Chapter 14

Molecular Approaches for Analysis of *Drosophila* MicroRNAs

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Abstract

MicroRNAs (miRNAs) belong to a class of small non-coding endogenous RNAs that regulate gene expression at the post-transcriptional level. These small RNAs recognize sequences within 3′ untranslated regions of target mRNAs in complexes referred to as a miRNA-induced silencing complex (miRISC). *Drosophila melanogaster* has served as an indispensable model system for defining the diverse biological roles of miRNAs, their mechanism of action as well as the role of miRNA biogenesis factors. In this chapter we describe some of the assays used for molecular analysis of *Drosophila* miRNAs.

Key words MiRNAs, Luciferase sensors, Post-transcriptional, *Drosophila*, Northern analysis, TaqMan assays

1 Introduction

Drosophila melanogaster has served as an important experimental organism for understanding the role of miRNAs in diverse cellular processes. Currently there are 258 *Drosophila melanogaster* miRNAs listed in the miRNA database (miRbase 22 release) that presumably regulate several hundreds of mRNA targets, thereby modulating a significant proportion of cellular proteins. In this section, we summarize the miRNA biogenesis pathways and functional roles of miRNAs that have been studied in *Drosophila melanogaster*.

1.1 Biogenesis of miRNAs

Mature miRNAs arise from longer precursor transcripts that are characterized by the presence of hairpins and are processed by two RNAse III cleavage reactions [1]. The first step in the generation of a processed miRNA is a transcription of its primary miRNA transcript (pri-miRNA) by RNA polymerase II [2]. These primary transcripts are cleaved by a microprocessor complex that contains the nuclear RNAse III enzyme, Drosha, to result in 50–70-nucleotide-long stem-loop precursor (pre) miRNAs [3, 4]. The

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pre-miRNAs undergo a second round of cleavage in the cytoplasm by a second RNAse III enzyme, Dicer-1, to generate 21–23 nucleotide miRNA duplexes [5–7]. One strand of this miRNA duplex preferentially associates with a miRNA-induced silencing complex (miRISC) and directs the effector complex to complementary sites in target mRNA(s) [8–12]. Analogous to other RNA polymerase II transcripts, the expression of mature miRNAs is regulated at both transcriptional and post-transcriptional levels. Northern, in situ and transcriptome analysis of pri-miRNAs in *Drosophila* have indicated that transcription of miRNAs is spatially and temporally regulated [13–16]. Studies have also uncovered post-transcriptional mechanisms that can regulate miRNA processing in a gene-specific or global manner. RNA binding proteins such as Lin-28, hnRNP A1 and KSRP can bind to pri- and pre-miRNAs and influence their processing in a positive or negative manner [17–20]. Changes in expression level and post-translational modifications of core miRNA biogenesis factors such as Drosha and DGCR8 have been shown to regulate miRNA processing activity [21–23]. Additionally, adenosine-to-inosine (A-to-I) RNA editing of miRNA sequences by the *a*denosine *d*eaminase *a*cting on *R*NA (ADAR) enzyme can alter stability, biogenesis and target recognition of miRNAs [24, 25]. Thus, precise regulation at multiple levels is required for achieving the dynamics of miRNA production and degradation [26]. In higher eukaryotes, defects in miRNA processing have been linked to several diseased states, including cancer. Thus, understanding conserved molecular mechanisms that control miRNA production in model organisms like *Drosophila melanogaster* is important to identify novel regulatory molecules that could serve as potential targets for therapeutic interventions.

1.2 Biological Functions of miRNAs

Drosophila melanogaster has contributed immensely towards an understanding of the diverse functional roles of miRNAs and the alterations in regulatory pathways that are caused by these non-coding RNAs [27, 28]. These small non-coding RNAs are involved in either fine-tuning the levels of their target mRNAs or are involved in silencing of noisy target genes [12, 29–33]. Analysis of miRNA mutants has revealed a role for *Drosophila* miRNAs in cell fate specification and differentiation, ageing, neurodegeneration, metabolism, circadian rhythm, stem cell maintenance and proliferation, behaviour, tissue growth and robustness [30, 31, 34–50]. Some of the currently available resources for analysis of *Drosophila* miRNAs include resources for overexpression of 165 miRNAs and a large collection of miRNA knockout mutants that encompass 130 individual miRNAs [51–54]. One of the key challenges for the future is to identify mRNA targets that are most responsive to each miRNA and to understand the roles of miRNA in large biochemical networks [27, 55]. In this chapter we have described detailed protocols of four molecular techniques that have been used extensively by fly researchers for functional characterization of miRNAs.

