cat. no. P6148-500G) **! CAUTION** PFA is toxic on inhalation and skin contact; wear gloves and work in a fume hood.
- Trizma-HCl (Sigma-Aldrich, cat. no. T6666)
- Trizma base (Sigma-Aldrich, cat. no. T6791)
- Trisodium citrate 2 hydrate (Panreac, cat. no. 141655.1211)
- NaCl (Sigma-Aldrich, cat. no. S7653)
- Triton X-100 (Sigma-Aldrich, cat. no. X100)
- Donkey serum (Chemicon, cat. no. S30-100ML)
- DAPI (Invitrogen, cat. no. D21490) **! CAUTION** Do not breathe the dust, and avoid contact with eyes and skin. Wear gloves when handling this reagent.
- Glycine (Sigma-Aldrich, cat. no. G7126)
- NaOH (Panreac, cat. no. 131687_1210)
- $NaN_3$ (sodium azide; Merck, cat. no. 1.06688.0100) **! CAUTION** $NaN_3$ is very toxic; avoid contact with eyes and skin. Wear gloves when handling this compound.
- Ethanol, 96% vol/vol (Panreac, cat. no. 361085)
- Ethanol, 100% vol/vol (Panreac, cat. no. 361086)
- Paraffin (Vogel, cat. no. VO-95-1001)
- Xylene (Panreac, cat. no. 251769)
- Hematoxylin (Panreac, cat. no. 253949)
- Eosin Y (Panreac, cat. no. 131299)
- Knockout Dulbecco's modified Eagle's medium (Gibco, Life Technologies, cat. no. 10829-018)
- GlutaMAX (Gibco, Life Technologies, cat. no. 35050-038)
- 2-Mercaptoethanol (Gibco, Life Technologies, cat. no. 31350-010)
- Basic fibroblast growth factor (bFGF) (Chemicon, cat. no. GF003AF-MG)
- Non-essential amino acids (Cambrex, Lonza, cat. no. BE13-114E)
- KnockOut serum replacement (Gibco, Life Technologies, cat. no. 10828028)
- Penicillin-streptomycin (Gibco, Life Technologies, cat. no. 15140122)
- Propyl gallate (Sigma-Aldrich, cat. no. P3130)
- Glycerol (Merck, cat. no. 1.04093.1000)
- AP staining kit (Sigma-Aldrich, cat. no. AB0300)
- Basic M.O.M. kit (Vector, cat. no. BMK-2202)
- TUNEL kit (Roche Diagnostics, cat. no. 11684817910)
- Tween-20 (Sigma-Aldrich, cat. no. P5927)
- EDTA (Sigma-Aldrich, cat. no. E5134)
- Proteinase K (Sigma-Aldrich, cat. no. P2308)
- Sodium cacodylate (EMS, cat. no. 12310) **! CAUTION** Sodium cacodylate is toxic; avoid contact with eyes and skin. Wear gloves when handling this compound.
- Cobalt (II) chloride anhydrous (Panreac, cat. no. QP213648)
- D-(+)-Sucrose (Fluka, cat. no. 84105)
- Tissue Tek/OCT (Sakura, cat. no. 4583)
- PAP pen (Ted Pella, cat. no. 22309)
- Permanent pen (Ted Pella, cat. no. 27175)
- SuperFrost Plus slides (Menzel-Gläser SuperFrost Plus, cat. no. J1800AMNZ)
- Coverslips with usual thickness (0.15 mm; Knittel Glass, cat. no. ACE180015024X)
- Coverslips with special thickness for high-magnification objectives (0.17 mm; Menzel-Gläser, cat. no. D102460)

- Target retrieval solution, pH = 9, 10× (Dako, cat. no. S2367)
- Target retrieval solution, pH = 6, 10× (Dako, cat. no. S2031)
- AP live stain (500×; Invitrogen, cat. no. A14353)
- QIAamp DNA mini kit (Qiagen, cat. no. 51304)
- AmpFlSTR identifier PCR amplification kit (PE Applied Biosystems Corona, cat. no. 4322288)
- Lipa HLA kit (Murex Immunogenetics)
- PCR kit for sequence-specific primers method (PCR-SSP, Olerup kit)
- miRNeasy mini kit (Qiagen, cat. no. 217004)

**Antibodies** Note that not all antibodies will be needed for every experiment. Check the procedure to determine which of the antibodies you will need:

- Oct4 (1.1) mouse IgG (Santa Cruz, cat. no. sc-5279)
- SSEA3 (1.2) rat IgM (Hybridoma Bank, cat. no. MC-631)
- Sox2 (1.3) rabbit IgG (Fisher Thermo Scientific, cat. no. PAI-16968)
- SSEA4 (1.4) mouse IgG (Hybridoma Bank, cat. no. MC-813-70)
- Tra-1-60 (1.5) mouse IgM (Chemicon, cat. no. MAB4360)
- Nanog human (1.6) goat IgG (R&D, cat. no. AF1997)
- Tra-1-81 (1.7) mouse IgM (Chemicon, cat. no. MAB4381)
- Nanog mouse (1.8) goat IgG (R&D Systems, cat. no. AF2729)
- SSEA1 (1.9) mouse IgM (Hybridoma Bank, cat. no. MC-480)
- GFP (1.10) chicken IgY (AVES, cat. no. GFP-1020)
- α-1-Fetoprotein (1.11) rabbit IgG (Dako, cat. no. A0008)
- hHNF-3β/FoxA2 (1.12) goat IgG (R&D Systems, cat. no. AF2400)
- β-III-Tubulin Tuj1 (1.13) mouse IgG (Covance, cat. no. MMS-435P)
- GFAP (1.14) rabbit IgG (Dako, cat. no. Z0334)
- α-Smooth muscle actin (1.15) mouse IgG (Sigma-Aldrich, cat. no. A5228)
- α-Sarcomeric actin (1.16) mouse IgM (Sigma, cat. no. A2172)
- α-Actinin sarcomeric (1.16B) mouse IgG (Sigma, cat. no. A7811)
- Gata4 (1.17) rabbit IgG (Santa Cruz, cat. no. sc-9053)
- Brachyury (1.18) goat IgG (R&D Systems, cat. no. AF2085)
- Pax7 (1.19) mouse IgG (R&D Systems, cat. no. MAB1675)
- Pax6 (1.20) rabbit IgG (Covance, cat. no. PRB-278P)
- Sox1 (1.21) rabbit IgG (Chemicon, cat. no. AB15766)
- MAP2 (1.22) mouse IgG (Santa Cruz, cat. no. 32791)
- Chondroitin sulfate (1.23) mouse IgM (Sigma-Aldrich, cat. no. C8035)
- Sox9 (1.24) goat IgG (R&D Systems, cat. no. AF3075)
- Fibronectin (1.25) mouse IgG (Sigma-Aldrich, cat. no. F0791)
- Ki67 (1.26) rabbit IgG (Thermo Scientific, cat. no. RM-9106-S)
- PCNA (1.27) mouse IgG (Sigma-Aldrich, cat. no. P8825)
- phH3 (1.28) rabbit IgG (Upstate, cat. no. 06-570)
- RFP (1.29) rabbit IgG (AbCam, cat. no. ab34771)
- FITC/Oregon-Alexa Fluor 488 (1.30) goat IgG (Invitrogen, cat. no. A11096)
- Neurofilament200 (1.31) rabbit IgG (Sigma, cat. no. N4142)
- E-cadherin (1.32) rat IgG (Sigma-Aldrich, cat. no. U3254)
- Connexin43 (1.33) mouse IgG (Becton Dickinson, cat. no. 610061)
- Tra-1-60 PE (1.34) mouse IgM (eBioscience, cat. no. 128863-82)
- Tra-1-81 FITC (1.35) mouse IgM (Macs, cat. no. 09-0069)
- Oct-4 (1.36) goat IgG (Santa Cruz, cat. no. sc-8630)
- Sox2 AF488 (1.37) mouse IgG1 (Becton Dickinson, cat. no. 561593)
- Tra-1-60 AF647 (1.38) mouse IgM (Becton Dickinson, cat. no. 560122)
- Goat anti-mouse IgG Cy2 (2.1) Fcγ fragment specific (Jackson, cat. no. 115-225-071)
- Goat anti-rat IgM Cy3 (2.2) μ-chain specific (Jackson, cat. no. 112-165-020)
- Donkey anti-rabbit IgG Cy2 (2.3; Jackson, cat. no. 711-225-152)
- Goat anti-mouse IgG Cy3 (2.4) Fcγ fragment specific (Jackson, cat. no. 115-165-071)
- Goat anti-mouse IgM Cy5 (2.5) μ-chain specific (Jackson, cat. no. 115-175-075)
- Donkey anti-goat IgG Cy2 (2.6; Jackson, cat. no. 705-225-147)
- Donkey anti-mouse IgM Cy3 (2.7) μ-chain specific (Jackson, cat. no. 715-165-140)
- Donkey anti-mouse IgG Cy3 (2.8; Jackson, cat. no. 715-165-151)
- Donkey anti-chicken IgY Cy2 (2.9; Jackson, cat. no. 703-225-155)
- Goat anti-rat IgM Cy5 (2.10) μ-chain specific (Jackson, cat. no. 112-175-020)
- Donkey anti-rabbit IgG Cy3 (2.11; Jackson, cat. no. 711-165-152)
- Donkey anti-mouse IgG Cy5 (2.12; Jackson, cat. no. 715-175-151)
- Donkey anti-goat IgG Cy3 (2.13; Jackson, cat. no. 705-165-147)
- Rabbit anti-mouse IgM Cy5 (2.14) μ-chain specific (Jackson, cat. no. 315-175-049)
- Donkey anti-mouse IgG Cy2 (2.15; Jackson, cat. no. 715-225-151)
- Goat anti-mouse IgM Cy3 (2.16) μ-chain specific (Jackson, cat. no. 115-165-075)
- Donkey anti-rabbit IgG FITC (2.17; Jackson, cat. no. 711-095-152)
- Donkey anti-rabbit IgG Cy5 (2.18; Jackson, cat. no. 711-175-152)
- Donkey anti-mouse IgM Cy2 (2.19) μ-chain specific (Jackson, cat. no. 715-225-140)
- Goat anti-mouse IgG2b Alexa Fluor 568 (2.20; Invitrogen, cat. no. A21144)
- Goat anti-mouse IgG1 Alexa Fluor 488 (2.21; Invitrogen, cat. no. A21121)
- Goat anti-mouse IgG2a Alexa Fluor 647 (2.22; Invitrogen, cat. no. A21241)
- Goat anti-mouse IgG2b Alexa Fluor 647 (2.23; Invitrogen, cat. no. A21242)
- Donkey anti-goat IgG Cy5 (2.24; Jackson, cat. no. 705-175-147)

**EQUIPMENT**

- Pascal system (pressure cooker; Dako cytomation, cat. no. S2800)
- Automatic tissue processor (Leica, cat. no. TP1020)
- Microtome (Leica, cat. no. RM2255)
- Cryostat (Leica, cat. no. CM 3050)
- Autostainer (Leica, cat. no. XL)
- Eppendorf tube
- Aluminum foil
- Nail polish
- Pasteur pipettes
- Conical tubes
- Stereomicroscope (Leica, cat. no. Z16APO) with objectives (Planapo ×1.0/WD 97 mm) and camera (Leica DFC 490)
- Optical microscope (Leica, cat. no. DM6000) with objectives (HC PL APO ×10/0.40/0.17/A 506284); (HC PL PLAN APO ×20/0.70/0.17/C 506166); (HCX PL APO ×40/0.85 Corr/0.11-0.23/C 506294); (HCX PL APO ×63/1.40–0.60 OIL/0.17/E 506187); (HCX PL APO ×100/1.40–0.70 OIL/0.17/D 506220); and camera (Leica DFC 500)
- Inverted microscope (Leica, cat. no. DMI4000) with objectives (N PLAN ×5/0.12 pH 0); (N PLAN ×10/0.25 pH 1); (HCX PL FLUOTAR L ×20/0.40/C pH 1); (N PLAN L ×40/0.55/C pH2 506294); (HCX PL FLUOTAR ×63/1.25 OIL/C pH3 506186) and camera (Leica DFC 500)
- Confocal microscopes (Leica, cat. no. TCS SP5 AOBS) and objectives (HC PL APO 10.0 × 0.30 507902); (HCX PL APO ×20.0/0.70 IMM UV 11506191); (HCX PL APO CS ×40.0/1.25 OIL UV 11506251); (HCX PL APO lambda blue ×63.0/1.40 OIL UV) and lasers (diode/488 nm argon/561 nm diode-pumped solid state (DPSS), 633 nm HeNe)
- Stereomicroscope (Leica, cat. no. SE6)
- Cell sorter: MoFlo (Dako)
- Karyotyping system: software CytoVision/Genus (Applied Imaging) on an Olympus BX51 microscope
- MagNA Pure LC machine (Roche Applies Sciences)
- MetaMorph software

**REAGENT SETUP**

**TBS, 10× (pH 7.4–7.5)** Mix 855 ml of distilled water, 132.2 g of Trizma-HCl, 19.4 g of Trizma base and 90.0 g of NaCl. Keep the solution in a refrigerator (i.e., at 4 °C) and use it within 2 weeks.

