3’ UTR (untranslated region) Reporter Clone and its vector, pMirTarget

Application Guide

OriGene Technologies, Inc

Package Contents and Storage Conditions

“3’ UTR reporter clone” as 10ug lyophilized plasmid DNA in a 2-D bar-coded Matrix tube. Shipped at ambient temperature. Once DNA is resuspended in water, store at -20ºC.

100 pmole of Forward (pMirTarget_F 5’AGAAGCTGCGCGGTGGTGTTGTG3’) and reverse (pMirTarget_R 5’CTGGAGGATCATCCAGCCGGCGT3’) DNA vector sequencing primers; dried onto the bottom of screw cap tubes. Store at room temperature. Once DNA is resuspended in water or TE, store at -20ºC.

Related Products

1. MicroRNA expression plasmid (SC4XXXXX), OriGene offers human and mouse microRNA clones individually or complete sets.

2. Positive controls: Positive control microRNA targeting reporter vector (5 repeats of the reverse complement of mature microRNAs sequence in pMirTarget vector).

3. Seeding sequence mutagenesis services: Seeding sequence of the 3’UTR clones could be mutated to test the specificity of microRNA and its targets’ interactions.

4. MicroRNA preparation kit

5. MircroRNA qPCR Detection System

6. Transfection reagent: Transfection reagents must be selected and optimized based on the cell type being used. OriGene suggests transfection reagents like MegaTran 1.0 (TT00003, OriGene), TurboFectin 8.0 (TF81001, OriGene) or FuGENE 6 (Roche), which have been shown to transfect most commonly used cell types.

8. Cell line and cell culture supplies: user preference

9. Luciferase assay kit: SKU: PR300001

10. LB-kan (25 ug/ml) liquid culture media and LB-kan (25 ug/ml) agar plates.

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund.

Introduction

MicroRNAs (miRNAs) are important regulators for target mRNA stability and translation. Although interactions among miRNAs and their target mRNAs are critical, very little experimental data is available. Researchers have to depend on computational predictions. However, with the lack of experimental data, those computational algorithms are not always accurate. 3'UTR-luciferase reporter constructs have been used by many individual researchers as a standard method to validate interactions between miRNAs and their targets. Different from shRNA or siRNA, most of the microRNAs only lower the expression of their targets about 30%. In high over-expression systems, small effects of microRNA usually are not detected. OriGene has used a new design adapted from C.P.Petersen et al. 2006, to dramatically increase the sensitivity of detection by decreasing the 3'UTR-luciferase reporter expression to a very low level (several hundred times lower than the CMV promoter). This design has been validated with human mir205 and its four (4) known and six (6) predicted 3' UTR targeting sequences.

OriGene's 3'UTR-luciferase reporter clones are used in two ways to study the interactions between microRNAs and their targets. First, the expression of certain microRNA in the cell line is known to exist. The 3'UTR-luciferase reporter clone, and the control (either empty pMirTarget vector or 3' UTR mutated clone), was independently transfected into the cell line. Luciferase activities were compared between the samples from the 3'UTR-luciferase reporter clone transfected cells and the control transfected cells as shown in diagram 1.

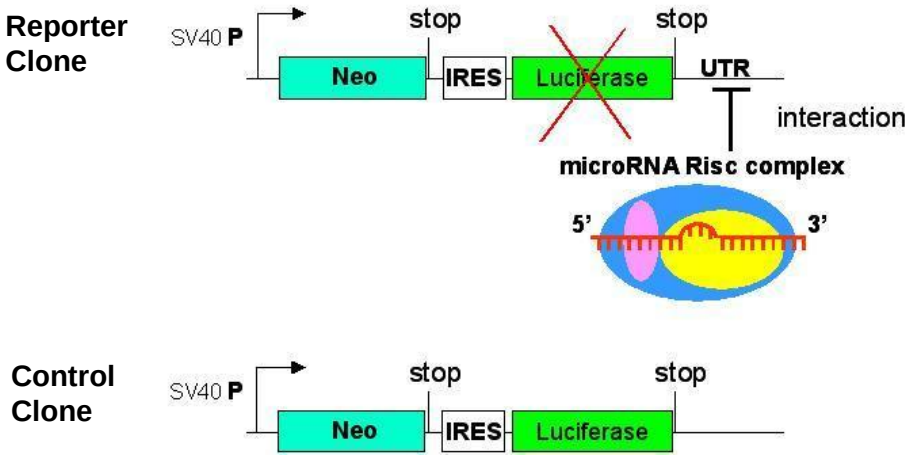


Diagram 1. 3'UTR-luciferase reporter clones are used in assaying for endogenous microRNA activity.

