Chapter 18

Discovery of Novel microRNAs in Aging *Caenorhabditis elegans*

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Abstract

The rapid development of deep sequencing technologies over the last few years and concomitant increases in sequencing depth and cost efficiencies have opened the door to a ever-widening range of applications in biology—from whole-genome sequencing, to ChIP-seq analysis, epigenomic and RNA transcriptome surveys. Here we describe the application of deep sequencing to the discovery of novel microRNAs and characterization of their differential expression during adulthood in *Caenorhabditis elegans.*

Key words *Caenorhabditis elegans*, microRNA, Differential gene expression, Deep sequencing

1 Introduction

microRNAs (miRNAs) are short, endogenous RNAs with functions in post-transcriptional regulation in a wide variety of eukaryotes [1]. As a novel class of gene regulatory elements, miRNAs have been implicated in a wide variety of functions during development in plants and animals and accumulating evidence points to functions of certain miRNAs as oncogenes and tumor suppressors [2]. Recent breakthroughs expand the repertoire of post-developmental functions for miRNAs and suggest that alteration of miRNA levels can directly affect the health span and longevity of organisms. In work pioneered in *C. elegans* we have shown that mutations to certain miRNAs can significantly lengthen or shorten nematode life-span [3, 4]. At least four of these miRNAs were first identified as miRNAs that are up-regulated during adulthood in *C. elegans* [4]. Importantly, these miRNAs function in longevity at least partially through conserved pathways such as the insulin-like and DNA damage response pathways. We have shown that these functions are at least partially mediated by miRNA repression of genes in these pathways, consistent with stereotypical miRNA-mediated regulation. These results expand the universe of known

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functions for miRNAs but it is clear that much remains to be discovered. Surveys of miRNA mutations in *C. elegans* have so far identified only a handful of miRNAs with obvious phenotypes, and the vast majority of miRNAs have unknown functions [5–7]. Furthermore, many other miRNAs remain undiscovered. In our deep sequencing surveys of aged nematodes, we identified and validated the expression of 17 novel miRNAs [4, 8]. Finally, miRNAs have the potential to target a vast number of genes. Together, these results suggest possible functional roles of many new miRNAs during adulthood and emphasize the power of deep sequencing technologies in uncovering these new regulatory factors or their targets. Here we discuss methods for discovery and differential expression analysis of miRNAs during adulthood in *C. elegans*.

2 Materials

2.1 C. elegans Maintenance and Growth

1. Strains: Obtain *C. elegans* strains from the *Caenorhabditis Genetics Center (CGC)*. Some useful strains for the discovery of novel miRNAs during aging: wild-type N2 (Bristol), *daf-2(e1370)* and *alg-1(gk214)*. All mutant strains are backcrossed against the reference N2 strain at least three times before further characterization.
2. Nematode growth medium (NGM): Mix 6 g NaCl, 34 g agar and 5 g BACTO peptone in 2 L of water. After autoclaving, let cool to 55 °C in a water bath for 15 min and then mix the following, in order, allowing each to mix completely: 50 ml 1 M potassium phosphate pH 6.0 (108.3 g KH_2PO_4, 35.6 g K_2HPO_4, in 1 L of water); 2 ml 5 mg/ml cholesterol, 2 ml 1 M $MgSO_4$ and 2 ml 1 M $CaCl_2$. Finally, dispense the NGM solution into petri dishes. Fill plates 2/3 full of agar (for small 6 cm plates, that would be about 10 ml of NGM).
3. M9 buffer: Mix 3 g of KH_2PO_4, 5 g NaCl, and 6 g Na_2HPO_4 in 1 L water. After autoclaving, add 1 ml 1 M $MgSO_4$.
4. FUDR: Prepare 40× stock solution of 5′-fluoro-2′deoxyuridine (Roche) in water (4 mg/ml) and filter-sterilize through 0.2 μm filter discs. Store individual aliquots at 20 °C. Once thawed, discard unused FUDR solution.

2.2 RNA Isolation, Cloning, Deep Sequencing, and qRT-PCR

1. Siliconized, RNase/DNase-free microcentrifuge tubes.
2. TRIzol reagent (Roche).
3. miRVana miRNA isolation kit (Ambion).
4. Superscript III Reverse Transcriptase (Invitrogen).
5. Turbo DNAfree kit (Ambion).
6. DGE-Small RNA Sample Prep Kit ver. 1.0 (Illumina).

