Transfection of pluripotent stem cells with Lipofectamine Stem Transfection Reagent in StemFlex Medium

PSC growth medium, passaging reagents, and complexation medium

Component	Cat. No.
StemFlex Medium	A3349401
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413302
rhLaminin-521	A29248
Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium	14190144
Versene Solution	15040066
TrypLE Select Enzyme (1X), no phenol red	12604013
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™ StemFlex™ Medium on Gibco™ Geltrex™ matrix or on a defined substrate such as rhLaminin-521, is ideal 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 or rhLaminin-521, in StemFlex 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 Gibco™ Versene™ Solution for expansion, the replating of large clumps of 5–10 cells promotes reattachment and survival in StemFlex Medium without the need to add Gibco™ RevitaCell™ Supplement. PSCs can be expanded in StemFlex Medium for subsequent transfection on Geltrex matrix or rhLaminin-521.

Precoating 24-well plates with Geltrex matrix or rhLaminin-521

Coating 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.

Coating with rhLaminin-521

1. Prepare a 1:40 dilution of rhLaminin-521 by adding 300 µL of rhLaminin-521 stock solution (0.5 mg/mL) to 12 mL of DPBS, for a final concentration of 2.5 µg/mL.
2. Add 400 µL of diluted rhLaminin-521 to each well of a 24-well plate, and incubate at 37°C for ≥2 hours to coat the wells with 0.5 µg/cm^2 of rhLaminin-521.

- **Important:** The optimal coating concentration of rhLaminin-521 can depend on the PSC line and ranges from 0.5 to 2 µg/cm^2. Increase the concentration if you observe areas of incomplete cellular attachment.
- **Tip:** Plates coated with Geltrex matrix or rhLaminin-521 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. To maximize transfection efficiency, seeding a single-cell suspension of PSCs prepared with Gibco™ TrypLE™ Select Enzyme is recommended.

- **Important:** As the plating efficiency of PSCs dissociated into single cells is lower than the passaging efficiency of clumped cells, we recommend adding RevitaCell Supplement for overnight replating in StemFlex Medium onto Geltrex matrix or rhLaminin-521 for transfecting the following day.

2. When feeder-free PSC cultures are less than 85% confluent, remove the StemFlex Medium and gently wash the cells twice with 2 mL of DPBS per well in a 6-well plate.
3. Add 1 mL of TrypLE Select Enzyme to each well, swirl to evenly coat the PSCs, and incubate at 37°C for 3–5 minutes.
4. Using a 1 mL pipette, gently triturate the cell suspension 5–10 times to dissociate into single cells.
5. Transfer the cell suspension into a 15 mL conical tube containing 3 mL of StemFlex Medium to inactivate the TrypLE Select Enzyme.
6. Centrifuge the cell suspension at 200 x *g* for 4 minutes.
7. Aspirate the supernatant and resuspend the pellet to a single-cell suspension in 3 mL of StemFlex Medium with RevitaCell Supplement.
8. Perform a total viable cell count with the Invitrogen™ Countess™ II Automated Cell Counter or another method.
9. Dilute with additional StemFlex Medium with RevitaCell Supplement to a final concentration of 100,000 cells/mL.
10. Aspirate the Geltrex matrix or rhLaminin-521 from the wells of a precoated 24-well plate.

- **Important:** Proliferating PSC cultures need room to expand during transfection, so plate the recommended starting number of cells (step 11) to achieve 30% confluence on the day of transfection.

11. Add 0.5 mL of the PSC suspension in StemFlex Medium with RevitaCell Supplement to plate 50,000 cells/well in the precoated 24-well plate.
12. Return the plate to the incubator and culture the cells at 37°C with 5% CO_2, overnight.

Changing medium on the day of transfection

Prepare a solution of of Gibco™ Opti-MEM™ I medium with RevitaCell Supplement. Aspirate the StemFlex medium, and add 0.5 mL of the supplemented Opti-MEM I medium to each well just before transfection.

- **Important:** Transfect in Opti-MEM I Medium with RevitaCell Supplement, not in StemFlex Medium, which can inhibit transfection.

