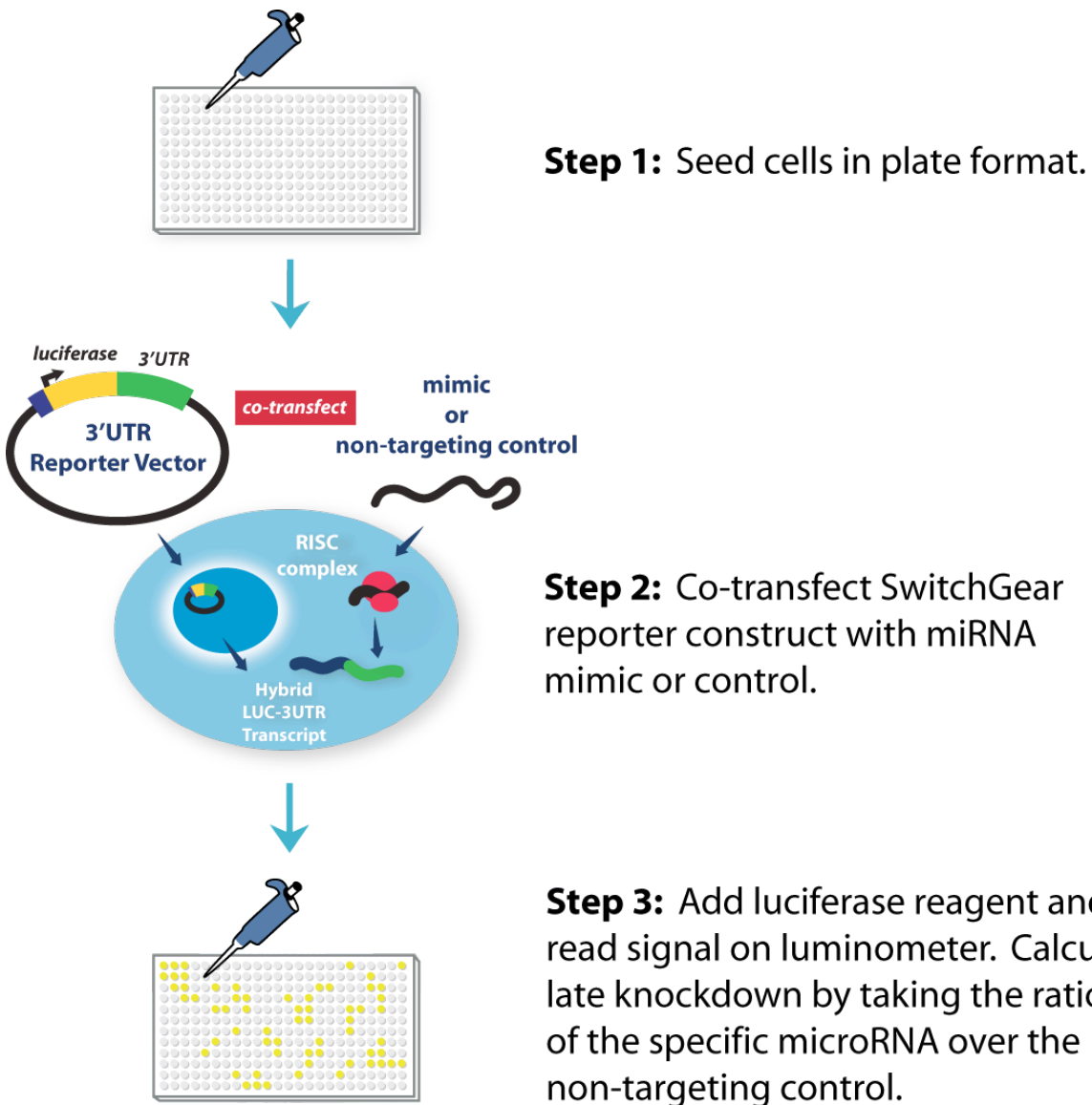


PROTOCOL FOR UTR CONSTRUCTS WITH CO-TRANSFECTION OF miRNA MIMIC OR INHIBITOR FOR ADHERENT CELLS

Important notes on experimental design:

- Co-transfection of any oligos/plasmids with SwitchGear reporter constructs reduces overall luciferase signals in a non-specific manner. Therefore, it is important to perform the following controls: reporter only, reporter + non-targeting control miRNA, and reporter + specific miRNA.
- Our empty vector (no UTR) provides a very high level of luciferase signal and serves as a positive control. In addition, we recommend using the SwitchGear housekeeping gene vectors and random control vectors containing random genomic fragments for accurately separating sequence-specific vs. non-specific effects.

SwitchGear 3'UTR reporter assay workflow



DAY 1

Goal: Seed cells to yield ≥80% confluence at time of transfection.

- Seed cells in a 96-well white assay plate in 100 µl total volume.
- In parallel, seed the appropriate number of cells in a clear 96-well plate for assessing confluence.

