Research Square

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MicroRNome by methylation-dependent sequencing (mime-seq)

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Method Article

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Abstract

The description of precise miRNA expression patterns is crucial to understand what these small RNAs contribute to animal development and physiology. High-throughput sequencing of miRNAs from individual developmental stages has provided insight into temporal regulation but often lacks the cellular resolution to link miRNA-function to the biology of distinct cell types within complex tissues. Here, we provide a protocol for _microRNome by methylation-dependent sequencing_ \(mime-seq), a chemical small RNA-tagging approach followed by chemoselective, high-throughput sequencing that enables the identification of tissue- and cell type-specific miRNA profiles in animals in a sensitive and reproducible manner. Using this strategy, we have uncovered the miRNA composition of specific cells and tissues within _C. elegans_ and _Drosophila_, providing insights into miRNA spatial distribution at high resolution. This protocol accompanies Alberti et al \(Nature Methods, published online February 26, 2017; 10.1038/nmeth.4610)

Introduction

Mime-seq relies on the cell-specific 3´ terminal methylation of animal miRNAs by selective expression of the small RNA duplex-specific methyltransferase HEN1, which is responsible for miRNA methylation in plants. Because methylated miRNAs are resistant to chemical ribose oxidation, tissue-specific animal miRNAs can be recovered by established methylation-specific cloning protocols, followed by high-throughput sequencing. See "Figure 1":http://www.nature.com/protocolexchange/system/uploads/6331/original/Figure_1.png

Reagents

Reagents • 10 cm and 15 cm peptone agar plates: \(www.wormbook.org/chapters/www_transformationmicroinjection/transformationmicroinjection.html#d0e878) • Sodium hypochlorite solution 10% \(Honeywell Fluka, 71696) • Sodium hydroxide \(NaOH, Sigma, S5881) • Standard _Drosophila_ maintenance medium: cornmeal/yeast/agar base supplemented with various carbohydrates and preservatives \(http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm) • Streptomycin Sulfate \(Amresco, 0382) • TRIzol™ Reagent \(Ambion, Life Technologies, cat. no. 15596018) • Chloroform \(Amresco, 0757) • Acid phenol chloroform \(5:1 solution, pH=4.5, Ambion) • 2-Propanol \(Sigma-Aldrich, cat. no. 59300) • Glycogen \(20 mg/ml, G1767-1VL Sigma) • Ethanol absolute \(VWR Chemicals, cat. no 20821.321) • DEPC-treated water \(Ambion, AM9920) • Ultra-Pure SequaGel UreaGel System \(National Diagnostic, cat. no. EC-833) • Ammonium Persulfate \(APS, Sigma, A3678) • N,N,N',N'-Tetramethylethylenediamine \(TEMED, Sigma, T9281) • Gel loading buffer II \(Ambion, AM8547) • 18-mer \(Integrated DNA Technologies, IDT, see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf for the sequence) • 30-mer \(Integrated DNA Technologies, IDT, see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf for the sequence) • 60-mer \(see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf for the sequence) • SYBR gold nucleic acid gel stain \(Life Technologies, S11494) • Standard transparent heavyweight sheet protector • DNA LoBind tubes 1.5 mL \(Eppendorf, G172215J) • Borax \(or Sodium tetraborate) powder \(Sigma, 71997) • Boric acid powder \(Sigma, B6768) • Sodium periodate \(Sigma, 311448) • 3M Sodium Acetate pH=5.5 \(Ambion, AM9740) • 10x T4 RNA Ligase Reaction Buffer \(NEB, B0216S) • 3´

linker Solexa \(Integrated DNA Technologies, IDT, see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf), gel purified on 15% urea-acrylamide gels \(0.4 mm thickness), resuspended to 50 µM in DEPC water • 5´ linker Solexa \(Integrated DNA Technologies, IDT, see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf), gel purified on 15% urea-acrylamide gels \(0.4 mm thickness), resuspended to 100 µM in DEPC water • T4 RNA Ligase 2, Truncated K227Q \(NEB, M0351L) • T4 RNA Ligase 1 \(ssRNA Ligase) \(NEB, M0204L) • Polyethylene glycol \(PEG) 8000 50% \(NEB, B1004A) • Sodium chloride \(NaCl, Merk, cat. no. 106404) • 20% Sodium Dodecyl Sulfate \(SDS) solution Ultrapure MB Grade \(Affymetrix, 75832) • ATP 10mM \(NEB, P0756S) • Century RNA Marker \(1mg/ml; Ambion, AM7140) • 10x RT buffer \(Invitrogen, 53032) • 10 mM dNTP solution mix \(NEB, N0447S) • Magnesium chloride \($MgCl_2$, Thermo Fisher, 00313561) • Small RNA seq RT primer \(see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf for the sequence) • Superscript III reverse transcriptase \(Invitrogen, 56575). 0.1 M DTT is provided together with the enzyme \(Invitrogen, y00147) • KAPA Real Time Library Amplification Kit \(KAPA BIOSYSTEM, KK2702) • Truseq Universal Adapter \(Solexa_PCR_fwd primer, see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf for the sequence) • Trueseq Index reverse primers \(Solexa_IDX_rev, see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf for the sequence) • Optical Flat 8-Cap strips \(BioRad, TCS0803) • Individual PCR Tubes 8-Tube strip, clear \(BioRad, TLS0801) • Certified^{TM} Low Range Ultra Agarose \(BioRad, cat. no 1613107) • GeneRuler 50 bp DNA Ladder \(Thermo Scientific, SM0373) • Zymoclean^{TM} Gel DNA Recovery Kit \(ZYMO RESEARCH, Catalog nos. D4001, D4002, D4007 & D4008) **Buffers** • 1x M9 buffer: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, H_2O to 1 liter. Sterilize by autoclaving. Add 1 ml of sterile 1 M $MgSO_4$ • Bleaching solution: for 10 mL combine 1 mL of 10% sodium hypochlorite solution, 1 mL of NaOH 10 M, 8 mL water • 5x Borate Buffer \(pH=8.6): 150 mM borax, 150 mM boric acid • Elution buffer: 0.3 M NaCl, 0.1% SDS

