A protocol for removal of antibiotic resistance cassettes from human embryonic stem cells genetically modified by homologous recombination or transgenesis

Richard P Davis, Magdaline Costa, Catarina Grandela, Andrew M Holland, Tanya Hatzistavrou, Suzanne J Micallef, Xueling Li, Adam L Goulburn, Lisa Azzola, Andrew G Elefanty & Edouard G Stanley

Monash Immunology and Stem Cell Laboratories, STRIP 1, Building 75, Level 3, Monash University, Clayton, Victoria 3800, Australia. Correspondence should be addressed to E.G.S. (ed.stanley@med.monash.edu.au).

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The first step in the generation of genetically tagged human embryonic stem cell (HESC) reporter lines is the isolation of cells that contain a stably integrated copy of the reporter vector. These cells are identified by their continued growth in the presence of a specific selective agent, usually conferred by a cassette encoding antibiotic resistance. In order to mitigate potential interference between the regulatory elements driving expression of the antibiotic resistance gene and those controlling the reporter gene, it is advisable to remove the positive selection cassette once the desired clones have been identified. This report describes a protocol for the removal of *loxP*-flanked selection cassettes from genetically modified HESCs by transient transfection with a vector expressing Cre recombinase. An integrated procedure for the clonal isolation of these genetically modified lines using single-cell deposition flow cytometry is also detailed. When performed sequentially, these protocols take ~1 month.

INTRODUCTION

Genetically tagged embryonic stem cell (ESC) reporter lines generated though transgenesis or gene targeting greatly facilitate the objective analysis of protocols aimed at directing differentiation toward specific lineages[1–7]. We recently described the generation of *MIXL1*$^{GFP/w}$ human ESCs (HESCs) in which expression of GFP identifies mesendoderm progenitors during the early stages of differentiation[8]. This line was created by site-specific insertion of a GFP reporter gene into the *MIXL1* locus using homologous recombination in HESCs. The gene-targeting vector used in this study included a positive selection cassette that incorporated a constitutively active promoter (phosphoglycerate kinase) upstream of a neomycin phosphotransferase gene (*neo*) that confers resistance to the drug G418. Applying G418 selection to HESCs electroporated with this vector allowed isolation of cells that had stably integrated the DNA construct into their genome. These G418-resistant clones were then screened for a gene-targeting event using a PCR-based screening protocol[8].

Previous studies have shown that positive selection cassettes can interfere with the expression of neighboring genes (refs. 9–11; and R.P.D. *et al.*, unpublished data). In the context of genetic-tagging studies, such interference could lead to the misexpression of the reporter gene, resulting in misidentification of cell types marked by the reporter. In general, deletion of the positive selection cassette requires these sequences to be flanked by either *loxP* or *FRT* sequences, enabling them to be removed by transient expression of the site-specific recombinases, Cre or Flp, respectively. Removal of the selection marker is usually confirmed by either PCR analysis or sensitivity of the ESCs to the selecting agent[12,13].

This report describes a protocol for the excision of sequences flanked by *loxP* sites from genetically modified HESCs that is an alternative to the transduction of a recombinant-modified Cre-recombinase protein into cells[14]. Instead, this method utilizes a vector encoding both Cre recombinase and puromycin resistance (*puro*), enabling the separation of Cre-expressing cells from non-transfected HESCs following 24–48 h of selection in puromycin-containing media[8,15]. While use of a cell-permeant Cre-recombinase protein ensures that ectopic chromosomal integration of foreign DNA cannot occur[14], the transduced HESC colonies consist of a heterogenous population of cells that have either excised or retained the loxP-flanked sequences.

The transfection protocol described in this report has been applied to multiple clones from six independent genetically tagged HESC lines, with the desired excision event routinely observed in nearly 100% of correctly targeted colonies emerging following puromycin selection[8]. While we have routinely found that individual colonies are homogeneous with respect to loss or retention of the loxP-flanked selectable marker, this in itself does not exclude the possibility that a given colony could have arisen from more than one cell. Therefore, we also describe an accompanying protocol for the clonal isolation of the genetically tagged HESC line using single-cell deposition by flow cytometry. The second procedure can be performed in conjunction with excision of the selectable marker, reducing the time required to establish fully characterized lines suitable for further experiments. These procedures complement recent protocols describing the expansion, genetic modification and differentiation of HESCs[16,17].

While these protocols were developed for removing selectable marker cassettes from HESCs that have stably integrated reporter genes, it is envisaged that this methodology could also be used to excise other genomic sequences flanked by loxP sites. However, this protocol would not be suitable for the Cre-mediated conditional activation of transgenes or conditional gene knockouts in HESCs, where finer control of Cre expression may be required[18]. Furthermore, this protocol would also be inappropriate for situations

where the HESCs were already puromycin resistant. In this instance, transduction of recombinant Cre-recombinase protein into the cells should be considered as an alternative approach[14].

