

## Protocol

# Use of the Sleeping Beauty Transposon System for Stable Gene Expression in Mouse Embryonic Stem Cells

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## INTRODUCTION

Sleeping Beauty (SB) transposon-based transfection is a two-component system consisting of a transposase and a transposon containing inverted repeat/direct repeat (IR/DR) sequences that result in precise integration into a TA dinucleotide. The transposon is designed with an expression cassette of interest flanked by IR/DRs, and SB transposase mediates stable integration and reliable long-term expression of the gene of interest. It has recently been demonstrated that SB efficiently mediates gene transfer and stable gene expression in human embryonic stem (ES) cells. Here, we describe a method for transfecting and establishing stable cell lines in mouse embryonic stem (mES) cells with the SB system.

## RELATED INFORMATION

See Figure 1 for a diagram of the SB transposon system. More information about the SB transposon system and its use in gene transfer can be found in Ivics et al. (1996, 1997) and Wilbur et al. (2007). Protocols are available for **Estimation of Cell Number by Hemocytometry Counting** (Sambrook and Russell 2006a), **Amplification of cDNA Generated by Reverse Transcription of mRNA** (Sambrook and Russell 2006b), **Southern Blotting: Capillary Transfer of DNA to Membranes** (Sambrook and Russell 2006c), **Southern Blotting: Simultaneous Transfer of DNA from a Single Agarose Gel to Two Membranes** (Sambrook and Russell 2006d), **Southern Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Membranes** (Sambrook and Russell 2006e), and **Inverse PCR** (Sambrook and Russell 2006f).

## MATERIALS

**CAUTIONS AND RECIPES:** Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

### Reagents

D3 mES cells (ATCC collection or University of Michigan Transgenic Core)  
Expand High Fidelity PCR System (Roche)  
Hanks' Balanced Salt Solution (1X HBSS) (without CaCl<sub>2</sub>, MgCl<sub>2</sub>, or MgSO<sub>4</sub>) (GIBCO/Invitrogen)  
<I>Hygromycin B (Invitrogen)

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Lipofectamine (Invitrogen)  
Lipofectamine Plus Reagent (Invitrogen)  
<R>mES cell growth medium  
<R>mES cell growth medium with Leukemia Inhibitory Factor (LIF)  
OPTI-MEM (+HEPES, +sodium bicarbonate, +glutamine) (GIBCO/Invitrogen)  
Sleeping Beauty transposon and transposase plasmids (available from the Center for Genome Engineering, University of Minnesota, <http://www.cge.umn.edu>)

*It is important to include a selection marker for the isolation of clones that harbor the transposon. The vector described in this protocol contains a hygromycin cassette for selection of stable clones. Alternative selection markers can be chosen from various antibiotic resistance genes or fluorescent markers such as enhanced green fluorescent protein (EGFP). Prepare plasmid DNA (~1 µg/µL) using a QIAGEN miniprep kit. To obtain high concentration yields, use a 5-mL overnight bacterial culture, wash the prep with the optional wash provided in the kit, and elute the DNA twice (see also Hermanson et al. 2004). For a diagram of the expression construct used in this protocol, see Figure 2.*

<I>Trypsin/EDTA (0.25% trypsin [GIBCO/Invitrogen] in 1 mM EDTA [Lonza], sterile filtered)

## Equipment

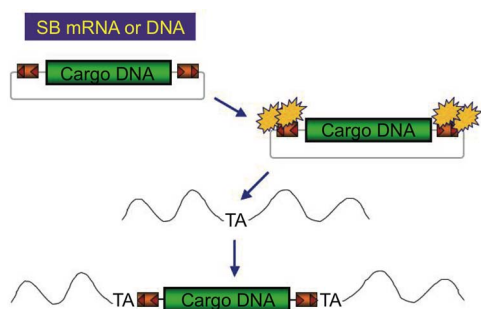
Aspirator  
Centrifuge (benchtop)  
Hemocytometer  
Hood (sterile for tissue culture)  
Incubator (humidified, 37°C, 5% CO<sub>2</sub>)  
Micropipettors (e.g., Gilson Pipetman P10, P20, P200, P1000) and disposable barrier tips (sterile, DNase/RNase-free)  
Microscope (inverted, equipped with bright-field optics)  
Pipettes (sterile serological; 2-, 5-, 10-, and 25-mL)  
Plates, dishes, and flasks (sterile tissue culture, gelatin-coated; 48-well, 24-well, 12-well, six-well, 10-cm, T25)  
*Prepare gelatin-coated plasticware just before use by coating the bottom of each dish with 0.1% (w/v) gelatin solution (Sigma). Incubate the dishes for 15 min in a humidified, 5% CO<sub>2</sub> incubator at 37°C, and wash twice with 1X HBSS. For a 10-cm dish, use 5 mL of gelatin solution and 2 × 5 mL of HBSS. Use the 10-cm dishes as soon as they are prepared; T25 flasks can be stored at 4°C for up to a month.*  
Tubes (sterile conical, 15-mL; Falcon)  
Tubes (sterile microcentrifuge, 1.5-mL)