More often, the assay is performed by co-transfection of the 3'UTR-luciferase reporter clones and microRNA clones, as shown in the diagram 2 was also used in OriGene's human mir205 studies. The interaction between microRNA and its targets are measured by comparing the results from the co-transfection of a

3’UTR-luciferase reporter clone and microRNA clone with that from the co-transfection of a 3’UTR-luciferase reporter and the empty pMir vector.

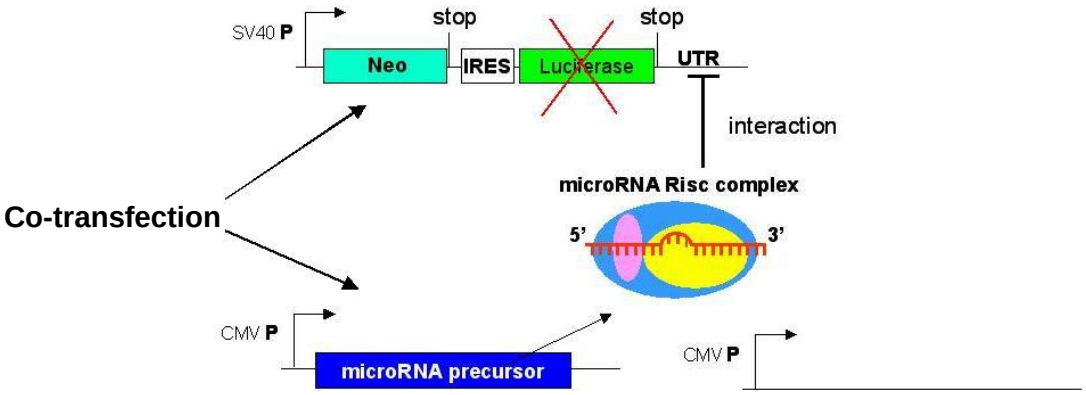


Diagram 2. 3’UTR-luciferase reporter clones are used in assaying for overexpressed microRNA by co-transfection.

In all cases, only two bases difference in the targeting region, many be enough to disrupt the interaction of the putative miRNA and its target. This must be considered to further validate the interaction.

To validate this system, OriGene’s 3’UTR-luciferase reporter clones have been used in a study of the interactions between human mir205 and its target clones. There was no detectable mir205 expression in HEK293T cells but it was detectable after the overexpression of OriGene’s pMir-mir205 clone, as shown in figure 1.

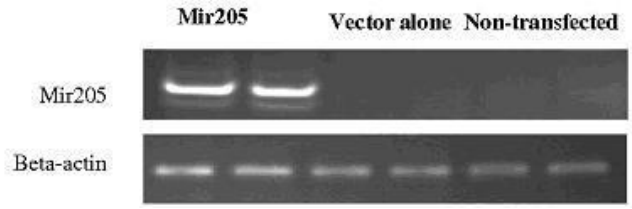


Figure 1. Overexpression of mir205 in HEK293 cells(RT-PCR amplification assessed by agarose gel electrophoresis).

The interaction of mir205 and its target, the reverse complement sequence of mir205, was confirmed after the mir205 was shown to down regulate GFP expression when its target was cloned after the stop codon of an IRES expressed GFP gene (Figure 2).