**TBS, 1× (pH 7,4–7,5)** Mix 900 ml of distilled water and 100 ml of 10× TBS. Keep the solution in a refrigerator and use it within 2 weeks.

**TBS, 1×, + 0.1% (vol/vol) Triton X-100** Mix 1,000 ml of 1× TBS and 1 ml of Triton X-100. Keep the solution in a refrigerator and use it within 1 week.

**TBS, 1×, + 0.5% (vol/vol) Triton X-100** Mix 1,000 ml of 1× TBS and 5 ml of Triton X-100. Keep the solution in a refrigerator and use it within 1 week.

**Blocking buffer** Blocking buffer is 1× TBS containing 0.5% (vol/vol) Triton X-100 and 3% or 6% (vol/vol) donkey serum. ▲ **CRITICAL** To avoid contamination, keep the 1× TBS with Triton X-100 at 4 °C and add the serum just before you start the immunodetection. Use only on the day of preparation.

**TBS++** TBS++ is 1× TBS containing 0.1% (vol/vol) Triton X-100 and 3% or 6% (vol/vol) donkey serum. ▲ **CRITICAL** To avoid contamination, keep the 1× TBS with Triton X-100 at 4 °C and add the serum just before you start the immunodetection. Use only on the day of preparation.

**Target retrieval solution for cryosections (10 mM sodium citrate buffer, pH 8.5)** Add 2.94 g of trisodium citrate 2-hydrate to 1,000 ml of distilled water. Mix the contents until the tri-sodium citrate 2-hydrate dissolves. Adjust the pH to 8.5 with 1 N HCl or 1 N NaOH. This solution can be stored at 4 °C for 1 month. Before use, preheat the hotplate and put a thermometer in the citrate buffer. When the citrate buffer reaches 80 °C, maintain this temperature and immerse the slides in the buffer. Maintain the temperature and keep the slides in the buffer for 40 min. Leave the slides in the solution and let the solution cool down to room temperature (RT, ~24 °C) for 20 min.

**Fluorescence mounting medium** Mix 4.2 g of glycine, 0.21 g of NaOH, 5.1 g of NaCl, 0.3 g of $NaN_3$, 50 g of propyl gallate, 300 ml of distilled water

and 700 ml of glycerol. Dissolve the contents by heating to a maximum temperature of 40 °C; let the solution cool down, aliquot it (1.5 and 10 ml) and keep the aliquots at −20 °C protected from light for 1 year. Thawed aliquots can be kept in a refrigerator for months.

**Blocking buffer for TUNEL (1× TBS + 0.2% (vol/vol) Triton X-100 + 0.05% (vol/vol) Tween-20)** Mix 100 ml of 1× TBS, 200 µl of Triton X-100 and 50 µl of Tween-20. Keep the buffer in the refrigerator and use it within 2 weeks.

**Tris-HCl, 10 mM, + 5 mM EDTA** Dissolve 0.788 g of Trizma-HCl and 0.9306 g of EDTA in 500 ml of distilled water. Adjust the pH to 8 with 1 N HCl or 1 N NaOH. Keep the solution in the refrigerator and use it within 2 weeks.

**EDTA (5 mM)** Dissolve 0.9306 g of EDTA in 500 ml of distilled water. Keep EDTA in the refrigerator and use it within 2 weeks.

**HES medium** Prepare KnockOut Dulbecco's modified Eagle's medium supplemented with 2 mmol per liter GlutaMAX, 0.05 mmol per liter 2-mercaptoethanol, 8 ng $ml^{-1}$ bFGF), 1% (vol/vol) non-essential amino acids, 20% (vol/vol) KnockOut serum replacement and 0.5% (vol/vol) penicillin-streptomycin.

**TdT buffer** Dissolve 1.182 g of Trizma-HCl in 250 ml of distilled water. Adjust the pH to 7.2 with 1 N HCl or 1 N NaOH. Add 7.49 g of sodium cacodylate and 0.032 g of cobalt (II) chloride anhydrous. Adjust the pH to 7.75 with 1 N HCl or 1 N NaOH. Keep the buffer in the refrigerator and use it within 2 weeks.

**EDTA (5 mM) in standard saline citrate (SSC)** Dissolve 0.9306 g of EDTA, 8.757 g of NaCl and 4.412 g of trisodium citrate 2-hydrate in 500 ml of distilled water. Keep the solution in the refrigerator and use it within 2 weeks.

## PROCEDURE

### AP staining ● TIMING 30 min

**1|** Culture ESC or iPSC colonies in a six-well plate for 4–5 d (colonies of small size are preferred). Add a previously characterized cell line as positive control and feeders as negative control. Before beginning the assay, we suggest taking pictures of the colonies with an inverted microscope.

**2|** Remove the medium and quickly fix the colonies by incubating each well with 2 ml of 4% (wt/vol) PFA for 1–2 min at RT.
**! CAUTION** PFA is toxic. Wear gloves and work in a fume hood.
**▲ CRITICAL STEP** Do not wash before fixation.
**▲ CRITICAL STEP** Avoid exceeding the fixation time because the AP activity could be inactivated. A longer fixation time will cancel the signal.

**3|** Aspirate the PFA and wash the wells once with 2 ml of 1× PBS at RT.

**4|** If you are assaying human cells, aspirate the 1× PBS and add new 1× PBS (previously warmed to 64 °C). Incubate the mixture for 20 min at 64 °C. Prepare the mixed solution A + B (mix solutions A and B of the AP staining kit in a 1:1 ratio) in these 20 min.
**▲ CRITICAL STEP** Only incubate human pluripotent stem cells at 64 °C.

**5|** Aspirate off and discard the 1× PBS and wash the wells twice with 1 ml of the mixed solution A + B.
**▲ CRITICAL STEP** The phosphate of the PBS can disturb the reaction of the enzyme with its substrates; therefore, you should wash the wells with the kit mixture.

**6|** Add 1.5 ml per well of the mixed solution A + B. Incubate the mixture in the dark at RT, checking the color of the colonies each minute with a stereomicroscope.
**▲ CRITICAL STEP** The blue signal should appear in 2–3 min and reach a plateau within 6 min. Exceeding the staining time will increase the background.

**7|** Remove the solution once colonies show a clear blue color, and wash the wells with 2 ml per well of 1× PBS.

**8|** Add 2 ml per well of 1× PBS and count the number of colonies that express AP (blue-dyed colonies) versus the number of differentiated colonies (undyed colonies). If required, take pictures.
**▲ CRITICAL STEP** Take pictures of dyed colonies immediately, as the fixation process is quite weak and the morphology of the colonies may change quickly.

**9|** Determine whether the colonies show AP activity. To be deemed positive for AP activity, more than 90% of the colonies should be positive for the blue staining. If colonies are positive, proceed to the next step.

### Pluripotency detection ● TIMING 2 d

**10|** Culture the cells in SlideFlasks for 5–7 d (colonies should reach a size at which they are not touching one another and no signs of differentiation spots are observed in the center of the colony). The number of SlideFlasks required depends on the number of antibody combinations being used (see Step 15).

**11|** Remove the culture medium and fix the samples with 2 ml of 4% (wt/vol) PFA for 20–30 min at RT.
**▲ CRITICAL STEP** Remove the culture medium and immediately add the fixative without any washing.

**12|** Wash the samples three times for 5 min each with 1× TBS. If necessary, perform antigen retrieval (**Box 4**).
■ **PAUSE POINT** If you do not immediately proceed with immunostaining, you may leave the SlideFlask with 3 ml of 1× TBS at 4 °C before using it in the next 2 weeks.

**13|** Incubate with blocking buffer (1× TBS plus 0.5% (vol/vol) Triton X-100 plus 6% (vol/vol) donkey serum) for 30 min to 1 h at RT, in order to permeabilize the plasma membrane and to decrease the nonspecific binding of the antibodies. Use ~2 ml per SlideFlask.
▲ **CRITICAL STEP** Remember to use only donkey serum, not goat serum, as well as not to use BSA, as there is one primary antibody made in goat.

**14|** Remove the plastic lid from the SlideFlask, and mark the borders of the slide with the PAP pen.
▲ **CRITICAL STEP** Before removing the lid, remember to mark the slide with a permanent marker in order to clearly identify the samples (as usually the sample names are on the lid). We usually write the name of the cell line, the antibody combination (A1, A2 or A3) and a reference number (we always write this number on everything connected to an experiment to link all the documents and samples from the same experiment).
**? TROUBLESHOOTING**

**15|** Choose the antibody combinations to use. The combinations depend on whether the sample is from humans or mice and whether the sample is GFP positive or DsRed positive. **Tables 1** and **2** show the different combinations we use and when each should be used. For example, for hESCs and hiPSCs, we prepare three combinations of antibodies: A1, A2 and A3.

**16|** Prepare one Eppendorf tube for each combination.

**17|** Calculate the total amount of solution you need for each combination, taking into account that a 200-µl volume is added per SlideFlask. The volumes given in **Table 1** are for a single SlideFlask. For example, a total volume of 400 µl is needed in each Eppendorf tube to characterize two cell lines.
▲ **CRITICAL STEP** In the case of SSEA3-specific antibody, the volume to be diluted is 50 µl; therefore, the amount of TBS++ added to the Eppendorf tube is 150 µl, achieving 200 µl of total solution per slide.

**18|** Take the last antibody aliquot you have used from the refrigerator, or thaw a new one from the freezer if the volume is insufficient. Do not freeze any remaining aliquot again, but keep it in the refrigerator.

**19|** Centrifuge the antibody aliquot briefly to be sure to recover all of the solution, and then vortex to homogenize it.

**20|** Add in each tube the quantity of antibody you need, taking into account the dilution and the number of slides. For example, if two cell lines have to be characterized, use 6.8 µl of the Oct4-specific antibody for combination A1 (3.4 µl × 2 slides).

**21|** Add the primary antibodies diluted in TBS++ inside the PAP pen–marked areas, and incubate the slides overnight at 4 °C in a humid chamber. Add some TBS++ drops without primary antibodies in the negative control area.
▲ **CRITICAL STEP** Ensure that your refrigerator is well leveled and do not let the slides dry.
■ **PAUSE POINT** Although overnight incubation at 4 °C is preferable, the incubation time can be adjusted: either 37 °C for 2 h or 4 °C over the weekend, adding extra water to the humid chamber.
**? TROUBLESHOOTING**

**22|** (Optional) Take the slides out of the refrigerator and leave them for 2 h at RT. Although this step is optional, it can improve the intensity of the immunodetections without increasing the background.

**23|** Wash the slides three times for 5 min each with TBS++.

**24|** Determine which secondary antibodies should be used. **Tables 3** and **4** show the combinations we use following the corresponding combinations of primary antibodies described in **Tables 1** and **2**. Attention must be paid to the different species of the primary antibodies and to the different fluorochromes to be used.

**25|** Prepare one Eppendorf tube for each combination.

**TABLE 1** | Primary antibody combinations for pluripotency detection in human ESCs and iPSCs.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| Human (A1) | Oct4 (1.1) | Mouse IgG | 1:60 | 3.4 |
| | SSEA3 (1.2) | Rat IgM | 1:3 | 50 |
| | TBS++ | | | 150 |
| Human (A2) | Sox2 (1.3) | Rabbit IgG | 1:100 | 2 |
| | SSEA4 (1.4) | Mouse IgG | 1:3 | 50 |
| | Tra-1-60 (1.5) | Mouse IgM | 1:200 | 1 |
| | TBS++ | | | 150 |
| Human (A3) | Nanog (1.6)[a] | Goat IgG | 1:25 | 8 |
| | Tra-1-81 (1.7) | Mouse IgM | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/GFP$^+$ (A4) | GFP (1.10) | Chicken IgY | 1:250 | 0.8 |
| | Oct4 (1.1) | Mouse IgG | 1:60 | 3.4 |
| | SSEA3 (1.2) | Rat IgM | 1:3 | 50 |
| | TBS++ | | | 150 |
| Human/GFP$^+$ (A5) | GFP (1.10) | Chicken IgY | 1:250 | 0.8 |
| | Sox2 (1.3) | Rabbit IgG | 1:100 | 2 |
| | SSEA4 (1.4) | Mouse IgG | 1:3 | 50 |
| | TBS++ | | | 150 |
| Human/GFP$^+$ (A6)[b] | GFP (1.10) | Chicken IgY | 1:250 | 0.8 |
| | Nanog (1.6)[a] | Goat IgG | 1:25 | 8 |
| | Tra-1-81 (1.7) | Mouse IgM | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/RFP$^+$ (A7) | Oct4 (1.1) | Mouse IgG | 1:60 | 3.4 |
| (DsRed, cherry or orange) | RFP (1.29) | Rabbit IgG | 1:400 | 0.5 |
| | SSEA3 (1.2) | Rat IgM | 1:3 | 50 |
| | TBS++ | | | 150 |
| Human/RFP$^+$ (A8) | Sox2 (1.3) | Rabbit IgG | 1:100 | 2 |
| (DsRed, cherry or orange) | Tra-1-60 (1.5) | Mouse IgM | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/RFP$^+$ (A9)[b] | Nanog (1.6)[a] | Goat IgG | 1:25 | 8 |
| (DsRed, cherry or orange) | Tra-1-81 (1.7) | Mouse IgM | 1:200 | 1 |
| | TBS++ | | | 200 |

[a]Nanog (1.6) antibody is specific only to human cells. [b]In combinations A8 and A9, as a rabbit anti-RFP antibody is used, it cannot be combined with the rabbit anti-Sox2. Therefore, we do not add the anti-RFP antibody, and we do not use any secondary antibody conjugated with red fluorescence.