2 Materials

2.1 RNA Preparation

1. Polypropylene pestles (Sigma Z359947).
2. Disposable gloves (Kimberly Clark).
3. Pipettes.
4. Barrier tips.
5. Microcentrifuge tube stand.
6. TRIzol Reagent (ThermoFisher Scientific 15596026).
7. Chloroform (Sigma 650471).
8. 2-Propanol (Sigma I9516).
9. 75% ethanol (prepared by diluting absolute ethanol in DEPC water).
10. GlycoBlue coprecipitant (15 mg/ml) (ThermoFisher Scientific AM9515).
11. 1.5 ml microcentrifuge tubes (RNase-DNase free).
12. DNAse I (RNAse-free) (NEB M303S).
13. Diethyl pyrocarbonate (DEPC) (Sigma D5758).
14. DEPC water (prepared by adding 0.1% DEPC to milliQ water and stirring overnight at RT followed by autoclaving).
15. Phenol: Chloroform (Sigma P1944).
16. 3M sodium acetate pH 5.3: Dissolve 246.1 g of sodium acetate in 500 mL of DEPC treated water. Adjust the pH to 5.3 with glacial acetic acid. Allow the solution to cool overnight. Adjust the pH once more to 5.3 with glacial acetic acid. Adjust the final volume to 1 L with DEPC-treated water and filter sterilize.
17. Vortex Genie 2 (Scientific Industries SI-0236).
18. Microcentrifuge.
19. −80 °C freezer (Eppendorf CryoCube −86 °C).
20. Eppendorf mixer (Eppendorf 2231000574).
21. Autoclave.

2.2 Small RNA Northern Blot

1. TEMED (Sigma T9281).
2. Heat block (95 °C).
3. UV Stratalinker (Stratagene model UV Stratalinker 1800).
4. 10% ammonium persulfate: Prepare by dissolving 1gm of ammonium persulfate (Sigma A3678) in 10 ml DEPC-treated milliQ water, and store the solution at 4 °C.
5. Electrophoresis and blotting apparatus (BioRad Mini-PROTEAN tetra cell and blotting module).

6. Amersham Hybond-N+ membrane (GE Healthcare Life Sciences RPN203B).
7. Phosphorimager (Amersham Typhoon 5 Biomolecular Imager 29187191).
8. Phosphorimager screen and cassettes (GE Healthcare Life Sciences).
9. [α-^{32}P] dATP (6000 Ci/mmol, 10 mCi/mL).
10. Exo- Klenow DNA polymerase (NEB M0212S).
11. Hybridization oven.
12. Hybridization bottles.
13. UV lamp hand-held.
14. Starfire oligo (Integrated DNA Technologies; designed based on miRNA to be detected) and Starfire Universal template (Integrated DNA Technologies).
15. Sephadex TM G-25 (Cat. No. 17-0033-01).
16. 5X TBE: 1 litre is prepared by mixing 54 g Tris base, 27.5 g boric acid and 20 ml of EDTA (pH 8.0).
17. Ethidium bromide 10 mg/ml (Sigma E1510).
18. 20 X SSPE: 1 l is prepared by mixing 175.3 g of sodium chloride and 27.6 g of sodium phosphate in monobasic in 800 ml water and then adjusting pH to 7.4 with sodium hydroxide and adjusting the volume to 1 litre followed by autoclaving for 20 min.
19. 2X gel loading dye is prepared by mixing 95% v/v deionized formamide, 20 mM EDTA (pH 8.0), 0.025% bromophenol blue, 0.025% xylene cyanol and 0.025% SDS.
20. Blotting paper.
21. Acrylamide: bisacrylamide 19:1, 40% w/v (Fisher BP1406-1).
22. Urea (Sigma U6504).
23. 20% sodium dodecyl sulphate (SDS) (Sigma-Aldrich L3771) is prepared by dissolving 20 gm of SDS in 90 ml milliQ water. Heat to 60 °C and stir with a magnetic stirrer to dissolve SDS. Adjust the volume to 100 ml. Filter sterilize and store at room temperature.
24. Sodium phosphate dibasic heptahydrate (Sigma-Aldrich S9390).
25. TES buffer is prepared by mixing Tris-Cl (10 mM, pH 7.6), EDTA (1 mM, pH 8.0) and sodium dodecyl sulphate (0.5%, w/v).
26. Micro Bio-spin chromatography columns (Bio-Rad, 7326204).
27. TE buffer is prepared by mixing Tris-Cl (10 mM, pH 8.0) and EDTA (1 mM, pH 8.0).

2.3 TaqMan miRNA Assays

1. Superscript III (ThermoFisher Scientific 18080051).
2. SUPERase-In (ThermoFisher Scientific AM2696).
3. 25 mM dNTPs (Prepared by mixing equal volumes of 100 mM dATP, dCTP, dGTP, dTTP) (Fermentas Life Sciences R0181).
4. RNAse-free water (Prepared by treating milliQ water with DEPC).
5. Vortex Genie2 (Scientific Industries SI-0236).
6. PCR machine (Bio-Rad Laboratories C1000 Touch Thermal Cycler).
7. QuantStudio 6 Real-Time PCR system (ThermoFisher Scientific 4485697).
8. Microcentrifuge (Eppendorf 5424R).
9. MicroAmp Clear Adhesive Film (ThermoFisher Scientific 4306311).
10. MicroAmp Fast Optical 96-well Reaction Plate, 0.1 ml (ThermoFisher Scientific 4346907).
11. TaqMan microRNA assays comprise of an RT primer and a TaqMan probe (ThermoFisher Scientific catalog no. determined by the miRNA to be detected).
12. TaqMan Fast Advanced mix (ThermoFisher Scientific 4444557).
13. Barrier Pipette tips.

