Test MicroRNA Target with *in vitro* Cell Culture Luciferase Assay

Nathalie Coré*

UMR 7288, IBDM, Aix-Marseille University, CNRS, Marseille, France

*For correspondence: nathalie.core@univ-amu.fr

[Abstract] MicroRNAs (miRNAs) are small non-coding RNAs of 21-24 nucleotides in length that modulate gene expression by targeting the untranslated region (UTR) of mRNA. This protocol is to be used to test the binding and activity of miRNA on putative UTR target sequences. It is based on the expression of Luciferase as a reporter gene fused to the UTR sequence in the presence of plasmids containing pre-miRNA of interest or synthetic miRNA to test in an *in vitro* cell culture assay.

Materials and Reagents

1. HEK293T cells (ATCC, catalog number: CRL-11268™)
2. DMEM (high glucose) (GutaMAX™) (Life technologies, catalog number: 61965)
3. Fetal bovine serum (FBS) heat inactivated (Sigma-Aldrich, catalog number: F9665)
4. Penicillin-Streptomycin (Life technologies, catalog number: 15140)
5. pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation, catalog number: E1330)
6. miScript miRNA Mimic (QIAGEN, catalog number varies depending on your interest miRNA)
7. Lipofectamine® 2000 Transfection Reagent (Life Technologies, catalog number: 11668)
8. Dual-Glo Luciferaseassay system (Promega Corporation, catalog number: E2920)
9. Culture medium (see Recipes)

Equipment

1. Luminometer (Berthold Technologies)
2. White 96-well plates (Greiner, catalog number: 655074)

Procedure

1. Clone the putative UTR sequence to test into the pmirGLO Dual-Luciferase miRNA Target Expression Vector.
2. Plate 1 x 10^4 cells per well in 50 µl culture medium from a sub-confluent HEK293T cell suspension (cell density < 1.3 x 10^5 cells/cm^2). Plate 3 wells for each condition to produce triplicates (conditions should include negative controls for miRNA and UTR sequence and positive control when existing such as known miRNA for the target or known target for the miRNA).
3. Transfect cells after 5-16 h in culture. The following is transfection condition for 1 well:
   a. Dilute 300 ng of reporter plasmid (an example of vector is pmirGLO Dual-Luciferase miRNA Target Expression Vector) in combination with miRNA Mimic (such as miScript miRNA Mimic) at a final concentration of 5 to 50 nM in 25 µl of pure DMEM.
   b. Dilute 0.5 µl Lipofectamine in 25 µl of pure DMEM.
   c. Incubate both diluted solutions 5 min at room temperature.
   d. Combine diluted nucleic acids with diluted Lipofectamine (50 µl final), mix gently and incubate complexes 20 min at room temperature.
   e. Add the transfection complexes (50 µl) to the well containing plated cells in 50 µl medium.

   Note: Usually cells are plated in the morning for a few hours and transfected in the afternoon. 20 nM is a usual working concentration for miRNA. Instead of mimic, plasmid encoding pre-miRNA could also be used (300 ng/well).
4. Incubate transfected cells at 37 °C in a 5% CO_2 incubator for 48 h without medium change.
5. Measure luciferase using the Dual-Glo Luciferase assay system as recommended by the manufacturer:
   a. Prepare the stop solution, according to the number of wells, by diluting Dual-Glo® Stop &Glo® Substrate (1:100) in Dual-Glo® Stop &Glo® Buffer.
   b. Due to evaporation of the medium with time, the final volume per well is less than 100 µl after 48 h in culture. Measure the volume of the medium left in one well by pipetting and discard the appropriate amount of medium to reduce the volume to 50 µl from the well by pipetting out.
   c. Add 50 µl of Dual-Glo® Luciferase Reagent to each well (equal volumes). Avoid depositing Reagent solution along the walls of the well. Mix gently by tapping the plate. Because the plate is not shaken, when a drop of reagent is on the wall of the well, the volume of reagent in contact with the culture medium is reduced and this has an impact on the measurement of the luciferase.

d. Incubate 10 min in the dark at room temperature without shaking.
e. Measure firefly luciferase activity using the luminometer.
f. Remove the plate from the luminometer and add 50 µl of Dual-Glo® Stop&Glo® Reagent (stop solution prepared in a.) per well. Mix gently.
g. Incubate 10 min in the dark at room temperature.
h. Measure Renilla luciferase activity using the luminometer.
Note: Dual-Glo® Luciferase and Dual-Glo® Stop&Glo® Reagents are stable for 2 h.

6. Calculate the ratio of luminescence from the experimental reporter (firefly) to luminescence from the control reporter (Renilla). Calculate the mean ratio for each triplicate and normalize this ratio to the ratio of control wells. Experimental conditions will include a miRNA negative control (which does not target the UTR), a reporter plasmid devoid of UTR or containing an irrelevant UTR sequence.

Recipes

1. Culture medium
DMEM, high glucose, GutaMAX™
10% FBS
1% Penicillin-Streptomycin (optional, medium either with or without antibiotics will not affect transfection efficiency)

Acknowledgments

I thank Ute Bissels (Miltenyi Biotec) and Philipp Follert for providing the pmirGlo vector and for advice on luciferase assays. Funding was provided by the Agence National de la Recherche (ANR, FORDOPA), Fondation pour la Recherche Médicale (Label Equipe FRM), Fondation de France and the European Commission (Marie-Curie: ITN AXREGEN and IAPP DopaNew).

References

1. de Chevigny, A., Core, N., Follert, P., Gaudin, M., Barbry, P., Beclin, C. and Cremer, H. (2012). miR-7a regulation of Pax6 controls spatial origin of forebrain dopaminergic neurons. *Nat Neurosci* 15(8): 1120-1126.