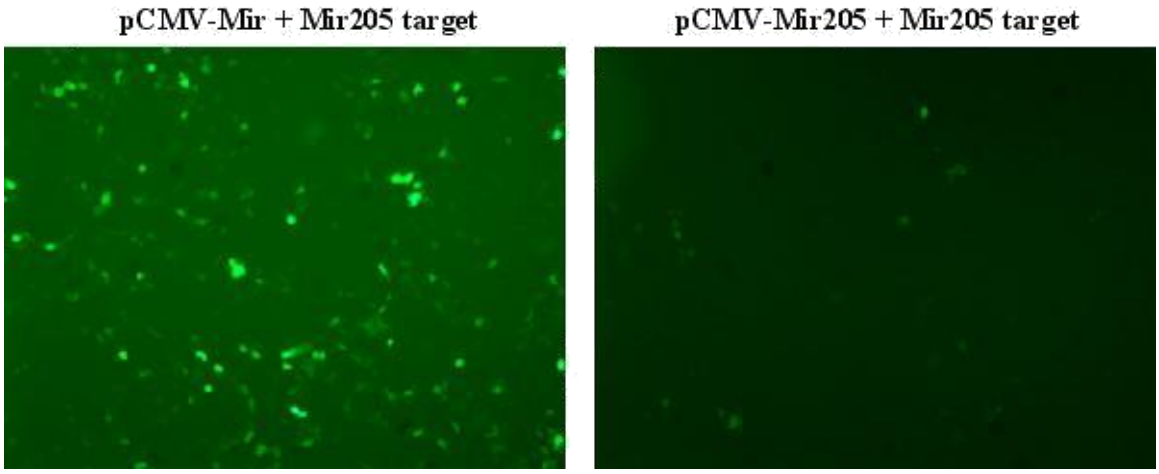


Figure 2. Mir205 down-regulated GFP expression when the GFP transcript was fused to the mir205 target sequence

To further validate OriGene's design of 3'UTR-luciferase reporter assays, 4 known mir205 targets and 6 predicted targets in pMirTarget were included in the mir205 study (Table 1).

Table 1. 3'UTR-luciferase reporter clones in mir205 targets study

Sample number	mr205 targeted clone in pMirTarget	Target Source	Target site
1	NM_001982	known	3' UTR
2	NM_030751	known	3' UTR
3	NM_001567	known	3' UTR
4	NM_014795	predicted	ORF
5	NM_001002814	predicted	3' UTR
6	NM_001025376	known	3' UTR
7	NM_005433	predicted	3' UTR
8	NM_014962	predicted	3' UTR
9	NM_024830	predicted	3' UTR
10	NM_019084	predicted	3' UTR
11	Mir205 Rev. comp.	CAGACTCCGGTGGAATGAAGGA	
12	Empty reporter		

We showed that all 4 known targets and 4 predicted targets caused measurable amount of down regulation of luciferase activity (Figure 3). Effects of mir205 on its targets were calculated by percentage of down regulation comparing the light emission from the mir205 vs. pMir cotransfected with the mir205 targets. Two individual experiments and four replicated transfections were performed. The statistical relevance of the effects were analyzed by the t-test (* $t<0.01$, ** $t<0.005$, *** $t<0.001$)

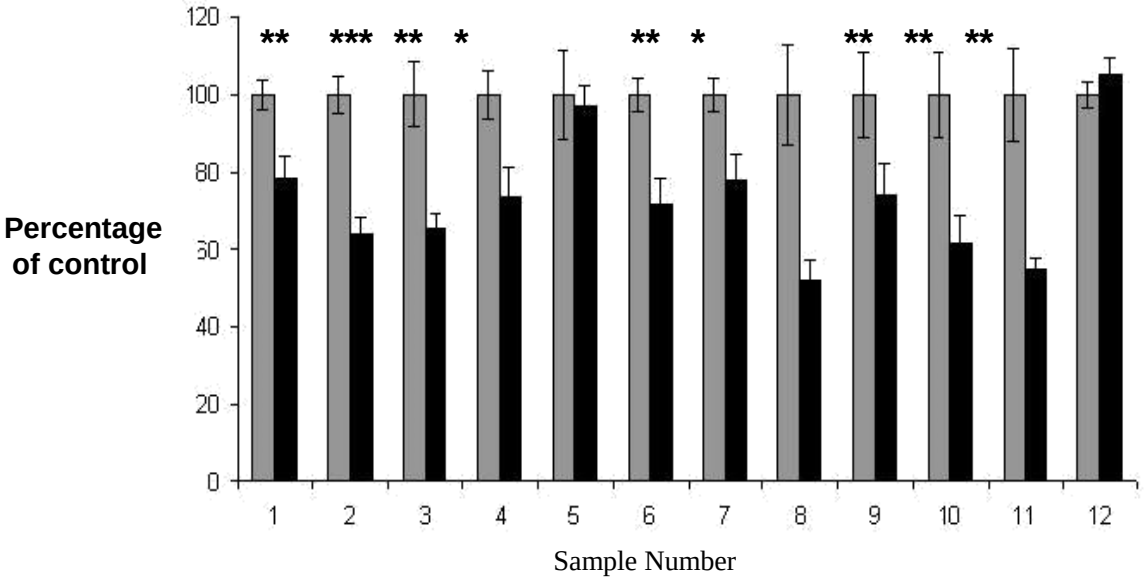


Figure 3. Mir205 down-regulated luciferase activity when the luciferase was fused to mir205 target sequences (grey bar, control with 3' UTR reporter clones; black bar, mir205 with 3' UTR reporter clones.)