2.4 Luciferase Sensor Assays

1. psiCHECK2 vector (Promega C8021).
2. Dual luciferase reporter assay system (Promega E1910).
3. Turner Model TD-20/20 luminometer or GloMax® 20/20 Luminometer (Promega E5311).
4. Polypropylene tubes 8 × 50 mm or 96-well plate of luminometer tubes.
5. Haemocytometer.
6. Effectene (Qiagen 301425).
7. Kc167/S2 cell line (DGRC).
8. CCM3 medium with glutamine (Hyclone SH30065.02).
9. 48-well tissue culture plates (Corning Costar TC-treated multiple well plates, CLS 3548).
10. 10× phosphate-buffered saline (PBS) is prepared by dissolving 80 g of sodium chloride, 2.0 g of potassium chloride, 14.4 g of disodium hydrogen phosphate and 2.4 g of potassium dihydrogen phosphate in 800 ml of milliQ water. Adjust the volume to 1 l and filter sterilize. Dilute to 1X for working solution.

3 Methods

3.1 Total RNA Preparation

TRIzol method of RNA extraction has been effectively used for isolating small RNAs such as miRNAs from cells, tissues and whole flies. The basic set-up for RNA preparation includes gloves, microfuge tube stand, pipettes, barrier tips, DEPC water, chloroform, 2-propanol and polypropylene pestle (Fig. 1). The name TRIzol is derived from the fact that this reagent facilitates purification of RNA, DNA and protein from a single sample.

1. Collect samples (5–10 whole larvae or adult fruit flies) in a 1.5 ml microfuge tube. Add 200 μl of TRIzol reagent to sample and homogenize with polypropylene pestles (autoclaved).
2. Add 800 μl of TRIzol reagent and shake vigorously by vortexing at room temperature for 10 min.
3. Add 200 μl of chloroform and vortex the sample briefly. Spin the sample for 15 min at 12,000 rpm at 4 °C.
4. Transfer the supernatant by pipetting to a new microcentrifuge tube and add 500 μl of 2-propanol. Add 1.5 μl GlycoBlue coprecipitant and mix by inverting the tube. Incubate the sample at room temperature for 10 min.

   Note: Using GlycoBlue coprecipitant aids in the recovery of RNA from limited tissue samples by precipitating with the RNA. In addition, the presence of the blue dye increases the visibility of the pellet.
5. Spin the sample for 15 min at maximum speed (12,000 rpm) at 4 °C.
6. Remove the supernatant and wash the RNA pellet with 500 μl of 75% ethanol. (Optional: The sample can be stored at −80 °C for overnight at this step.) Spin the sample at maximum speed for 5 min at 4 °C. Remove the 75% ethanol by pipetting and air-dry the RNA pellet.
7. Resuspend the dried pellet in 90 μl of DEPC-treated water, and vortex for 10 min to dissolve the RNA. Set up DNAse I

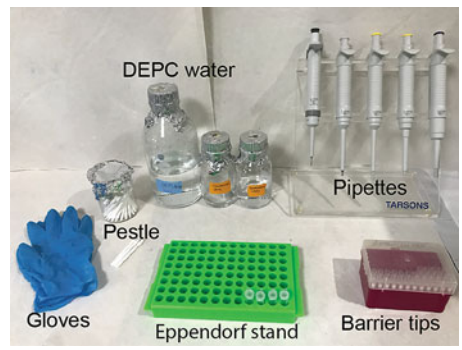

Fig. 1 The basic set-up for RNA preparation

digestion by adding 10 μl of 10X DNAse I buffer and 1 μl DNAse I enzyme. Incubate the sample at 37 °C for 30 min.

8. Perform Phenol: Chloroform extraction as follows. Add 170 μl of DEPC treated water and 30 μl of 3 M sodium acetate (pH 5.3) and 250 μl of phenol: chloroform (pH 4.3–4.7). Vortex and spin for 5 min at RT at max speed. Remove the top aqueous layer into a new tube (autoclaved) and 750 μl (3 volumes) of 100% ethanol. Incubate at −80 °C for 2 h or overnight.
9. Centrifuge at 4 °C for 15 min at 12,000 rpm. Remove supernatant and add 500 μl of 75% ethanol (removes salt from preparation). Spin at 12,000 rpm for 5 min.
10. Remove ethanol and air-dry pellet.
11. Resuspend in 15–50 μl (depending on the amount of starting material) of DEPC water.
12. Quantitate RNA.