## METHOD

*Use standard aseptic technique for all procedures.*

### Passaging mES Cells onto Gelatin-Coated Dishes for Transfection

1. Grow D3 mES cells in a gelatin-coated T25 tissue culture flask in a humidified, 5% CO<sub>2</sub> incubator at 37°C until 90%-95% confluent (2 d).  
*D3 mES cells may also be grown in 10-cm, gelatin-coated tissue culture dishes or flasks. Other ES cell lines may require different culture methods. For example, mouse embryonic fibroblasts (MEFs) may be used as an alternative to gelatin, in combination with LIF (Nagy et al. 2003).*
2. Wash the cells twice with 1X HBSS.
3. Add 2 mL of trypsin/EDTA to the flask and incubate for 2 min in a humidified, 5% CO<sub>2</sub> incubator at 37°C.
4. Add 4 mL of mES cell growth medium to the flask to stop the trypsin. Triturate (pipette up and down) with a 5-mL pipette to mechanically dissociate the cells from the flask.
5. Transfer the dissociated mES cells to a 15-mL conical tube, and centrifuge at 1200 rpm for 5 min at room temperature.



**FIGURE 1.** The SB Transposon System. SB is a two-component system consisting of a transposase source (mRNA or DNA) and a transposon plasmid containing IR/DR sequences that flank any cargo DNA of interest. Once expressed, the transposase recognizes and binds the IR/DRs and catalyzes the precise integration into a small TA dinucleotide target site. Upon integration, the TA dinucleotides are duplicated and the transgene is inserted into the genome as a single copy.

6. Aspirate the supernatant. Resuspend the pelleted cells in 1 mL of mES cell growth medium with LIF; dissociate the cells mechanically into a single-cell suspension.
7. Count the cells using a hemacytometer (see **Estimation of Cell Number by Hemocytometry Counting** [Sambrook and Russell 2006a]).  
*At least  $6 \times 10^6$  cells are needed to proceed with the transfection.*
8. Plate  $3 \times 10^6$  mES cells in 10 mL of mES cell growth medium with LIF into a gelatin-coated, 10-cm tissue culture dish. Prepare two 10-cm dishes per transfection.
9. Incubate the cultures in a humidified, 5% CO<sub>2</sub> incubator overnight at 37°C.

#### Transfection of mES cells

10. Grow the mES cells from Step 9 to 50%-80% confluence.
11. Prepare the Lipofectamine transfection mixture:
  - i. In a microcentrifuge tube labeled "A," add 750  $\mu$ L of OPTI-MEM, 20  $\mu$ L of Lipofectamine Plus Reagent, 3  $\mu$ g of transposon plasmid, and 1  $\mu$ g of transposase expression plasmid.
  - ii. In a microcentrifuge tube labeled "B," add 750  $\mu$ L of OPTI-MEM and 30  $\mu$ L of Lipofectamine.
  - iii. Incubate both tubes in a sterile tissue culture hood for 15 min at room temperature.
  - iv. Combine the contents of tubes A and B and incubate in a sterile tissue culture hood for 15 min at room temperature.
12. Aspirate the growth medium from the plated cells and add the Lipofectamine mixture from Step 11.iv to the cells.
13. Incubate the cells with the Lipofectamine mixture in a humidified, 5% CO<sub>2</sub> incubator for 6 h at 37°C.
14. Replace the Lipofectamine mixture with 10 mL of mES cell growth medium with LIF. Continue to incubate the cells in a humidified, 5% CO<sub>2</sub> incubator at 37°C.
15. 48 h after transfection, add hygromycin B at a final concentration of 200  $\mu$ g/mL to the culture to select for transfected cells. Maintain the cells in a humidified, 5% CO<sub>2</sub> incubator at 37°C, and split them at a 1:2 ratio into four gelatin-coated, 10-cm dishes when they are 90%-95% confluent.  
*See Troubleshooting.*

#### Isolation of Individual Clones

16. When colonies are ready to be picked, aspirate the medium from each 10-cm dish and replace it with 2 mL of 1X HBSS.



**FIGURE 2.** The pT2/EF1 $\alpha$ -MCS-Hygro expression construct. This expression construct contains a ubiquitous EF1 $\alpha$  promoter, a multiple cloning site (MCS) for inserting a gene of interest, an internal ribosome entry site (IRES) for producing a bicistronic message, hygromycin for selection of stable mES clones, and a poly(A) tail.

