Transfection of pluripotent stem cells with Lipofectamine Stem Transfection Reagent in Essential 8 Medium

PSC growth medium, passaging reagents, and complexation medium

Component	Cat. No.
Essential 8 Medium	A1517001
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	A14700
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 Gibco™ Essential 8™ Medium on a defined substrate such as vitronectin is ideal for efficient transfection. Subculturing or passaging with Gibco™ Versene™ Solution is also recommended during expansion and seeding or replating for transfection. Gentle EDTA-based dissociation of PSC cultures can be used to 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 vitronectin, for culture in Essential 8 Medium or Gibco™ Essential 8™ Flex 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 and survival in Essential 8 Medium on vitronectin without the need to add Gibco™ RevitaCell™ Supplement. PSCs can be expanded in Essential 8 and Essential 8 Flex media interchangeably to support a flexible feeding schedule. Expansion and transfection in Essential 8 Medium can also be successfully performed on Gibco™ rhLaminin-521 or Gibco™ Geltrex™ matrix.

Precoating 24-well plates with vitronectin

1. Prepare a 1:100 dilution of vitronectin by diluting a 60 µL aliquot of vitronectin stock solution (0.5 mg/mL) in 6 mL of DPBS without magnesium or calcium, resulting in a working concentration of 5 µg/mL.

2. Add 200 µL of diluted vitronectin to each well, and incubate at room temperature for 1 hour to coat a 24-well plate with 0.5 µg/cm^2 vitronectin.

- **Tip:** Vitronectin-coated plates can be prepared ahead of time and stored for up to 1 week at 4°C. Equilibrate at room temperature for at least 1 hour before plating cells.

Seeding cells for transfection

1. When feeder-free PSC cultures are less than 85% confluent, remove the Essential 8 Medium and gently wash the cells twice with 2 mL of DPBS (without calcium and magnesium) per well.

- **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.

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 Essential 8 Medium (with RevitaCell Supplement) 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 a microscope.

- **Important:** Some single cells may also be generated by this method, and their survival will be promoted by the inclusion of RevitaCell Supplement.

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 Essential 8 Medium with RevitaCell Supplement to a final concentration of 100,000 cells/mL to account for differences in plating efficiencies.

11. Aspirate the vitronectin solution from the wells of a precoated 24-well plate.

12. Add 0.5 mL of the PSC suspension in Essential 8 Medium (with RevitaCell Supplement) to plate 50,000 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 Essential 8 Medium (with or without RevitaCell Supplement) in each well.

- **Important:** Transfect in Essential 8 Medium, not Essential 8 Flex Medium, which can inhibit DNA transfection.

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

DNA transfection protocol

On the day of transfection perform the following steps, which have been optimized for using Invitrogen™ Lipofectamine™ Stem Transfection Reagent in Essential 8 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 cells; 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:** Overlay an additional 0.5 mL of Essential 8 Medium the following day 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 plasmid 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 to being able to assess transfection efficiency; some promoters such as the cytomegalovirus (CMV) promoter can be transcriptionally silenced in PSCs.

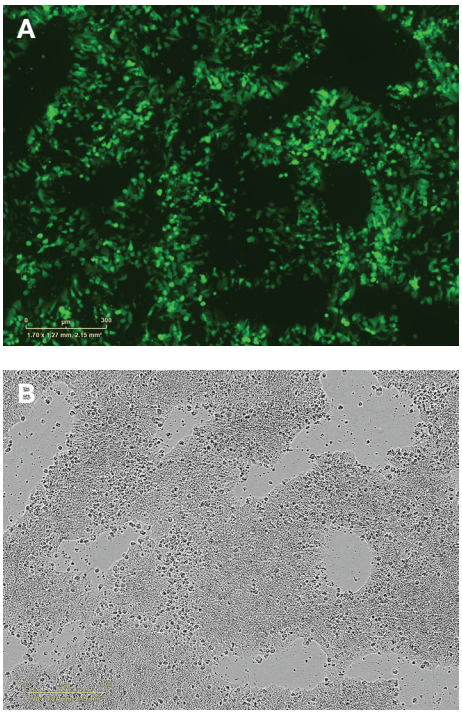

Figure 1. Posttransfection analysis of iPSCs. (A) Fluorescence image demonstrating 75% transfection efficiency, and **(B)** bright-field image. iPSCs are shown 44 hours after transfection with 500 ng of a 6 kb EF1α–GFP plasmid and 1 µL of Lipofectamine Stem reagent in Essential 8 Medium on vitronectin.

mRNA transfection protocol

On the day of transfection perform the following steps, which have been optimized for using Lipofectamine Stem reagent in Essential 8 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)	250–500 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 cells; 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:** Overlay an additional 0.5 mL of Essential 8 Medium the following day 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 at 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 250 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 translational repression and toxicity.
  - Invitrogen™ mMESSAGE mMACHINE™ kits use an ARCA capping system that incorporates the 5′ cap only in the correct orientation, and Invitrogen™ MEGAclear™ columns can be used to eliminate uncapped transcripts and small unincorporated nucleotides that can contribute to cytotoxicity.