Experimental design

Cre recombinase-expression vector. The vector (*pEFBOS-CreIRESpuro*[8]; GenBank accession number EU693012) expresses the genes for Cre recombinase (*Cre*) and puromycin N-acetyltransferase (*puro*). An internal ribosome entry site (IRES) sequence between the two genes permits the translation of two open-reading frames from one mRNA transcript. The elongation factor α promoter (*EF1α*) was chosen to drive the expression of the downstream genes because it has previously been successfully utilized for the expression of foreign genes in HESCs[19]. The *puro* gene was employed because puromycin can kill HESCs within 48 h, enabling selection of cells that transiently express the vector, yet still providing sufficient time for Cre-mediated recombination to occur.

PCR screening the puromycin-resistant colonies. It is recommended that the HESC colonies that arise following puromycin selection are screened by PCR to confirm that both the *loxP*-flanked selection cassette has been excised and that the Cre-expression vector has not integrated into the genome (see Steps 30–33). While we have used a 39-base primer as part of the screen to detect integrated *pEFBOS-CreIRESpuro* vector, it is anticipated that a shorter primer directed against the 5′ region of the *puro* gene, in combination with an appropriate reverse primer, would suffice.

Culturing the HESCs. It is essential that the HESCs are adapted to enzymatic passaging using either trypsin or TrypLE Select before performing these procedures. Detailed protocols describing the enzymatic passaging of HESCs have been described previously[16,20].

The authors have successfully adapted five different HESC lines (HES-2, -3, -4, MEL-1, -2) to enzymatic passaging for short periods of time (up to 25 passages) without the appearance of cells with an abnormal karyotype or changes that can be detected morphologically or by flow cytometric analysis. The authors recommend that enzymatic passaging be only used for the generation of sufficient cell numbers required for experiments rather than as a method for routine long-term HESC maintenance. The cells should be enzymatically passaged twice a week regardless of the confluence of the HESCs on the flask to prevent difficulties in dissociating the cells. Typically a confluent 150-cm^2 flask will contain ~8 × 10^6 HESCs[20].

Controls for the FuGENE transfection. The use of a negative control is recommended to provide an objective measure of the degree of cell death associated with the complete absence of puromycin resistance. In practice, all the HESCs in this control culture die after 48 h of puromycin selection. The negative control may consist of either a dish of untransfected HESCs, or HESCs transfected with a vector expressing a reporter gene, such as *GFP* or *RFP*, but lacking *puro*.

Single-cell deposition of HESCs by flow cytometry. Adding propidium iodide (PI) to the disaggregated HESCs provides a way of identifying the viable cells to be clonally isolated using the single-cell deposition function of a flow cytometer. In addition, this strategy can be combined with gating based on the cells forward- and side-scatter properties. The cloning efficiency of the HESCs could also be further improved by the addition of 10 μM of the Rho-associated kinase inhibitor, Y-27632, to the culture media 1 h before dissociating the HESCs, as well as to the cells following single-cell deposition[21].

MATERIALS

REAGENTS

- Vector for transfection, *pEFBOS-CreIRESpuro*[8] (GenBank accession number EU693012): this vector is available on request
- HES2, HES3 or HES4 cells: these lines can be obtained from ES Cell International and are on the National Institutes of Health Stem Cell Registry
- MEL1 cells: this cell line can be obtained from Millipore (cat. no. SCC020)
- Mitotically inactivated mouse embryonic fibroblasts (MEFs)[22]
- PBS, without $CaCl_2$ and $MgCl_2$ (CMF-PBS; Invitrogen, cat. no. 14190-144)
- FuGENE 6 Transfection Reagent (Roche, cat. no. 11814443001)
  ▲ **CRITICAL** No other lipofection solutions have been optimized for use with this protocol.
- Puromycin solution (Sigma, cat. no. P9620)
- TrypLE Select (Invitrogen, cat. no. 12563-029)
- PI (Sigma, cat. no. P4864)
- Gelatin, 0.1% (wt/vol) solution (Sigma, cat. no. G1890)
- Phenol:chloroform:isoamyl alcohol (25:24:1) solution saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma, cat. no. P2069) ! **CAUTION** Avoid contact with skin and eyes. Do not breathe vapor and avoid prolonged or repeated exposure. Work in a fume hood wearing compatible chemical-resistant gloves and chemical safety goggles.
- Ethanol (Merck AnalaR, cat. no. 410230) ! **CAUTION** Keep away from sources of ignition.
- Platinum *Taq* DNA polymerase (Invitrogen, cat. no. 10966.034)
- MassRuler DNA Ladder (Fermentas, cat. no. SM0403)
- DMEM/F12 (Invitrogen, cat. no. 11320-033)
- 20% Knockout serum replacer (Invitrogen, cat. no. 10828-028)
- Non-essential amino acids (Invitrogen, cat. no. 11140-050)
- L-Glutamine (Invitrogen, cat. no. 25030-081)
- Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)
- 2β-Mercaptoethanol (Invitrogen, cat. no. 21985-023)
- Fibroblast growth factor 2 (FGF2; Peprotech, cat. no. 100-18B)
- DMEM (Invitrogen, cat. no. 11960-044)
- Heat-inactivated FCS (JRH Biosciences, cat. no. 12003)
- Trypsin–EDTA (TE; 0.25% (wt/vol) trypsin, EDTA · 4Na) (Invitrogen, cat. no. 25200-056)
- Chicken serum (Hunter Antisera, cat. no. 110)
- Tris, pH 8.0 (Amresco, cat. no. 0497)
- NaCl (Merck AnalaR, cat. no. 1.06404)
- EDTA (Merck AnalaR, product no. 10093.5V)
- SDS (Sigma-Aldrich, cat. no. L4390) ! **CAUTION** Avoid contact with skin and eyes. Do not inhale dust. Keep away from sources of ignition. Wear a dust mask, type N95 (US) or type P1 (EN 143) respirator, gloves and safety glasses when handling the powder.
- Proteinase K (Sigma-Aldrich, cat. no. P2308)