7. Taqman miRNA Assays (Applied Biosystems).
8. Custom Taqman miRNA Assays (Applied Biosystems).
9. Taqman miRNA Reverse Transcription Kit (Applied Biosystems), containing Multi-scribe RT (50 U/μl), RNase Inhibitor (20 U/μl), 10 mM dNTP mix.
10. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems).
11. miScript II RT Kit (Qiagen), containing 5× miScript HiFlex Buffer.
12. miScript Primer Assays (Qiagen), including RNA6B control.
13. miScript SYBR Green PCR Kit (Qiagen).

2.3 Bioinformatic Analysis

1. Required input files:

   C. elegans genome: cel_ws201.fa from wormbase: ftp://ftp.wormbase.org/pub/wormbase/genomes/c_elegans/sequences/dna/

   C. elegans mature miRNA sequences, mature_cel.fa in fasta format obtained from miRBase (mirbase.org):

   C. elegans precursor miRNA sequences, mature_other.fa, obtained from miRBase.

   mature miRNA sequences of other species, precursor_cel.fa, obtained from miRBase.

   Deep sequencing reads, in fastq or fasta format.

2. Software packages:

   Differential expression of miRNAs and novel miRNA discovery: miRDeep2 [9]. Other prerequisite software can be installed automatically or manually (*see* miRDeep2 documentation and tutorial).

   Statistical analysis of differential expression: Any of a number of software packages: DESeq, DEGseq, edgeR, baySeq, mirZ, or SAMseq [10–15].

3 Methods

3.1 RNA Isolation from Synchronized Populations of Adult C. elegans

Routine *C. elegans* culture procedures are carried using standard, published protocols [16].

1. Allow animals to grow for at least three generations on *E. coli* strain OP50 without starvation. In order to obtain 15–20 μg of RNA, a mixed population of animals (medium density population, unstarved, on a NGM plate) are harvested from 10 to 20 small (6 cm) NGM plates or 1–2 large (15 cm) NGM plates using 10–15 ml of M9 buffer (*see* **Note 1**). The worm pellets

are washed 3–4 times with M9 to remove OP50. These animals are then bleach-treated in 0.1 % (vol/vol) sodium hypochlorite and 0.5 M NaOH in total volume of 5 ml in a 15 ml falcon tube for a maximum of 5 min and washed with M9 buffer 5–6 times (*see* **Note 2**). The remaining eggs are then resuspended in 20–50 ml of M9 buffer and incubated overnight at 20 °C (or appropriate temperature for temperature sensitive mutants) with gentle shaking/nutation in a 200 ml Erlenmeyer flask covered loosely with aluminum foil (to prevent hypoxia).

2. Synchronized, starved L1-stage larvae are plated on large NGM plates containing OP50 and grown at 20 °C (or appropriate temperature) until late L4-early adulthood stage. At this point, animals are transferred using M9 to plates containing 5′-fluoro-2′-deoxyuridine (FUDR) (0.1 mg/ml) (to prevent progeny production) and maintained at 20 °C (or appropriate temperature). At selected time points of adulthood, animals are harvested using M9, washed 6 times with M9 buffer, flash frozen in liquid N2, and stored at −80 °C for later analysis.
3. Worm lysis for RNA extraction: Worms can be lysed by traditional mortar and pestle grinding of frozen worm pellets or by alternative methods. Currently, we lyse worm preps using Zirconia beads (1 mm, Biospec) in a Fastprep24 homogenizer (MP Biomedicals), which permits rapid and uniform lysis of multiple *C. elegans* samples at one time.
4. Small RNA extraction: Isolate total RNA by guanidine thiocyanate hydrochloride/phenol method or using the commercial TRIzol reagent (Roche) (*see* **Note 3**). Small RNAs can be harvested from total RNA by size selection in the presence of ^{32}P-labeled RNA oligonucleotide size markers using polyacrylamide gel electrophoresis (PAGE) [4, 17]. Alternatively, isolate small RNAs using specialized commercial kits, such as the miRVana miRNA isolation kit (Ambion) following the manufacturer's protocols. We have had good success extracting RNA enriched for small RNAs by either method [4, 8].
5. Small RNA cloning: cDNA libraries of small RNAs are prepared using the DGE-Small RNA Sample Prep Kit ver. 1.0 (Illumina) following the manufacturer's recommended protocols. Small RNAs corresponding to sizes of 10–30 nucleotides are selectively purified and ligated to adapters and amplified by RT-PCR (*see* **Notes 4** and **5**). Consider the use of multiplexing in order to substantially decrease the cost of sequencing and increase the number of samples to be sequenced [18]. Purified DNA is then loaded on an Illumina Flow Cell for cluster generation and sequenced according to the manufacturer's instructions. For miRNA sequencing, 36-cycle, single-end read sequencing is appropriate—following the manufacturer's protocols for the DGE-Small RNA Cluster Generation Kit and 36 Cycle Solexa (Illumina) Sequencing Kit.