In order to test the specificities of the interactions between mir205 and its targets, two nucleotides in the seeding sequences of four possible mir205 targets were mutated. All the tested clones showed a reversal of the down regulation (Figure 4). Effects of mir205 on its targets and mutated targets were measured by percentage of down regulation comparing the light emission from mir205 vs. pMir cotransfected with mir205 targets. Two individual experiments and four replicated transfections were performed.

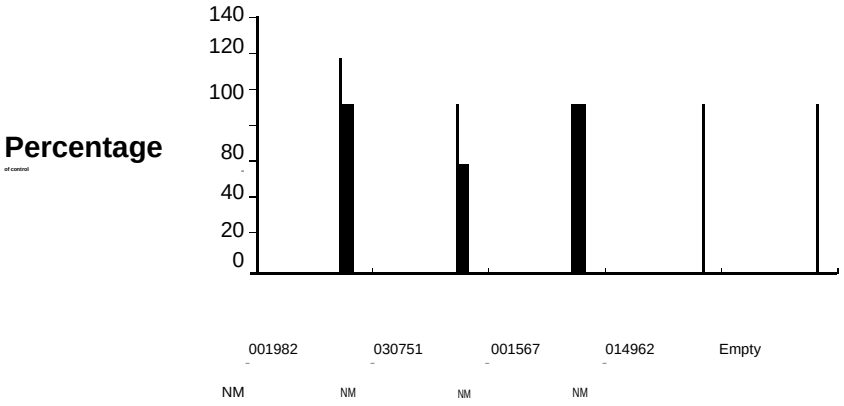


Figure 4. Abolishment of Mir205 effects by mutated seeding sequences in the 3'UTR-luciferase reporter clones (grey bar, control with 3' UTR reporter clones; darker grey bar, mir205 with 3' UTR reporter clones; black bar, mir205 with mutated 3' UTR reporter clones.)

To further analyze the interactions between mir205 and its targets, we asked whether multiple interacting sites could enhance the mir205 effects on its targets. Five (5) repeats of the targeting sequences were generated for the 3'UTR of NM_005433 as NM_005433 5X. One and 5 repeats of mir205 reverse complementary sequences were also generated as the 205 sponge and 205 sponge 5X. All of those constructs were cloned into the pMirTarget vector. After co-transfection with mir205 and using pMirTarget as a negative control, we showed that multiple target sequences could further decrease the luciferase activity (Figure 5). Effects of mir205 on its targets were measured by percentage of down regulation comparing the light emission from mir205 vs. pMir cotransfected with mir205 targets. Two individual experiments and four replicated transfections were performed.