EQUIPMENT

- 75-cm^2 Tissue culture flask with vented cap (Iwaki, cat. no. 3123-075)
- 60 × 15-mm^2 tissue culture dishes (Falcon, cat. no. 353002)
- 96-well flat-bottom tissue culture-treated plates and lids (Falcon, cat. no. 353072)
- Center-well organ culture dishes, 60 × 15-mm^2 (Falcon, cat. no. 353037)
- 15-ml Centrifuge tube (Iwaki, cat. no. 2325-015)
- 5-ml Sterile round-bottom polystyrene tubes (12 × 75 mm^2) with cell-strainer caps (Falcon, cat. no. 352235)
- 5-ml Sterile round-bottom polystyrene tubes (12 × 75 mm^2) with snap lids (Falcon, cat. no. 352054)
- 1.5-ml Microtubes (Axygen Scientific, cat. no. MCT-175), or equivalent
- Parafilm M (Pechiney Plastic Packaging, cat. no. PM-996), or equivalent
- 26G × 1/2″ (0.45 × 13 mm^2) needles (Terumo, cat. no. NN*2613R)

- 1-ml Syringe (Terumo, cat. no. SS+01T)
- Tissue culture incubator at 5% CO_2
- Refrigerated centrifuge (4K15; Sigma)
- Microfuge (1-15; Sigma)
- Single-cell deposition function of a FACSVantageSE-DiVa system (Becton Dickinson) or equivalent
- Stereomicroscope (Leica MZ6; Leica)

REAGENT SETUP

HESC medium DMEM/F12, 20% Knockout serum replacer, 10 mM Non-essential amino acids, 2 mM L-glutamine, 1× penicillin/streptomycin, 50 mM 2β-mercaptoethanol and 10 ng ml^{-1} FGF2. Filter sterilize the solution and store up to 1 week at 4 °C. *Note*: After single-cell deposition of HESCs, the concentration of the FGF2 is increased to 40 ng ml^{-1}.

MEF medium DMEM, 10% (vol/vol) heat-inactivated FCS, 2 mM L-glutamine and 1× penicillin/streptomycin. Filter sterilize the solution and store up to 1 week at 4 °C.

Trypsin Trypsin–EDTA (0.25% (wt/vol) trypsin, EDTA · 4Na) with 2% (vol/vol) chicken serum. Diluted 1:2 with CMF-PBS[22]. Decant solution into 10-ml aliquots and store at −20 °C until use. Once thawed, the solution can be stored for up to 4 weeks at 4 °C.

DNA lysis buffer 100 mM Tris pH 8.0, 200 mM NaCl, 5 mM EDTA and 0.2% (wt/vol) SDS. Store at room temperature (20–25 °C) indefinitely. Make a 20 mg ml^{-1} stock solution of proteinase K in H_2O, decant and store 1-ml aliquots at –20 °C. Add 200 μg ml^{-1} proteinase K to the above solution at the time of use.

TE 10 mM Tris pH 8.0 and 1 mM EDTA pH 8.0. Store the solution at room temperature indefinitely.

PROCEDURE

Removal of the antibiotic selection marker cassette from genetically modified HESCs ● TIMING 12–17 d

1| The day before FuGENE transfection, enzymatically passage HESCs using trypsin or TrypLE Select, as previously described[20], onto 5 × 6-cm dishes containing MEFs preseeded at a density of 3×10^4 per cm^2. Generally, a 75-cm^2 flask should provide ∼4×10^6 HESCs. Approximately 8×10^5 HESCs should be seeded per 6-cm dish in 4 ml HESC medium. Incubate the dishes in a humidified incubator at 37 °C, 5% CO_2 overnight.

▲ CRITICAL STEP The HESC density needs to be low enough to allow the cells to proliferate for 48 h before the addition of puromycin to the dishes.

2| Approximately 2 h before transfection of the Cre recombinase-expression vector into the HESCs, replace the medium on the dishes with 4 ml of HESC medium. The HESCs should be ∼70% confluent (**Fig. 1a**).

