Transfection of pluripotent stem cells with Lipofectamine Stem reagent in mTeSR1 Medium on Geltrex matrix

PSC growth medium, passaging reagents, and complexation medium

Component	Cat. No.
mTeSR1 Medium (STEMCELL Technologies)	05850
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	1413302
Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium	14190144
Versene Solution	15040066
RevitaCell Supplement	A2644501
Opti-MEM I Reduced Serum Medium	31985062

Starting with undifferentiated human pluripotent stem cells (PSCs) expanded in a feeder-free culture system such as mTeSR™1 Medium (STEMCELL Technologies) on a substrate such as Gibco™ Geltrex™ matrix is ideal for efficient transfection. PSCs grown in such a culture system can be successfully subcultured or passaged with Gibco™ Versene™ Solution. This gentle EDTA-based dissociation method is recommended during expansion and seeding, or replating for transfection. This method helps to ensure that PSCs do not differentiate or die during passaging, and generate a homogeneous starting population of small clumps of cells for efficient transfection.

Passaging

- Maintain PSCs in the format of your choice, such as 6-well plates, 60 cm dishes, or T-75 flasks coated with Geltrex matrix, for culture in mTeSR1 Medium. Propagating PSCs in 6-well plates and transfecting in 24-well plates are convenient formats used in this protocol.
- Passage PSCs every 3 to 5 days, before they reach ~85% confluence.
- **Tip:** For routine passaging of PSCs with Versene solution for expansion, the replating of large clumps of 5–10 cells promotes reattachment on Geltrex matrix and survival in mTeSR1 Medium without the need to add Gibco™ RevitaCell™ Supplement.

Precoating 24-well plates with Geltrex matrix

1. Prepare a 1:100 dilution of Geltrex matrix in cold Gibco™ DMEM/F-12 Medium with GlutaMAX™ Supplement (Cat. No. 10565).
2. Add 300 µL of diluted Geltrex matrix to each well of a 24-well plate and incubate at 37°C for ≥1 hour, before use.

- **Tip:** Geltrex matrix–coated plates can be prepared ahead of time and stored for up to 2 weeks at 4°C. Equilibrate at room temperature for 1 hour before plating cells.

Seeding cells for transfection

1. When feeder-free PSC cultures are less than 85% confluent, remove the mTeSR1 Medium and gently wash the cells twice with 2 mL of DPBS (without calcium and magnesium) per well in a 6-well plate.

- **Important:** Use DPBS without calcium and magnesium, as these ions can interfere with the effects of the Versene solution. Work with no more than 1 to 3 wells at a time to be able to accurately time the dissociation process.

2. Add 1 mL of Versene solution per well and incubate at 37°C for 3–5 minutes.

- **Important:** If using another EDTA-based dissociation reagent or an enzymatic dissociation method, optimal incubation time may vary.

3. Observe cultures under a microscope, and aspirate the Versene solution when individual cells have contracted and are visible within colonies but remain attached to the well.

4. Add 1 mL of mTeSR1 Medium (with or without RevitaCell Supplement or a rho kinase inhibitor) to each well to inactivate the Versene solution.

- **Important:** Unlike for routine passaging, PSC cultures should be dissociated into small clumps of 3–5 cells to promote efficient transfection. Colonies in clusters that are too large (>10 cells) will transfect efficiently only around the outer edges.

5. Flush the cells off the surface of the well with a 1 mL pipette, and triturate in the plate 3 times to dissociate into small clusters of 3–5 cells.

6. Confirm under the microscope that there are no remaining large clusters.

7. Triturate again 3 times if needed, and view under the microscope.

- **Important:** Some single cells may also be generated by this method, and their survival will be promoted by the inclusion of RevitaCell Supplement or a rho kinase inhibitor. Use of other enzymatic dissociation reagents may require the inclusion of a rho kinase inhibitor to promote adequate survival.

8. Collect the contents of each well in a 15 mL conical tube.

9. Perform a total viable cell count, including single cells as well as individual cells within small clusters.

- **Important:** Proliferating PSC cultures need room to expand during transfection, so plate the recommended starting number of cells (step 12) to achieve 30–60% confluence on the day of transfection.

10. Dilute with additional mTeSR1 Medium with RevitaCell Supplement to a final concentration of 100,000 cells/mL; if RevitaCell Supplement is omitted, dilute to 150,000 cells/mL to account for differences in plating efficiencies.