3.2 Northern Blots for miRNAs

This protocol has been used for detection of miRNAs (19–24 nucleotides) and precursor miRNAs (50–70 nucleotides) by hybridization of membrane blots [56, 57]. The technique involves separation of RNAs on a denaturing polyacrylamide gel, followed by its transfer to membrane. The RNA is fixed onto the membrane by UV cross-linking and incubated with antisense radioactive probes (Fig. 2). Several variations of this technique, including LNA probes and chemical cross-linking of RNA, have been utilized to increase its sensitivity and allow detection of less abundant miRNAs [58, 59]. We describe here a protocol that has been used for detection of miRNAs from *Drosophila melanogaster* whole animals, dissected tissues from different stages and cell lines [14, 25, 31].

1. Prepare 1 l 15% PAGE; 7.5 M Urea gel by as follows:

5X TBE	200 ml
Acrylamide: bisacrylamide (40%)	375 ml
Urea	450 g
MilliQ water	To 1 l

Stir the mixture until the urea dissolves (may need to heat to 45 °C), and filter and store in a dark-coloured glass bottle at 4 °C.

2. Assemble the gel casting unit, and pour gel into the glass mini gel set-up after mixing 15 ml 15% PAGE; 7.5 M Urea mix with 15 μl TEMED and 150 μl of 10% ammonium persulfate. Use a 10–12-well comb based on the volume of RNA to be loaded. Allow the gel to polymerize for 20–30 min.

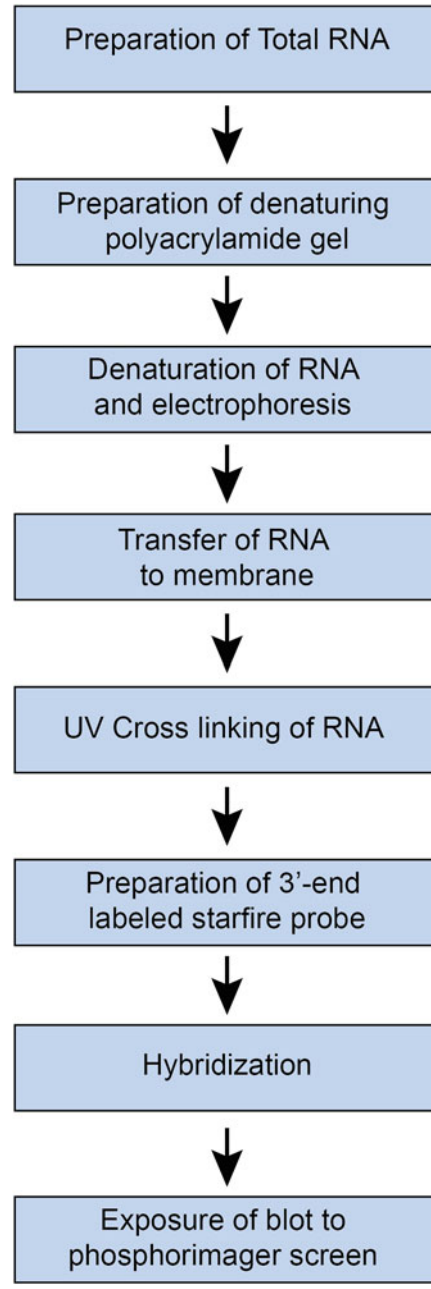

Fig. 2 Workflow for small RNA northern blot analysis. The key steps in the workflow are the preparation of total RNA, preparation of denaturing polyacrylamide gel, denaturation of RNA and electrophoresis, transfer of RNA to the membrane, UV cross-linking of the membrane, preparation of probe, hybridization, exposure of blot to phosphorimager screen and analysis

3. Mix 15–20 μg of total RNA with an equal volume of 2X formamide dye, and heat the samples at 90 °C for 4–5 min. The samples can be stored in ice until ready to load.
4. Remove the gels from casting unit and assemble the gel-running set-up. Fill the tanks with 1X TBE. Wash the wells prior to loading the gel and load the denatured RNA sample in the lane.
5. Run the gel with constant stirring (by loading gel box on a magnetic stirrer) in 1X TBE at 125 V (constant V) for 1.5 h (until the bromophenol blue reaches the bottom of the gel).
6. Cut the Hybond N+ membrane and two blotting papers to the dimension of the small glass plate, and soak the membrane in 0.5X TBE until the gel run is complete.
7. After the gel run, disassemble the glass plates and lift the gel on a blotting paper after wetting it with 0.5X TBE. Wear gloves while loading and setting up transfer to prevent RNAse contamination.
8. Stain the gel with ethidium bromide to confirm uniform loading in all lanes.
9. Prepare the set-up required for transfer as follows:
   (i) Place a sponge pad that has been wetted with 0.5X TBE on one of the sides of the cassette.
   (ii) Place the filter paper with gel on the fibre/sponge pad.
   (iii) Place the prewetted membrane on top of the gel using forceps. Remove air bubbles with a roller.
   (iv) Place another wet blotting paper on top of the membrane.
   (v) Place the prewetted fibre/sponge pad on top of the blotting paper.
   (vi) Close the cassette carefully, trying to avoid moving the gel or membrane, and lock the cassette by sliding the white latch.
   (vii) Place the cassette in the transfer module, and assemble the second cassette if two gels were run.
   (viii) Add the frozen blue cooling unit and fill the tank with chilled 0.5X TBE.
   (ix) Add a magnetic stir bar and place the tank on a magnetic stirrer. The stir bar aids in maintaining uniform buffer temperature and ion distribution in the tank during transfer.
   (x) Place the lid and plug the cables and transfer at 80 V (constant V) for 1 h. Buffer from running gel can be diluted to 0.5X and reused for transfer.

