DSRNAI EXPERIMENTS IN DROSOPHILA S2 CELL CULTURE

1. Date: 9.23.99, Contact: Carolyn Worby at cdworby@umich.edu

2. FAQ:Frequently Asked Questions

I. PREPARATION OF THE TEMPLATE DNA

1. Design two oligos for your gene of interest.

Each should incorporate a 5' T7 RNA polymerase binding site, resulting in a PCR product of approximately 700 bp.

2. T7 RNA polymerase binding site:

TTA ATA CGA CTC ACT ATA GGG AGA

3. Purify the PCR DNA template.

It should be free of RNases and inhibitors such as high salt, detergents or EDTA.For example, we use the High Pure PCR Product Purification kit from Roche/Boehringer Mannheim (cat. #1732-668).

4. Quantify the PCR product on an agarose gel or OD_{260nm} to a concentration of 125 ng/μl or greater.

The dsRNA reaction detailed below requires 1 μg of template in a volume of 8 μl or less.

II. PREPARATION OF THE DSRNA

1. We use the Ambion MEGAscript T7 kit, cat. # 1334. We modify the supplied MEGAscript protocol as follows:

Prepare the template as described above.Thaw reaction components, except Enzyme mix, at RT. Do not put on ice. Precipitates may form if reagents are chilled.Add the following reagents from the kit in the order shown into a 1.5 microfuge tube at RT for a 1X reaction, (which can be scaled up according to your needs):X μl Nuclease free water to a final volume of 20 μl 2 μl 10X Reaction Buffer 2 μl each, ATP, CTP, GTP, UTP mixes X μl linearized DNA template (1 μg total) 2 μl Enzyme mixMix tube gently by hand, spin contents down and incubate at 37° C for 2 to 6 hours.Ethanol precipitate your reactions.Spin and remove EtOH, air dry 15 minutes. For a 1X reaction, resuspend the pellet in 40 μl or less of RNase-free H2O. You will want the dsRNA at a final concentration of 3ug/ul.Heat your reaction tubes at 65° C for 30 minutes. Place tubes in a beaker of 65° C H_2O on the benchtop and slowly cool to room temperature to anneal the RNA.Adjust the final concentration of your dsRNA to 3 μg/ul, using this calculation:OD_{260nm} 1 = 45ug/ml.Run 1-2 μg of your dsRNA on a 1% agarose gel to check the integrity and size of the dsRNA. A 1X reaction typically yields 150 μg to 200 μg of dsRNAStore dsRNA at -20° C. dsRNA appears to be stable for a minimum of 4 months at -20° C, with no loss of efficacy.

III. PROCEED WITH DSRNAI EXPERIMENT

1. Day 1

Plate Drosophila S2 cells into 6-well, 35 mm dishes using Serum-free Expression medium at a concentration of:1 x 10^6 cells/1 ml/wellImmediately add 15 μg dsRNA (5 μl of 3 μg/ul stock)Swirl plates well to mix RNA and cellsIncubate at RT for 30 - 60 minutesAdd 2 mls media + serum and return plates to RT incubator

2. Days 2 -4

Recovery and growth period

3. Day 4

Harvest the cells by scraping to dislodge the cells from the dish and transfer directly into eppendorf tubes.Spin 1000 rpm, 5 minutes and discard the supernatant. Lyse the cells in RIPA buffer, pipet up/down.Spin at 15K for 20 minute at 4°C and save supernatant to a new tube.Check the level of your protein of interest from the extracts prepared above, which can also be immunoprecipitated by standard Western Blot protocol.

RIPA BUFFER

1.

Stock solution	Need	Final Concentration
5M NaCl	15 ml	150mM NaCl
100% NP.40	5 ml	1% NP.40
Deoxycholate	2.5 g	0.5% Deoxycholate
10% SDS	5 ml	0.1% SDS
2M Tris pH 8.0	12.5 m	50mM Tris
200mM NaVanadate	500 μl	0.2mM NaVO
1M NaF	5 ml	10mM Naf
500mM EDTA pH 8.0	400 μl	0.4mM EDTA
100% Glycerol	50 ml	10% Glycerol
dd H_2O	Total Volume to 500 ml	

Sterile filter and store at 4°C

DROSOPHILA S2 CELL CULTURE REAGENTS

1. Growth medium:

Schneider's Drosophila medium, Gibco/BRL, cat. # 11720-034 10% Fetal Bovine Serum Pen/Strep

2. Grow S2 cells at room temperature, no CO_2 requirement, on dishes, in flasks, or in spinner culture.

S2 cells grow both as cells atttached to the tissue culture dish and as free floating cells in the media.

3. Serum-free medium for dsRNAi experiments:

DES Serum-free Expression medium, Invitrogen, cat.# Q510-01 2mM Glutamine

FAQ:Frequently Asked Questions

Reference: Worby CA, Simonson-Leff N, Dixon JE. (2001) RNA interference of gene expression (RNAi) in cultured Drosophila cells. **Sci STKE (95):PL1**

Downloadable PDF file of the Sci STKE (95):PL1