Protocol

RNAi in Mammalian Cells by siRNA Duplex Transfection

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Long dsRNA cannot be used in most cultured mammalian cells because it triggers the interferon response, causing widespread changes in gene expression and apoptosis. This protocol describes a method for delivering into mammalian cells siRNA duplexes that are too short to elicit the sequence-nonspecific responses associated with long dsRNA.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

DharmaFECT 4 Transfection Reagent (Dharmacon, catalog no. T-2004)
Dulbecco's modified Eagle's medium (DMEM)
Dulbecco's phosphate-buffered saline (PBS), lacking calcium and magnesium
Heat-inactivated fetal bovine serum (FBS)
Mammalian cell lines (e.g., HeLa and NTera2)
Penicillin and streptomycin
siRNA duplexes (10 μM) (see Protocol: **Preparation of siRNA Duplexes** [Li and Zamore 2019])
Trypsin–EDTA solution

Equipment

Centrifuges
Conical tubes (50 mL)
Equipment for immunofluorescence and western blotting, quantitative RT-PCR, or northern hybridization (see Step 10)
Laminar flow hood (Class II)
Microcentrifuge tubes (1.5 mL)
Stereomicroscope
Tissue culture dishes (10 cm)
Tissue culture incubator (37°C, 5% CO_2), humidified
Tissue culture plates (24 well)

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METHOD

This protocol is designed for cells grown in 24-well plates. If other multiwell plates, flasks, or dishes of a different diameter are used, scale the cell density and reagent volumes according to the surface area of the well (see Table 1).

Preparation of Cells for Transfection

1. Grow cells in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO_2 humidified incubator to a density of ~90% confluence.
2. Treat cells with trypsin–EDTA to release them from the plastic substrate, and then resuspend the cells in antibiotic-free DMEM supplemented with 10% FBS. Seed 500 µL of cell suspension into each well of a 24-well plate.
3. Incubate the cultured cells overnight at 37°C in a 5% CO_2 humidified incubator so that they will reach 30%–40% confluence (~5×10^4 cells) at the time of transfection.

   The optimal cell density will vary according to the growth characteristics of the cell line. Consult the transfection reagent manufacturer's manuals for cell-line-specific recommendations, or determine the optimal cell density experimentally.

Preparation of Transfection Medium

All volumes are shown for a single-well experiment. However, performing at least triplicate experiments is recommended. For multiple-well transfections, add 10% extra mix to compensate for losses during pipetting.

4. Add 1 µL (10 pmol) of siRNA duplexes to 24 µL of antibiotic- and serum-free DMEM in a fresh sterile microcentrifuge tube, and mix by pipetting gently up and down.
5. Add 1 µL of DharmaFECT 4 with 24 µL of antibiotic- and serum-free DMEM in another fresh sterile microcentrifuge tube, and mix by pipetting gently up and down.

   High transfection efficiency is a prerequisite for a potent gene silencing. In this protocol, we use Thermo Scientific DharmaFECT 4, which was developed for siRNA transfection and delivers siRNAs effectively to a variety of mammalian cell cultures with low toxicity (http://www.dharmacon.com/product/productlandingtemplate.aspx?id=239&tab=1).
6. Incubate both mixtures for 5 min at room temperature. Slowly add the siRNA mix to the DharmaFECT 4 mix. Mix by pipetting gently up and down, and incubate for 20 min at room temperature to allow for formation of siRNA–lipid complexes.

Transfection and Analysis of Knockdown

7. Replace the growth medium for the cells (Step 3) with 450 µL of antibiotic-free DMEM supplemented with 10% FBS that has been warmed to 37°C.
8. Add 50 µL of the siRNA–lipid transfection medium (from Step 6) into each well of the cultured cells. Try to distribute the drops evenly over the well, and mix by gently agitating after the transfection medium is added.

TABLE 1. Volumes of cells, DharmaFECT 4, and siRNAs (or ASOs) used for transfection of cultured mammalian cells

Culture plate or dish	24 well	12 well	6 well	6 cm	10 cm
Surface area per well (cm^2)	2	4	10	20	60
DMEM + 10% FBS (mL)	0.45	0.9	1.8	4.5	9
Tube A					
DMEM (µL)	24	48	96	240	480
10 µM siRNA duplexes or 12.5 µM ASOs (µL)	1	2	4	10	20
Tube B					
DMEM (µL)	24	48	96	240	480
DharmaFECT 4 (µL)	1	2	4	10	20
Total growth medium (mL)	0.5	1	2	5	10

9. Incubate the cultured cells for 1–4 d at 37°C in a 5% CO_2 humidified incubator.

   If cell toxicity is observed after 1 d, replace the transfection medium with fresh antibiotic-free DMEM supplemented with 10% FBS and continue the incubation.

10. Analyze knockdown effects by assessing the reduction of target protein level by immunofluorescence and western blotting using antibody that specifically recognizes the target protein and measuring the reduction of the target mRNA level by quantitative reverse transcription-polymerase chain reaction (RT-PCR) or northern hybridization.

    Mostly, a reduction in target RNA is accompanied by a decrease of target protein level. Some protein might have a long half-life, which will extend the time course of protein knockdown.

    See Troubleshooting.

TROUBLESHOOTING

Problem (Step 10): There is no gene knockdown.

Solution: This might result from low transfection efficiency or siRNA degradation. The following solutions can be used.

- Repeat the transfection with a fluorescent siRNA duplex.
- Try other transfection reagents with a high transfection efficiency of siRNAs in the cell line under investigation. In case a particular cell line shows low transfection using DharmaFECT 4, try other transfection reagents such as DharmaFECT 1, 2, and 3 (Dharmacon, catalog nos T-2001, T-2002, and T-2003) or Lipofectamine RNAiMAX and Lipofectamine 2000 (Life Technologies, catalog nos 13778-075 and 11668-019), and TransIT-TKO siRNA Transfection Reagent (Mirus, catalog no. MIR 2150).
- Check siRNA duplexes on a nondenaturing polyacrylamide gel.
- In the case of degradation, prepare siRNA duplexes again and repeat the entire experiment.

Problem (Step 10): siRNAs are less potent than predicted.

Solution: The concentration of the siRNA duplex used in this protocol is 20 nM. Optimize the concentration of the siRNA duplex from 1 to 100 nM. Different siRNAs targeting the same gene generally vary in their silencing efficiency. Perform experiments with several other siRNA duplexes targeting different regions of the given mRNA.

REFERENCES

Li C, Zamore PD. 2019. Preparation of siRNA duplexes. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot097444.

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