10. After the transfer, UV cross-link membrane with the auto-cross-link setting of Stratalinker 1800 (twice). The membrane should be dry prior to cross-linking.
11. Prepare hybridization buffer as follows:

DEPC-treated MilliQ water	25 ml
0.5 M Na_2HPO_4 pH 7.0	40 ml
20% SDS	35 ml
Total volume	100 ml

12. For prehybridization, place the cross-linked membrane into a hybridization tube with the RNA side facing inwards. For each blot to be probed, add 10 ml of prehybridization solution. Prehybridize the blot at 33–35 °C for 15 min to 1 h with rotation.
13. Prepare the probe by assembling the labelling reaction in a 0.5 ml tube as follows:
    (i) 0.5 μl of Starfire primer
    (ii) 0.5 μl of Starfire template
    (iii) 0.5 μl of NEB exo-Klenow 10X reaction buffer
    (iv) Vortex and incubate/denature in a heat block set at 94 °C for 1 min. Spin the tube in a centrifuge and let it cool at room temperature for 5 min. Then add 3 μl of [α-^{32}P] dATP (6000 μCi/ml) and 0.5 μL of NEB exo-Klenow.
14. Incubate the tube at room temperature for at least 1 h.
15. Stop the reaction by adding 45 μl of TES buffer.
16. Remove unincorporated [α-^{32}P] dATP with a Sephadex G-25 spin column as follows:
    (i) Put the micro bio-spin column in an empty 1.5 ml microfuge tube.
    (ii) Pipette 500 μl of a 50% suspension of completely hydrated Sephadex G-25 into the column and spin at 3000 rpm in a microcentrifuge for 2 min. Remove the eluted water and wash two times with 50 μl of TE buffer. Discard the flow through and tube.
    (iii) Place the micro bio-spin column in a new 1.5 ml microfuge tube, and apply 50 μl of the labelled reaction from Step 15.
    (iv) Centrifuge the column for 3 min at 3000 rpm.
    (v) Add an additional 50 μL of TES buffer and re-elute. Discard the column in radioactive waste.
    (vi) The eluted sample can be directly used in hybridization.
17. After prehybridization, add the entire probe to the prehybridization buffer. Hybridize at 33–35 °C for ~20 h.

18. After hybridization, the hybridization solution can be transferred to a 50 ml conical tube and stored at −20 °C.
19. Wash the blots in several volumes excess of 2xSSPE, 0.1% SDS for a total of three times 20 min or more at 33 °C.
20. Wrap the washed blots in a clear plastic wrap and expose to phosphorimager screen overnight.
21. Read the hybridization signals by scanning the screen in a phosphorimager, and analyse with the appropriate software.
22. For analysis of relative abundance, the blot can be stripped and reprobed with an internal control such as U6 snRNA. The hybridization signal from the internal control can be used for normalization.
23. Northern blots can be reused after stripping the probe by boiling the membrane in 0.1% SDS and then cooling it to room temperature. The blot can be exposed to determine whether the probe is completely stripped. The blot can then be probed for another abundantly expressed miRNA or a normalization control.

3.3 TaqMan miRNA Assays

The TaqMan miRNA assays are extensively utilized for detection and quantification of mature miRNAs. Some of the advantages of these assays are that they are relatively easy and fast to perform. In addition, these assays provide high specificity and sensitivity and allow accurate quantitation and detection of miRNAs from a very limited amount of RNA [60]. The assay is performed using a two-step RT-PCR (Fig. 3). The first step is the reverse transcription, where single-stranded cDNA is reverse transcribed from total RNA using a specific primer for the miRNA.

