Chapter 14

Gene Reporter Assay to Validate MicroRNA Targets in Drosophila S2 Cells

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Abstract

Bioinformatics programs have helped tremendously in identifying the targets of microRNAs, which are small noncoding RNAs that regulate gene expression posttranscriptionally. However, the partial complementarity between miRNAs and their targets hinders the accuracy of target prediction, necessitating the use of experimental validation procedures. Here, we describe a gene reporter assay typically used in our lab to validate putative miRNA–mRNA interactions in Drosophila S2 cells.

Key words Reporter assay, Target validation, Luciferase, S2, Drosophila

1 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs of 17–25 nucleotides in length that posttranscriptionally regulate gene expression by perfectly or imperfectly base pairing with their target mRNAs in plants and animals, respectively [1, 2]. MicroRNAs are involved in a number of fundamental cellular processes ranging from metabolism to cell growth and differentiation to apoptosis [3–5]. The current estimate is that over half of the mammalian protein-coding genes are controlled posttranscriptionally through various mechanisms by miRNAs [6]. A single miRNA can regulate the expression of multiple target genes, but at the same time, one target gene can be independently or cooperatively regulated by multiple miRNAs.

Advances in sequencing technology have led to a great progress in the identification of novel miRNAs in a wide range of species [7–9]. This has been further supplemented with sophisticated target prediction algorithms to effectively predict miRNA–mRNA interactions [10–13]. Since the base pairing between miRNAs and their targets is imperfect in animals, the interactions predicted by bioinformatics tools have to be validated experimentally to avoid potential false-positive target predictions.

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A number of different approaches have been used in the experimental identification of putative miRNA–mRNA interactions [14, 15]. Broadly, these approaches have been categorized under transcriptome analyses, biochemical approaches, and proteome analyses. The choice primarily depends upon the intended number of targets. For example, if the aim is to identify multiple miRNA targets at once, a genomics approach such as transcriptome or proteome analyses, following miRNA knockdown or overexpression, would be highly desirable. The handicap of this approach is that it would be cumbersome to distinguish between direct or indirect effects of miRNAs on target expression. To avoid indirect miRNA effects in a genomics target screen, AGO or RISC immunoprecipitation can be combined with microarray or deep-sequencing analysis of total RNA isolated from the immunoprecipitate in the presence and absence of an miRNA of interest ([16, 17], Chapter 6). By doing so, the changes in the mRNA contents, thus miRNA targets, of RISC complexes can be directly identified when miRNAs are over-expressed or knocked-down.

Direct demonstration of miRNA targets usually involves experimental identification of target mRNAs individually. This approach nicely eliminates indirect off-target effects since the function of the reporter mRNA is determined exclusively by a physical interaction between the miRNA of interest and the reporter construct. One disadvantage of this approach is that it is quite labor-intensive. The results should still be interpreted carefully especially when miRNAs are over-expressed transiently. Any superficial increase in the intracellular miRNA concentration beyond its physiological concentration could potentially generate false-positive results by interfering with the interaction between other miRNAs and RISC complexes [18].

In this chapter, a gene reporter assay is described in which the 3′UTR of a target mRNA is cloned into an expression vector bearing a reporter gene (e.g., luciferase). When co-expressed with the miRNA, the miRNA-mediated suppression of the reporter function, miRNA:target interaction, can easily be measured. Cells without miRNA are used as negative controls. Additionally, constructs containing 3′UTRs with mutated target sites serve as additional negative controls. This approach can be further solidified by using miRNA inhibitors in the experimental design.

2 Materials

2.1 Molecular Cloning Components

- PCR Cloning Kit (Fermentase InsTAclone™ PCR cloning kit #K1213 which contains the TA cloning vector pTZ57R/T, 5× ligation buffer, T4 DNA ligase and nuclease-free water).
- Fragment isolation kit (Invitrogen PureLink gel extraction kit, K2100-12).

- Plasmid purification Kits (Fermentas GeneJET™ plasmid miniprep kit K0503; Invitrogen PureLink™ HiPure plasmid filter midiprep kit K2100-15).
- Restriction enzymes.
- Taq DNA polymerase (Fermentas).
- Squishing buffer (10 mM Tris–Cl, pH 8.2, 1 mM EDTA, 25 mM NaCl, and 200 μg/ml proteinase K).
- SOC medium (0.5 % yeast extract, 2 % Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose).