**TABLE 2** | Primary antibody combinations for pluripotency detection in mouse ESCs and iPSCs.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| Mouse (A10) | Sox2 (1.3) | Rabbit IgG | 1:100 | 2 |
| | Oct4 (1.1) | Mouse IgG2b | 1:60 | 3.4 |
| | TBS++ | | | 200 |
| Mouse (A11) | Nanog (1.8)[a] | Goat IgG | 1:25 | 8 |
| | SSEA1 (1.9) | Mouse IgM | 1:3 | 50 |
| | TBS++ | | | 150 |
| Mouse/GFP$^+$ (A12) | GFP (1.10) | Chicken IgY | 1:250 | 0.8 |
| | Sox2 (1.3) | Rabbit IgG | 1:100 | 2 |
| | Oct4 (1.1) | Mouse IgG2b | 1:60 | 3.4 |
| | TBS++ | | | 200 |
| Mouse/GFP$^+$ (A13) | GFP (1.10) | Chicken IgY | 1:250 | 0.8 |
| | Nanog (1.8)[a] | Goat IgG | 1:25 | 8 |
| | SSEA1 (1.9) | Mouse IgM | 1:3 | 50 |
| | TBS++ | | | 150 |
| Mouse/RFP$^+$ (A14)[b,c] | Sox2 (1.3) | Rabbit IgG | 1:100 | 2 |
| | SSEA1 (1.9) | Mouse IgM | 1:3 | 50 |
| | TBS++ | | | 150 |
| Mouse/RFP$^+$ (A15) | Oct4 (1.1) | Mouse IgG2b | 1:60 | 3.4 |
| | RFP (1.29) | Rabbit IgG | 1:400 | 0.5 |
| | Nanog (1.8)[a] | Goat IgG | 1:25 | 8 |
| | TBS++ | | | 200 |

[a]Nanog (1.8) antibody is specific only to mouse cells. [b]Combination A14: as mentioned above for human samples, even with mouse cells the two rabbit antibodies anti-Sox2 and anti-RFP cannot be used together. Therefore, the anti-RFP antibody is not added in this combination. [c]Combination A14: we have not found a suitable anti-mouse IgM-Cy5 made in donkey, only in goat or rabbit. For this reason, the mouse IgM anti-SSEA1 cannot be mixed with the goat anti-Nanog and the rabbit anti-RFP. It can only be used with the rabbit Sox2 using a secondary antibody made in goat.



**26|** Calculate the total amount of solution needed for each combination, considering that a total of 200 μl will be added in each SlideFlask. For example, to characterize two cell lines, 400 μl of TBS++ will be added in each Eppendorf tube.

**27|** Take the last aliquot you have used for each antibody from the refrigerator, or thaw a new one if the volume is insufficient. Keep the leftover aliquot in the refrigerator, but do not freeze it again.
▲ **CRITICAL STEP** Fluorescent secondary antibodies must be protected from light by aluminum foil.

**28|** Centrifuge the aliquot briefly to be sure to recover all of the solution, and then vortex to homogenize it.

**29|** Add the necessary amount of antibody in each Eppendorf tube, taking into account the dilution and the number of slides. For example, to characterize two cell lines in the tube for the combination A1′, you should add 2 μl of the anti-mouse IgG Cy2 antibody (1 μl × 2 slides).

**TABLE 3** | Secondary antibody combinations for pluripotency detection in human ESCs and iPSCs.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| Human (A1′)[a,b] | Anti-mouse IgG Cy2 (2.1) | Goat IgG | 1:200 | 1 |
| | Anti-rat IgM Cy3 (2.2) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human (A2′) | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgG Cy3 (2.4) | Goat IgG | 1:200 | 1 |
| | Anti-mouse IgM Cy5 (2.5) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human (A3′)[c] | Anti-goat IgG Cy2 (2.6) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgM Cy3 (2.7) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/GFP$^+$ (A4′) | Anti-chicken IgY Cy2 (2.9) | Donkey IgG | 1:50 | 4 |
| | Anti-mouse IgG Cy3 (2.4) | Goat IgG | 1:200 | 1 |
| | Anti-rat IgM Cy5 (2.10) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/GFP$^+$ (A5′) | Anti-chicken IgY Cy2 (2.9) | Donkey IgG | 1:50 | 4 |
| | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgG Cy5 (2.12) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/GFP$^+$ (A6′) | Anti-chicken IgY Cy2 (2.9) | Donkey IgG | 1:50 | 4 |
| | Anti-goat IgG Cy3 (2.13) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgM Cy5 (2.14) | Rabbit IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/RFP$^+$ (A7′) | Anti-mouse IgG Cy2 (2.1) | Goat IgG | 1:200 | 1 |
| (DsRed, cherry or orange) | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1 |
| | Anti-rat IgM Cy5 (2.10) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/RFP$^+$ (A8′) | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1 |
| (DsRed, cherry or orange) | Anti-mouse IgM Cy5 (2.5) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Human/RFP$^+$ (A9′) | Anti-goat IgG Cy2 (2.6) | Donkey IgG | 1:200 | 1 |
| (DsRed, cherry or orange) | Anti-mouse IgM Cy5 (2.14) | Rabbit IgG | 1:200 | 1 |
| | TBS++ | | | 200 |

[a]Combination A1′: the anti-mouse IgG Cy2 must be a preabsorbed with rat serum and the anti-rat IgM Cy3 must be a preabsorbed with mouse serum to avoid cross-linking. Check this information in the antibody's data sheet. [b]Combination A1′: in this case, the primary antibodies contain IgM and IgG. Thus, the secondary antibodies have to recognize the μ- and γ-chains, respectively. [c]Combination A3′: when a goat primary antibody is used in a double immunodetection, check that the secondary against the other primary antibody has not been made in goat. Because the anti-goat antibody will recognize the other secondary antibody, leading to cross-linking between the two signals.

**TABLE 4** | Secondary antibody combinations for pluripotency detection in mouse ESCs and iPSCs.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| Mouse (A10′)[a] | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgG2b Alexa Fluor 568 (2.20) | Goat IgG | 1:100 | 2 |
| | TBS++ | | | 200 |
| Mouse (A11′) | Anti-goat IgG Cy2 (2.6) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgM Cy3 (2.7) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Mouse/GFP$^+$ (A12′) | Anti-chicken IgY Cy2 (2.9) | Donkey IgG | 1:50 | 4 |
| | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgG2b Alexa Fluor 647 (2.23) | Goat IgG | 1:100 | 2 |
| | TBS++ | | | 200 |
| Mouse/GFP$^+$ (A13′) | Anti-chicken IgY Cy2 (2.9) | Donkey IgG | 1:50 | 4 |
| | Anti-goat IgG Cy3 (2.13) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgM Cy5 (2.14) | Rabbit IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Mouse/RFP$^+$ (A14′) | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgM Cy5 (2.5) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Mouse/RFP$^+$ (A15′)[b] | Anti-mouse IgG Cy2 (2.15) | Donkey IgG | 1:200 | 1 |
| | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1 |
| | Anti-goat IgG Cy5 (2.24) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |

[a]For combination A10′, use a specific antibody for the immunoglobulin isotype of the primary antibody (IgG2b in this case) or M.O.M component to decrease the nonspecific background.
[b]For combination A15′, the anti-mouse IgG2b antibody cannot be used to decrease the background because it was made in goat and the same combination uses the goat anti-Nanog. Therefore, if the signal in the negative control is not lower than in the area of detection, the M.O.M reagent must be used.



**30|** Add the secondary antibody diluted in TBS++ inside the PAP pen–marked areas and incubate the slides for 2 h at 37 °C in a humid chamber. Ensure that you also add the secondary antibodies in the negative control areas. If possible, leave the slides at RT for 30 min before starting the washes.
▲ **CRITICAL STEP** Before adding the antibodies, dry the PAP pen lines and, if necessary, draw the lines again.

**31|** Wash the slides with 1× TBS three times for 5 min each.

**32|** Incubate the slides with DAPI at a 1:10,000 ratio for 10 min at RT (add 200 μl on each slide).

**33|** Remove the DAPI.

**34|** Add two or three drops of mounting medium.

**35|** Cover the slide with a coverslip (24 mm × 60 mm).
▲ **CRITICAL STEP** To obtain optimal resolution, take into consideration the thickness of the coverslip. If a 0.17-μm labeled objective is used, coverslips of the same thickness must be used, and not the usual 0.15-μm coverslips.

**36|** For each slide, dry the excess mounting medium and cover the borders with nail polish to fix the coverslip to the slide.
■ **PAUSE POINT** Keep the slides at 4 °C in darkness until microscopic observation. During the two weeks after mounting, the fluorescence will be well preserved. After this time, fluorescence staining could be fainter or diffused.

**37|** Take images with a confocal microscope of each cell line for each antibody combination. If pluripotency markers are expressed as expected for a pluripotent stem cell, proceed with Step 38.
**? TROUBLESHOOTING**

**Differentiation detection in EBs**

**38|** Culture the pluripotent-positive cells in suspension in order to obtain EBs[19,20]. Either detect the differentiation markers in the EBs in suspension (option A) or in SlideFlasks (option B). The number of SlideFlasks required depends on the number of antibody combinations being used (**Table 5**). Ensure that at least five EBs are in each SlideFlask. Let them attach to the surface and grow until different structures appear around the primitive EBs (check by inverted microscope). Normally it takes around 2–3 weeks in culture. Once EBs are obtained, detect the differentiation markers in the SlideFlask (option B).

**(A) Differentiation detection with EBs in suspension ● TIMING 5 d**

(i) Remove the EBs in suspension from the culture plate with a Pasteur pipette and put them in a six-well plate with 1 ml of PBS.
(ii) In the fume hood, add 1 ml of 8% (wt/vol) PFA and cover the plate with the lid. Fix for 2 h at 4 °C.
(iii) Remove the fixative in the fume hood, taking care not to aspirate the EBs. Add 1× TBS. Repeat this step twice. If necessary, do not remove the entire fixative and dilute the remaining fixative with more washes.
(iv) Prepare a 96-well plate and add 100 µl of 1× TBS in the wells you are going to use, depending on the antibody combinations selected (**Table 5**). Combinations B1, B2 and B3 are the most common markers used. Some variations, which are shown in combinations B4–B12, can be used. Include wells for the negative controls.
(v) With the Pasteur pipette, transfer from the six-well plate at least three EBs into each well of the 96-well plate using a stereomicroscope. Put EBs in the negative control wells as well.
(vi) Wash the samples again with 1× TBS four times for 30 min each. Place the 96-well plate over a shaker during the wash time.
■ **PAUSE POINT** The immunodetection can be started immediately, or the 96-well plates can be left at 4 °C with 1× TBS for up to 2 weeks.
(vii) Incubate the samples with blocking buffer (1× TBS plus 0.5% (vol/vol) Triton X-100 plus 6% (vol/vol) donkey serum) for 2 h at RT plus 24 h at 4 °C plus 2 h at RT. Use a minimum volume of 200 µl per well. Place the 96-well plate over a shaker when possible.
(viii) Remove the blocking buffer and put the primary combination chosen in each well (**Table 5**) according to the general guidelines explained in Steps 15–20. Prepare only 100 µl of solution per combination. In the negative control wells, add only 100 µl of TBS++. Incubate the samples in TBS++ for 2 h at RT, plus 48 or 72 h at 4 °C, plus 2 h at RT.
(ix) Wash the samples with TBS++ four times for 30 min each at RT. Place the 96-well plate over a shaker.
(x) Add secondary antibody combinations depending on the ones used in Step 38A(viii) (**Table 6**) according to the general guidelines explained in Steps 24–30. Prepare only 100 µl of solution per combination. Remember to add each secondary antibody selected in one of the negative control wells. Incubate the samples in TBS++ 2 h at RT, plus overnight at 4 °C, plus 2 h at RT.
(xi) Wash the wells with 1× TBS four times for 30 min each at RT. Place the 96-well plate over a shaker.
(xii) Incubate the samples with DAPI at a 1:1,000 ratio for 1 h at RT. Place the 96-well plate over a shaker.
(xiii) Remove the DAPI solution.
(xiv) Add a few drops of mounting medium diluted with 1× TBS 1:1 for 30 min at RT.
▲ **CRITICAL STEP** The mounting medium must be around and inside the sample. For this reason, in this thick sample, an intermediate step must be added in order to dilute the viscous mounting medium with 1× TBS.
(xv) Prepare one slide for each antibody combination. Add a few drops of mounting medium in each slide and, with the help of the stereomicroscope if necessary, transfer the EBs from the 96-well plate to the slide.
(xvi) Place a coverslip over the EBs. If the EBs are quite big, and the 3D structure has to be observed, it is better to put a coverslip on each edge of the slide, seal it with nail polish and then put the EBs between them with mounting medium. Cover the slide with another coverslip, supported by the former, and seal it with nail polish. In some cases, two coverslips must be used to increase the thickness and avoid flattening of the EBs.
**? TROUBLESHOOTING**

**(B) Differentiation detection with EBs in a SlideFlask ● TIMING 3 d**

(i) Remove the culture medium and fix the samples with 2 ml of 4% (wt/vol) PFA for 20–30 min at RT.
(ii) Wash the samples three times for 5 min each with 1× TBS.
■ **PAUSE POINT** At this point, you may start the immunostaining or leave the SlideFlask with 3 ml of 1× TBS at 4 °C before using it in the next 2 weeks.