DNA transfection protocol

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

Step	Tube	Complexation component	Amount per well (24-well plate)
1	Tube 1	Opti-MEM I medium	25 µL
		Lipofectamine Stem reagent	2 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	Aspirate the StemFlex Medium and add 0.5 mL of Opti-MEM I medium with RevitaCell Supplement per well, just before transfection.		
6	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.		
7	Return culture dish to incubator and culture the cells at 37°C with 5% CO_2, for 4 hours. **Important:** After 4 hours of transfection, add 0.5 mL of StemFlex Medium warmed to room temperature to each well, return plate to incubator, and culture the cells at 37°C with 5% CO_2, overnight.		
8	The following day, aspirate the StemFlex Medium and transfection complexes, and add 0.5 mL of fresh StemFlex 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.
- 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.
- 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.

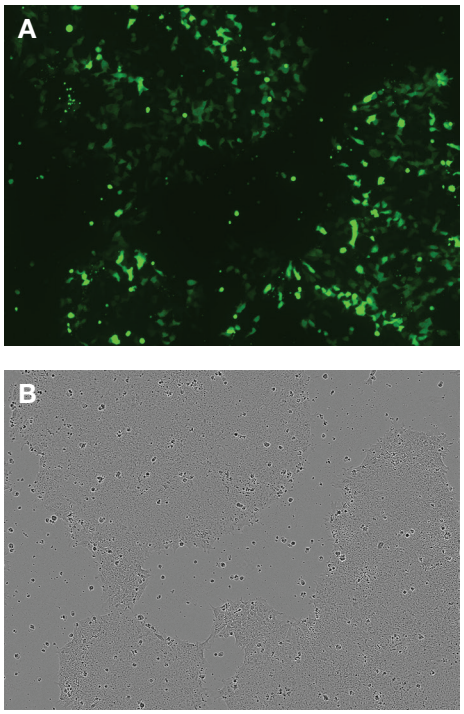

Figure 1. Posttransfection analysis of iPSCs. **(A)** Fluorescence image demonstrating 71% transfection efficiency, and **(B)** bright-field image. iPSCs are shown 44 hours after transfection with 500 ng of an 11.2 kb EF1α-GFP plasmid and 2 µL of Lipofectamine Stem reagent in StemFlex Medium on Geltrex matrix.

mRNA transfection protocol

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

Step	Tube	Complexation component	Amount per well (24-well plate)
1	Tube 1	Opti-MEM I medium	25 µL
		Lipofectamine Stem reagent	2 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	Aspirate the StemFlex Medium and add 0.5 mL of Opti-MEM I medium with RevitaCell Supplement per well, just before transfection.		
6	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.		
7	Return culture dish to incubator and culture the cells at 37°C with 5% CO_2, for 4 hours. **Important:** After 4 hours of transfection, add 0.5 mL of StemFlex Medium warmed to room temperature to each well, return plate to incubator, and culture the cells at 37°C with 5% CO_2, overnight.		
8	The following day, aspirate the StemFlex Medium and transfection complexes, and add 0.5 mL of fresh StemFlex 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 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 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.

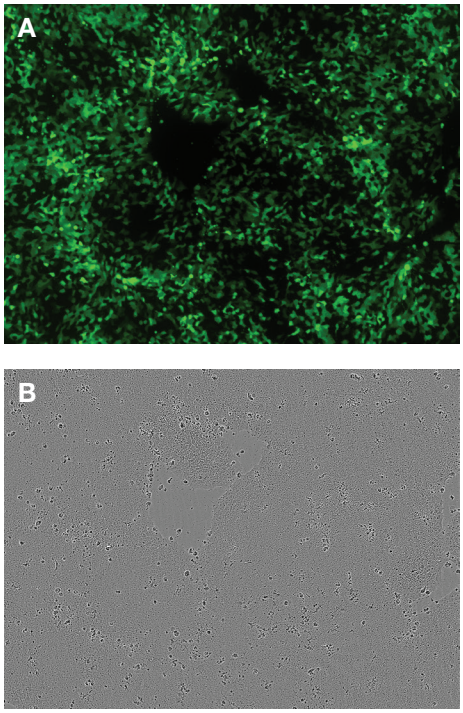

Figure 2. Posttransfection analysis of iPSCs. **(A)** Fluorescence image demonstrating 68% transfection efficiency, and **(B)** bright-field image. iPSCs (NCRM1) are shown 44 hours after transfection with 250 ng of GFP mRNA and 2 µL of Lipofectamine Stem reagent in StemFlex Medium on Geltrex matrix.