2.2 S2 Cell Maintenance and Transfection Components

- Schneider's Drosophila medium (Invitrogen 11720-034).
- Heat inactivated fetal bovine serum (Invitrogen, GIBCO 10500).
- Penicillin G (Biochrom AG, A321-44), Streptomycin (Biochrom AG, A331-44).
- Calcium-phosphate transfection kit (Invitrogen K2780-01), (2× HEPES-buffered saline (HBS), 2 M $CaCl_2$, tissue culture sterile water).

Drosophila Schneider 2 embryonic stem cells are maintained in Drosophila Schneider medium (Invitrogen) supplemented with L-Glutamine, 10 % FBS (GIBCO) and 2 % Penicillin–Streptomycin (Biochrom AG) at 25 °C without CO_2. Passages of cells are performed twice a week.

2.3 Luciferase Assay Components

- Luciferase assay kit (Promega, E1960).
- Passive cell lysis buffer (glycerol (50–75 %), CDTA (1.0–5.0 %), *N*,*N*-Bis(3-D-glukonamidopropyl) cholamide (<1.00 %)).
- 1× PBS [(1) 8 g NaCl, (2) 0.2 g KCl, (3) 1.44 g Na_2HPO_4, (4) 0.24 g KH_2PO_4, (5) in 800 ml of distilled H_2O, (6) adjust the pH to 7.4 with HCl. Add H_2O to 1 l].

3 Methods

Three main constructs should be prepared to perform a complete luciferase dual reporter assay. The first construct contains the miRNA precursor, which is expressed under the control of a potent promoter such as actin. The second one is a chimeric construct carrying the open reading frame of the luciferase firefly gene fused to the 3′UTR of the target mRNA of interest. The third construct carries the luciferase renilla which is used to normalize the transfection efficiency.

3.1 MicroRNA Overexpression Constructs

There are at least three different ways of over expressing miRNAs in cell lines such as Drosophila S2 cells. Several commercial companies provide experimentally validated synthetic mature miRNAs or their precursors (pre-miRNA). Transient transfection of these molecules is easy and quick but requires dose kinetics to determine expression levels comparable to their endogenous concentrations in the cell line used. The third approach involves the expression of miRNAs on an expression vector carrying a strong promoter. The use of an inducible promoter would be desirable, if possible, to modulate the expression levels of miRNA transcripts. In this section, the third approach will be described.

The cloning of an miRNA gene into an expression vector can be carried out in two ways. In the first option, the PCR-amplified miRNA sequence is directly cloned into the expression vector. This approach saves time by eliminating a second cloning step. However, the preparation of the fragments and the vector carrying the matching restriction sites can be difficult especially for beginners. In this situation, it may be easier to clone the miRNA sequence into a TA cloning vector such as pTZ57R/T (Fermentase). The fragment can then be easily transferred from this sub-cloning vector into the expression vector. Unless specified by the product provider, the protocols described by Sambrook and Russell are usually followed [19].

3.1.1 PCR Amplification of an MicroRNA Gene from Genomic DNA and Cloning into Expression Vectors

Although the isolation procedure may vary depending upon the cell/tissue, singly fly genomic DNA isolation procedure [20] was used to prepare the genomic DNA for PCR amplification of the miRNA precursor. The same protocol was applied to isolate genomic DNA from S2 cells. The following procedure has given excellent results especially with Drosophila embryos or adult flies.

(a) A single fly is mashed five to ten times by a pipet tip in 100 µl squishing buffer.

(b) The mixture is incubated at 37 °C for 30 min following the addition of proteinase K (200 µg/ml). The powder proteinase K should be dissolved in pure water, divided in aliquots, and stored at −20 °C until use to minimize the loss of activity.

(c) Proteinase K is then inactivated by heating the sample at 95 °C for 2 min. As an option, RNAse can be added to the mixture followed by phenol–chloroform extraction to eliminate contaminating RNAs. Usually, RNA should not interfere with DNA amplification, though. The genomic DNA can be stored at −20 °C until use.

(d) Forward and reverse primers are designed to amplify candidate miRNA precursors by PCR. An example is presented in Fig. 1. Both forward and reverse primers can be about 20–25 nucleotides in length. A restriction enzyme recognition site should be placed on the 5′-ends of primers to facilitate cloning in the subsequent steps. Having the same restriction site on both primers makes it easy to prepare the fragments for cloning.