1. 25 ng of total RNA is used per 10 μl reverse transcription (RT) reaction.
2. The RT reaction is prepared as follows:
   (i) Thaw the reagents on ice, and add the following reagents in a 1.5 ml microcentrifuge tube:

Reagent	Volume in a 10 μl reaction
25 mM dNTPs	0.1 μl
10X RT Buffer	1 μl
25 mM $MgCl_2$	2 μl
0.1 M DTT	1 μl
Superscript III	0.1 μl
Superase-In	0.1 μl
Total	4.3 μl per reaction

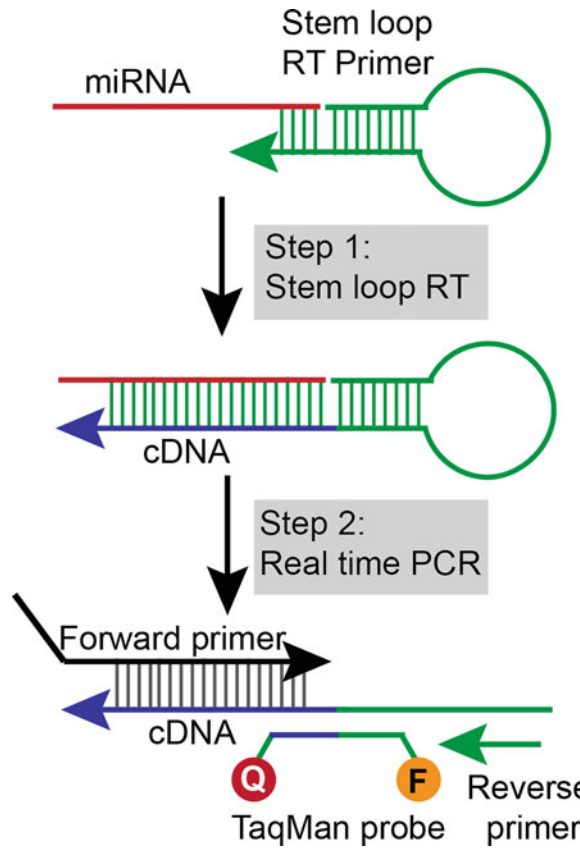

Fig. 3 TaqMan quantitative real-time PCR. TaqMan-based miRNA assays are performed in two steps. The first step is the stem-loop RT reaction in which stem-loop RT primers bind to the 3′end of the miRNA and are reverse transcribed to single-stranded cDNA. In the second step, the RT product is quantitated using TaqMan assays that include a tailed miRNA-specific forward primer, reverse primer and a TaqMan probe. The tailed primer is designed at the 5′ end to increase the melting temperature of the primer

(ii) To each 10 μl reaction, dispense 4.3 μl of the RT reaction mix and 1 μl of RNA sample up to a total of 3 μl RT primer (0.75 μl each of up to 4 miRNA RT primers can be added to each reaction) and RNAse-free water to a total volume 5.7 μl in a 0.2 ml polypropylene tube.

3. Mix the contents in the tube by tapping, and centrifuge briefly to bring the components to the bottom of the tube.
4. Use the following conditions to perform the reverse transcription in the thermal cycler.

Temperature (°C)	Time (min)
16	30
42	30
85	5
4	∝

5. Set the reaction volume to 10 μl and place your tubes in the thermal cycler.
6. Start the reverse transcription program run.
7. The second step is the PCR reaction, where the cDNA is amplified using the TaqMan miRNA assay and PCR master mix.
8. Thaw the miRNA assays on ice and centrifuge briefly to bring spin down. Prepare 1:5 dilution of the cDNA template.
9. The PCR reaction should be performed in two technical replicates for each RT reaction. The PCR reactions are set in a 96-well plate with each PCR reaction containing the following reagents:

Component	Volume (μl)/10 μl reaction[a]
TaqMan probe	0.5
TaqMan mix	5
cDNA	1
RNAse-free water	3.5
Total	10

[a]Scale the volumes to 2.25 reactions for two technical replicates and 12.5% extra to account for pipetting losses

10. Mix the contents and centrifuge briefly to bring the solution to the bottom of the tube.
11. Transfer 10 μl of the PCR reaction for each cDNA to each well of the PCR reaction plate. Seal the reaction plate with an adhesive cover after transferring all the PCR reactions.
12. Centrifuge the reaction plate at 1200 rpm for 1 min to spin down the contents.
13. Load the reaction plate in the PCR machine and set the following cycling conditions:

Temperature	Time	Cycles
95 °C	20 s	1
95 °C 60 °C	1 s 20 s	40

14. Set the appropriate reaction volume for the reaction plate and start the run.
15. In order to use the comparative C_t method, endogenous controls such as 2S rRNA can be used for normalization of expression levels of the target miRNAs.

Note: TaqMan assays are extremely sensitive, and care should be taken to avoid any fluorescent contamination on the outside of the plate or gloves as those will be detected as a signal by the PCR machine block. Hence, extreme caution should be exercised while setting up the reaction, so that the block is not contaminated with the reaction cocktail or RT product. One recommended way to monitor contamination is to employ "no template controls" (NTC) in each experiment.