3.2 Analysis Using miRDeep2

1. Collect sequencing data and ensure that it is in a format that is compatible with miRDeep2 [9]. Typically, sequencing data from Illumina will be in fastq format, which is compatible with miRDeep2. However, if necessary (*see* miRDeep2 documentation for information on compatible input formats), convert the sequencing file into an acceptable input format using any number of appropriate tools—one option is the online web server, Galaxy [19].
2. To facilitate sequence alignment, obtain the *C. elegans* genome from Wormbase and build an index of it using Bowtie and the Bowtie-build command included in miRDeep2: bowtie-build cel_genome.fa cel_genome

   This will generate several .ebwt files which miRDeep2 will use.
3. Create a text file, config.txt, containing the filenames of your samples, and a chosen sample ID (three-letter format), with one sample per line, in the general format:

Filename	SamplenameID (3 letter format)
N2_young.fa	N2y
N2_old.fa	N2o
daf2_young.fa	d2y
daf2_old.fa	d2o

4. Collapse deep sequencing reads and map against reference genome by running script "mapper.pl" (part of miRDeep2 package) from command line:

   mapper.pl config.txt -d -k AGCAGTGACGTGTGTGTGT -c -m -i -j -l 17/-p genome_cel -s reads.fa -t reads_vs_genome.arf

   Explanation of parameters:

   config.txt contains list of samples (*see* Subheading 3.3, **step 3**).

   -d tells mapper to use config file for names of input files to process.

   -c specifies that read samples are in fasta format.

   -m collapses the reads.

   -k trims the 3′ adapter sequence.

   -i converts rna to dna.

   -j removes any sequence other than ATGC.

   - l 17 removes any sequences shorter than 17 bases.

-p genome_cel specifies the reference genome.

-s reads.fa specifies the filename of the output.

-t prints read mappings to this file (which is used in subsequent steps).

The output of this mapping procedure will be a "reads.fa" file (or whatever was chosen as the output filename) containing all the reads that match the reference genome in collapsed format. The name of each read will contain the samplenameID and a_x index, where x represents the number of identical, collapsed reads found in the sequencing data.

5. To determine differential expression, run the miRDeep2 script "quantifier.pl":

quantifier.pl -p precursor-cel.fa -m mature12-cel.fa -r reads.fa -c config.txt -g 1 -t cel

where -p precusor-cel.fa specifies the file containing known miRNA precursors from mirbase.

-m mature-cel.fa specifies the file containing mature miRNA sequences.

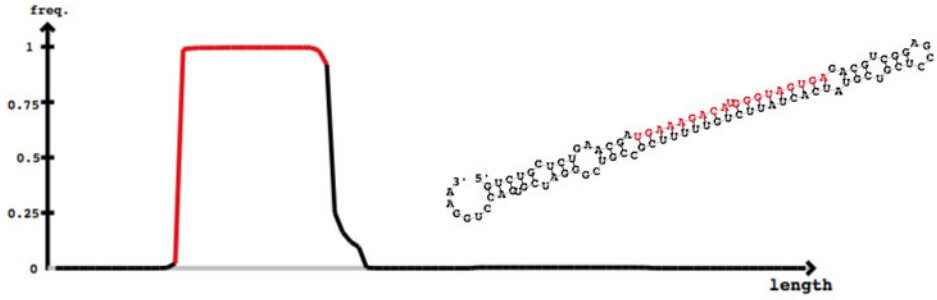
-r reads.fa specifies the input file of deep sequencing reads (generated by mapper.pl).

-c config.txt contains list of samples and sample code (3-letter).

-g 1 allows one mismatch.

-t cel specifies the reference genome (3-letter species code).