5'-CAGCACACAGGTCACCATTCCAAAAGAGGTGGGCGCATATATTTTCATGATTT
ATAGAATTTAACTAATATAGTGTTCGTTGTGTTTCAGCTGGCTGGTGCCATCATTG
GTAAGGGAGGTGGCCGCATCCGTCGCATCCGCAACGAGTCCAGTGCGTACATCAC
CTCGACGAGCCCCTGCCAAACTCGAACGATCGTAT*CATCACCATCTCGGGCACGC*C
GAAGCAAATACAAATGGCCCAGTATCTGCTGCAACAGAGGTTGGTGTCGCAATCT
GAATAAGAAGTATGCACATCCTATCATTTGTCTAACCACCCATCCCCACAATAAC
ACTCTAACACAAAAAAAAAAAAAAACGCGTGATTAATTTGGAAGGAAAGGTGTC
TGCTGTGCGTCCCGGTCCCGAGTGTTAAAATATGTGCTTGATCGTAACTCCATCCA
AACTCGATATTAACTAACCGATTGGTCCTCCTGGGAGTGCATTCCGTATGGAAGA
CTAGTGATTTTGTTGTTTGGTCTTTGGTAATAACAATAAATCCCTTGTCTTCTTACG
GCGTGCATTTGTGCTCTTCATTCTATCGATGGTTAACCAATAAAACTAAACACGG
CATTGGAAACTACCTAACTAAACGTGTCACAATTCATCCTTGTCCCAGCTCGCAA
AAAAAAAAAAAATAATAATCATCCATCGTGTATAACTATTTCACCCAAAAACGA
ACTCCCCAGTACCCTCCCATCCCACTACATCCACATCCACATTCATTTAGCTGTAT
CTGCTGTTCATTAAAAAGTTCATTAATTATGTTTTCCTTTGGCTTGCTGCCATCCTG
CCGCCAACTCTTTCCATTGCAGCGTACA*CGAGAATGGCAGGCGAAACA*TTTAAGTG
GGCAAGGACTTGAACAGCTACAACAGCAATAGCAACACCATTCGACAAAACAAC
AAATTCAACAACTACAATAACCATCGAGAATTGCTGCGTTTCACGTTTAAATTTA
AATGT

Fig. 1 Primer design to PCR-amplify the *dme*-miR7 gene. The miRNA sequence was obtained from flybase. Placing the mature miRNA sequence in the middle (*gray-shadowed* and *underlined*), approximately 150–200 bp from each region is amplified. Sequences complementary to the primer sequences are *italicized* and *underlined*. One of the restriction sites that does not cut the miRNA gene but is present in the multiple cloning site of the expression vector is then placed on each primer with a few extra nucleotide sequences (depending on the restriction site chosen). Such a design would result in a forward primer 5′ GG**GGATCC**CATCACCATCTCGGGCACGC 3′ and a reverse primer 5′ GG**GAGCTC**TGTTTCGCCTGCCATTCTG 3′. The restriction sites are *bolded* in the primer sequences. The two additional 5′ G residues are used to facilitate more efficient restriction digestion

However, the fragment can be cloned in either orientation, which requires verification of the correct orientation. To avoid this problem, different restriction sites should be placed on each primer. The selected restriction sites should be present only in the multiple cloning site of the expression vector and absent in the other regions of the vector or the candidate miRNA gene. Such non-cuter sites can be detected by programs freely available on the web (e.g., RESTRICTION MAPPER: http://www.restrictionmapper.org/). Primers are designed to amplify at least a region 150–200 bp flanking from each side of the mature miRNA sequence.

(e) PCR amplification may require optimization of the annealing temperature and the template amount but the setup described below may be a good start. Mix the following in a clean Eppendorf tube: 2.5 µl 10× taq buffer (Fermentase), 1.5 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTP mix (Fermentase), forward primer (5–10 mM), reverse primer (5–10 mM), 0.75–1 unit Taq polymerase (Fermentase), template genomic DNA (50–500 ng), and dH_2O to 25 µl.

(f) Amplify the miRNA gene in a thermocycler by using the following program: initial denaturation at 94 °C for 5 min followed by 25 cycles for initiation at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 1 min.

The program is finished with a final extension at 72 °C for 10 min. The PCR products are run on 1 % agarose gel to check the size of the fragment against a marker. Note that the annealing temperature depends upon the melting temperature of the primers, which will vary depending on the primer sequence.