11. Aspirate the Geltrex matrix from the wells of a precoated 24-well plate.

12. Add 0.5 mL of the PSC suspension in mTeSR1 Medium (with or without RevitaCell Supplement) to plate 50,000–75,000 cells/well in the precoated 24-well plate.

13. Return the plate to the incubator and culture at 37°C with 5% CO_2, overnight.

- **Important:** Plate PSCs only 1 day before transfection, to prevent seeded colonies from growing too large.

Changing medium on the day of transfection

On the following day, aspirate the spent medium and replace with 0.5 mL of fresh mTeSR1 Medium (with or without RevitaCell Supplement) in each well.

- **Tip:** Inclusion of RevitaCell Supplement or a rho kinase inhibitor on the day of transfection is not necessary but can promote survival during transfection.

DNA transfection protocol

Perform the following steps, which have been optimized for using Invitrogen™ Lipofectamine™ Stem Transfection Reagent in mTeSR1 Medium:

Step	Tube	Complexation component	Amount per well (24-well plate)
1	Tube 1	Opti-MEM I medium	25 µL
		Lipofectamine Stem reagent	1 µL
2	Tube 2	Opti-MEM I medium	25 µL
		DNA (0.5–5 µg/µL)	500 ng
3	Add tube 2 solution to tube 1, and mix well.		
4	Incubate mixture from step 3 for 10 minutes at room temperature.		
5	Add 50 µL of complex from step 4 to each well; gently swirl plate to ensure even distribution of the complex across the entire well.		
6	Return culture dish to incubator and culture at 37°C with 5% CO_2, overnight. **Important:** The following day, overlay an additional 0.5 mL of mTeSR1 Medium per well, if PSCs are going to be transfected for 48 hours; passage before they reach 85% confluence.		

Analysis of transfection efficiency

Observe PSCs transfected with a GFP reporter construct at 24 and 48 hours posttransfection by fluorescence microscopy or flow cytometry for endpoint analysis (Figure 1).

Tips and tricks

- The amount of Lipofectamine Stem reagent required for optimal transfection depends on the amount of PSCs plated and the amount of DNA used.
- If the starting confluence of PSCs on the day of transfection is ~60%, using 2 µL of Lipofectamine Stem reagent can improve transfection efficiency.
- If cytotoxicity from the DNA preparation is evident, reducing the amount of DNA to 250 ng per well can improve survival while maintaining efficient transfection.
- Using a plasmid with a promoter that is active in human PSCs, such as the EF1α promoter, is critical for assessing transfection efficiency; some promoters such as the cytomegalovirus (CMV) promoter can be transcriptionally silenced in PSCs.

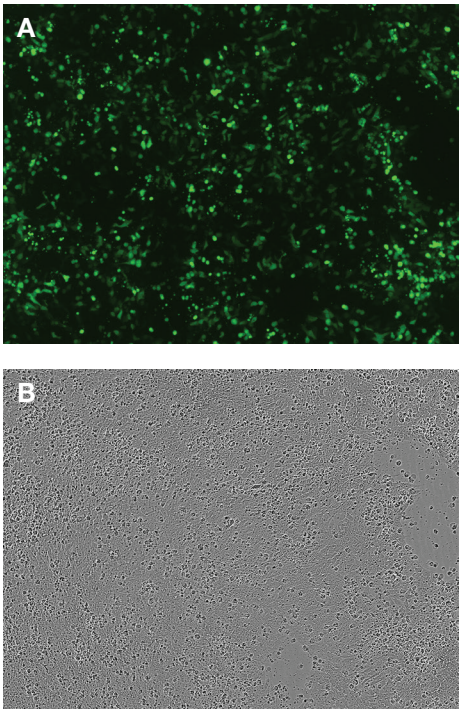

Figure 1. Posttransfection analysis of iPSCs. (A) Fluorescence image demonstrating 69% transfection efficiency, and **(B)** bright-field image. NCRM1 iPSCs are shown 48 hours after transfection with 500 ng of an 11.2 kb EF1α-GFP plasmid and 1 µL of Lipofectamine Stem reagent in mTeSR1 Medium on Geltrex matrix.

mRNA transfection protocol

Perform the following steps, which have been optimized for using Lipofectamine Stem reagent in mTeSR1 Medium:

Step	Tube	Complexation component	Amount per well (24-well plate)
1	Tube 1	Opti-MEM I medium	25 µL
		Lipofectamine Stem reagent	1 µL
2	Tube 2	Opti-MEM I medium	25 µL
		mRNA (0.5–5 µg/µL)	250 ng
3	Add tube 2 solution to tube 1, and mix well.		
4	Incubate mixture from step 3 for 10 minutes at room temperature.		
5	Add 50 µL of complex from step 4 to each well; gently swirl plate to ensure even distribution of the complex across the entire well.		
6	Return culture dish to incubator and culture at 37°C with 5% CO_2, overnight. **Important:** The following day, overlay an additional 0.5 mL of mTeSR1 Medium per well, if PSCs are going to be transfected for 48 hours; passage before they reach 85% confluence.		

Analysis of transfection efficiency

Observe PSCs transfected with a fluorescent mRNA at 24 and 48 hours posttransfection by fluorescence microscopy or flow cytometry for endpoint analysis (Figure 2).

Tips and tricks

- The amount of mRNA required to generate a specific biological readout will vary by user application; Lipofectamine Stem reagent efficiently delivers mRNA into PSCs across a range of dosages.
- Including an independent GFP mRNA (50 ng) in addition to your transcript of interest allows an independent assessment of transfection efficiency.
- If cytotoxicity from the mRNA preparation is evident, reducing the amount of mRNA to 125 ng per well can improve survival while maintaining efficient transfection.
- The method of generation and purification of *in vitro* transcribed (IVT) mRNA can contribute to toxicity as well as translational repression.
  - An anti-reverse cap analog (ARCA) system, included in the Invitrogen™ mMESSAGE mMACHINE™ Kit for *in vitro* transcription, and Invitrogen™ MEGAclear™ columns can be used to eliminate uncapped transcripts and small unincorporated nucleotides that can contribute to cytotoxicity.

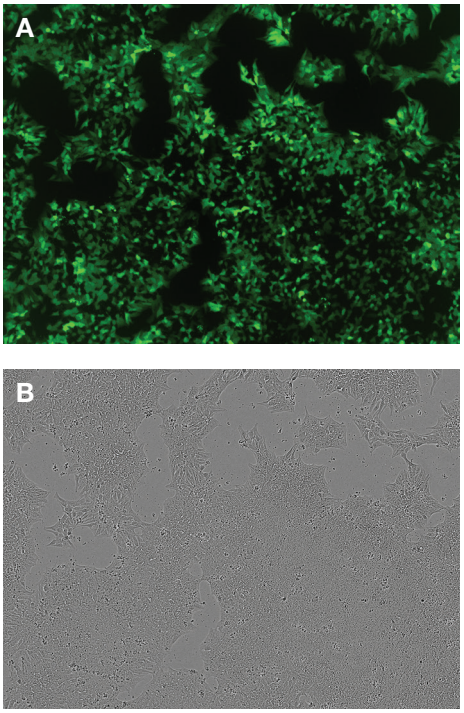

Figure 2. Posttransfection analysis of human embryonic stem cells (H9 hESCs). **(A)** Fluorescence image demonstrating 70% transfection efficiency, and **(B)** bright-field image. H9 hESCs are shown 48 hours after transfection with 250 ng of GFP mRNA and 1 µL of Lipofectamine Stem reagent in mTeSR1 Medium on Geltrex matrix.

Ribonucleoprotein (RNP) transfection protocol

RNP complex components:

- Invitrogen™ GeneArt™ Platinum™ Cas9 Nuclease (Cat. No. B25641)
- gRNA (see "Designing and generating gRNA by *in vitro* transcription")

On the day of transfection (1 day after plating PSCs in a single well of a 24-well plate), perform the following steps, which have been optimized for using Lipofectamine Stem reagent in mTeSR1 Medium:

Step	Tube	Complexation component	Amount per well (24-well plate)
1	Tube 1	Opti-MEM I medium	25 µL
		Lipofectamine Stem reagent	1 µL
2	Tube 2	Opti-MEM I medium	25 µL
		Cas9 nuclease	500 ng
		gRNA (0.1–5 µg/µL)	125 ng
3	Add tube 2 solution to tube 1, and mix well.		
4	Incubate mixture from step 3 for 10 minutes at room temperature.		
5	Add 50 µL of complex from step 4 to each well; gently swirl plate to ensure even distribution of the complex across the entire well.		
6	Return culture dish to incubator and culture at 37°C with 5% CO_2, overnight. **Important:** The following day, overlay an additional 0.5 mL of mTeSR1 Medium per well, if PSCs are going to be transfected for 48 hours; passage before they reach 85% confluence.		