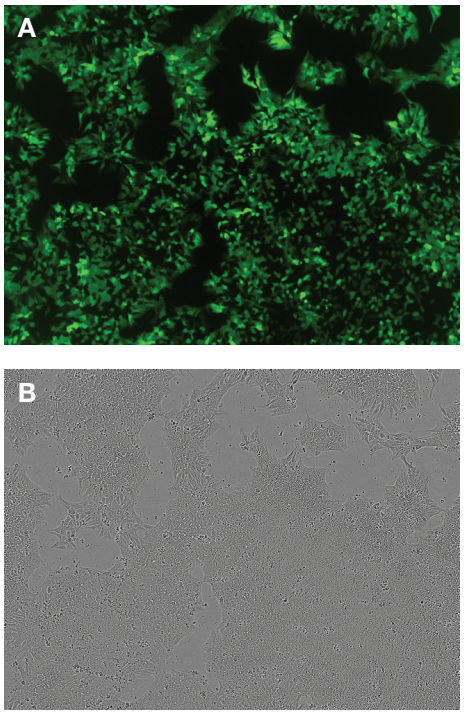

Figure 2. Posttransfection analysis of H9 hESCs. (A) Fluorescence image demonstrating 74% transfection efficiency, and **(B)** bright-field image. H9 hESCs are shown 24 hours after transfection with 250 ng of GFP mRNA and 1 µL of Lipofectamine Stem reagent in Essential 8 Medium on vitronectin.

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 Essential 8 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 (0.1–0.5 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 cells; 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:** Overlay an additional 0.5 mL of Essential 8 Medium the following day if PSCs are going to be transfected for 48 hours; passage before they reach 85% confluence.		

Transfection efficiency analysis

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 formation using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit or 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.
- In addition to transfection efficiency, the efficiency of insertion or deletion (indel) formation at a given locus can depend on gRNA design.
- PSCs can also be reverse transfected during replating in Essential 8 Medium on vitronectin. Increase the amount of cells seeded to 150,000 per well, and double the amount of Lipofectamine Stem reagent to 2 µL during complex formation. Aspirate the coating, 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.

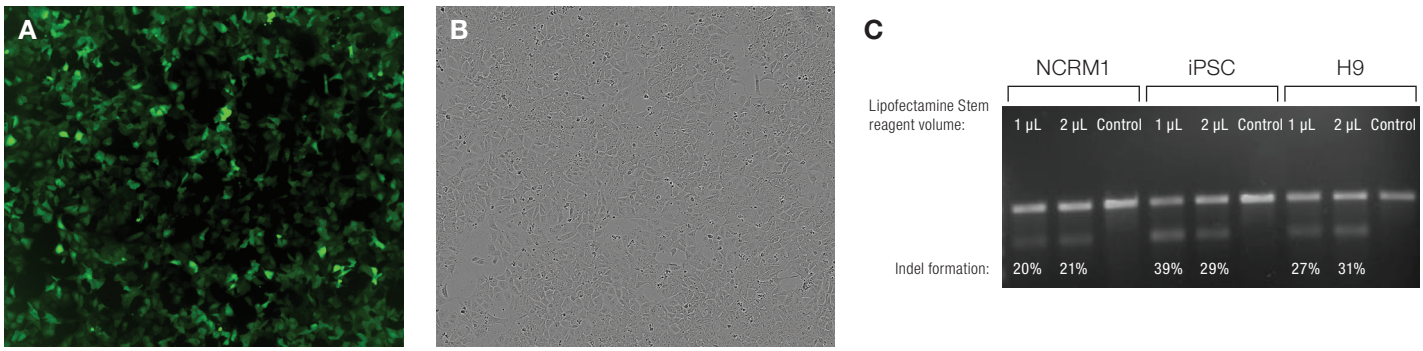

Figure 3. Posttransfection analysis of H9 hESCs. (A) Fluorescence image demonstrating 75% transfection efficiency, and **(B)** bright-field image. H9 hESCs are shown 24 hours after transfection with 500 ng of GeneArt Platinum Cas9 Nuclease, 125 ng of gRNA, 50 ng of GFP mRNA, and 1 µL of Lipofectamine Stem reagent in Essential 8 Medium on vitronectin. **(C)** Genomic cleavage detection analyses of NCRM1 cells, Gibco™ iPSCs, and H9 hESCs 48 hours posttransfection, demonstrating 20–39% indel formation within the *HPRT* locus.

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Designing and generating gRNA by *in vitro* transcription

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™ 3.0 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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