**TABLE 5** | Primary antibody combinations for the *in vitro* differentiation test in mouse and human EBs.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| *Guided differentiation* | | | | |
| Endoderm (B1) | α-1-Fetoprotein (1.11) | Rabbit IgG | 1:400 | 0.75 |
| | FoxA2 (1.12) | Goat IgG | 1:50 | 6 |
| | TBS++ | | | 300[a] |
| Ectoderm (B2) | β-III-Tubulin Tuj1 (1.13) | Mouse IgG | 1:500 | 0.6 |
| | GFAP (1.14) | Rabbit IgG | 1:1000 | 0.3 |
| | TBS++ | | | 300 |
| Mesoderm (B3) | α-Smooth muscle actin (1.15) | Mouse IgG | 1:400 | 0.75 |
| | TBS++ | | | 300 |
| *Spontaneous differentiation* | | | | |
| Ecto/endo (B4) | β-III-Tubulin Tuj1 (1.13) | Mouse IgG | 1:500 | 0.6 |
| | FoxA2 (1.12) | Goat IgG | 1:50 | 6 |
| | TBS++ | | | 300 |
| Meso/endo (B5) | α-Smooth muscle actin (1.15) | Mouse IgG | 1:400 | 0.75 |
| | α-1-Fetoprotein (1.11) | Rabbit IgG | 1:400 | 0.75 |
| | TBS++ | | | 300 |
| *Early differentiation* | | | | |
| (B6) | Brachyury (1.18) | Goat IgG | 1:25 | 12 |
| | α-Smooth muscle actin (1.15) | Mouse IgG | 1:400 | 0.75 |
| | TBS++ | | | 300 |
| (B7) | Pax7 (1.19) | Mouse IgG | 1:25 | 12 |
| | α-Sarcomeric actin (1.16) | Mouse IgM | 1:400 | 0.75 |
| | TBS++ | | | 300 |
| (B8) | Pax6 (1.20) | Rabbit IgG | 1:100 | 3 |
| | MAP2 (1.22) | Mouse IgG1 | 1:50 | 6 |
| | TBS++ | | | 300 |
| (B9) | Sox1 (1.21) | Rabbit IgG | 1:200 | 1.5 |
| | MAP2 (1.22) | Mouse IgG1 | 1:50 | 6 |
| | TBS++ | | | 300 |
| *Specific differentiation to cardiomyocytes* | | | | |
| (B10) | Gata4 (1.17) | Rabbit IgG | 1:50 | 6 |
| | α-Actinin sarcomeric (1.16B) | Mouse IgG | 1:100 | 3 |
| | TBS++ | | | 300 |

(continued)

**TABLE 5** | Primary antibody combinations for the *in vitro* differentiation test in mouse and human EBs (continued).

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| *Specific differentiation to neurons* | | | | |
| (B11) | MAP-2 (1.22) | Mouse IgG1 | 1:50 | 6 |
| | Pax6 (1.20) | Rabbit IgG | 1:100 | 3 |
| | β-III-Tubulin Tuj1 (1.13) | Mouse IgG2a | 1:500 | 0.6 |
| | TBS++ | | | 300 |
| (B12) | MAP-2 (1.22) | Mouse IgG1 | 1:50 | 6 |
| | Neurofilament200 (1.31) | Rabbit IgG | 1:100 | 3 |
| | β-III-Tubulin Tuj1 (1.13) | Mouse IgG2a | 1:500 | 0.6 |
| | TBS++ | | | 300 |

[a]It is better to use 300 μl for each slide (because the EBs may be thick) instead of the 200 μl used in the pluripotency immunodetection, and to increase the time of primary antibody incubation to 48 h.



(iii) Incubate the samples with 2 ml of blocking buffer (1× TBS plus 0.5% (vol/vol) Triton X-100 plus 6% (vol/vol) donkey serum) for 30 min–1 h at RT.
(iv) Remove the plastic lid from the SlideFlask.
(v) Mark the borders of the slide with a PAP pen. It is recommended to create an area for a negative control (drawing a vertical line with the PAP pen) with at least one EB.
(vi) Choose the antibody combinations to use (**Table 5**) and prepare one Eppendorf tube for each combination, as described previously (Steps 15–20).
(vii) Add appropriate primary antibodies diluted in TBS++ (1× TBS plus 0.1% (vol/vol) Triton X-100 plus 6% (vol/vol) donkey serum) in each slide and incubate them for 48 h at 4 °C in a humid chamber. Add only TBS++ in the negative control area.
(viii) (Optional) Take the slides out of the refrigerator and leave them for 2 h at RT. Although this step is optional, it can improve the intensity of the immunodetections without increasing the background.
(ix) Wash the slides three times for 5 min each with TBS++.
(x) Determine which secondary antibodies should be used. **Table 6** shows the combinations we use after the corresponding combinations of primary antibodies described in **Table 5**.
(xi) Prepare one Eppendorf tube for each combination, calculating a total of 300 μl per slide. Follow the guidelines described previously (Steps 24–30).
(xii) Incubate the slides for 2 h at 37 °C in the humid chamber.
(xiii) Wash the slides with 1× TBS three times for 5 min each.
(xiv) Incubate the samples with DAPI at a 1:10,000 ratio for 10 min at RT (add 300 μl on each slide).
(xv) Remove the DAPI.
(xvi) Add two or three drops of mounting medium.
(xvii) Cover the slide with a coverslip (24 mm × 60 mm).
▲ **CRITICAL STEP** To obtain optimal resolution, take into consideration the thickness of the coverslip. If a 0.17-μm labeled objective is used, coverslips of the same thickness must be used instead of the usual 0.15-μm coverslips.
**? TROUBLESHOOTING**
(xviii) Dry the excess mounting medium and cover the borders with nail polish to fix each coverslip to the slide.

**39|** For each antibody combination, take images of each cell line with a confocal microscope. If differentiation markers of the three germ layers are expressed, proceed with the *in vivo* differentiation test.

**Differentiation detection in teratomas ● TIMING 4 d**

**40|** Once it is confirmed that a cell line is able to differentiate to the three germ layers *in vitro*, the *in vivo* test can be performed. Inject around 1 million ESCs or IPSCs into the testis of a SCID mouse. If a teratoma grows, it should be removed after 2 or 3 months.

**TABLE 6** | Secondary antibody combinations for the *in vitro* differentiation test in mouse and human EBs.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| *Guided differentiation* | | | | |
| Endoderm (B1′) | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1.5 |
| | Anti-goat IgG Cy3 (2.13) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| Ectoderm (B2′) | Anti-mouse IgG Cy2 (2.15) | Donkey IgG | 1:200 | 1.5 |
| | Anti-Rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| Mesoderm (B3′) | Anti-mouse IgG Cy2 (2.15) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| *Spontaneous differentiation* | | | | |
| Ecto/Endo (B4′) | Anti-mouse IgG Cy2 (2.15) | Donkey IgG | 1:200 | 1.5 |
| | Anti-goat IgG Cy3 (2.13) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| Meso/Endo (B5′) | Anti-mouse IgG Cy2 (2.15) | Donkey IgG | 1:200 | 1.5 |
| | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| *Early differentiation* | | | | |
| (B6′) | Anti-goat IgG Cy2 (2.6) | Donkey IgG | 1:200 | 1.5 |
| | Anti-mouse IgG Cy3 (2.8) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| (B7′) | Anti-mouse IgG Cy2 (2.1) | Goat IgG | 1:200 | 1.5 |
| | Anti-mouse IgM Cy3 (2.16) | Goat IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| (B8′) | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1.5 |
| | Anti-mouse IgG Cy3 (2.8) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| (B9′) | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1.5 |
| | Anti-mouse IgG Cy3 (2.8) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |
| *Specific differentiation to cardiomyocytes* | | | | |
| (B10′)[a] | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1.5 |
| | Anti-mouse IgG Cy3 (2.8) | Donkey IgG | 1:200 | 1.5 |
| | TBS++ | | | 300 |

(continued)

**TABLE 6** | Secondary antibody combinations for the *in vitro* differentiation test in mouse and human EBs (continued).

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| *Specific differentiation to neurons* | | | | |
| (B11′) | Anti-mouse IgG1 Alexa Fluor 488 (2.21) | Goat IgG | 1:100 | 3 |
| | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1.5 |
| | Anti-mouse IgG2a Alexa Fluor 647 (2.22) | Goat IgG | 1:100 | 3 |
| | TBS++ | | | 300 |
| (B12′) | Anti-mouse IgG1 Alexa Fluor 488 (2.21) | Goat IgG | 1:100 | 3 |
| | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1.5 |
| | Anti-mouse IgG2a Alexa Fluor 647 (2.22) | Goat IgG | 1:100 | 3 |
| | TBS++ | | | 300 |

[a]Combination B10: for this detection, it is preferable to fix with 2% (wt/vol) PFA for 15 min and ensure that the fixative is at 37 °C. Moreover, add the fixative directly at 4% (wt/vol) to the same amount of culture medium. Without these precautions, the actinin sarcomeric staining can be lost.



**41|** Put the teratoma in a 50-ml conical tube with 4% (wt/vol) PFA, ensuring that the teratoma is totally covered by ~20 ml of fixative solution. Fix it from 4 h to overnight at 4 °C.

**42|** Remove the fixative completely.

**43|** Wash with 1× PBS three times for 30 min each in a shaker.

■ **PAUSE POINT** The samples can be kept in the refrigerator for a maximum of 1 week before being embedded.

**44|** To detect the three germ layers with the best morphology preservation, embed the teratoma in paraffin by following option A. Alternatively, to detect proliferation markers or proliferation versus apoptosis studies, cryoembed the teratoma in OCT by following option B or C. The same samples can be divided into two pieces to allow two embedding options to be performed on one sample.

**(A) Embedding in paraffin** ● **TIMING 6–8 d**

(i) Embed the teratoma in paraffin by following the classic protocol. Briefly, start the dehydration process with 30% (vol/vol) ethanol for 1 h at RT. Move up the gradient of ethanol in a series over time (50%, 1 h; 70%, 1 h; 96%, 1 h; repeat 96%, 1 h; 100%, 1 h; repeat 100%, 1 h). Remove the ethanol and immerse the teratoma in xylene (two 1-h incubations). Finally, remove the xylene and transfer the teratoma to liquid paraffin (two 1-h incubations).

(ii) Transfer the teratoma to a paraffin cassette and add liquid paraffin prewarmed at 60 °C. Allow the paraffin to cool down and harden.

■ **PAUSE POINT** The paraffin blocks can be stored in cassette storage drawers at RT for a long time (years).

(iii) Obtain sections with a rotary microtome (5 μm).

▲ **CRITICAL STEP** We usually section the blocks sequentially, starting with ten slides and putting the first section on the first slide, the second section on the second slide and so on. We do not use a continuous method (i.e., the first strip of sections on the first slide, the second strip on the second and so on). If after the first ten slides there is still sample in the block, we start a second series of ten slides, and two slides of each series are used for immunodetection.

▲ **CRITICAL STEP** Use StarFrost slides to recover the sections.

(iv) Leave the sections you are going to use overnight at 37 °C or for 1 h at 60 °C.

(v) *Dewax and rehydrate*. Dewax by immersing the slides in xylene three times for 5 min each. Start rehydration with 100% ethanol, two times for 5 min each. Move down the ethanol gradient in a series over time (96%, 2 × 5 min; 70%, 5 min; 50%, 5 min; 30%, 5 min; distilled water, 5 min).

(vi) Allocate one slide for H&E staining and three for immunodetection.

(vii) Perform an antigen retrieval by incubating the slides with 1× citrate buffer (pH 9) for ~1 h with a Pascal system or a pressure cooker at 103 kPa per 15 p.s.i. (20 min between 27 °C and 125 °C, 1 min at maximum temperature, 20 min between 125 °C and 90 °C, 1 min at 90 °C).
(viii) Let the slides cool down in the 1× citrate buffer for 20 min.
(ix) Wash the slides in 1× TBS three times for 5 min each.
(x) Incubate the slides with 1 ml of blocking buffer over the slides (1× TBS with 0.5% (vol/vol) Triton X-100, 3% (vol/vol) donkey serum) for 30 min at RT.
(xi) Mark the borders of the slides with a PAP pen and create an area with one section for the negative control.
(xii) For human and mouse samples, choose the primary antibody combinations to use (**Table 7**).
(xiii) Prepare primary antibodies diluted in TBS++ as previously described (Steps 15–20).
(xiv) Add 200 µl of each combination in each slide. Incubate the slides overnight at 4 °C in a humid chamber. Add only TBS++ in the negative control area.
(xv) Wash the slides in TBS++ three times for 5 min each.
(xvi) Select the secondary antibodies shown in **Table 8**.
(xvii) Prepare one Eppendorf tube for each combination, calculating a total of 200 µl per slide, and following the guidelines described in Steps 24–30.
(xviii) Incubate the slides for 2 h at 37 °C in the humid chamber.
(xix) Wash them with 1× TBS three times for 5 min each.
(xx) Incubate the samples with DAPI at a 1:10,000 ratio for 10 min at RT (add 300 µl on each slide).
(xxi) Remove the DAPI.
(xxii) Add two or three drops of mounting medium.
(xxiii) Cover each slide with a coverslip (24 mm × 60 mm).
(xxiv) Dry the excess mounting medium and cover the borders with nail polish to fix the coverslips to the slides.
■ **PAUSE POINT** Keep the slides at 4 °C in darkness until microscopic observation. During the two weeks after mounting, the fluorescence is well preserved. After this time, fluorescence staining could be fainter or diffused.
(xxv) For each antibody combination, take images with a confocal microscope of each cell line. If differentiation markers of the three germ layers are expressed, the capacity of the cell line to differentiate *in vivo* will be confirmed.