(g) The PCR-amplified fragment should be purified from agarose gel to eliminate contaminating genomic DNA and incomplete PCR products. The purelink quick gel extraction kit (Invitrogen) yielded consistent results in our lab. Since the company provides detailed instructions, this particular procedure will not be explained here. It is important to keep in mind that elution of the fragment into distilled water increases the efficiency of downstream enzymatic reactions.

(h) The PCR-amplified and gel-purified products of miRNA precursor includes adenine residues at their 3′ ends due to extension by Taq polymerase. By taking advantage of this property, the fragments are easily cloned into TA cloning vectors using the instructions of the manufacturer. We commonly use pGEM T easy (Promega) or pTZ57R/T (Fermentase) for this purpose. 50 ng vector and 4.5–5 ng extracted PCR product of approximately 300–500 bp is sufficient to set up an efficient ligation reaction. Note that the ligation reaction can be completed in 1 h or left overnight if desired.

3.1.2 Transformation of Ligation Products to DH5α Competent Cells

Since blue-white colony screening is performed to select the correct transformant carrying the miRNA precursor, 40 µl X-Gal, and 7 µl IPTG should be spread onto an agar plate before starting the transformation procedure. If commercial competent cells are used, we advise that the manufacturer's instructions are followed. The following procedure originally described by Sambrook and Russell [18] works well for home-made competent cells. Remember to prepare a water-bath at 42 °C before you start the transformation. [AU1]

(a) Immediately place 50 µl competent cells into an ice-cold Eppendorf tube and let it thaw on ice.

(b) Gently add 5 µl ligation mixture into the competent cells and incubate on ice for 20 min after mixing the tube content gently.

(c) Heat shock the cells at 42 °C for 45 s and immediately place the tube on ice for 2 min.

(d) Add 950 µl SOC medium into the tube and shake 1 h in a shaker (100 RPM at 37 °C).

(e) Spread 100 µl onto an agar plate containing the appropriate selectable antibiotic. Incubate the plate in an incubator at 37 °C overnight (16–24 h).

(f) A single white colony is then streaked onto an agar plate again to obtain a pure single colony. One of the colonies is inoculated into 8 ml LB medium and the plasmid is purified via a plasmid purification kit (e.g., Fermentase GeneJET™ plasmid miniprep kit K0503) according to the manufacturer's instructions.

3.1.3 Transfer of the MicroRNA Precursor from the Cloning Vector into an Expression Vector

The fragment containing the miRNA precursor sequence is released from the sub-cloning vector by digesting it with the appropriate restriction enzymes (e.g., 5′ *Bam*HI and 3′ *Sal*I). The expression vector is also digested by the same enzymes to generate sticky ends for the ligation of the miRNA precursor released from the sub-cloning vector. The last step involves ligating the miRNA-carrying fragment into the expression vector with the sticky ends. The basic molecular biology protocols described by Sambrook and Russell [18] can be used to complete this step. Two control reactions should be included to minimize the number of false-positive transformants. Control ligation I is basically the same as the test reaction but does not contain any ligase enzyme. Control ligation II should not contain any insert. When transformed into *E. coli*, no transformants should be observed from these two control ligation reactions if the expression vector is prepared properly. Following the transformation, the cloning efficiency can be checked as in Subheading 3.1.2.

3.2 Construction of a Chimeric Gene Carrying the Open Reading Frame of the Luciferase Firefly Gene Fused to the 3′ UTR of a Target mRNA

The chimeric gene includes firefly luciferase gene whose 3′UTR is replaced with that of the miRNA target which contains a single or multiple binding sites for the miRNA of interest. This chimera is cloned into an expression vector (e.g., pAct-5c for expression in Drosophila S2 cells) to examine the effect of miRNA:target 3′UTR interaction on the expression of the reporter luciferase activity.

(a) Forward and reverse primers are designed as in Subheading 3.1.1, **step a** to amplify firefly luciferase without its 3′UTR. The selected restriction sites should be present in the correct orientation in the expression vector. The pGL4.12[luc2CP] construct can be used as a template to amplify firefly luciferase as in Subheading 3.1.1. The only difference is that the template DNA is pGL4.12 for this reaction.

(b) Double-digest the expression vector pAct-5c and the PCR products with the same restriction enzymes included in the primers to amplify the firefly luciferase and extract from 1 % agarose gel by a fragment isolation kit.