3| Transfect the HESCs on four of the 6-cm dishes with the *pEFBOS-CreIRESpuro* vector using FuGENE 6 Transfection Reagent. The fifth 6-cm dish will serve as a negative control. Pipette 470 μl DMEM/F12 medium into a sterile 1.5-ml microtube. Directly pipette 30 μl FuGENE 6 Transfection Reagent into this medium. Flick the tube to mix and incubate at room temperature for 5 min. Set aside 100 μl of this mixture to incubate in a separate microtube for use on the negative control dish. Add 4 μl of 1 μg μl^{-1} vector DNA into the tube containing 400 μl DMEM/F12 plus FuGENE 6, flick the tube to mix and incubate at room temperature for 40 min. Add 100 μl of this DNA mixture to each of the four dishes in a dropwise manner while swirling the dish. To the fifth 6-cm dish, add the 100 μl of DMEM/F12 plus FuGENE 6 without DNA in a similar manner. Return the dishes to the humidified incubator (37 °C, 5% CO_2).

▲ CRITICAL STEP A FuGENE 6:DNA (μl:μg) ratio of 6:1 is recommended. In our experience, a 3:1 ratio is also effective, but we observed fewer colonies after antibiotic selection.

4| Apply antibiotic selection to the HESCs 24 h after FuGENE transfection. At this stage, the HESCs will be ∼90% confluent. The recommended final concentration of puromycin is 2 μg ml^{-1}.

▲ CRITICAL STEP The antibiotic concentration may need to be optimized for different HESC lines. A titration of puromycin concentration is recommended to determine the concentration of antibiotic that eliminates untransfected HESCs.

5| Maintain puromycin selection for 48 h and change medium daily. After 48 h, there should be no viable HESCs in the negative control culture.

▲ CRITICAL STEP If the MEFs are sensitive to puromycin, the dishes will need to be supplemented with additional MEFs after selection (**Fig. 1b**). After selection, the MEFs are maintained at a density of ∼2×10^4 per cm^2.

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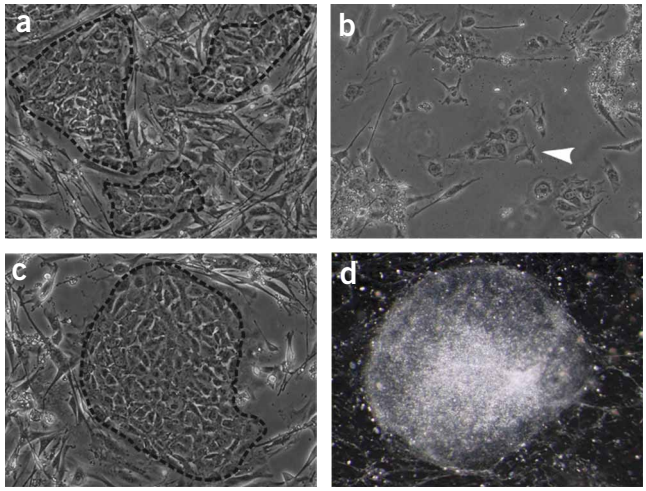

Figure 1 | Morphology of human embryonic stem cells (HESCs) prepared for transfection of the *pEFBOS-CreIRESpuro* vector by FuGENE 6 Transfection Reagent and following selection in puromycin. (**a**) Enzymatically passaged HESCs growing on a layer of mouse embryonic fibroblasts (MEFs) on the day of FuGENE transfection. Groups of HESCs are outlined by a black dotted line. Original magnification, ×100. (**b**) Puromycin-resistant HESCs (white arrow) following 48 h of selection with 2 μg ml^{-1} puromycin. The MEFs did not survive selection as they were puromycin sensitive. Original magnification, ×100. (**c**) A HESC colony (outlined by a black dotted line) grown for 6 d after removal of selection. Original magnification, ×100. (**d**) A HESC colony 13 d after removal of selection that is ready to be manually dissected and passaged, in duplicate, into 48-well plates. Original magnification, ×25.

6| If the transfected HESCs are to be cloned using single-cell deposition flow cytometry (Steps 8–29) before screening for excision of the positive selection cassette (Steps 30–33), allow the colonies to grow until they consist of around 250 cells (**Fig. 1c**) (~5–7 d after selection), and proceed to Step 8. Otherwise (if the colonies are to be used directly for DNA isolation and screening, without further cloning), allow the colonies to grow for ~12 d until they reach organ culture size (~2 mm in diameter) (**Fig. 1d**).

7| The transfer of colonies and subsequent preparation of DNA from the colonies is described in detail in a previous *Nature Protocol*[16]. After the isolation of DNA from the HESCs, proceed to Step 30.

Cloning of the genetically modified HESC line using single-cell deposition by a flow cytometer ● TIMING ~25 d

8| On the day before cloning the HESC line, coat each well of 10 × 96-well plates by adding 50 µl of 0.1% (wt/vol) gelatin solution. Remove the gelatin solution after 20 min.

9| Seed the gelatinized 96-well plates with 3×10^4 MEFs per cm^2 in 50 µl MEF medium/well (~1×10^4 MEFs per well).

10| Incubate the plates in a 37 °C, 5% CO_2 humidified incubator overnight.

11| The following day, replace the MEF medium on the MEF-containing 96-well plates with 100 µl HESC medium supplemented with 40 ng ml^{-1} FGF2.
▲ CRITICAL STEP The concentration of FGF2 is increased to assist in the clonal growth and expansion of the HESCs, while also reducing spontaneous differentiation[23,24].