**(B) Cell proliferation in teratomas ● TIMING 6–8 d**

(i) Cryoprotect the sample with 1× PBS plus 30% (wt/vol) sucrose overnight at 4 °C.
(ii) Embed the sample in OCT with a cryomold and freeze it at −80 °C overnight.
(iii) Make sections in a sequential way with the cryostat (10 µm).
▲ **CRITICAL STEP** For a quantification assay, make sure to study all the areas of the teratoma. In the case of a large teratoma, cut it into smaller pieces to ensure that all its areas are studied (principal, medium and final). Put the different pieces in the same block and cut it sequentially. If the teratoma is small, put it all in one block, but be sure to cut it in its entirety (in a sequential way).
▲ **CRITICAL STEP** Use SuperFrost slides to recover the sections.
(iv) Let the sections dry overnight.
(v) Choose two slides from each teratoma, one for each combination.
■ **PAUSE POINT** The slides can be frozen at −80 °C and kept for years.
(vi) Wash the slides with 1× TBS three times for 5 min each.
(vii) Perform the antigen retrieval. For Ki67 detection, use 1× citrate buffer (pH 6) with the Pascal system as in Step 44A(vii), or boil the slides at 100 °C for 20 min in 1× citrate buffer (pH 6). For PCNA and phH3 detection, use a bain-marie system with citrate buffer (pH 8.5) at 80 °C for 40 min.
(viii) Let the slides cool down in the same buffer (~20 min).
(ix) Wash the slides with 1× TBS three times for 5 min each.
(x) Incubate the slides with blocking buffer (1× TBS plus 0.5% (vol/vol) Triton X-100 plus 3% (vol/vol) donkey serum) for 30 min at RT.
(xi) Mark the borders of the slide and create a negative control area with one section.
(xii) Prepare primary antibodies in TBS++ (1× TBS plus 0.1% (vol/vol) Triton X-100 plus 3% (vol/vol) donkey serum) as shown in the table below. Follow the guidelines described in Steps 15–20.

**TABLE 7** | Primary antibody combinations for the *in vivo* differentiation test in human and mouse cells.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| Endoderm (C1) | α-1-Fetoprotein (1.11) | Rabbit IgG | 1:400 | 0.5 |
| | FoxA2 (1.12) | Goat IgG | 1:50 | 4 |
| | TBS++ | | | 200 |
| Ectoderm (C2) | β-III-Tubulin Tuj1 (1.13) | Mouse IgG | 1:500 | 0.4 |
| | GFAP (1.14) | Rabbit IgG | 1:1,000 | 0.2 |
| | TBS++ | | | 200 |
| Mesoderm (C3) | α-Smooth muscle actin (1.15) | Mouse IgG | 1:400 | 0.5 |
| | α-Sarcomeric actin (1.16) | Mouse IgM | 1:400 | 0.5 |
| | TBS++ | | | 200 |
| *Other possible combinations* | | | | |
| Mesoderm (C4) | Chondroitin sulfate (1.23) | Mouse IgM | 1:200 | 1 |
| | Fibronectin (1.25) | Mouse IgG | 1:100 | 2 |
| | TBS++ | | | 200 |
| Mesoderm (C5) | Chondroitin sulfate (1.23) | Mouse IgM | 1:200 | 1 |
| | Sox9 (1.24) | Goat IgG | 1:50 | 4 |
| | TBS++ | | | 200 |
| Mesoderm (C6) | Pax7 (1.19) | Mouse IgG | 1:25 | 8 |
| | α-Sarcomeric actin (1.16) | Mouse IgM | 1:400 | 0.5 |
| | TBS++ | | | 200 |
| Ectoderm (C7) | Pax6 (1.20) | Rabbit IgG | 1:100 | 2 |
| | MAP2 (1.22) | Mouse IgG1 | 1:50 | 4 |
| | TBS++ | | | 200 |

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| (D1) | Ki67 (1.26) | Rabbit IgG | 1:100 | 2 |
| | TBS++ | | | 200 |
| (D2) | PCNA (1.27) | Mouse IgG | 1:500 | 0.4 |
| | phH3 (1.28) | Rabbit IgG | 1:100 | 2 |
| | TBS++ | | | 200 |

(xiii) Remove blocking buffer and add the primary antibody diluted inside the PAP pen–marked areas and TBS++ in the negative area. Incubate the slides in a humid chamber for 72 h at 4 °C for Ki67 (combination D1) or 24 h at 4 °C for PCNA/phH3 (combination D2).

(xiv) Wash the slides with TBS++ three times for 5 min each.

(xv) Prepare secondary antibodies as explained in the table below and add each combination at appropriate points on the slide. Follow the guidelines described previously (Steps 24–30).

**TABLE 8** | Secondary antibody combinations for the *in vivo* differentiation test in human and mouse cells.

| Combination | Antibody | Source | Dilution | Volume to add per slide |
|---|---|---|---|---|
| Endoderm (C1′) | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1 |
| | Anti-goat IgG Cy3 (2.13) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Ectoderm (C2′) | Anti-mouse IgG Cy2 (2.15) | Donkey IgG | 1:200 | 1 |
| | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Mesoderm (C3′) | Anti-mouse IgG Cy2 (2.1) | Goat IgG | 1:200 | 1 |
| | Anti-mouse IgM Cy3 (2.16) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| *Other possible combinations* | | | | |
| Mesoderm (C4′) | Anti-mouse IgM Cy2 (2.19) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgG Cy3 (2.4) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Mesoderm (C5′) | Anti-mouse IgM Cy2 (2.19) | Donkey IgG | 1:200 | 1 |
| | Anti-goat IgG Cy3 (2.13) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Mesoderm (C6′) | Anti-mouse IgG Cy2 (2.1) | Goat IgG | 1:200 | 1 |
| | Anti-mouse IgM Cy3 (2.16) | Goat IgG | 1:200 | 1 |
| | TBS++ | | | 200 |
| Ectoderm (C7′) | Anti-rabbit IgG Cy2 (2.3) | Donkey IgG | 1:200 | 1 |
| | Anti-mouse IgG Cy3 (2.8) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| (D1′) | Anti-rabbit IgG FITC (2.17) | Donkey IgG | 1:50 | 4 |
| | TBS++ | | | 200 |
| (D2′) | Anti-mouse IgG Cy2 (2.15) | Donkey IgG | 1:200 | 1 |
| | Anti-rabbit IgG Cy3 (2.11) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |

(xvi) Incubate the slides for 2 h at 37 °C in a humid chamber.
(xvii) Wash them with TBS++ three times for 5 min each.

(xviii) If you are using the combination D1′, add the third incubation D″ (see table below) and incubate it for 2 h at 37 °C in a humid chamber.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| (D1″) | Anti-FITC/Oregon Alexa Fluor 488 (1.30) | Goat IgG | 1:100 | 2 |
| | TBS++ | | | 200 |

(xix) If you have used a third incubation, wash in 1× TBS three times for 5 min each.
(xx) Incubate the slides with DAPI at a 1:10,000 ratio for 10 min at RT (add a minimum volume of 200 μl to each slide).
(xxi) Remove the DAPI solution.
(xxii) Add two or three drops of mounting medium.
(xxiii) Cover each slide with a coverslip (24 mm × 60 mm).
(xxiv) Dry the excess mounting medium and cover the borders with nail polish to fix the coverslips to the slides.
■ **PAUSE POINT** Keep the slides at 4 °C in darkness until microscopic observation. During the two weeks after mounting, the fluorescence is well preserved. After this time, fluorescence staining could be fainter or diffused.
(xxv) Take images with a confocal microscope.
▲ **CRITICAL STEP** Select three sections per slide (one of each area) and take ten images of each (three at the top of the section, four in the middle area and three at the bottom). As a result, you will have a total of 60 images per combination for each teratoma.
(xxvi) Use MetaMorph software to count the green (Ki67) versus blue (total nucleus) signal in one slide, or the green (PCNA) and red (phH3) versus blue (DAPI, total nuclei) signal in the other slide.

**(C) Proliferation versus cell death detection in teratomas** ● **TIMING 7 d**

(i) Cryoprotect the sample with 1× PBS plus 30% (wt/vol) sucrose overnight at 4 °C.
(ii) Embed the sample in OCT with a cryomold and freeze it at −80 °C overnight.
(iii) Make sections in a sequential way with the cryostat (10 μm).
▲ **CRITICAL STEP** Use SuperFrost slides to recover the sections.
(iv) Let the sections dry overnight.
(v) Choose two slides from each teratoma, one for each combination.
■ **PAUSE POINT** The slides can be stored at −80 °C for years.
(vi) Wash the slides with 1× TBS three times for 5 min each.
(vii) Perform antigen retrieval in citrate buffer (pH 8.5) with a bain-marie system for 40 min at 80 °C.
(viii) Let the slides cool down in the same buffer (~20 min).
(ix) Wash the slides with 1× TBS three times for 5 min each.
(x) Incubate the slides with blocking buffer (1× TBS plus 0.5% (vol/vol) Triton X-100 plus 3% (vol/vol) donkey serum) for 30 min at RT.
(xi) Remove the buffer and mark the borders of the slide with a PAP pen, creating one area with one section for a negative control.
(xii) Prepare the primary antibody combination as described below the guidelines previously described (Steps 15–20). Add primary antibodies and incubate overnight at 4 °C in a humid chamber.

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| (E1) | PCNA (1.27) | Mouse IgG | 1:500 | 0.4 |
| | phH3 (1.28) | Rabbit IgG | 1:100 | 2 |
| | TBS++ | | | 200 |

(xiii) Wash the slides with TBS++ three times for 5 min each.
(xiv) Prepare secondary antibody combinations as described below according to the guidelines previously described (Steps 15–20).

| Combination | Antibody | Source | Dilution | Volume to add per slide (μl) |
|---|---|---|---|---|
| (E1′) | Anti-mouse IgG Cy3 (2.8) | Donkey IgG | 1:200 | 1 |
| | Anti-rabbit IgG Cy5 (2.18) | Donkey IgG | 1:200 | 1 |
| | TBS++ | | | 200 |

(xv) Add secondary antibodies and incubate for 2 h at 37 °C in a humid chamber.
(xvi) Wash the slides with 1× TBS three times for 5 min each.
(xvii) Incubate the slides with DAPI at a 1:10,000 ratio for 10 min at RT (add 200 µl on the slide).
(xviii) Remove the DAPI solution.
(xix) Add 1 ml of 4% (wt/vol) PFA to each slide and incubate for 50 min at RT.
(xx) Wash the slides with 1× TBS five times for 5 min each.
(xxi) Incubate the slides with 1× TBS plus 0.2% (vol/vol) Triton X-100 plus 0.05% (vol/vol) Tween-20 for 30 min at RT.
(xxii) Wash the slides with 1× TBS twice for 5 min each.
(xxiii) Add 10 mM Tris-HCl plus 5 mM EDTA in distilled $H_2O$ (pH 8) to the samples and incubate them for 5 min at RT.
(xxiv) Add proteinase K at a concentration of 20 µg $ml^{-1}$ in 10 mM Tris-HCl plus 5 mM EDTA for 15 min at RT.
(xxv) Stop the reaction by adding 5 mM EDTA twice for 5 min each at RT.
▲ **CRITICAL STEP** To avoid damaging the sample, remember to stop the proteinase K reaction with EDTA immediately after 15 min.
(xxvi) Incubate the samples with TdT buffer (pH 7.75) for 10 min at RT.
(xxvii) Remove the buffer and add the FITC solution from the TUNEL kit and incubate the samples for 2 h at 37 °C.
▲ **CRITICAL STEP** Initially, use the dilutions suggested in the kit (10 µl of the enzyme reaction with 90 µl of the nucleotide-FITC solution for each slide). The concentration of the enzyme could be lowered to half without affecting the results. Do not decrease the concentration of the nucleotide. We prepare 5 µl of the enzyme reaction and 5 µl of TdT with 90 µl of the nucleotide-FITC solution for each slide.
(xxviii) Add SSC plus EDTA twice for 10 min each at RT.
(xxix) Wash the slides with 1× TBS twice for 10 min each.
(xxx) Wash them again with TBS++ for 10 min.
(xxxi) Prepare the combination E1″.

| Combination | Antibody | Source | Dilution | Volume to add per slide (µl) |
|---|---|---|---|---|
| (E1″) | Anti-FITC/Oregon Alexa Fluor 488 (1.30) | Goat IgG | 1:100 | 2 |
| | TBS++ | | | 200 |

(xxxii) Add the antibody and incubate the slides overnight at 4 °C in a humid chamber.
(xxxiii) Wash the slides with 1× TBS three times for 5 min each.
(xxxiv) Incubate the samples with DAPI at a 1:10,000 ratio for 10 min at RT (add 200 µl to the slide).
(xxxv) Remove the DAPI solution.
(xxxvi) Add two or three drops of mounting medium.
(xxxvii) Cover the slides with a coverslip (24 mm × 60 mm).
(xxxviii) Dry the excess mounting medium and cover the borders with nail polish to fix the coverslips to the slides.
■ **PAUSE POINT** Keep the slides at 4 °C in darkness until microscopic observation. During the two weeks after mounting, the fluorescence is well preserved. After this time, fluorescence staining could be fainter or diffused.
(xxxix) Take images with a confocal microscope.
(xl) Use MetaMorph software to count the green signal (TUNEL), red signal (PCNA) and far red signal (phH3), all versus the blue signal (DAPI, total nuclei).