17. Manually pick the colonies using an inverted microscope equipped with bright-field optics:
  - i. Draw up 20  $\mu$ L of 1X HBSS with each colony and place it into one well of a gelatin-coated, 48-well plate with 50  $\mu$ L of trypsin/EDTA.
  - ii. Gently pipette up and down several times to dissociate the colony into single cells.
  - iii. Use the microscope to verify that there are no clumps.
18. Add mES cell growth medium with LIF to a final volume of 300-350  $\mu$ L per well to inactivate the trypsin. Grow the cells in a humidified, 5% CO<sub>2</sub> incubator at 37°C until they are 90%-95% confluent. Change the medium every 2 d.
19. Passage the cells of each clone into one well of a gelatin-coated, 24-well sterile tissue culture plate. Use a final per-well volume of 500  $\mu$ L of mES cell growth medium with LIF plus 200  $\mu$ g/mL hygromycin B. Grow the cells in a humidified, 5% CO<sub>2</sub> incubator at 37°C until they are 90%-95% confluent. Change the medium every 2 d.
20. Passage the cells of each clone into one well of a gelatin-coated, 12-well sterile tissue culture plate. Use a final per-well volume of 1 mL of mES cell growth medium with LIF plus 200  $\mu$ g/mL hygromycin B. Grow the cells in a humidified, 5% CO<sub>2</sub> incubator at 37°C until they are 90%-95% confluent. Change the medium every 2 d.
21. Passage the cells of each clone into one well of a gelatin-coated, six-well sterile tissue culture plate. Use a final per-well volume of 2 mL of mES cell growth medium with LIF plus 200  $\mu$ g/mL hygromycin B. Grow the cells in a humidified, 5% CO<sub>2</sub> incubator at 37°C until they are 90%-95% confluent. Change the medium every 2 d.
22. Passage the cells of each clone at a 1:2 ratio into two wells of a gelatin-coated, six-well sterile tissue culture plate. Use a final per-well volume of 2 mL of mES cell growth medium with LIF plus 200  $\mu$ g/mL hygromycin B. Grow the cells in a humidified, 5% CO<sub>2</sub> incubator at 37°C until they are 90%-95% confluent. Change the medium every 2 d.
23. Passage the cells of each clone (two wells of a six-well sterile tissue culture plate) into a gelatin-coated T25 flask. Use a final volume of 3 mL of mES cell growth medium with LIF plus 200  $\mu$ g/mL hygromycin B. Continue to maintain the cells in a humidified, 5% CO<sub>2</sub> incubator at 37°C.

#### Verification of Gene Expression

24. Check the gene expression of each clone by standard reverse transcriptase polymerase chain reaction (RT-PCR). See **Amplification of cDNA Generated by Reverse Transcription of mRNA** (Sambrook and Russell 2006b).

#### Determination of Transposon Integration Number by Southern Blot Analysis

25. Isolate genomic DNA from the transgenic mES cells, perform a restriction digest of the DNA, and use Southern blot analysis to determine the number of transposons that have integrated into the genome. For details of these procedures, see **Southern Blotting: Capillary Transfer of DNA to Membranes** (Sambrook and Russell 2006c), **Southern Blotting: Simultaneous Transfer of DNA from a Single Agarose Gel to Two Membranes** (Sambrook and Russell 2006d), and **Southern Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Membranes** (Sambrook and Russell 2006e).

#### Determination of the Transposon Integration Site with Inverse PCR

26. Verify that the integration is SB-mediated by performing inverse PCR with genomic DNA from the transgenic mES cells. For details of this procedure, see **Inverse PCR** (Sambrook and Russell 2006f).

*We recommend the Expand High Fidelity PCR System (see also Hermanson et al. 2004). Splinkerette PCR is an alternative method for identifying the transposon insertion site (Dupuy et al. 2002).*

## TROUBLESHOOTING

**Problem:** Cell death occurs during transfection.

[Step 15]

**Solution:** To reduce cell death, try the following:

1. Transfect ES cells at higher confluence.
2. Use less Lipofectamine.
3. Use less DNA.
4. Include serum at concentrations used to maintain the cells normally (10% serum for mES cells).

**Problem:** Transfection efficiency is low.

[Step 15]

**Solution:** To increase transfection efficiency, consider the following:

1. The plasmids might be degraded. Verify that the plasmids are intact by running each plasmid on an agarose gel before transfecting.
  - i. If the plasmids are intact, check that the plasmid concentration is at least 1  $\mu\text{g}/\mu\text{L}$ .
  - ii. If the plasmids are degraded, regrow the plasmid and isolate a fresh plasmid preparation.
2. The ES cells might have differentiated. To avoid transfecting differentiated cells, only grow the cells for 24 h. Increase the plating density if the cells are not confluent enough within this 24-h period.
3. *Mycoplasma* could be contaminating the ES cells. To detect *Mycoplasma* contamination, use a commercially available PCR kit.
4. Lengthen the time that the cells are exposed to Lipofectamine.
5. Retransfect the cells.

## DISCUSSION

The use of viral vectors for gene transfer, although highly efficient, is not always an ideal choice due to recombination, immune responses, and other safety concerns associated with these vectors. DNA transposons offer an effective, alternative method for nonviral gene transfer in mES cells, because they avoid the safety concerns associated with viral vectors and are more efficient than plasmid-based methods. The result of using a transposon system is stable integration and long-term expression of a transgene. SB as described in this protocol is an advantageous method for gene delivery and integration in mES cells. It is important to identify the insertion locus and confirm that the integration is SB-mediated. For additional applications, such as delivering expression cassettes that are >10 kb or mutagenic vectors to mES cells, other transposon systems such as Tol2 and piggyBac may also be considered.

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