12| Disaggregate the HESCs (from Step 6) to a single-cell suspension using either trypsin or TrypLE Select, as previously described[16]. Briefly, aspirate the HESC medium from the 6-cm dishes and rinse the dishes with 3 ml CMF-PBS. Add 2 ml of the dissociating agent and incubate the dishes at 37 °C for 2–3 min. Typically, a proportion of the MEFs will peel off the dish as a layer before the HESC colonies begin to detach. Carefully remove the MEFs by aspiration. Add a further 2 ml of the dissociating agent and return the dishes to the 37 °C incubator for another 2–3 min. After this time, the HESCs will have detached from the dishes, forming a near–single-cell suspension. Collect the cells from the four *pEFBOS-CreIRESpuro*-transfected dishes in a 15-ml centrifuge tube. Wash the dishes with a total of 6 ml of HESC medium. Transfer this HESC medium to the same centrifuge tube containing the disaggregated HESCs and pellet cells for 3 min at 480*g* in a refrigerated centrifuge at 4 °C.
▲ CRITICAL STEP Removal of the MEF layer reduces the possibility that these cells will entrap the HESCs.

13| Aspirate the supernatant from the pelleted cells and resuspend the HESCs in 1 ml HESC medium.

14| Filter the cell suspension using a sterile fluorescence-activated cell sorting (FACS) tube with a cell-strainer cap by gently adding the cells to the cap. Pipette the cells up and down until they start to pass through the cell strainer. Cover the caps with a small square of Parafilm, and centrifuge the tube for 1 min at 480*g* in a refrigerated centrifuge at 4 °C to pellet the HESCs.
▲ CRITICAL STEP This step removes any cell clumps and cellular debris and ensures that the pelleted HESCs form a single-cell suspension that will not block the flow cytometer.

15| Carefully remove the HESC medium from the cell pellet and resuspend in ~700 µl HESC medium containing 40 ng ml^{-1} FGF2 and 1 µg ml^{-1} PI. Replace the cell-strainer caps with sterile FACS tube caps and store the dissociated HESCs on ice or at 4 °C until required.

16| Using the single-cell deposition function of a sterilized flow cytometer, place a single HESC into each well of the 96-well plates (from Step 11).

17| Incubate the 96-well plates in a humidified incubator at 37 °C, 5% CO_2. Four days after seeding the plates with HESCs, top up each well with an additional 50 µl of HESC medium containing 40 ng ml^{-1} FGF2.

18| Wells containing colonies of viable HESCs should be visible by microscopy 7–9 d after single-cell deposition (**Fig. 2a**). Identify these wells and supplement with additional MEFs at 1×10^4 per cm^2.
? TROUBLESHOOTING

19| After the identification of wells containing HESC colonies, change the HESC medium (supplemented with 40 ng ml^{-1} FGF2) in these wells daily.

20| Allow the colonies to grow for an additional ~7 d, by which time they should be ~2 mm in diameter (**Fig. 2b**).

21| Between 16 and 18 d after the single-cell deposition, passage the HESC colonies. Using a pipettor with 200-µl plugged tips, scrape the bottom of the wells until the colonies have fragmented. A stereomicroscope can be used to ensure that the

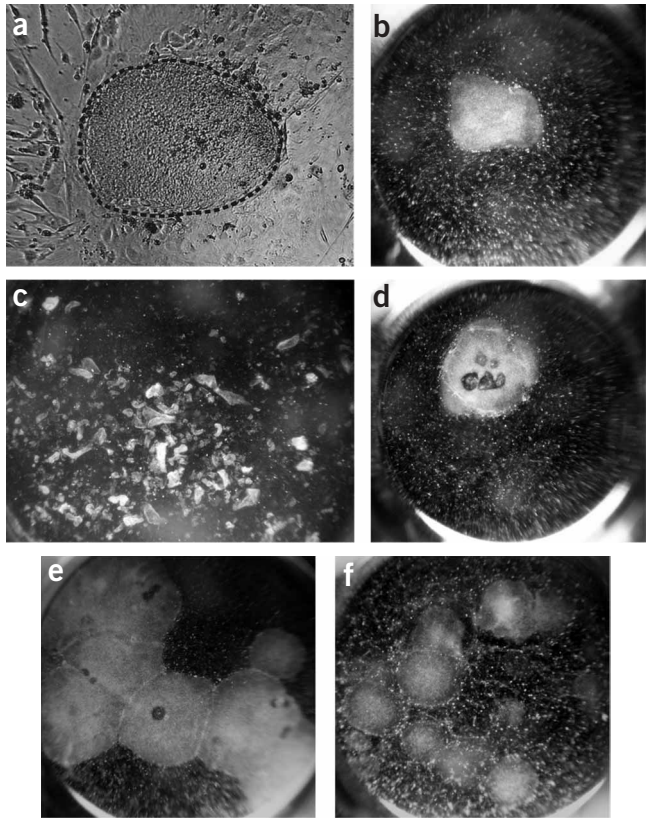

Figure 2 | Photomicrographs of human embryonic stem cells (HESCs) cloned by single-cell deposition. (**a**) A HESC colony (outlined by a black dotted line) 9 d after seeding a well with a single HESC. Original magnification, ×50. (**b**) Low-power view of the HESC colony from **a** after a further 7 d of culture in the original 96-well plate. The colony is ~2 mm in maximum dimension. Original magnification, ×12.5. (**c**) Clumps of HESCs resulting from fragmentation of a colony using a 200-µl pipette tip. Original magnification, ×16. (**d**) A HESC colony that has begun to differentiate in its center, 16 d after single-cell deposition. This colony should be passaged to minimize the extent of differentiation. Original magnification, ×12.5. (**e**,**f**) Wells from a 48-well plate containing HESC colonies ready to be passaged. The HESC colonies in **e** are large enough to transfer only one-third of the cells to a new well in a 48-well plate, while one-half of the cells in **f** should be replicated. Original magnification, ×12.5.