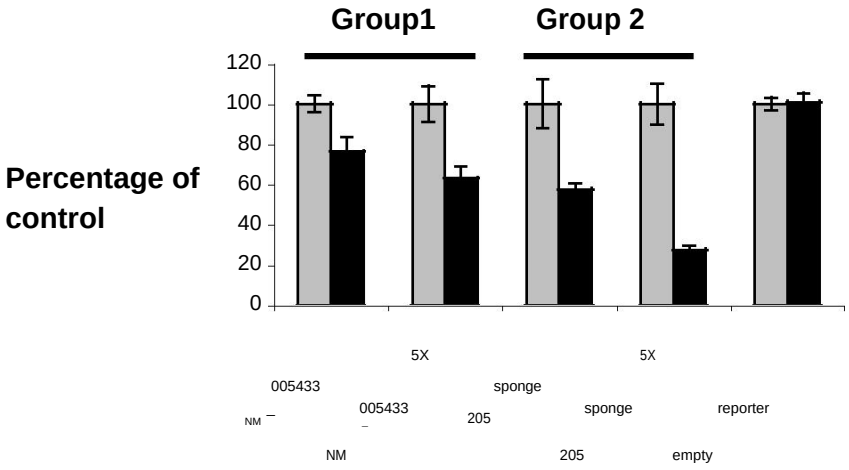


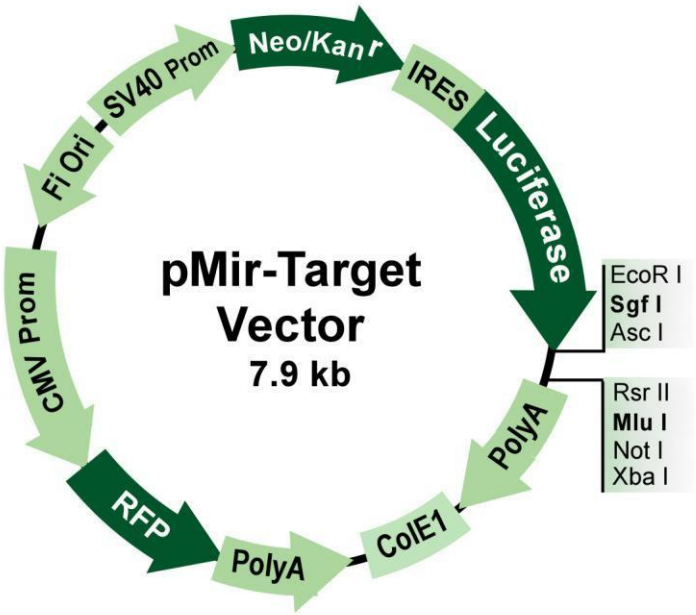
Figure 5. Enhanced interaction between mir205 and its targets by 5X multiple mir205 targeting sequences (grey bar, control with 3' UTR reporter clones; black bar, mir205 with 3' UTR reporter clones.)

Vector Information

OriGene's pMirTarget vector was adapted from C.P.Petersen et al.'s design in 2006. Firefly luciferase with 3' UTR microRNA targeting sequence was transcribed by SV40 promoter but was translated with an internal ribosome entering sequence (IRES). The IRES design doesn't affect the interaction of microRNA and its target, nor does it affect microRNA on its targets (OriGene internal research data 2010 and Petersen, C.P., Bordeleau, M., Pelletier, J. and Sharp, P.A., 2006). However since the IRES design dramatically decreases the expression level of luciferase, small changes in activity can now be detected. Our data suggests it is a suitable design to study the interaction between microRNAs and their targets.

pMirTarget vector contains other helpful features. A red fluorescent protein (RFP) under a CMV promoter is very helpful in monitoring the transfection and normalizing transfection efficiency. Neomycin under an SV40 promoter is useful for stable transfections.

pMirTarget Map

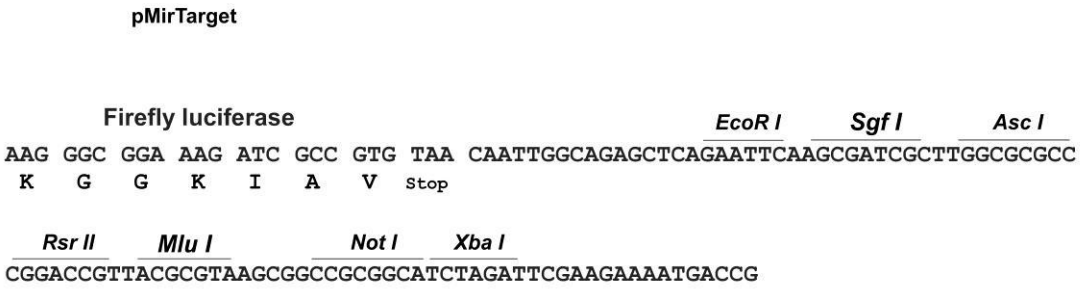


3' UTR reporter clone Insert Description

The 3' UTR sequence indicated by NCBI reference sequence is PCR amplified from OriGene's library clones with a high fidelity PCR enzyme. PCR products are cloned into pMirTarget after the stop codon of luciferase. All inserts are fully sequenced to ensure the clone largely matches this NCBI "reference" sequence.

Please note that the sequence provided by OriGene may contain SNPs and the final insert sequence is found on OriGene's web site. The clone can be sequenced with the included pMirTarget F and pMirTarget R (sequencing primers) to confirm the clone after plasmid amplification/purification in E.coli.

Multiple Cloning Sites of the pMirTarget vector:



Product Application Protocols

Introduction of reporter constructs into mammalian cells via transfection:

- Add 100 uL of dH2O into each of the tubes containing the 3'UTR expression plasmids. Vortex the tubes briefly to resuspend the DNA. The concentration of this solution is 100 ng/uL. Store at -20ºC.