DAY 2

Goal: Prepare reporter constructs, miRNAs, and transfect into the confluent cells

Prepare constructs

- Thaw Switchgear constructs (plasmid DNA) at room temperature. Mix well.
- Centrifuge to remove condensation.

Prepare microRNAs

- Thaw microRNAs (specific and non-targeting controls) at room temperature and centrifuge.
- Dilute to a working concentration of 2 µM in RNase-free water.
- Always include an experimental miRNA mimic and a non-targeting miRNA control.

Perform transfections

1. For each transfection combine the following reagents:

Mixture 1

Individual reporter (30ng/µL)	3.33 µL
microRNA	Varies*
Serum-free media	to 10 µL

Perform at least three replicate transfections for each treatment/UTR combination.

- Include some additional volume to allow for pipetting error.
- For each reporter, set up replicates for the following: reporter only, reporter + non-targeting control miRNA and reporter + specific miRNA.
 - Set up transfections for the targeting microRNA at the same time as those for a non-targeting control.

*Effective concentration of miRNA can vary from 10 nM-100 nM. Consult miRNA manufacturer for appropriate concentration.

2. Make up the DharmaFECT DUO (Dharmacon) mixture as follows for each transfection:

Mixture 2

DharmaFECT Duo	0.15 µL
Serum free media	9.85 µL

- The amount of DharmaFECT Duo to be added may be cell line dependent
 - The amounts indicated above are appropriate for HT-1080 cells.
 - Consult the manufacturer’s documentation to determine the appropriate amount for your cell line.

Tip: Make a large transfection mixture by multiplying the volumes above by the number of reporters, the number of replicates and the number of miRNAs to be tested. When calculating the amount of transfection mix to prepare, be sure to include a small amount

extra to account for pipetting errors and evaporation. See Experiment Design Example in the Appendix.

3. Allow the DharmaFECT Duo mixture to incubate at room temperature for 5 minutes.
4. After 5 minutes, add 10 μL of the DharmaFECT Duo mixture (Mixture 2) to each prepared tube of plasmid and/or miRNA (Mixture 1).
 - Each tube should have a volume of 20 μL (per replicate).
5. Incubate each mixture for 20 minutes at room temperature.
6. After 20 minutes, add 80 μL of pre-warmed (37°C), antibiotic-free, complete media to each tube
 - A deep-well block can be used for preparation of many replicates and samples simultaneously.
 - Mix the solution by pipetting up and down.
7. Remove the seeded plate from the incubator
 - Verify earlier that cells are at least 80% confluent.
8. Carefully pipet off the media from each well.
9. Add 100 μL of the transfection mixture to each well.
10. Place plate in incubator overnight.

DAY 3

Goal: Measure luciferase activity

1. Remove plate from incubator 24 hours after transfection completed.
2. Add 100 μL Steady-Glo Luciferase Assay Reagent (Promega) to each well and cover with lid or with foil tape, and incubate for 15 to 30 minutes in a dark area.
3. Read on luminometer.
4. Calculate the knockdown by calculating luciferase signal ratio for each construct for specific miRNA over the non-targeting control (luminescence = specific miRNA/non-targeting control). Use the data from housekeeping, random, and empty constructs to control for non-UTR specific treatment effects.

APPENDIX

Experiment Design Example

1. For 3.5 replicates of a single reporter with and without 100nM of miRNA use the following amounts:

	Plasmid Only	Plasmid + Non-targeting miRNA	Plasmid + miRNA
Individual reporter (30ng/ μ L)	11.67 μ L	11.67 μ L	11.67 μ L
2 μ M miRNA stock	0.00 μ L	17.50 μ L	17.50 μ L
Serum free media	23.33 μ L	5.83 μ L	5.83 μ L
Total Volume	35.00 μ L	35.00 μ L	35.00 μ L

2. For 3.5 replicates with the DharmaFECT Duo mixture (please also include additional volume for pipetting error):

DharmaFECT Duo	1.58 μ L
Serum free media	103.43 μ L
Total Volume	105.00 μ L

3. Incubate the DharmaFECT Duo mixture for 5 minutes at room temperature.

4. Add 35 μ L of the DharmaFECT Duo mixture to each tube for a total volume of 70 μ L.

5. Incubate for 20 minutes.

6. Add 280 μ L of pre-warmed (37°C), antibiotic-free, complete media to each tube.

7. Remove seeded plate from incubator.

8. Carefully pipet off media from each well.

9. Add 100 μ L of the transfection mixture to each well.

10. Return plate to incubator.

Example catalog numbers

Item	Vendor	Catalog Number
White Tissue Culture Plates (96-well solid bottom)	VWR	82050-736
Clear Tissue Culture Plates (96-well) with lid	VWR	353072
Steady-Glo Luciferase Assay Reagent	Promega	E2510, E2520
DharmaFECT® Duo Transfection Reagent (0.75mL)	Dharmacon	T-2010-02
Foil Plate Sealing Tape	E&K Scientific	T592100
Breathable Plate Sealing Tape	E&K Scientific	T896100-S
Plate Luminometer	Molecular Devices	LMaxII-384