entire HESC colony has been detached and broken up into sufficiently small cell clumps (**Fig. 2c**). The colony pieces can then be collected and transferred onto 48-well plates containing MEFs seeded at a density of 2×10^4 per cm^2.
▲ **CRITICAL STEP** If the HESC colonies begin to differentiate in the 96-well plates, it is advisable to transfer them to the 48-well plates, irrespective of the size of the colony (**Fig. 2d**). This step will remove some of the differentiated cells.
? TROUBLESHOOTING

22| Change the media in the wells daily. The concentration of FGF2 in the HESC medium can be returned to 10 ng ml^{-1} following the transfer.

23| The HESC clones are large enough to passage 7–9 d after the initial transfer to the 48-well plates. Break the clones up as described in Step 21, and transfer between one-third and one-half of each clone onto a new 48-well plate containing MEFs seeded at a density of 2×10^4 per cm^2. This 48-well plate is used to maintain the HESC clones. Place the remaining pieces representing one-half to two-thirds of each clone into individual 1.5-ml microtubes.
▲ **CRITICAL STEP** The proportion of a clone that is required for replication is dependent on the density and confluence of the clone in the initial 48-well plate (**Fig. 2e,f**). If the well is <40% confluent and the density of the colonies is low, then one-half of the contents of the well should be transferred to the new 48-well plate. For more confluent wells with larger heaped colonies, one-third of the well contents is sufficient to ensure the line is maintained.

Preparation of DNA for PCR screening ● TIMING ~7 h

24| Pellet the colony pieces in the microtubes using a microfuge (480*g*, room temperature, 1 min), and carefully aspirate the media.

25| Add 100 µl DNA lysis buffer containing proteinase K to the microtubes and incubate at 55 °C for 3 h.
■ **PAUSE POINT** Alternatively, the microtubes can be incubated at 37 °C overnight, and the DNA preparation can be continued the following day.

26| Add 100 µl of phenol:chloroform:isoamyl alcohol solution to each tube, mix well and centrifuge the tubes at 12,000–15,000*g* in a microfuge for 10 min, at room temperature.

27| Carefully transfer the upper aqueous layer containing the DNA to a fresh microfuge tube containing 250 µl 100% ethanol. Mix the solutions until the DNA precipitates. At room temperature, pellet the DNA by centrifugation at 12,000–15,000*g* in a microfuge for 10 min.

28| Gently decant the supernatant and wash the DNA pellet by adding 250 µl 70% ethanol. Spin the microtubes again at 12,000–15,000*g* for 10 min, at room temperature.

29| Remove the 70% ethanol and allow the DNA pellets to air dry. Add 50 µl TE to each microtube and incubate at 55 °C for 2–3 h to facilitate dissolving the DNA. If time permits, this DNA solution may be incubated overnight in a 55 °C incubator.
■ **PAUSE POINT** The DNA solutions can be stored at 4 °C indefinitely.

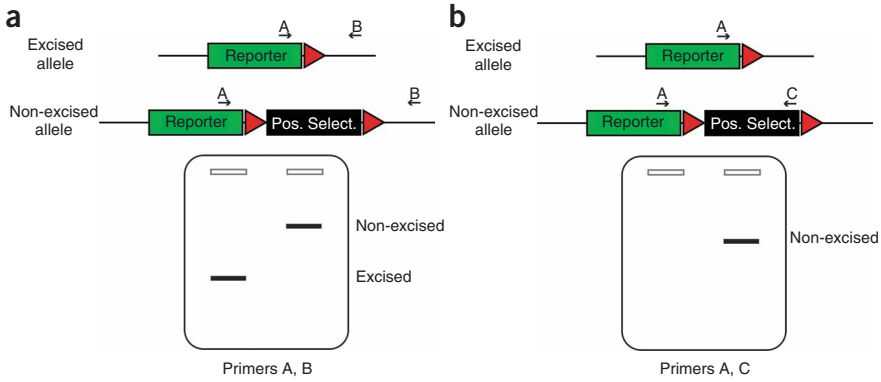

Figure 3 | Possible primer locations for human embryonic stem cell (HESC) colony screening for excision of the positive selection cassette. (**a**) PCR analysis using a primer (primer A) that corresponds to sequences within the reporter gene (Reporter) paired with a primer (primer B) that anneals to sequences 3′ of the positive selection marker (Pos. Select.) identify HESC colonies in which the selection cassette is removed. Both excised and non-excised alleles will generate PCR products, but the PCR product amplified from the excised allele will be shorter by an amount corresponding to the length of sequence between the two *loxP* sites. (**b**) PCR analysis using primer A paired with a primer that anneals to sequences within the selection cassette (primer C) can be used to identify HESC colonies that contain cells which have retained the positive selection marker. The PCR products can be visualized by agarose gel electrophoresis.