- Plate the appropriate transfected cell line (e.g. HEK293 for human, NIH3T3 for mouse or OLN-93 for rat shRNA validation) cells at 3 X 10^5 in 2 ml into a well of a 6-well plate. Grow the cells overnight in a 5% CO_2 incubator to achieve 50% confluence.

- In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results. Serum-free DMEM 10 uL TurboFectin 8 solution (**0.3 uL**). Reporter plasmid DNA (**50ng**). Testing microRNA expression plasmid **100ng** (optional, available at OriGene)

- *Note: Add the TurboFectin 8.0 (or equivalent) directly into the serum-free media. DO NOT let the transfection reagent touch any plastic other than the pipette tip.*

- *For reporter construct and microRNA construct co-transfection, adjust the ratio of each (we suggests using 2:1 ratio of microRNA vs. reporter)*, *Mix the tube contents gently. Do NOT vortex!*

- Incubate at room temperature for 15-45 minutes.

- Add the DNA-TurboFectin 8.0 mix to the 6-well plate directly without removal of the culture media. Mix by gently swirling the plate.

  Incubate the cells in a 5% CO_2 incubator for 24 to 48 hrs. Then perform the luciferase assays (SKU: PR300001).

Creation of stable cell lines:

- Transfect the cells with the reporter plasmid DNA using the standard protocol for transient transfections. After transfection, do not change the medium until the cells are ready to be passaged.

- Passage the transfected cells into a fresh vessel containing growth medium and 0.5-1mg/ml G418. An appropriate kill curve over this range should be used to determine the minimum concentration of antibiotic

needed to obtain 75% cell death in 48-72hrs. Continue to grow and passage the cells as necessary, maintaining selective pressure by keeping G418 in the growth medium. After 1-2 weeks, a large number of the cells will be killed by the antibiotic, indicating that they did not take up or have lost the plasmid with the neomycin resistance cassette. The cells that remain growing in the neomycin -containing medium have retained the reporter plasmid, which has stably integrated into the genome of the targeted cells.

- Select clonal populations of surviving cells by transferring a well-isolated single clumps of cells (the clonal ancestor and cells divided from it) into separate wells of a 24-well plate. Select 5-10 clonal populations. Continue growing these cells in selection medium for 1-2 additional passages. At this time, each well contains a clonal population of stably transfected cells, which can be maintained in normal growth medium without the pressure of G418 (although you may wish to grow the cells under “light pressure” 0.5mg/mL G418). These populations can be used for experiments or stored under liquid nitrogen in growth medium with 10% DMSO and 20% FBS for future use (see proper storage conditions for the cell type used).

Plasmid DNA amplification (Optional)

- If desired, customers can amplify the reporter plasmid. Add 100 uL of dH2O into each tube. Vortex the tubes briefly to resuspend the DNA. Pipette 1 uL of this solution to another tube and add 99 uL dH2O. The concentration of the DNA solution should be around 1 ng/uL. The plasmid solution should be stored at –20 ^{O}C.

- Thaw transformation competent *E. Coli* cells (standard laboratory DH5alpha) on ice. Perform a transformation reaction with 1-2 uL of the diluted plasmid.

- Plate out the transformants on an LB-Kan (25ug/mL) plate and incubate overnight at 37 ^{O}C, until colonies appear.

- The following day, inoculate single bacterial colonies into 5 ml of LB-Kan (25ug/mL) media and grow them overnight.

- Purify the DNA plasmids from the culture using a miniprep DNA isolation kit. Resuspend the DNA in 50 uL of TE solution and determine the DNA concentration of the samples. Store the solution at –20^{O}C.

Assay for endogenous microRNA in cell lines

In this assay design, we use all the plasmids and reagents from OriGene Technology Inc. The user of this kit could use similar plasmids and reagents of their own in a similar setting.

To assay the interaction of endogenous microRNA using a 3'UTR reporter plasmid, we suggest following the initial set-ups for screening. **The same amount and quality of DNA should be used for each transfection. You may also use RFP to normalize the variation between transfections.**

Transfections	Control 1	Experiment	Control 2
pMirTarget	+	-	-
pMirTarget-3UTR	-	+	-
pMirTarget-mir sponge	-	-	+

For positive hits, further validation should be carried out in the following settings. Be sure to compensate for the amount of DNA difference with a common plasmid, such as pEntry (Cat# PS100001) from OriGene.