(c) Ligation, transformation, and verification of the insert can be performed as in Subheading 3.1.

[AU2]

(d) **Steps 3.2a–d** are repeated but with a pair of primers complementary to the 3′UTR of target mRNA to insert the 3′UTR of the miRNA target downstream from the firefly luciferase.

3.3 Construction of the Normalization Vector Containing Renilla Luciferase

It is important to normalize the transfection efficiency in all transfection reactions. This is accomplished by transfecting the cells with a different reporter gene such as luciferase renilla. The cloning rationale is almost the same as in Subheading 3.1 except for the template and the pair of primers. The commercial plasmid pGL4.74(hRluc/TK, Promega) is used as a template instead.

3.4 S2 Cell Transfection

(a) 24 h before transfection, Drosophila S2 cells (5×10^5 per well) are seeded in a 6-well plate (Jet Biofil, TCP011006) in complete medium containing 10 % FBS and 2 % Penicillin–Streptomycin (At least four wells should be seeded to measure one miRNA-target dual reporter assay—referred to as well 1, 2, 3, and 4). Well 1 contains the control S2 cells that are not transfected with any plasmids. It is used to assess the effect of the transfection reagent on S2 cells. Well 2 is another control that is used for transfection with the empty expression vector (pAct-5c) to measure the effect of plasmids, if any, to the cells. Well 3 contains S2 cells transfected with the chimeric reporter gene (firefly luciferase and target mRNA 3′UTR). Well 4 contains the S2 cells transfected with the miRNA expression plasmid (e.g., pAct-5c-miRX) and the chimeric reporter gene. The cells in the wells 2, 3, and 4 are also transfected with equal amounts of renilla luciferase to normalize the transfection efficiency.

(b) For transfection, the calcium-phosphate transfection kit (Invitrogen) offers high transfection rate, and 10 μg plasmid DNA is sufficient. It is very important to use a Pasteur pipette to slowly add the solutions A2, A3, and A4 (dropwise) to the solutions B2, B3, and B4, respectively, while bubbling air through by another Pasteur pipette. This is a slow process executed over 1 or 2 min.

3.5 Measurement of Luciferase Reporter Function

The manufacturer company's instructions were followed to measure the luciferase activity. The measurement consists of three main steps: (1) cell lysis, (2) measurements of luciferase firefly, and (3) luciferase renilla.

(a) 48 h after transfection, the cell medium in the wells is removed and cells are rinsed with 1× PBS at least twice. The rinsing solution should be removed completely.

(b) 500 μl PLB is added into each well and the cell culture plate is placed on an orbital shaker (~50 RPM) for 15 min at room temperature. If desired, cells are scraped in PLB directly and rinsed with 1× PBS in microfuge tubes. 500 μl PLB is added and cells are lysed by pipetting.

(c) Luciferase reporter function can be conveniently measured in a 96-well plate using a luminometer (VarioScan, Thermo). The luminometer is programmed to read a 2-s premeasurement delay followed by a 10-s measurement period for each reporter assay. 20 μl cell lysate is usually sufficient for each assay.

(d) Cells may possess very little amount of luciferase. Thus, the background enzymatic activity should be subtracted from the total luminescence. For this purpose, non-transfected control cells are lysed in PLB and used as a negative control. Any luminescence obtained from this sample is subtracted from all other readings.

4 Conclusion

Advances in sequencing technology have nearly exhausted the identification of potential miRNAs in mammals. Nowadays, it has become more important to identify potential targets of miRNAs and to understand the significance of miRNA:mRNA interactions in biological systems. Although sophisticated target prediction algorithms effectively predict miRNA–mRNA interactions, experimental validation is essential in animals due to imperfect pairing between miRNAs and their targets.

Typically, transcriptome analyses, biochemical approaches, and proteome analyses are used to identify miRNA:mRNA interactions. Gene reporter assays are preferred over other approaches as they nicely avoid off-target effects associated with transcriptome or proteome analyses. Direct demonstration of a single miRNA:mRNA interaction increases specificity. Reporter assays are also attractive due to their simplicity and cost-effectiveness.

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Author Queries

Chapter No.: 14 0002058779

Queries	Details Required	Author's Response
AU1	The author names "Sambrook and Russell" in the sentence "The following procedure…" mismatch with reference citation [18]. Please check.	
AU2	Please check the cross reference "Steps 3.2a–d" for correctness.	