PCR screen for the removal of the positive selection cassette ● TIMING 2 d

30| Using primers specific to sequences flanking the positive selection cassette, screen the DNA by PCR for the loss of the positive selection marker. The 5′ primer should be chosen such that it anneals to sequences within the reporter gene and the 3′ primer to sequences 3′ of the positive selection marker (**Fig. 3a**). The PCR conditions will be dependent on the sequence being amplified. Typically, the PCR conditions used in the initial identification of the genetically modified HESC line will suffice. Include a sample of DNA from the genetically modified HESC line before removal of the positive selection marker as a control. HESC colonies in which the selection marker has been removed will yield a smaller PCR product compared to the parental HESC line. Visualize the PCR products by agarose gel electrophoresis, as previously described[25]. The PCR product can also be sequenced to confirm the fidelity of the excision process.

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31| Identify the colonies in which the positive selection marker has been excised. If these HESC colonies have not been cloned using single-cell deposition flow cytometry, the above PCR screen will not exclude the presence of residual cells that still contain the selection cassette. Perform a second PCR with at least one of the primers corresponding to sequences within the positive selection cassette (**Fig. 3b**). Visualize the PCR products by agarose gel electrophoresis. No PCR product is obtained if the HESC colony contains only cells from which the positive selection cassette has been excised. DNA from HESCs that still contain the selection cassette is used as a positive control.

32| Perform a third PCR screen with primers directed against sequences in the *pEFBOS-CreIRESpuro* expression vector to confirm that the plasmid did not integrate into the genome of the resulting colonies. We recommend the following primers and PCR. Forward primer: 5′-CTGGCGCGCCTTACCGAGTACAAGCCCACGGTGCGCCTC-3′ and reverse primer: 5′-CGTGGGAGACCTGATACTCTCAAG-3′. A sample of the *pEFBOS-CreIRESpuro* vector serves as a positive control:

Component	Amount (per reaction) (μl)	Final
dH_2O	12.9	
10× PCR buffer, minus Mg	2	1×
50 mM $MgCl_2$	1.2	4 mM
DMSO	1	5%
10 mM dNTPs	0.4	200 μM
250 ng μl^{-1} forward primer	0.2	2.5 ng μl^{-1}
250 ng μl^{-1} reverse primer	0.2	2.5 ng μl^{-1}
5 U μl^{-1} Platinum *Taq* DNA polymerase	0.1	0.025 U μl^{-1}
50–200 ng μl^{-1} genomic template DNA	2	5–20 ng μl^{-1}

33| Perform the PCR using the following conditions:

Cycle number	Denature	Anneal	Extend
1	94 °C for 3 min		
2–30	94 °C for 20 s	56 °C for 30 s	68 °C for 70 s
31			68 °C for 10 min

Note: If the vector has integrated into the genome a PCR product of ~1 kb will be amplified.

? TROUBLESHOOTING

34| Choose two or three genetically modified HESC colonies in which the positive selection marker is excised and the *pEFBOS-CreIRESpuro* vector has not integrated into the genome. Transfer these colonies from the 48-well plate to 2× organ culture dishes containing MEFs seeded at 6×10^4 per cm^2. Fragment the colonies as described in Step 21. The new lines should be maintained in organ culture as previously described[16].

▲ **CRITICAL STEP** Newly generated genetically modified HESC lines should be karyotyped, and examined for expression of stem cell markers and teratoma formation. If the line has not been cloned by single-cell deposition, this should also be performed to ensure the line does not represent a mixed population of HESCs. In addition, stocks of the clones should be frozen in liquid nitrogen using methods outlined previously[26].

● **TIMING**

Step 1, preparation of HESCs for transfection: ~ 30 min
Steps 2 and 3, transfection of HESCs: ~1 h 30 min
Steps 4–7, generation and expansion of puromycin-resistant HESCs: 7–12 d
Steps 8–10, seeding of 96-well plates with MEFs: ~30 min per ten plates
Steps 11–16, preparation and cloning of HESCs by single-cell deposition: ~2–3 h per ten plates
Steps 17–20, expansion and identification of clonal HESCs: ~10 d
Step 21, passaging of HESCs from a 96-well plate: ~1 h per 30 colonies
Step 22, changing of HESC medium: ~10 min per 48-well plate
Step 23, consolidation of HESC colonies for PCR screening: ~1 h per 30 colonies
Steps 24–29, preparation of DNA for PCR screening: ~7 h
Steps 30–33, PCR screening of HESC clones: ~2 d
Step 34, passaging of HESCs from 48-well plate: ~10 min per well