3.4 Luciferase Reporter Assays

A critical step in functional analysis of a miRNA is the identification of its functionally relevant endogenous target(s), and a number of approaches are being utilized by researchers to reliably identify and validate targets that have been predicted using computational tools [12, 61–64]. One widely used experimental technique for validation of miRNA targets and quantitation of the effects of specific miRNA-target interactions in the *Drosophila* cell culture system is the 3′UTR-luciferase reporter assay [25, 31, 51, 65–67]. In this assay, the 3′UTR from a potential miRNA target is fused to the end of a luciferase reporter gene. If a 3′UTR harbours a miRNA binding site, the reporter activity will change with variation in the concentration of the functional miRNA. This reporter system can also be used to assess the relevance of a particular miRNA binding site by engineering mutations to disrupt binding of a miRNA. Other controls that can be used for the luciferase miRNA sensor assay include the empty vector control (no 3′UTR) or control with the 3′UTR of *Drosophila melanogaster white* gene lacking any miRNA binding sites. This serves as a control for transfection efficiency and will provide a high luciferase signal. In addition, a synthetic 3′UTR harbouring the perfect miRNA binding sites may be utilized as a control for miRNA expression or miRNA mimic activity. The key steps in the luciferase assay include designing the constructs, transfection of reporter constructs and pri-miRNA reporters, luciferase reporter assays and analysis of data (Fig. 4a).

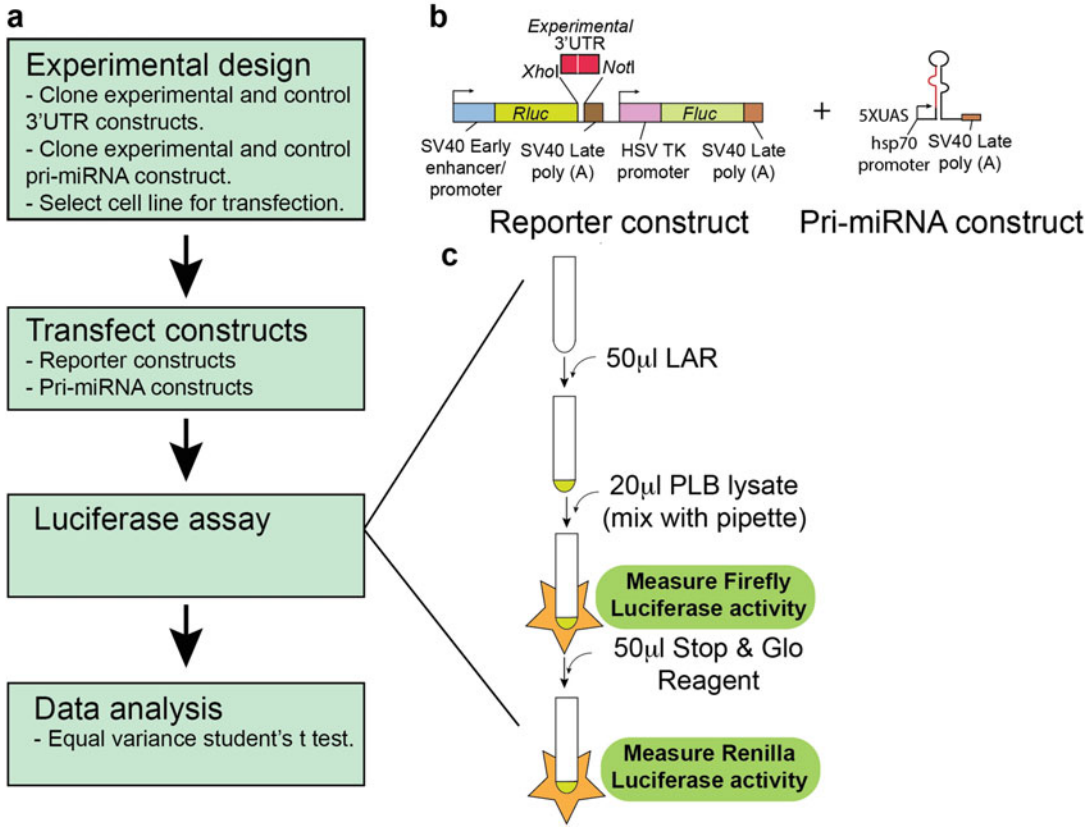

Fig. 4 Workflow for luciferase miRNA sensor assays. (**a**) The key steps in this workflow are experimental design, transfection of the reporter and pri-miRNA constructs, luciferase assay and data analysis. (**b**) Schematic of the psiCHECK 2 vector used for cloning the experimental and control 3′UTR sequences (left) and the pri-miRNA construct cloned in pUAST attB vector (right). (**c**) Format of the dual luciferase assay using a manual luminometer

1. Clone the 3′ UTR of the potential miRNA target into siCHECK-2 series of vectors. The primary reporter gene in this vector is a synthetic version of Renilla luciferase, *hRluc*. To facilitate fusion of the target gene sequences or synthetic miRNA binding sites to Renilla luciferase gene, a multiple cloning region has been engineered downstream of its stop codon. The restriction sites in the multiple cloning site can be used to create genetic fusions of the Renilla reporter with any gene of interest (Fig. 4b).
2. These vectors also include the firefly luciferase gene as a second reporter gene that can be utilized for normalization of the Renilla luciferase signal (Fig. 4b).
3. In addition to cloning the 3′UTR of the target gene, the pri-miRNA gene of interest also needs to be cloned into a vector that allows high expression (e.g. pUASTattB)

(Fig. 4b). Alternatively, miRNA mimics (synthetic mature miRNAs from Dharmafect) can be transfected along with luciferase reporters.