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 StemFlex Medium:

Step	Tube	Complexation component	Amount per well (24-well plate)
1	Tube 1	Opti-MEM I medium	25 µL
		Lipofectamine Stem reagent	2 µL
2	Tube 2	Opti-MEM I medium	25 µL
		Cas9 nuclease	1.5 µg
		gRNA (0.1–0.5 µg/µL)	375 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	Aspirate the StemFlex Medium and add 0.5 mL of Opti-MEM I medium with RevitaCell Supplement per well, just before transfection.		
6	Add 50 µL of complex from step 4 to each well; gently swirl plate to ensure even distrbution of the complex across the entire well.		
7	Return culture dish to incubator and culture the cells at 37°C with 5% CO_2, for 4 hours. **Important:** After 4 hours of transfection, add 0.5 mL of StemFlex Medium warmed to room temperature to each well, return plate to incubator, and culture the cells at 37°C with 5% CO_2, overnight.		
8	The following day, aspirate the StemFlex Medium and transfection complexes, and add 0.5 mL of fresh StemFlex 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 Opti-MEM I Medium on vitronectin, but the number of cells seeded should be increased to 150,000 per well. Dissociate the PSCs and then prepare the transfection complex as recommended above. 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.

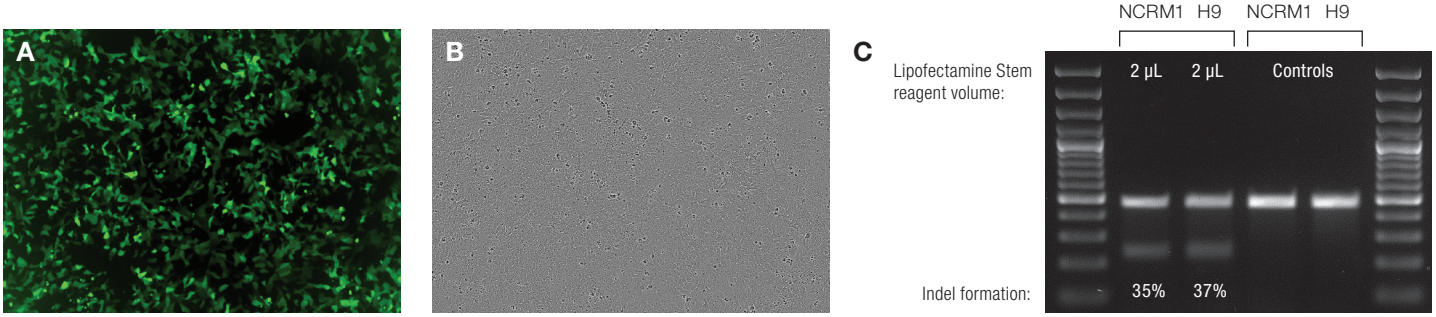

Figure 3. Posttransfection analysis of iPSCs. (A) Fluorescence image demonstrating 60% transfection efficiency, and **(B)** bright-field image. iPSCs (NCRM1) are shown 24 hours posttransfection with 1.5 µg of GeneArt Platinum Cas9 Nuclease, 375 ng of gRNA, 50 ng of GFP mRNA, and 2 µL of Lipofectamine Stem reagent in StemFlex Medium on Geltrex matrix. **(C)** Genomic cleavage detection analysis of NCRM1 iPSCs and H9 hESCs 48 hours posttransfection, demonstrating 35% and 37% indel formation, respectively, within the *HPRT* 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. These 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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