

Transfecting Stealth™ RNAi or siRNA into Mouse D3 Embryonic Stem Cells Using Lipofectamine™ 2000

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Introduction

Lipofectamine™ 2000 Reagent is a proprietary formulation that facilitates highly efficient delivery of Stealth™ RNAi or short interfering RNA (siRNA) to mammalian cells for RNAi analysis. This reference provides general guidelines and an optimized procedure to transfect Stealth™ RNAi (or siRNA) into mouse D3 embryonic stem cells (ATCC, Cat. No. CRL-1934) using Lipofectamine™ 2000 (Cat. No. 11668-027).

Note: While transfection conditions have been optimized to allow highly efficient delivery of Stealth™ RNAi into D3 cells, other factors related to the target gene of interest including the transcription rate of the target gene, the stability of the resulting protein, and efficacy of the Stealth™ RNAi sequence chosen can influence the degree of target gene knockdown observed. Take these factors into consideration when designing your RNAi experiment.

Important Guidelines for Transfection

Follow these important guidelines when transfecting Stealth™ RNA (or siRNA) into D3 cells using Lipofectamine™ 2000:

1. Use 50 nM Stealth™ RNAi (or siRNA) complexed with 2 µg/ml Lipofectamine™ 2000 (stock solution is 1 mg/ml) for transfection. To increase accuracy and reduce assay variability, we recommend performing transfection **in triplicate** for each sample condition.
2. Maintain and transfect D3 cells on inactivated mouse embryonic fibroblast (MEF) feeder layers. Prepare mouse embryonic fibroblasts using standard protocols, and inactivate cells using mitomycin C or irradiation.
3. D3 embryonic stem cells grow in large clusters, and must remain as clusters to retain pluripotency. **To increase transfection efficiency, transfect cells at the time of plating.** Perform transfection in serum-free KSR complete medium containing leukemia inhibitory factor (LIF). See below for the composition of KSR complete medium.
4. Use Opti-MEM® I Reduced Serum Medium (Cat. No. 31985-062) to dilute Lipofectamine™ 2000 and Stealth™ RNAi (or siRNA) prior to complex formation.

Materials Needed

Have the following reagents on hand before beginning:

- D3 cells maintained on inactivated MEF feeder layers in KSR complete medium (*i.e.* KnockOut™ D-MEM (Cat. No. 10829-018) supplemented with 15% KnockOut™ Serum Replacement (Cat. No. 10828-028), 100 µM MEM Non-Essential Amino Acids (Cat. No. 11140-050), 2 mM glutamine (Cat. No. 25030-149), 10⁻⁴ M 2-mercaptoethanol (Cat. No. 21985-023), 50 U/ml penicillin, 50 µg/ml streptomycin (Cat. No. 15070-063), and 1000 U/ml LIF (*e.g.* ESGRO, Chemicon, Cat. No. ESG1106).
Note 1: Before adding D3 cells, maintain inactivated MEF cells in MEF growth medium (*i.e.* KnockOut™ D-MEM supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10% heat-inactivated, ES cell-qualified, fetal bovine serum (Cat. No. 16141-061).
Note 2: Use low-passage DE3 cells; make sure that cells are healthy and greater than 90% viable before transfection.
- Stealth™ RNAi (or siRNA) of interest (20 µM in annealing buffer)
- Lipofectamine™ 2000 Reagent (store at +4°C until use)
- Opti-MEM® I Reduced Serum Medium (pre-warm to 37°C before use)
- Appropriate tissue culture plates and supplies

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Transfection Procedure

Use this procedure to transfect Stealth™ RNAi (or siRNA) into D3 cells (plated on inactivated MEF feeder layers) using Lipofectamine™ 2000 in a **12-well format**. For other formats, see the table in **Recommended Reagent Amounts and Volumes** for the appropriate reagent amounts to add. **Tip:** To reduce well-to-well variability when transfecting multiple replicates (*e.g.* triplicates), proportionally scale up the reagent volumes to form complexes (Step 2), then aliquot an equal volume of complexes into each well.

- One day before transfection, plate 1.75×10^5 inactivated MEF cells in 1 ml of MEF growth medium per well.
- For each transfection sample**, prepare Stealth™ RNAi-Lipofectamine™ 2000 complexes as follows:
 - Dilute 50 pmole of Stealth™ RNAi (*i.e.* 2.5 μ l of 20 μ M Stealth™ RNAi) in 100 μ l of Opti-MEM® I Reduced Serum Medium. Mix gently.
 - Mix Lipofectamine™ 2000 gently before use, then dilute 2 μ l in 100 μ l of Opti-MEM® I Reduced Serum Medium. Mix gently and incubate for 15 minutes at room temperature.
 - After the 15-minute incubation, combine the diluted Stealth™ RNAi and the diluted Lipofectamine™ 2000 (total volume ~ 205 μ l). Mix gently and incubate for 15 minutes at room temperature to form complexes (solution may appear cloudy).
- During the 15-minute incubation (Step 2c), prepare D3 cells for plating. Select a flask of low passage, healthy D3 cells maintained on an inactivated MEF feeder layer. Dissociate cells by trypsinization, prepare a single cell suspension, and determine cell counts. Remove the plated inactivated MEF feeder cells from the CO₂ incubator (Step 1) and remove the MEF growth medium from the cells. Carefully rinse the cell monolayer once with KSR complete medium to remove residual serum. Seed D3 cells on the inactivated MEF feeder layer to a final density of 2×10^5 cells per well in 1 ml of KSR complete medium.
- Add the ~205 μ l of Stealth™ RNAi-Lipofectamine™ 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
- Incubate the cells at 37°C in a humidified CO₂ incubator for 16-24 hours or as appropriate until you are ready to assay for gene knockdown.

Recommended Reagent Amounts and Volumes

To transfect D3 cells in different tissue culture formats, vary the amounts of Stealth™ RNAi (or siRNA), Lipofectamine™ 2000, cells, and medium used in proportion to the relative surface area, as shown in the table. Remember to use inactivated MEF cells. **Note:** 20 μ M Stealth™ RNAi or siRNA = 20 pmole/ μ l.

Culture vessel	Relative surface area (vs. 24-well)	MEF cells plated per well	D3 cells plated per well	Volume of plating medium	Stealth™ RNAi (pmole) in media volume (μ l)	Lipofectamine™ 2000 (μ l) in media volume (μ l)
24-well	0.5	8.75×10^4	1×10^5	400 μ l	25 pmole in 50 μ l	1 μ l in 50 μ l
12-well	1	1.75×10^5	2×10^5	1 ml	50 pmole in 100 μ l	2 μ l in 100 μ l
6-well	5	4.4×10^5	5×10^5	2 ml	125 pmole in 250 μ l	5 μ l in 250 μ l

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