Transfections	Control	Exp1	Exp2	Exp3	Exp4
pMirTarget	+				
PMirTarget-3UTR		+		+	
PMirTarget-3UTR mutant			+		+
Mir sponge				+	+

The interactions between miR and the testing 3'UTRs are confirmed only when the mutation in seeding region abolish the interaction, and miR sponge could attenuate or abolish the interaction between miR and the testing 3'UTRs.

Assay for introduced miR and its targets

In this assay design, we use all the plasmids and reagents from OriGene Technology Inc. The user of this kit could use similar plasmids and reagents of their own in a similar setting.

Expression of the introduced miR in your specific cell line must be confirmed before you begin your experiment. We suggest that you use OriGene's miR detection kit for this experiment.

To assay the interaction of the introduced miR using a 3'UTR reporter plasmid, we suggest following the initial set-ups of a screening using OriGene's microRNA clone and pMir vector. **The same amount and quality of DNA should be used for each transfection. You may also use the RFP expression to normalize the variation between transfections.**

Transfection	Experiment	Control
pMirTarget-3UTR	+	+
miR in pMir	+	-
PMir(Empty Vector)	-	+

For positive hits, further validation should be carried out in following settings. Be sure to compensate the amount of DNA difference with a common plasmid.

Transfections	Exp1	Exp2	Control
pMirTarget-3UTR	+		+
pMirTarget-3UTR mutant		+	
MiR in pMir	+	+	
PMir(Empty Vector)			+

The interactions between miR and the testing 3'UTRs are confirmed when the mutation in seeding region could abolish the interaction.

Frequently Asked Questions

Why should I use OriGene's 3' UTR reporter clones in the pMirTarget vector?

OriGene has the largest collection of human full-length cDNA clones. Thus OriGene can provide you 3'UTR clones rapidly and economically. Our research has shown that the pMirTarget was more sensitive when compared to other 3'UTR-luciferase reporter systems.

Does the IRES design affect the interaction of microRNA and its targets?

No.

Based on published data by Philip A. Sharp's group at MIT and our own data, the microRNA represses the translation even if the translation is started from IRES.

How can I get consistent, reliable results doing transient transfection?

The key is to achieve the same transfection efficiency, which requires the same amount and same quality of experimental and control DNA. In the pMirTarget, RFP expression driven by the CMV promoter is a good indicator of transfection. If your transfections (experiment and control) showed the same level of RFP signal, your 3'UTR-luciferase reporter assays will be reliable.

What is the best positive control in a 3' UTR reporter assays?

To our experience, the microRNA sponge or tandem repeats (5X) of mature microRNA reverse complementary sequence downstream of the luciferase stop codon in the pMirTarget serve as excellent positive controls.

Do I have to mutate the seeding sequence to show the specificity of microRNA and its targets interaction?

Yes.

It is the best way to show that the interactions are specific. OriGene offers the service for mutant clone construction. On each wildtype 3'UTR clone page, there is a button of "Mutant clone request". To place an order for a mutant clone, click on the button and fill out the mutant sequence information. When ordered with the wildtype 3'UTR clones, the fee for the mutant clone is usually $400 unless quoted otherwise.

What does your disclaimer mean?

OriGene's disclaimer for the 3' UTR reporter clones reads as follows: "Our molecular clone sequence data has been matched to the accession number below as a point of reference. Note that the complete sequence of our molecular clones may differ from the sequence published for this corresponding accession number, e.g., by representing a single nucleotide polymorphism (SNP)." The NCBI RefSeq mRNA sequences are continuously being revised. These sequences are therefore used only as a "reference" and not as a "standard". OriGene's clones are isolated from full-length cDNA libraries and may differ from the reference sequence for this reason.

What is the 3'UTR clone Guarantee?

OriGene warrants that the product will meet the specifications listed. At OriGene's discretion, free replacement of any non-conforming product will be made if OriGene is notified within 30 days of product receipt. If you experience any difficulty with any OriGene product, please contact our Technical Support Staff at 888-267-4436, or 301-340-3188 outside the US.

References

Petersen, C.P. *et al.* Short RNAs repress translation after initiation in mammalian cells. ***Molecular Cell*** **21, 533–542 (2006)**