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 9**.

**TABLE 9** | Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 14 | The label of the slide is lost during the process | The permanent pen is not good | Use a truly permanent pen specifically designed for cell culture |
| 21 | Different intensities of the signal can be seen along the slide | The refrigerator or shelf is not level | Check them with a leveler |

(continued)

**TABLE 9** | Troubleshooting table (continued).

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 37 | The signal is observed only at the periphery of the colonies. At the confocal, positive cells are not seen even deep inside the colony | The colony is too thick | Make a very weak antigen retrieval (see **Box 4**) |
| | | | Increase the incubation time for the primary antibody (48 h or a whole weekend) |
| 38A(xvi) | There are bubbles in the slide | Incorrect mounting process | Add bigger mounting medium drops on the slide, hold the coverslip with two fingers over the slide, and then let it make contact with the slide by the other side. Go down very slowly with your fingers, increasing the area of contact between the slide and the coverslip. The mounting medium may push out the air. At the very last moment, use a needle to let the coverslip lay down completely |
| 38B(xvii) | The EB can be focused with the ×20 objective but not with higher magnification | The distance between the objective and the sample is too big | The sample must be right under the coverslip. If the EBs are too small, do not use other cover slides at the edges to avoid smashing them. Use a thinner surface |





● **TIMING**

Steps 1–9, AP staining: 30 min
Steps 10–37, pluripotency detection: 2 d
Step 38A, differentiation detection with EBs in suspension: 5 d
Step 38B, differentiation detection with EBs in a SlideFlask: 3 d
Step 39, imaging by confocal microscopy: 2 h per cell line
Steps 40–43, differentiation detection in teratomas: 4 d
Step 44A, embedding in paraffin: 6–8 d
Step 44B, cell proliferation in teratomas: 6–8 d
Step 44C, proliferation versus cell death detection in teratomas: 7 d
**Box 1**, AP live staining protocol: 45 min
**Box 2**, surface marker live detection: 30 min
**Box 3**, flow cytometry analysis: 3–4 h
**Box 4**, antigen retrieval: 1 h 30 min

## ANTICIPATED RESULTS

AP staining is the first stage for checking whether a cell line is pluripotent. A clear blue signal should be seen in the colonies, but no staining of the feeders should be observed (**Fig. 8**). Next, perform an immunodetection of the cells for a variety of stem cell markers. The nuclear markers Oct4, Nanog and Sox2 must be limited to the nuclei of the stem cells, without any signal in the cytoplasm or feeders. The SSEA- and the Tra- markers should be expressed in the membranes. Human and mouse cells will give different results both in marker expression and in morphology. Human colonies are flat with oval or triangular form (**Fig. 9**), whereas mouse colonies are thicker and round (**Fig. 10**).

If the immunostaining for stem cell markers suggests that cells are pluripotent, then the next stage is to grow EBs. The different structures of the three germ layers that grow in EBs are very characteristic. Usually, in the center of the EB, some globular structures can be observed, and give positive results for the endodermal markers (**Fig. 11a**,**b**). The ectoderm can

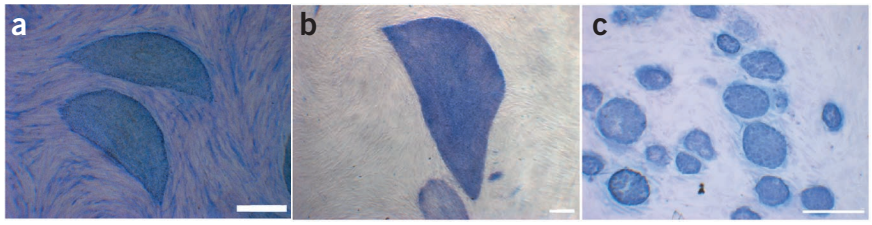


**Figure 8** | AP staining. (**a**,**b**) AP staining without the heating step (**a**) and with the heating step (**b**) in hESCs. (**c**) AP staining in mESCs without the heating step. *Image information*: Stereomicroscope: Leica Macrofluo Z16 Apo. Objective: (**a**,**b**) ×1.0—zoom ×4.0 **c**: ×1.0—zoom ×9.2. Bright field. Image pixels: (**a**,**b**) 1,088 × 816, (**c**) 2,176 × 1,632. Resolution: 24 bits. Image: *xy* plane. Scale bars, 500 μm.

be seen as filaments growing around the EBs (**Fig. 11c**,**d**). The mesoderm is characterized by very flat cells growing around the central EB (**Fig. 11e**,**f**). The same markers can be identified in the EBs analyzed *in toto*, as they were still growing in cell suspension (**Fig. 11g**,**h**).

Other markers can also be useful to detect the three germ layers, including the following: brachyury (nuclear) is one of the earliest markers of the mesoderm, present in few cells of the EBs (**Fig. 11i**); Gata4 (nuclear) and α-actinin sarcomeric (cytoplasmic) are perfect to identify myocardiocytes (**Fig. 11j**); and Pax6 (**Fig. 11k**) and Sox1 are nuclear markers for the



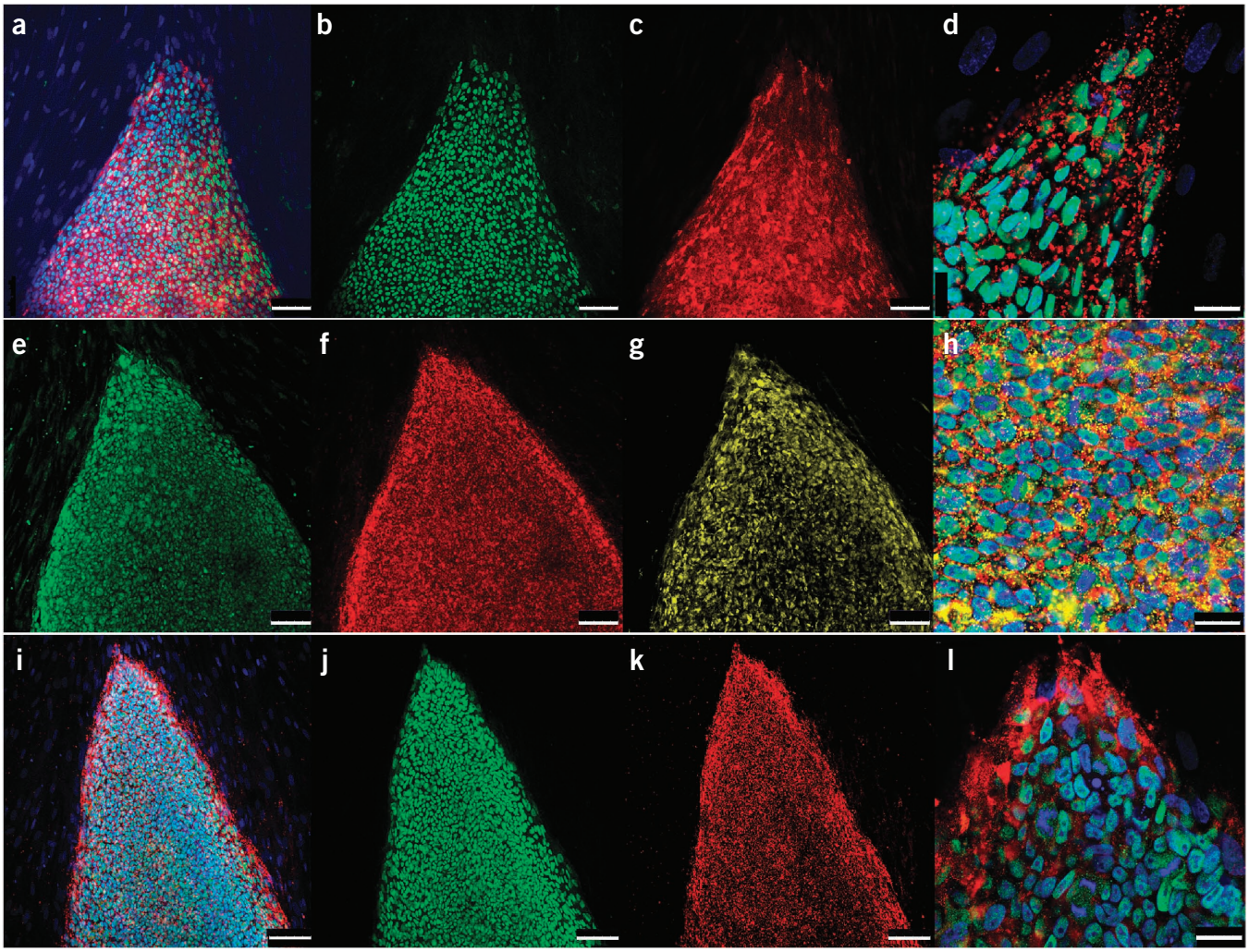


**Figure 9** | Pluripotency detection in hESCs. (**a**) Merged image with DAPI (blue), Oct4 (green) and SSEA3 (red). (**b**) Oct4 signal. (**c**) SSEA3 signal. (**d**) Merged image, detail of the colony. Note that only the colony is positive for the markers. (**e**–**g**) Sox2 (green) (**e**), SSEA3 (red) (**f**) and Tra-1–60 (yellow) (**g**). (**h**) Merged image, detail of the colony. (**i**) Merged image with DAPI (blue), Nanog (green) and Tra-1-81 (red). (**j**) Nanog signal. (**k**) Tra-1-81 signal. (**l**) Merged image, detail of the colony. *Image information*: Confocal microscope: Leica TCS SP5 AOBS. Objectives (**a**–**c**,**e**–**g**,**i**–**k**): HCX PL APO ×20.0/0.70 IMM UV 11506191. (**d**,**h**,**l**): HCX PL APO lambda blue ×63.0/1.40 OIL UV. Sequential mode. Laser: 405-nm diode/488-nm argon/561-nm DPSS/633-nm HeNe. AOBS detection system. Emission bandwidth: 415–529 nm/515–565 nm/582–689 nm/696–737 nm. Image pixels: 1,024 × 1,024. Resolution: 8 bits. Image: Maximum projection of a *xy*/*z* series. Scale bars (**a**–**c**, **e**–**g**, **i**–**k**), 100 μm; (**d**,**h**,**l**), 25 μm.