As output, the quantifier.pl script will generate a file named expression.html which is viewable in a browser and contains a summary of the data and links to pdfs that show the miRNA mappings, with pileup of reads, read counts, frequency diagrams, and signature and secondary structure of the precursor hairpins (Fig. 1). Optional parameters can be used in quantifier.pl to report mismatches, take star sequences into consideration, or alter the mapping parameters to the precursors sequences (*see* **Note 6**). The expression tables generated by quantifier.pl script will include both raw number of reads as well as normalized reads. It is important to consider which normalization method is appropriate to the biological question being studied (*see* **Note 7**) and to ensure that proper biological controls and replicates are integrated into the study design (*see* **Note 8**). The quantifier.pl program will also generate a .csv file with full read counts for all known miRNAs which can be exported to spreadsheet programs and/or to various packages for further statistical analysis (*see* **Note 9**).

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                                cel-miR-71
5'- gucugcucugaacgaugaaagacauggguaguga gacgucggagccucgucguaucacuauucuguuuuucgccgucgggaucgugaccuggaa  -3'   exp
    (((((.(((..(((.(((((((((.(((((((((.(((.(((....))).)))..)))))))))))))))))))).)))..)))..))).))).....   reads   mm   sample
    ..............Caugaaagacauggguagug.....................................................................   1       1    d2o
    ..............Aaugaaagacauggguaguga....................................................................   10      1    d2o
    ..............Aaugaaagacauggguagugag...................................................................   2       1    d2o
    ..............augaaagacauggguag.........................................................................   1       0    d2o
    ..............Cugaaagacauggguagu........................................................................   1       1    d2o
    ..............augaaagacauggguagu........................................................................   3       0    d2o
    ..............Cugaaagacauggguagug.......................................................................   2       1    d2o
    ..............Gugaaagacauggguagug.......................................................................   2       1    d2o
    ..............augaaagacauggguagug.......................................................................   7       0    d2o
    ..............augaaagacauggguagugU......................................................................   22      1    d2o
    ..............augaCagacauggguaguga.....................................................................   1       1    d2o
    ..............augaaagacaugggCaguga.....................................................................   1       1    d2o
    ..............Uugaaagacauggguaguga.....................................................................   2       1    d2o
    ..............augaaagacauggguaguga.....................................................................   111     0    d2o
    ..............Cugaaagacauggguaguga.....................................................................   53      1    d2o
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Fig. 1 Analysis of deep sequencing reads of known miRNAs using miRDeep2. Frequency diagram, pileup of reads, identification of mismatches (mm), 2° structure of precursor, and mature sequence highlighted in *red*

6. To identify novel miRNAs, use the "miRDeep2.pl" script:

   miRDeep2.pl reads.fa genome_cel.fa reads_vs_genome.arf mature-cel.fa \

   mature-other.fa precursors-cel.fa -t cel -b -2 2>report.log

   where reads.fa and reads_vs_genome.arf represent the files generated by mapper.pl; genome_cel.fa is the reference genome; and mature-cel.fa and precursors-cel.fa are the *C. elegans* mature miRNAs and precursors from miRBase. The file mature-other.fa contains miRNA sequences for other species, with potential overlap or homology with new candidate miRNAs.

   -b minimum cutoff score for predicted novel miRNAs to be displayed in table. Default is 0. Adjust or -1 or -2 to identify more candidates (including more false positives).

   The miRDeep2.pl script will generate various output files, including a table in html format that summarizes the information for the top candidate novel miRNAs identified by the program, including miRDeep score, *p*-values, read counts