Analysis of transfection efficiency

Observe PSCs transfected with a GFP reporter construct at 24 and 48 hours posttransfection by fluorescence microscopy or flow cytometry, and analyze double-stranded break (DSB) formation using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit or a similar assay (Figure 3).

Tips and tricks

- Adding 50 ng of GFP mRNA to the transfection complex along with the RNP complex can provide an independent measure of transfection efficiency.
- PSCs can also be reverse-transfected during replating in mTeSR1 Medium on Geltrex matrix. Double the number of cells seeded to 100,000 or 150,000 per well, and double the amount of Lipofectamine Stem reagent to 2 µL during complex formation. Aspirate the coating solution, add the cells in suspension to the well, overlay the transfection complex, and swirl to mix. The PSCs will start being transfected as they settle and attach.

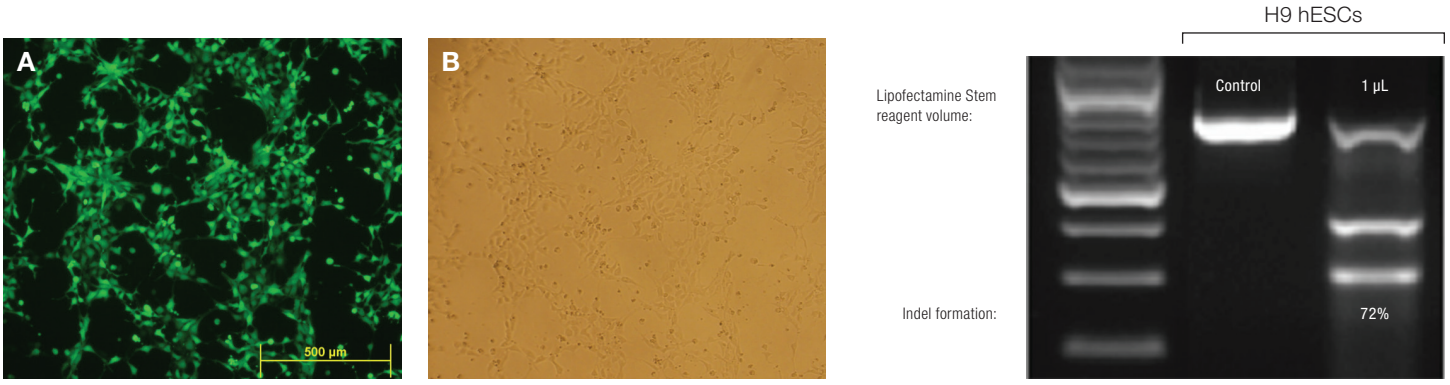

Figure 3. Posttransfection analysis of H9 hESCs. (A) Fluorescence image demonstrating 73% transfection efficiency, and **(B)** bright-field image. H9 hESCs are shown 24 hours posttransfection with 500 ng of GeneArt Platinum Cas9 Nuclease, 125 ng of gRNA, 50 ng of GFP mRNA, and 1 µL of Lipofectamine Stem reagent in mTeSR1 Medium on Geltrex matrix. **(C)** Genomic cleavage detection analysis of H9 hESCs 48 hours posttransfection, demonstrating 72% indel formation within the *EMX1* locus."

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Designing and generating gRNA by *in vitro* transcription

In addition to RNP transfection efficiency, the efficiency of DSB/indel formation at a given locus can depend on gRNA design. Use the Invitrogen™ GeneArt™ CRISPR Search and Design Tool, available at **thermofisher.com/crisprdesign**, to search our database of >600,000 predesigned gRNA sequences specific to every gene in the human genome. GeneArt™ predesigned gRNAs are optimized for gene knockout and typically target the first 3 transcribed exons per gene.

Clone and generate your own gRNA using the Invitrogen™ GeneArt™ Precision gRNA Synthesis Kit (Cat. No. A29377). gRNA concentration can be quantified on the Invitrogen™ Qubit™ 3 Fluorometer (Cat. No. Q33216) coupled with the Invitrogen™ Qubit™ RNA BR Assay Kit (Cat. No. Q10210).

Find out more at **thermofisher.com/transfection**

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