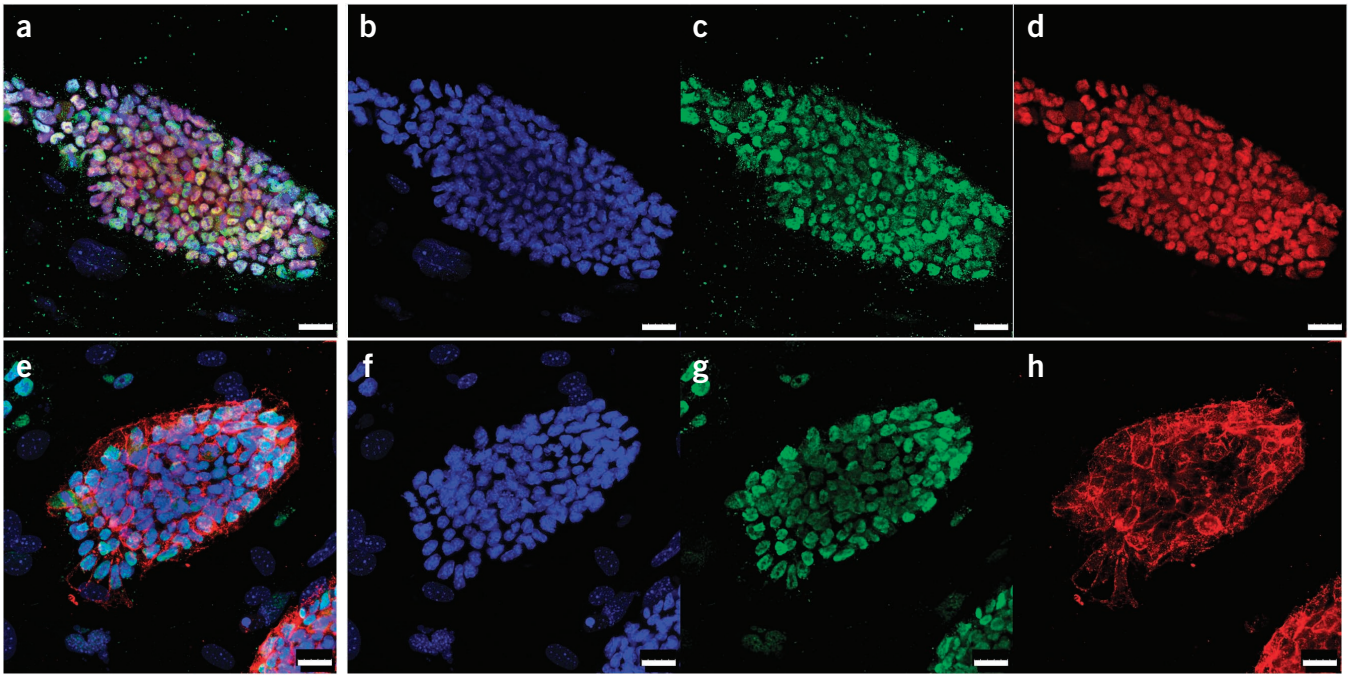


**Figure 10** | Pluripotency detection in mESCs. (**a**) Merged image with DAPI (blue), Sox2 (green) and Oct4 (red). (**b**) DAPI signal. (**c**) Sox2 signal. (**d**) Oct4 signal. (**e**) Merged image with DAPI (blue), Nanog (green) and SSEA-1 (red). (**f**) DAPI signal. (**g**) Nanog signal. (**h**) SSEA1 signal. *Image information*: Confocal microscope: Leica TCS SP5 AOBS. Objective: HCX PL APO lambda blue ×63.0/1.40 OIL UV. Sequential mode. Laser: 405-nm diode/488-nm Argon/561-nm DPSS. AOBS detection system. Emission bandwidth: 415–529 nm/515–565 nm/582–689 nm. Image pixels: 1,024 × 1,024. Resolution: 8 bits. Image: Maximum projection of a *xy/z* series. Scale bars, 25 μm.



primitive ectoderm (**Fig. 11l**). To study the different level of neuron differentiation, we suggest MAP2 for the neural progenitors, neurofilament200 for the differentiated postmitotic neurons and Tuj1 to stain all stages (**Fig. 11m–p**).

When you are analyzing the teratomas, first look at the H&E staining. On histological staining, you should see, at the external part of the section, the mouse tissue where the cells were injected (the muscle in the intramuscular ones, the dermis in the subcutaneous or the testis in the intratesticular, for example). If a teratoma has been generated from the injected stem cells, some irregular structures will be seen in the middle of the regular tissue with a characteristic morphology: columnar and mucin-producing goblet cells, neural rosettes, immature striated muscle or osteoid formations. The presence of these structures indicates that tissues derived from the three germ layers are present. It is recommended that an expert pathologist perform the H&E analysis. However, if this is not possible, immunohistochemistry on consecutive slides can be done to confirm the presence of these tissues. The positive cells for the endodermal markers (α-fetoprotein and FoxA2) should be localized where a columnar epithelium with clear secretion vacuoles and basal nuclei were detected in the H&E slide (**Fig. 12a–d**). The positive cells for ectoderm markers such as Tuj1 correspond in the H&E staining to round cells with round nuclei and a thin cytoplasmic elongation (**Fig. 12e–h**). The positive cells for the muscle markers (mesoderm) are observed by the H&E as long pink cells with elongated nuclei forming incipient muscle fibers (**Fig. 12i–l**). The positive cells for cartilage markers (chondroitin sulfate and Sox9) can be easily detected in the H&E slide as round and regular structures with holes and dark blue nuclei (**Fig. 12m–p**).

In some cases, if the teratoma is very small or the mouse was killed before the total generation of the teratoma, primitive markers such as Pax7 for mesoderm or Pax6 for ectoderm can be analyzed (**Fig. 13a**,**b**).

In some of the studies conducted in our lab, once the cell lines have been characterized we compare their proliferation capacity or the percentage of cells undergoing apoptosis. For example, a study looking at the inhibition of p53 expression as a tool for enhancing reprogramming efficiency needed this type of analysis[29]. **Figure 13** shows examples of the types of results obtained: nuclear staining of Ki67 (**Fig. 13c**), nuclear staining of the PCNA (**Fig. 13d**) and TUNEL (green), PCNA (red) and phH3 (yellow) analyzed concurrently (**Fig. 13e**).

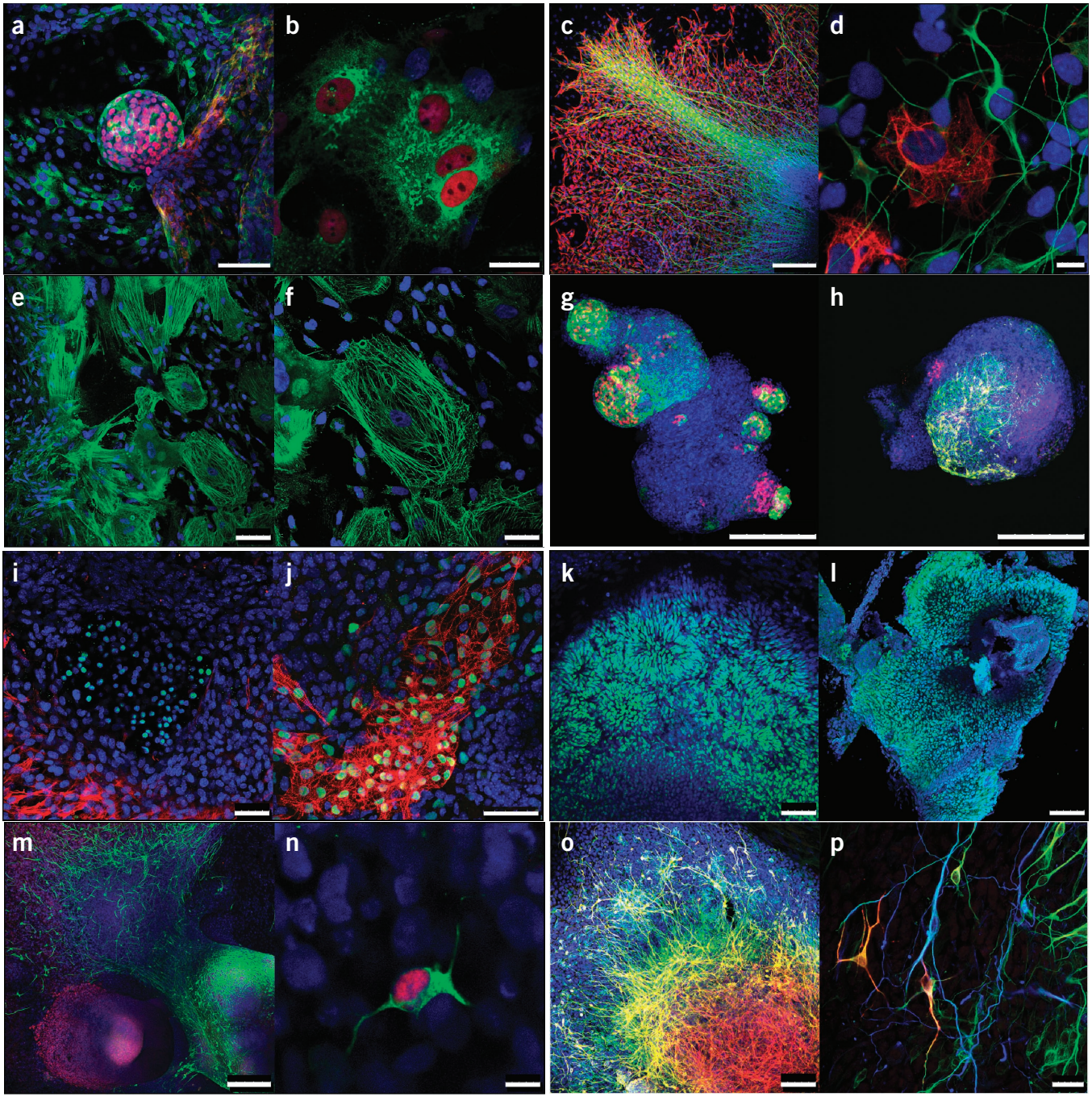


**Figure 11** | Differentiation test *in vitro*. The images show differentiation of hESCs in EBs, even though the differentiation structures on EBs from mESCs are identical. (**a**) Endoderm staining in an EB. DAPI (blue), AFP (green) and FoxA2 (red). We can distinguish the cytoplasmic AFP signal and the nuclear mark of FoxA2, normally in a tubular or globular structure. (**b**) Detail of **a**. (**c**) Ectoderm staining in EBs. DAPI (blue), Tuj1 (green) and GFAP (red). It is easy to see many axons of the neurons and, in some cases, the astrocytes below them. (**d**) Detail of **c**. (**e**) Mesoderm staining in EBs. DAPI (blue) and α-smooth muscle actin (ASMA; green). Usually, the flat cells growing around the EBs are ASMA-positive cells. (**f**) Detail of **e**. (**g**) *In toto* staining of an EB, AFP (green) and FoxA2 (red). (**h**) *In toto* staining of an EB, Tuj1 (green) and TH (red). (**i**) Brachyury in primitive mesoderm (green) with ASMA in red (DAPI in blue). (**j**) Gata4 (green) and α-sarcomeric actinin (red) in a differentiation to myocardiocytes (DAPI in blue). (**k**,**l**) Sox1 (**k**) and Pax6 (in green) (**l**) in primitive ectoderm, showing the neural rosettes (DAPI in blue). (**m**) Pax6 (red) and MAP2 (green). (**n**) Detail of **m** (DAPI in blue). (**o**) Tuj1 (green), MAP2 (red) and neurofilament (yellow) in EBs (DAPI in blue). It is common to find MAP2 in the center of the sphere and neurofilament in the most external part. (**p**) (detail of **o**) We have cropped the DAPI image and changed the colors to better show the different stages of the neurons. With Tuj1 (in green), we can see all the cells. With MAP2 (in red), we see the youngest neurons, and with neurofilament (blue) we can visualize the cellular body and the axons of the most mature neurons. Colocalization of Tuj1 and MAP2 is shown in yellow, and colocalization of Tuj1 and neurofilament is shown in cyan. *Image information*: Confocal microscope: Leica TCS SP5 AOBS. Objectives (**a**,**i**): HCX PL APO CS ×40.0/1.25 OIL UV 11506251. (**b**,**d**,**f**,**j**,**n**,**p**): HCX PL APO lambda blue ×63.0/1.40 OIL UV. C. (**m**): ACS APO ×10.0/0.30 507902. (**e**,**g**,**h**,**k**,**l**,**o**): HCX PL APO ×20.0/0.70 IMM UV 11506191. Sequential mode. Laser: 405-nm diode/ 488-nm argon/561-nm DPSS. AOBS detection system. Emission bandwidth: 415–529 nm/515–565 nm/582–689 nm. Image pixels: 1,024 × 1,024. Resolution: 8 bits. (**a**,**c**,**g**,**h**,**j**–**m**,**o**) Maximum projection of a *xy*/*z* series. (**b**,**d**–**f**,**i**,**n**) *xy* plane. Scale bars, (**a**) 75 μm, (**b**) 25 μm, (**c**) 250 μm, (**d**) 10 μm, (**e**) 100 μm, (**f**) 50 μm, (**g**,**h**) 250 μm, (**i**) 40 μm, (**j**) 50 μm, (**k**) 100 μm, (**l**) 100 μm, (**m**) 250 μm, (**n**) 75 μm, (**o**) 100 μm, (**p**) 25 μm.