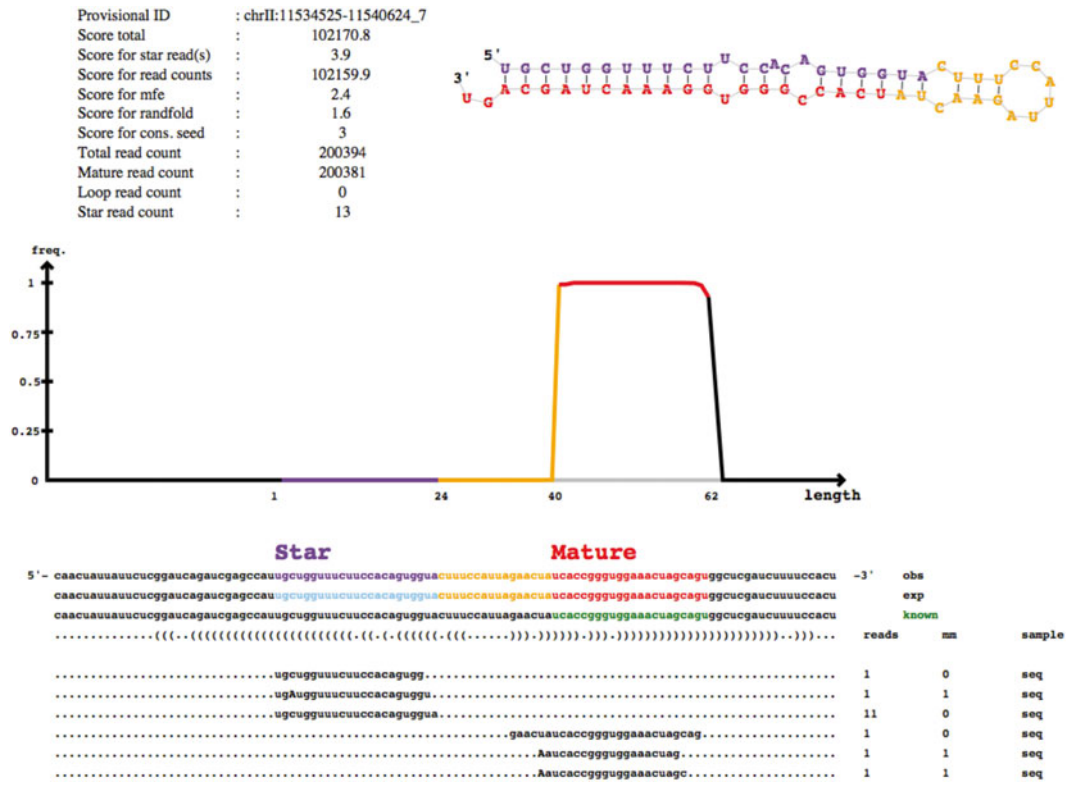

Fig. 2 Identification of novel miRNAs using miRDeep2. Frequency diagram, 2° structure of putative precursor, and identification of potential mature and star sequences from deep sequencing data

(mature, star, and loop regions), homology to other miRNAs, and sequences of predicted mature, star, and precursor hairpins. In addition, there will be links to read pileups, frequency diagrams, and secondary structure diagrams, including predicted mature and star sequences (Fig. 2). The reported miRDeep2 scores and associated information is used to narrow down the top candidate, novel miRNAs that should be further characterized (*see* **Note 10**).

3.3 Validation of miRNA Expression

For validation by qRT-PCR, extract total RNA as described before (Subheading 3.1, **step 4**). To confirm expression changes of known miRNAs during aging, RNA should be obtained from synchronized animals at relevant time-points during adulthood. To validate novel miRNAs, RNA should be obtained from synchronized wild-type N2 animals as well as *alg-1(gk214)* mutants in young adulthood and/or later time points (according to its expression pattern from deep-sequencing) (*see* **Note 11**).

1. Northern analysis of known miRNAs: For confirmation of the expression of known miRNAs, we use ~15–20 μg of total RNA

obtained from animals at different points of adulthood. We design probes using the StarFire Oligonucleotide Labelling Kit (from Integrated DNA Technologies) which are complementary to the mature sequences of miRNAs in question. We use a probe for U6 small nuclear RNA sequence as a normalization control (5′-GCA GGG GCC ATG CTA ATC TTC TCT GTA TT). As an additional control, we utilize a probe for *miR-66* (5′-TCA CAT CCC TAA TCA GTG TCA TG), whose expression remains constant during development [20, 21], during aging or in *daf-2(e1370)* mutants [4] (*see* **Note 12**).