4. Seed 200 µl cells (Kc167/S2) in 48-well plates (at a density of 1.0–3.5 $\times$ 10^6 cells/ml), on the day of transfection.

   Note: The cell line to be used for luciferase sensor assays should be highly transfectable and should preferably have low endogenous levels of the miRNA being analysed. If the endogenous level of the miRNA is high, it will be hard to detect the changes in luciferase activity.

5. Transfect reporter constructs and pri-miRNA constructs using the Effectene Transfection Reagent. The transfections are carried out in triplicates
6. Dilute 300 ng of the Gal4 plasmid (to drive expression of the primary miRNA transcript), UAS pri-miRNA and miRNA sensor in 50 µl of the DNA-condensation buffer, Buffer EC.
7. Add 6 µl of enhancer and mix by pipetting 3–5 times.
8. Incubate at room temperature (15–25 °C) for 5 min.
9. Add 12 µl Effectene Transfection Reagent to the DNA-Enhancer mixture. Mix by pipetting up and down five times.
10. Incubate the samples for 10 min at room temperature (15–25 °C) to allow transfection complex formation.
11. Add 70 µl growth medium (can contain serum and antibiotics) to the tube containing the transfection complexes. Mix by pipetting up and down twice, and immediately add 40 µL of the transfection complexes dropwise onto the cells in each well (three replicates). Gently swirl the dish to ensure uniform distribution of the transfection complexes.
12. Seventy-two hours post-transfection, remove the 48-well tissue culture plates with the Kc167/S2 cell line from the incubator.
13. Transfer the cells to 1.5 ml microcentrifuge tubes with a 1 ml pipette.
14. Spin down the cells in a microcentrifuge at 3000 rpm for 3 min, and aspirate the cell culture medium.
15. Wash the cells with 500 µl of phosphate-buffered saline (PBS). Spin down the cells at 3000 rpm for 3 min and aspirate the PBS.
16. Apply 50 µl of 1X passive lysis buffer (PLB) to the cell pellet, and resuspend with a P200 pipette to obtain a homogenous suspension.
17. Incubate the tubes at RT for at least 10 min.
18. Clear the lysate of cellular debris by spinning at 1200 rpm for 2 min. Use the supernatant for luciferase assay.

19. Prepare Luciferase Assay Reagent II (LAR II) by resuspending the provided lyophilized Luciferase Assay Substrate in 10 ml of the supplied Luciferase Assay Buffer II (Fig. 4c).

    It is recommended that once the luciferase assay reagent is reconstituted, working aliquots be made and stored at −70 °C for the longer term.

20. Prepare an adequate volume of Stop & Glo to perform the required number of assays (50 μl reagent per assay). Stop & Glo substrate is supplied at a 50X concentration. Add 1 volume of 50X Stop & Glo substrate to 50 volumes of Stop & Glo buffer in a polypropylene tube. The Stop & Glo Reagent should always be prepared immediately before use as the reconstituted reagent is not very stable at room temperature or at 4 °C.

21. Luciferase assays are carried out with a Dual-Glo luciferase assay kit in a Turner Model TD-20/20. Predispense 50 μl of LAR II into the required number of luminometer tubes and program the luminometer. For single-sample luminometers such as Turner Model TD-20/20, the equipment should be configured to measure light emission over a defined period. The company recommends programming the luminometer to provide a 2 s pre-read delay, followed by a 10 s measurement period.

22. Transfer 15–20 μl of cell lysate into the luminometer tube containing the LAR II and mix by pipetting. Take care to avoid bubbles. Vortexing is not recommended when mixing the lysate with LAR II as it may lead to the coating of the sides of the tube with a luminescent solution that can escape mixing with the subsequently added Stop & Glo solution reagent.

23. Place the tube in the luminometer and note the reading for firefly luciferase activity (Fig. 4c).

24. Dispense 50 μl of Stop & Glo reagent in the tube containing the sample lysate and LAR II, and mix by pipetting.

25. Replace the tube in the luminometer and record reading of Renilla luciferase activity (Fig. 4c).

26. Discard the tube and proceed to the next sample.

27. Fold repression by a miRNA is calculated by comparison with assays in which an empty vector (not expressing the miRNA) or a non-targeting control miRNA is used.

28. The assays are usually performed in three replicates, and the data are analysed by equal variance student's t test.

    Note: As an extension of the luciferase assays, researchers can also test the significance of individual miRNA binding sites by mutagenizing individual miRNA binding sites in these reporters, in a scenario where there are multiple miRNA binding sites or multiple miRNAs regulating the same target mRNA.

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