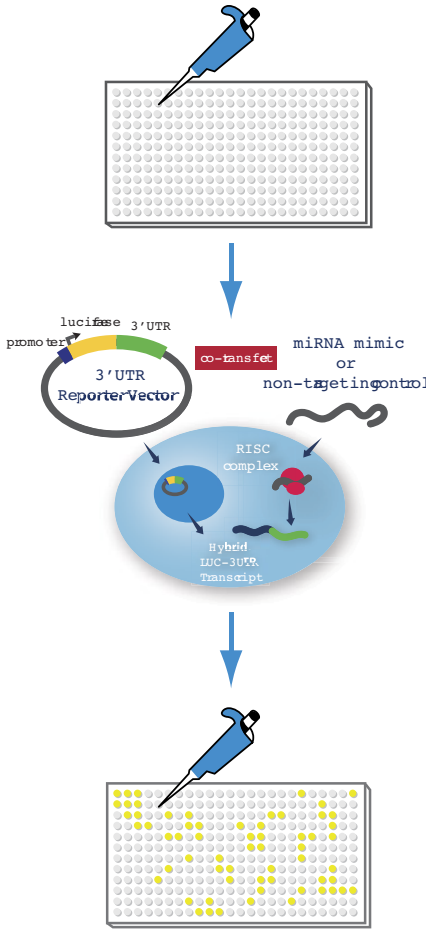
LiGHTSW!TCH
Luciferase Assay System

Protocol

Co-transfection of 3'UTR GoClones with LightSwitch miRNA Mimics or Inhibitors

LightSwitch Luciferase Assay System Workflow:
3'UTR Reporter GoClones



Step 1: Seed cells in plate format.

Step 2: Co-transfect SwitchGear GoClone reporter construct with LightSwitch miRNA mimic, inhibitor, or non-targeting control.

Step 3: Add LightSwitch Luciferase Assay Reagent and read signal on luminometer. Calculate knockdown by taking the ratio of the signal of the specific microRNA mimic over the non-targeting control.

All SwitchGear reporter constructs utilize RenSP, a destabilized form of a novel luciferase developed by SwitchGear Genomics for its reporter gene assays. The optimized luciferase protein has a half-life of ~1 hour enabling a detailed analysis of kinetic responses with a highly robust signal.

Important notes on experimental design:

- The LightSwitch Luciferase Assay System is a fully optimized reporter system. Use all components of the LightSwitch System as recommended to obtain optimal reporter assay results: 3'UTR GoClone reporters, LightSwitch miRNA Mimics or Inhibitors, and LightSwitch Assay Reagents.
- Co-transfection of any oligos/plasmids with SwitchGear GoClone 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 GoClone housekeeping controls and random controls for accurately separating sequence-specific vs. non-specific effects.
- This protocol uses DharmaFect Duo transfection reagent (Dharmacon).

DAY 1

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

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

DAY 2

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

Prepare constructs

1. Thaw GoClone constructs (plasmid DNA) at room temperature. Mix well.
2. Centrifuge to remove condensation from the cap.

Prepare LightSwitch microRNAs

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

Perform transfections

5. For each transfection combine the following reagents:

Mixture 1

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

* Effective concentration of miRNA can vary from 10 nM-100 nM. We recommend starting with a 50 nM concentration with LightSwitch miRNAs.

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.

6. 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 HT1080 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.

7. Allow the DharmaFECT Duo mixture to incubate at room temperature for 5 minutes.

8. After 5 minutes, add 10µL of the DharmaFECT Duo mixture (Mixture 2) to each prepared tube of plasmid and/or miRNA (Mixture 1).
   - Each tube should contain a volume of 20µL per replicate transfection.

9. Incubate each mixture for 20 minutes at room temperature.

10. After 20 minutes, add 80 µL of pre-warmed (37°C), antibiotic-free, complete media per replicate to each tube for a total of 100µL per replicate transfection.
    - A deep-well block may be used for preparation of many replicates and samples simultaneously.
    - Mix the solution by pipetting up and down.

11. Remove the seeded plate from the incubator.
    - Verify that cells are at least 80% confluent.

12. Carefully pipet off the media from each well.

13. Add 100µL of the transfection mixture to each well.

14. Place plate in incubator overnight.

DAY 3

Goal: Measure luciferase activity with LightSwitch Assay Reagents

Note: Luciferase assays may be conducted immediately or the plates may be frozen at -80˚C (freezing generally increases cell lysis and luciferase signal). If using frozen plates, thaw and bring to room temperature before assaying.

1. Remove plate from incubator and bring to room temperature.
2. Prepare LightSwitch Assay Reagents (for LS010 kit, protocols of other kit sizes may be found online):
   A. Reconstitute 100X Substrate by adding 100µL of Substrate Solvent to tube of lyophilized Assay Substrate. *Protect from light and minimize time at room temperature. 100X Substrate may be stored at -20C for 2-3 weeks. For best results, use freshly reconstituted substrate.*
   B. Prepare Assay Solution by thawing 10mL bottle of Assay Buffer in room temperature water bath and add 100µL of reconstituted 100X Substrate prior to use. *For best results, avoid additional freeze-thaw cycles. To thaw re-frozen buffer, incubate in a 37C water bath and mix well to ensure that all components go back into solution.*
3. Use a multi-channel pipettor to add 100µL LightSwitch Assay Solution (buffer+substrate) directly to each sample well in a white 96-well plate.
   If cells were grown in another plate or flask format, transfer samples to a white 96-well plate in 100µL total volume (media or PBS).
4. Cover plate, protect from light, and incubate for 30 minutes at room temperature.
   If assaying more than one plate, stagger addition of assay solution so that each plate incubates for 30 minutes before reading.
5. Read each well for 2 seconds in a plate luminometer (SpectraMax L or equivalent).
6. 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

Experimental Design Example

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

Mixture #1	Plasmid Only	Plasmid + Non-targeting miRNA	Plasmid + miRNA
Individual GoClone reporter (30ng/µL)	11.67 µL	11.67 µL	11.67 µL
2 µM miRNA stock	0.00 µL	17.50 µL	17.50 µL
Serum free media	23.33 µL	5.83 µL	5.83 µL
Total Volume	35.00 µL	35.00 µL	35.00 µL

2. Create Mix #2 (includes extra for pipetting error) that will be added to the 3 versions of Mix #1:

DharmaFECT Duo	1.84 µL
Serum free media	120.66 µL
Total Volume	122.5 µL

3. Incubate Mixture #2 for 5 minutes at room temperature.
4. Add 35 µL of Mixture #2 to each version of Mixture #1 for a total volume of 70µL.
5. Incubate for 20 minutes.
6. Add 280 µL 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 100µL of the transfection mixture to each well.
10. Return plate to incubator.

Related Products	Vendor	Catalog Number
White Tissue Culture Plates (96-well solid bottom)	VWR	82050-736
Clear Tissue Culture Plates (96-well) with lid	VWR	353072
LightSwitch miRNA Mimics	SwitchGear	MIM****
LightSwitch miRNA Inhibitors	SwitchGear	INH****
LightSwitch Luciferase Assay Reagent	SwitchGear	LS010
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	SpectraMax L