2. Quantitative RT-PCR of novel miRNAs: RNA samples (1–10 μg) are treated with Turbo DNAse according to the manufacturer's protocols (Ambion, Turbo DNAfree kir). The expression of miRNAs is then measured using Taqman small RNA Assays (Applied Biosystems). To validate candidate novel miRNAs, custom Taqman assays are purchased from Applied Biosystems and tested according to the manufacturer's protocols, except that we use a Lightcycler 480 qPCR instrument. We adapted qPCR cycling conditions appropriate for miRNA Taqman detection on LightCycler 480 instruments [22]:Enzyme activation: 95 °C for 10 min; amplification (45 cycles): 95 °C for 15 s (ramp: 4,4 °C/s, analysis mode: quantification), 60 °C for 60 s (ramp: 2,2 °C/s); cooling: 40 °C for 30 s (ramp: 2 °C/s). The detection format was set to "Mono Color Hydrolysis Probe" and the second derivative maximum method was used for absolute quantification. Expression levels are normalized against endogenous control, the small nucleolar RNA (snoRNA), U18 (Taqman). For purpose of validating the expression of these candidate miRNAs, we consider only miRNAs with amplification < 35 cycles and those miRNAs whose expression is reduced in *alg-1(gk214)* mutants (Fig. 3).

3. Quantitative RT-PCR of known miRNAs: For known miRNAs, in addition to the Taqman method we also use miRScript qRT-PCR (Qiagen). RNA samples (1–10 μg) are treated with Turbo DNase according to the manufacturer's protocols (Ambion, Turbo DNA-free). DNase-free RNA (1 μg) is converted to cDNA as per the manufacturer's protocols (Qiagen), using the "HighFlex" protocol, which allows measurement of the levels of small RNAs and large RNAs simultaneously. PCR cycling conditions are as per the manufacturer's protocols. Typically, we use primers against mRNA genes CDC-42, PMP-3, and Y45F10D.4 for Geometric Means Normalization (*see* **Note 13**).

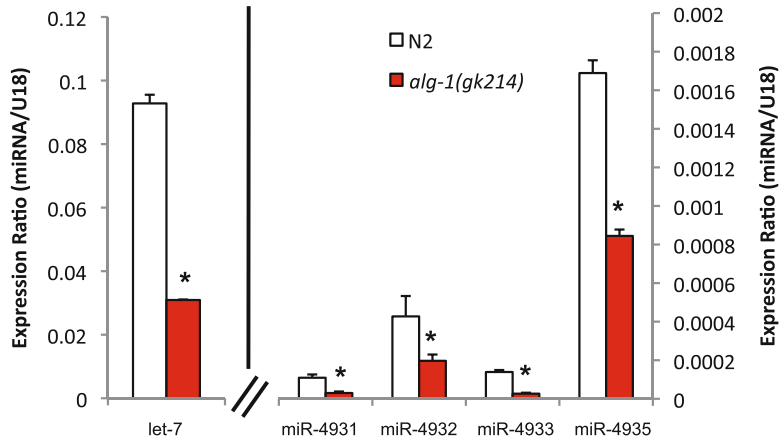

Fig. 3 Validation of expression of novel mi RNAs in aging *C. elegans*. The expression of four candidate miRNAs identified by deep sequencing was confirmed by Taqman qRT-PCR. Consistent with their classification as miRNAs, their expression was significantly reduced in *alg-1*(*gk214*) mutant animals. The expression of known miRNA *let-7* is shown as a positive control (image reproduced from [4] with permission from Elsevier)

4 Notes

1. RNA yields. Total RNA: 8 μg from 25 μl of packed pellet of worms (~300 worms from one small (6 cm) plate). On large plate (16 cm), 2500–3000 worms yielded ~100–150 μl packed worm pellet, which yielded ~40–100 μg of RNA.
2. For maximum yield of eggs, bleach treat a plate enriched for gravid adults. During bleach treatment, vortex tube every ~2 min and observe through microscope. Once gravid adults rupture (body will bend and split), arrest bleach treatment by adding 2 volumes of M9 buffer, spin down, and aspirate supernatant. It is important to then wash bleached worm pellets at least 4–6 times with M9 to remove any residual bleach.
3. Use reagent/conditions that do *not* deplete small RNAs (i.e., use regular TRIzol, not "LS TRIzol"). Make sure to use siliconized, RNase-free tubes throughout to prevent adsorption of RNA to tubing. Glycogen or Glycoblue can be utilized as a carrier to facilitate precipitation and visualization of RNA pellets after precipitation. RNA pellets after precipitations should be air-dried until ethanol is fully evaporated (~5–10 min) but they should not be excessively dried, or the pellet will become difficult to resuspend.
4. Cloning other small RNAs: Piwi-interacting RNAs (piRNAs, i.e., 21U-RNAs) will be also cloned by this procedure. For

cloning of other small RNAs, such as siRNAs, consider alternative protocols [23, 24].