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5	Puromycin-resistant colonies do not form	The *puro*-containing vector did not transfect into the human embryonic stem cells (HESCs)	Alter the FuGENE:DNA ratio
			Transfect a vector carrying a fluorescent reporter marker into the HESCs, to confirm the HESCs are being transfected
		Concentration of puromycin used for selection is too high	Perform a titration of the antibiotic to ensure the optimal concentration is added to the HESCs
		Culturing conditions may be suboptimal	Supplement dishes with more mouse embryonic fibroblasts (MEFs) at an earlier time point
	After selection, the dishes remain confluent with HESCs	Dishes are seeded with too many HESCs	Decrease the initial seeding density of HESCs on the dishes
		Concentration of puromycin used for selection is too low	Perform a titration of the antibiotic to ensure the optimal concentration is added to the HESCs
18	No clones are recovered after single-cell deposition	The cloning efficiency of the HESCs is very low	Increase the number of 96-well plates into which cells are deposited
			Treat the cells with the Rho-associated kinase inhibitor, Y-27632 (ref. 21)
		The ratio of MEFs:HESCs in the sorted population is too high	Remove some of the MEFs from the dishes before harvesting the HESCs
			Enzymatically passage the HESCs onto dishes with a low density of MEFs (1×10^4 MEFs per cm^2) the day before single-cell deposition
			Delay sorting of the HESCs until the dishes are more confluent

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Culturing conditions may be suboptimal	Change the media more frequently and increase the density of MEFs on the 96-well plates
21	HESC colonies differentiate	Culturing conditions may be suboptimal	Change the media more frequently and increase the density of MEFs on the plates
			Passage colonies earlier
30	No PCR product is obtained when HESC colonies are screened with primers flanking the positive selection cassette	Not enough DNA template was included in the PCR	Perform a PCR using primers specific for an endogenous gene to determine the optimal amount of DNA template to use
		Suboptimal PCR conditions	Alter the annealing temperature and include up to 5% DMSO in the reaction mixture
		The original genetically tagged HESC line consisted of a mixed population of cells that included nontargeted HESCs lacking either 5′ or 3′ primer binding sites	Screen the colonies by PCR after transfection of the Cre recombinase-expression vector before cloning the line to identify genetically tagged HESC clones
33	A PCR product is obtained from the HESCs indicating the Cre recombinase-expression vector is present	The expression vector has integrated into the genome	Select a different clone that has not integrated the expression vector

ANTICIPATED RESULTS

The protocols described in this article were used to remove the *neo* gene from the *MIXL1*$^{GFP/w}$ HESC lines and to clone one of these lines[8]. This protocol has also been used to remove the positive selection marker from an additional six genetically modified lines in our laboratory. On average, 85 puromycin-resistant colonies are obtained per 1×10^6 HESCs transfected with the *pEFBOS-creIRESpuro* vector (**Table 2**). If the genetically modified HESCs consist of a homogenous population of cells, generally >95% of the resulting puromycin-resistant colonies will have excised the *loxP*-flanked positive selection cassette (**Table 3**). This is irrespective of whether the genetic modification is a result of gene targeting or random integration. The cloning efficiency of the HESCs after single-cell deposition using flow cytometry does vary

TABLE 2 | Number of colonies arising after puromycin selection of four genetically modified HESC subclones transfected with the vector *pEFBOS-creIRESpuro*.

Type of genetic modification	Parental HESC line	Number of colonies per 1×10^6 HESCs
Gene targeting	MEL1	58
	MEL1	66
Transgenic	MEL1	98
	MEL1	127

HESC, human embryonic stem cell.

TABLE 3 | Number of HESC colonies after puromycin selection in which the *loxP*-flanked positive selection cassette was excised.

Targeted locus	HESC line	Positive selection cassette	Number of colonies picked	Number of colonies in which the selection marker was excised
Gene-targeted HESC lines				
Locus 1	HES2	*pMC1neo**	48	48
	HES3	*PGKneo*	16	14
	HES4	*pMC1neo**	72	72
	MEL1	*PGKneo*	24	24
Locus 2	HES3	*PGKhygro*	25	24
Locus 3	MEL1	*PGKneo*	6	6

Transgenic construct	HESC line	Positive selection cassette	Number of colonies picked	Number of colonies in which the selection marker was excised
Transgenic HESC lines				
Construct 1	HES3	*PGKneo*	76	74
Construct 2	MEL1	*PGKneo*	32	32

HESC, human embryonic stem cell. *The use of this positive selection cassette is not recommended because silencing of the *neo* gene was observed.

TABLE 4 | Single-cell cloning efficiency of gene-targeted HESCs.

Targeted locus	HESC line	Number of colonies per 960 individual HESCs plated	Cloning efficiency (%)
Locus 1	HES3	47	4.9
	MEL1	18	1.9
Locus 2	HES3	76	7.9
Locus 3	MEL1	44	4.6
		34	3.5
Locus 4	HES3	41	4.3

HESC, human embryonic stem cell.

between cell lines, but is usually between 1 and 8% (**Table 4**). All genetically modified HESC lines generated by our laboratory that have undergone these procedures have retained characteristic stem cell properties and remained karyotypically normal. If there is a possibility that the original gene-targeted HESC line is not clonal, it is recommended that the puromycin-resistant colonies are re-examined for the presence of the targeted allele before cloning by single-cell deposition flow cytometry.

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