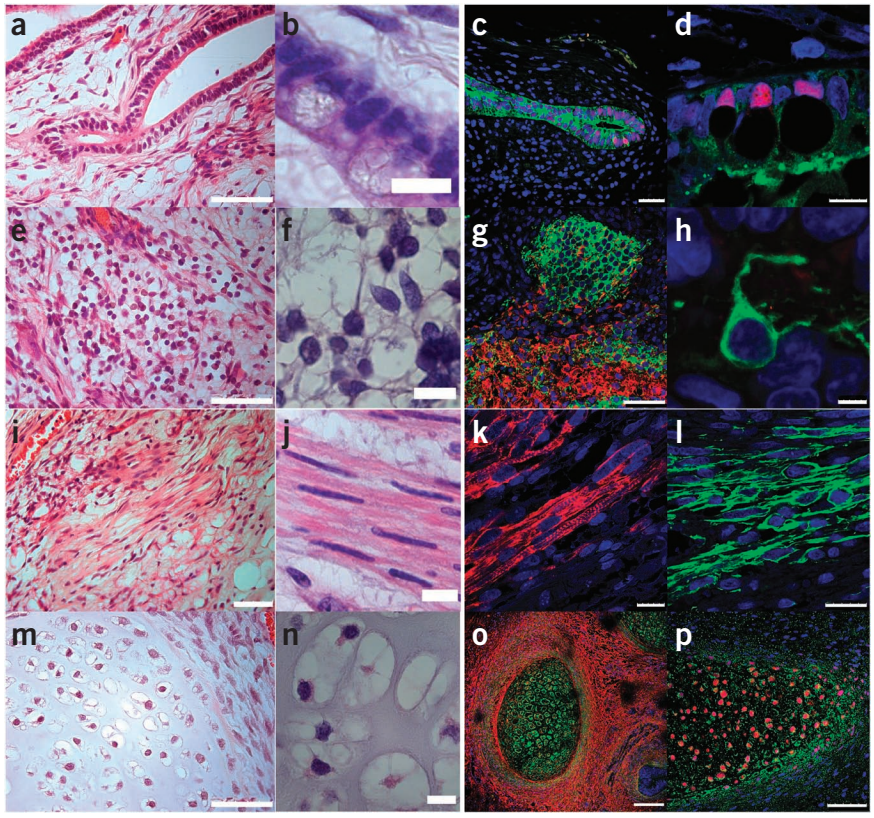


**Figure 12** | Differentiation test *in vivo*. H&E staining and immunofluorescence detection of the three germ layers in a teratoma from hESC lines. (**a**–**d**) Differentiation to endoderm: H&E staining (**a**, ×630; **b**, ×2,000), AFP (green) and FoxA2 (red) markers with DAPI in blue (**c**,**d**). (**e**–**h**) Differentiation to ectoderm: H&E staining (**e**, ×630; **f**, ×2,000); Tuj1 (green) and GFAP (red) markers (**g**); Tuj1 (**h**) (DAPI in blue). (**i**–**l**) Differentiation to mesoderm (muscle): H&E staining (**i**, ×400; **j**, ×2,000); ASA (**k**) and ASMA (**l**) markers (DAPI in blue). (**m**–**p**) Differentiation to mesoderm (cartilage), H&E staining (**m**, ×630; **n**, ×2,000), chondroitin sulfate (green) and fibronectin (red); chondroitin sulfate (green) (**o**) and Sox9 (red) markers (**p**) (DAPI in blue). *Image information*: (**a**,**b**,**e**,**f**,**i**–**k**,**m**,**n**) Optical Microscope Leica DM6000. Bright-field images: (**c**,**d**,**g**,**h**,**k**,**l**,**o**,**p**) confocal microscope Leica TCS SP5. Objectives: (**a**,**e**,**m**) HCX PL APO ×63/1.40–0.60 OIL/0.17/E 506187. (**b**,**f**,**j**,**n**) HCX PL APO ×100/1.40–0.70 OIL/0.17/D 506220. (**i**) HCX PL APO ×40/0.85 Corr/0.11–0.23/C 506294. (**c**) HCX PL APO CS ×40.0/1.25 OIL UV 11506251. (**d**,**g**,**h**,**k**,**l**,**p**) HCX PL APO lambda blue ×63.0/1.40 OIL UV. (**o**) HCX PL APO ×20.0/0.70 IMM UV 11506191. Sequential mode. Laser: 405-nm diode/488-nm argon/561-nm DPSS. AOBS detection system. Emission bandwidth: 415–529 nm/515–565 nm/582–689 nm. Image pixels: 1,024 × 1,024. Resolution: 8 bits. All the images are in *xy* plane except **h**, which is a maximum projection of an *xy/z* series. Scale bars, (**a**,**e**,**i**,**m**) 50 μm, (**b**,**f**,**j**,**n**) 10 μm, (**c**) 50 μm, (**d**) 10 μm, (**g**) 50 μm, (**h**) 10 μm, (**k**) 100 μm, (**l**) 100 μm, (**o**) 100 μm; (**p**) 75 μm. All animal experiments were conducted in accordance with experimental protocols previously approved by the Institutional Ethics Committee on Experimental Animals of the Barcelona Biomedical Research Park, in full compliance with Spanish and European laws and regulations.

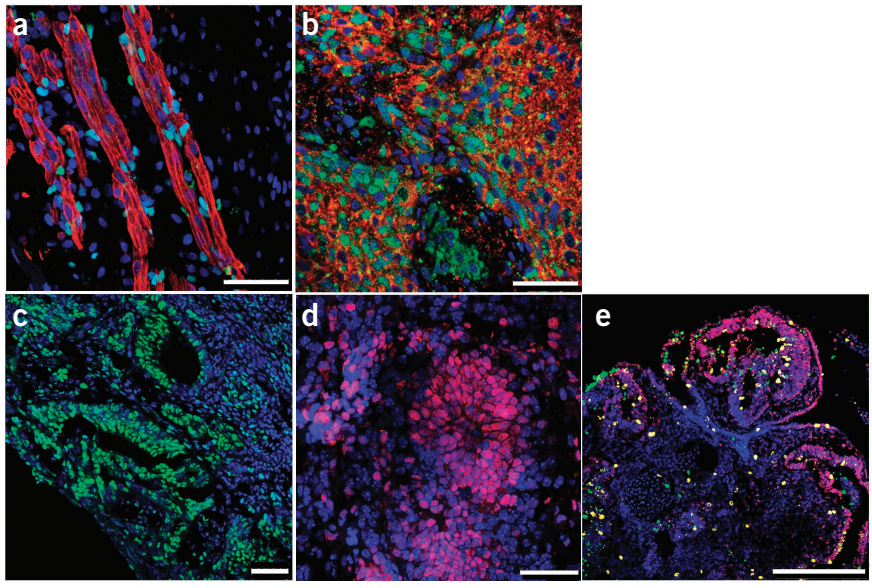


**Figure 13** | Analysis of teratomas. (**a**,**b**) Pax7 (green) and α-sarcomeric actin (red) detection in mesoderm (**a**) and Pax6 (green) and Tuj1 (red) in ectoderm (**b**); DAPI is shown in blue. Cell proliferation detection in teratomas. (**c**) DAPI (blue) and Ki67 (green) signal. (**d**) DAPI (blue) and PCNA (red) signal. (**e**) Cell proliferation detection versus apoptosis in teratomas; DAPI (blue), TUNEL (green), PCNA (red) and phH3 (yellow). All animal experiments were conducted in accordance with experimental protocols previously approved by the Institutional Ethics Committee on Experimental Animals, in full compliance with Spanish and European laws and regulations. *Image information*: Confocal microscope: Leica TCS SP5 AOBS. Objective: HCX PL APO lambda blue ×63.0/1.40 OIL UV. Sequential mode. Laser: 405-nm diode/488-nm argon/561-nm DPSS. AOBS detection system. Emission bandwidth: 415–529 nm/515–565 nm/582–689 nm/696–737 nm. Image pixels: 1,024 × 1,024. Resolution: 8 bits. Images: (**a**) maximum projection of an *xy/z* series. (**b**) *xy* plane. Scale bars, (**a**,**b**,**e**) 250 μm, (**c**,**d**) 50 μm. All animal experiments were conducted in accordance with experimental protocols previously approved by the Institutional Ethics Committee on Experimental Animals of the Barcelona Biomedical Research Park, in full compliance with Spanish and European laws and regulations.

*Note: Supplementary information is available in the online version of the paper.*

**ACKNOWLEDGMENTS** We are grateful to all the researchers who provided us their samples to be analyzed, with special mention to I. Rodriguez, A. Giorgietti, A. Consiglio, R. Vassena, N. Montserrat, C. Eguizabal and S. Menendez. Work in the laboratory of J.C.I.B. was supported by grants from TERCEL-ISCII-MINECO, CIBER, Fundacion Cellex, Sanofi, The Leona M. and Harry B. Helmsley Charitable Trust and the G. Harold and Leila Y. Mathers Charitable Foundation.

**AUTHOR CONTRIBUTIONS** All authors contributed equally to this work. M.M. designed the protocols, analyzed the data and wrote the paper; L.M. and C.P. performed and optimized the protocols; C.M. contributed to the microscope observations, live-cell staining and flow cytometry analysis; M.C. performed cell cultures and EB formation, and helped with the manuscript; L.L.-R. helped with the manuscript and gave conceptual advice; C.R.E. performed and optimized the protocols; and J.C.I.B. supervised the project and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS** The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nprot.2012.154. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.



1. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156 (1981).
2. Thomson, J.A. *et al.* Embryonic stem cells lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
3. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676 (2006).
4. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblast by defined factors. *Cell* **131**, 861–872 (2007).
5. Carpenter, M.K. & Bhatia, M. Characterization of human embryonic stem cells. in *Handbook of Stem Cells*, Vol 1: Embryonic stem cells (eds. Lanza, R. *et al.*) 407–411 (Elsevier/Academic Press, 2004).
6. Ohnuki, M., Takahashi, K. & Yamanaka, S. Generation and characterization of human induced Pluripotent stem cells. *Curr. Protoc. Stem Cell Biol.* **9**, 4A.2.1–4A.2.25 (2009).
7. Crook, J.M., Hei, D. & Stacey, G. International Stem Cell Banking Initiative: raising standards to bank on. *In Vitro Cell Dev. Biol. Anim.* **46**, 169–172 (2010).
8. Adewumi, O. *et al.* Characterization of human embryonic stem cell lines by the international stem cell initiative. *Nat. Biotechnol.* **25**, 803–815 (2007).
9. Neale, F., Clubb, J.S., Hotchks, D. & Posen, S. Heat stability of human placental alkaline phosphatase. *J. Clin. Pathol.* **18**, 359–363 (1965).
10. Henthorn, P., Zervos, P., Raducha, M., Harris, H. & Kadesch, T. Expression of a human placental alkaline phosphatase gene in transfected cells: use as a reporter for studies of gene expression. *Proc. Natl. Acad. Sci. USA* **85**, 6342–6346 (1988).
11. Maatman, R. *et al.* Aggregation of embryos and embryonic stem cells. Transgenic mouse. *Methods Mol. Biol.* **209**, 201–230 (2002).
12. Wang, J., Levasseur, D.N. & Orkin, S.H. Requirement of Nanog dimerization for stem cell self-renewal and pluripotency. *Proc. Natl. Acad. Sci. USA* **105**, 6326–6331.
13. Das, S., Jena, S. & Levasseur, D.N. Alternative splicing produces Nanog protein variants with different capacities for self-renewal and pluripotency in embryonic stem cells. *J. Biol. Chem.* **286**, 42690–42703 (2011).
14. Cauffman, G., Van de Velde, H., Liebaers, I. & Van Steirteghem, A. Oct-4 mPNA and protein expression during human preimplantation development. *Mol. Hum. Reprod.* **11**, 173–181 (2005).
15. Cauffman, G., Liebaers, I., Van Steirteghem, A. & Van de Velde, H. POU5F1 isoforms show different expression patterns in human embryonic stem cells and preimplantation embryos. *Stem Cells* **24**, 2685–2691 (2006).
16. Müller, F.J. *et al.* A bioinformatic assay for pluripotency in human cells. *Nat. Methods* **8**, 315–317 (2011).
17. Hentze, H. *et al.* Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. *Stem Cell Res.* **3**, 198–210 (2009).
18. Itskovitz-Eldor, J. *et al.* Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol. Med.* **6**, 88–95 (2000).
19. Aasen, T. *et al.* Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat. Biotechnol.* **26**, 1276–1284 (2008).
20. Aran, B. *et al.* Derivation of human embryonic stem cells at the Center of Regenerative Medicine in Barcelona. *In vitro Cell Dev. Biol. Anim.* **46**, 356–366 (2010).
21. Keller, G.M. *In vitro* differentiation of embryonic stem cells. *Curr. Opin. Cell Biol.* **7**, 862–869 (1995).
22. Cerdan, C., Hong, S.H. & Bhatia, M. Formation and hematopoietic differentiation of human embryoid bodies by suspension and hanging drop cultures. *Curr. Protoc. Stem Cell Biol.* **3**, 1D.2.1–1D.2.16 (2007).
23. Sundström, J. *et al.* Characterization of the model for experimental testicular teratoma in 129/SvJ-mice. *Br. J. Cancer* **80**, 149–160 (1999).
24. Gertow, K. *et al.* Isolation of human embryonic stem cell–derived teratomas for the assessment of pluripotency. *Curr. Protoc. Stem Cell Biol.* **3**, 1B.4.1–1B.4.29 (2007).
25. Campos, P.B., Sartore, R.C., Abdalla, S.N. & Rehen, S.K. Chromosomal spread preparation of human embryonic stem cells for karyotyping. *J. Vis. Exp.* **31**, e1512 (2009).
26. Schröck, E. *et al.* Multicolor spectral karyotyping of human chromosomes. *Science* **273**, 494–497 (1996).
27. Gonzalez, F. *et al.* Generation of mouse induced pluripotent stem cells by transient expression of a single non-viral polycistronic vector. *Proc. Natl. Acad. Sci. USA* **106**, 8918–8922 (2009).
28. Raya, A. *et al.* Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature* **460**, 53–59 (2009).
29. Kawamura, T. *et al.* Linking the p53 tumor suppressor pathway to somatic cell reprogramming. *Nature* **460**, 1140–1144 (2009).
30. Giorgetti, A. *et al.* Generation of induced pluripotent stem cells from cord blood using OCT4 and SOX2. *Cell Stem Cell* **5**, 353–357 (2009).
31. Montserrat, N. *et al.* Generation of feeder free pig induced pluripotent stem cells without Pou5f1. *Cell Transplant.* **21**, 815–825 (2011).