5. Small RNA cloning considerations: To avoid biases in miRNA representation, these are the most important factors to consider during cloning: maintain similar conditions for 3′- and 5′-adapter ligation, use similar concentrations of input RNA for all samples and avoid too many cycles of PCR after RT extensions [25].
6. Additional (optional) parameters in quantifier.pl: Using the -g option to allow mismatches between read and precursor mappings might allow the identification of interesting miRNAs isoforms (such as edited sites). Other possible options to consider are -s star.fa, to compare sequences against a file of star sequences from miRBase. This will allow the determination of mature and star sequences mapping to the precursors. Options -e and -f specify how far upstream (-e, default 2 nucleotides) and downstream (-f, default = 5 nucleotides) of the mature/star sequence the program should consider as a match to the sequencing sequences.
7. As of version 2.0.4 of miRDeep, quantifier.pl normalizes miRNA reads according to total number of miRNAs in each sample. If one suspects that there might be biological reasons for the total numbers of miRNAs to be different in different samples, one might normalize against total number of genome-matching reads. This can be done by recovering the raw miRNA count number from the .csv file generated by quantifier.pl. As an alternative, during RNA preparation one might consider "spiking" the pool with a known amount of one or more known oligonucleotide "calibrator" sequences (that do not match the reference genome) and which can be used as a normalization controls after sequencing [18, 26, 27].
8. Estimation of miRNA abundance: In general, it is not appropriate to compare the abundance of one miRNA versus another within the same sample due to biases inherent to the small RNA cloning procedures, and which depend on the secondary structure and sequence of each miRNA [25]. These biases, however, do not affect the estimation of the relative abundances of each miRNAs between samples [25]. Therefore, with appropriate controls and normalization it is generally possible to determine relative changes in expression of individual miRNAs across different samples.
9. Statistical analysis of differential expression: In order to determine statistical significance of changes in expression of miRNAs read counts determined by miRDeep2, one can export the .csv data into any of a number of tools for differential analysis of deep sequencing data, such as DESeq, DEGseq, edgeR, baySeq, mirZ, and SAMseq [10–15].

10. Analysis and curation of novel miRNA identifications by miRDeep2: The reported miRDeep2 scores and associated information is used to narrow down the top candidate, novel miRNAs that should be further characterized. In particular, we consider characteristics reported by miRDeep2 such as read scores, secondary structure of putative precursor hairpins, and homology with known miRNAs in other species. Manual curation is essential too. Although miRDeep2 attempts to exclude reads that overlap with annotated regions of the genome, it is important to confirm that the latest annotations are consistent with possible miRNA classification. For example, miRNAs should not exist within annotated coding region of an open reading frame (either sense or antisense), and it is unlikely that it would be encoded in 5′ or 3′ UTRs of a known gene. Therefore, we manually perform blast analysis of candidate miRNAs against the reference genome to ensure that a candidate miRNA does not overlap with already annotated regions and also to discover possible overlap with annotated small RNAs in other species. Sequences that survive this curation process and exhibit good secondary structures characteristics are then considered for validation by qRT-PCR.
11. Validation of novel miRNA expression: ALG-1 is a miRNA-associated factor and it is known that functional ALG-1 is required for mature miRNA accumulation [28]. Therefore, to confirm that a putative novel miRNA is indeed expressed, we check (a) if it is expressed by qRT-PCR, and (b) if its level is reduced in *alg-1(gk214)* animals. In our experience, we observe a two- to fivefold reduction in bona fide miRNA levels in *alg-1(gk214)* mutants as measured by qRT-PCR.
12. Validation of differential expression of miRNAs obtained by deep sequencing can be accomplished by typical methods of RNA expression analysis, such as Northern analysis or qRT-PCR. However, for miRNAs that are expressed at low levels, especially candidate novel miRNAs, it will be difficult to measure their expression by Northern. Therefore, we typically validate the expression of novel miRNAs using qRT-PCR.
13. Validation of differential expression of known miRNAs: The advantage of quantitative PCR using miScript is that it allows the quantification of small RNAs and large RNAs simultaneously from the same cDNA sample. This permits the use of multiple RNAs as endogenous controls, allowing for better normalization of data. We utilize the genes CDC-42, PMP-3, and Y45F10D.4, and perform geometric means normalization of our qRT-PCR data as recommended [29]. As an additional endogenous control we utilize the small RNA, RNA6B (Qiagen).

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