Cold Spring Harbor Protocols

Inducing RNAi in *Drosophila* Cells by Transfection with dsRNA

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Protocol

Inducing RNAi in *Drosophila* Cells by Transfection with dsRNA

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In *Drosophila* cells, RNA interference (RNAi) can be triggered by synthetic long double-stranded RNAs (dsRNAs). For many *Drosophila* cell lines and cell types, passive dsRNA uptake is inefficient. More complete silencing responses can often be obtained in *Drosophila* S2 cells using transfection, perhaps because higher levels of intracellular dsRNA are achieved. In this protocol, S2 cells are transfected with dsRNA using QIAGEN's Effectene reagent, which has proven to be reliable for many investigators. A plasmid DNA can also be included in the transfection mix to provide additional functionality. The plasmid DNA can encode, for example, a reporter of the activity of a pathway or specific transcription factor, or a marker that allows visualization of some cellular behavior or structure. It is also useful to include a plasmid that encodes a fluorescent protein simply to monitor transfection efficiency.

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.

RECIPES: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Complete S2 medium <R>

DNA as a transfection or functional marker (optional; see Step 3)

Drosophila S2 cells grown on plates or maintained in suspension culture

Cells that are healthy and growing exponentially give the best results. It may be necessary to test different lots of fetal bovine serum to find one that works well with S2 or other fly cells. Doubling times of 18–24 h are normal for most lines. Note especially that many S2 lines are persistently virus infected (e.g., with flock house virus [FHV]). Care must be taken to obtain and work with noninfected cells.

dsRNA at 0.5–10 µg/µL in nuclease-free H_2O or TE buffer (10 mM Tris [pH 8]/1 mM EDTA)

dsRNAs derived from different portions of target genes differ in their silencing efficiency. Therefore, it is generally a good idea to test several different dsRNAs in parallel for each gene to be silenced. See Discussion.

Transfection reagent

Effectene Transfection Reagent (QIAGEN) is used in this protocol. It is recommended that many different transfection reagents and protocols be tried in an effort to optimize knockdown efficiency, particularly when working with a new cell line. It would not be uncommon to scan a dozen different commercial reagents, including charged and neutral lipids, nanoparticles, and several different

Adapted from *RNA: A Laboratory Manual*, by Donald C. Rio, Manuel Ares Jr, Gregory J. Hannon, and Timothy W. Nilsen. CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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electroporation protocols (e.g., standard electroporation and Amaxa nucleofection) when developing a means to efficiently introduce dsRNA into a previously uncharacterized cell type.

Trypsin/EDTA in PBS <R>

Equipment

Centrifuge (low speed)

Culture incubator preset to 25°C

Hemocytometer for cell counting

Microcentrifuge tubes

Microscope

Tissue culture plates (six-well)

This protocol presents a method for six-well plates, but it can be scaled as needed based on the ratio of the surface area of the desired vessel to that of a well of a six-well plate (see Table 1).

Vortex mixer

METHOD

1. Collect S2 cells from adherent culture using trypsin/EDTA in PBS or harvest from a suspension culture and pellet cells by centrifuging at 800–1000*g* for 5 min at room temperature.

   S2 cells adhere weakly to plates. They can often be removed without the use of trypsin by incubation with PBS containing 1 mM EDTA and 1 mM EGTA.

2. Use a hemocytometer to resuspend and count the cells. Dilute cells in fresh complete S2 medium (typically to a density of 1×10^6 cells/mL). Dispense 2 mL of diluted cells into each well of a six-well plate.

   At this point, cells are generally left from several hours to overnight to adhere to the plate. If this is the case, exchange the medium before transfection. Cells can also be used immediately with some (e.g., Effectene) but not all (e.g., calcium phosphate coprecipitation) transfection methods.

3. Prepare the nucleic acid mix in a microcentrifuge tube according to the manufacturer's instructions. For each plate well, the mix consists of optional DNA (as a transfection or functional marker), dsRNA (total nucleic acid comprising ~2 µg), and EC buffer, a component of the Effectene Kit. Mix the total volume (should be ~200 µL) by inversion or gentle vortexing.

   It is typical to cotransfect a plasmid encoding green fluorescent protein (GFP) with and without a GFP dsRNA. It must be recognized, however, that it is often easier to reduce the levels of a cotransfected marker than an endogenous gene. Many cell lines are sensitive to the knockdown of thread, *an endogenous inhibitor of apoptosis orthologous to mammalian inhibitor of apoptosis (IAP), and die in the presence of a* thread *dsRNA. This is also an effective control for endogenous knockdown in many situations.*

4. Add Enhancer from the Effectine Kit to the mix so that there is 16 µL of Enhancer per well. Mix well by inversion and then incubate for 5 min at room temperature.

5. Prepare the transfection solution by adding Effectene to the mix so that there is 8 mL of Effectene per well. Mix by pipetting up and down 5–10 times or vortexing on high for 10 sec. Allow the solution to stand for 10 min at room temperature.

6. Dispense the mix dropwise into individual wells of six-well plates containing the cells from Step 2.

TABLE 1. Scaling up or down for different plate formats

Culture vessel	96-well	48-well	24-well	12-well	6-well	35 mm	60 mm	100 mm	150 mm	T25	T75
Surface area (cm^2)	0.3	0.7	2	4	10	10	20	60	140	25	75

Multiply all volumes and cell numbers by the surface area of the desired plate divided by the surface area of the six-well plate

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7. Incubate the plates in a humidified chamber in a tissue culture incubator for 2–6 d at 25°C.
8. Measure knockdown by quantitative polymerase chain reaction (PCR) or western blotting and determine the phenotypic consequences of knockdown as desired.

   It is important wherever possible to measure knockdown by quantifying changes in protein levels. Although it is easier to measure RNA knockdown, RNA and protein are not always equally affected nor does their decline follow the same time course.

DISCUSSION

In some circumstances (e.g., when close paralogs must be distinguished or if rescue experiments are planned), it is desirable to use a single synthetic small interfering (siRNA) rather than a long dsRNA. In these cases, use a transfection protocol similar to the one outlined above, but substitute a synthetic siRNA for the dsRNA. Choosing an siRNA sequence is a complex topic and is detailed elsewhere (see **RNAi in Cultured Mammalian Cells Using Synthetic siRNAs** [Chang et al. 2012]). Presently, there is no reason to suspect that the characteristics of effective siRNAs differ in vertebrates and invertebrates (although new evidence may emerge), and the same design procedures should be followed in both cases.

RECIPES

Complete S2 Medium

1× antibiotic-antimycotic solution (Invitrogen 15240-062)
10% fetal bovine serum

Prepare in Schneider's complete medium (Invitrogen 11720-034).

Trypsin/EDTA in PBS

0.25% trypsin
1 mM EDTA

Prepare in phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+}.

REFERENCE

Chang K, Marran K, Valentine A, Hannon GJ. 2012. RNAi in cultured mammalian cells using synthetic siRNAs. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot071076.