Equipment

• Standard molecular biology lab equipment \(such as centrifuge, microscope, thermocycler, qPCR machine) • gel electrophoresis apparatus • Illumina high-throughput sequencing machine \(such as Illumina HiSeqV4 SR50)

Procedure

Growing and harvesting of worms and flies For all mime-seq experiments using _C. elegans_, we profiled synchronized first larval stage \(L1) animals. Specifically, large populations of L1-stage animals \(2-5 millions) were obtained by culturing on solid 15 cm peptone agar plates seeded with 3 ml of concentrated, streptomycin resistant _E. coli_ strain HB101. Obtaining such numbers of animals typically required expansion for three generations starting from a few small plates. Worms were synchronized at the L1 stage at every generation using the standard bleaching protocol \(Sulston, J. E. & Hodgkin, J., 1988). Important numbers for _C. elegans_ experiments: • Plate 80,000-90,000 L1s on each 15 cm peptone agar plate seeded with 3ml of concentrated HB101. • It takes 3 days at 20˚C after plating L1s to obtain gravid adults for the next round of bleaching. • Aim to harvest 2-5 million L1s \(from roughly 10-15 15 cm plates) by centrifugating, removing all water,

resuspending in 600 µl TRIzol and flash freezing in liquid N_2. Proceed to RNA isolation or store at -80˚C. For mime-seq experiments using _Drosophila melanogaster_, fly lines were maintained by periodically transferring new adult flies to fresh media. Flies were kept in an incubator at 25˚C with constant humidity. In order to collect flies for the experiments: • Specific crosses were set up between muscle Gal-4 driver stocks and UAS::Ath-HEN1 stocks. At the same time, the control fly lines were transferred to fresh medium to ensure hatching at the same time as crosses. • After 3 days, the F_0 flies were removed from the cross-vials. The eclosed F_1 flies were collected over 3 days and placed into fresh vials to be aged for 2 days. The flies were sexed and genotyped at the same time. **Total RNA isolation** - Extract total RNA from S2 cells, flies and worms using TRIzol Reagent. For flies, collect 2-5 days old flies, snap-freeze them in liquid N_2 and separate heads from the bodies using metal sieves \(keeping all tools cooled by liquid nitrogen). Upon addition of TRIzol reagent to the tube containing snap-frozen fly bodies \(1 ml TRIzol per 20 fly bodies), disrupt the fly tissues using pestle tool. For _C. elegans_ L1s previously harvested in 600 µl TRIzol, freeze-crack 3 to 4 times \(by freezing in liquid N_2 and thawing to room temperature) and vortex to dissolve the cuticle - Add 200 µl of chloroform per 1 ml of TRIzol, agitate samples vigorously and centrifuge at full speed \(14,000 rpm) for 15 min at 4°C. The mixture separates into lower, red, phenolchloroform phase, an interphase, and a colorless aqueous phase - Carefully transfer the upper aqueous phase to a fresh tube - Perform an extra cleaning step by adding 1 volume of acid phenol chloroform \(5:1 solution, pH=4.5), vortexing and centrifugating for 3 minutes at full speed at 4°C - Transfer upper, aqueous phase to a new tube - Precipitate RNA by adding 1 µl of glycogen \(20 mg/ml) and 1 volume of isopropyl alcohol - Incubate at room temperature for 10 minutes and centrifuge at full speed for at least 30 minutes at 4˚C; RNA will form a gel-like pellet on the side and bottom of the tube - Decant supernatant - Wash by adding 300 µl of 80% cold ethanol, vortex and centrifuge 10 minutes at full speed at 4°C - Decant supernatant, collect remaining ethanol by quick centrifugation and pipette all remaining liquid - Dissolve RNA pellet in 20 µl \(or more, depending on the pellet size) of DEPC-treated water, to obtain a target RNA concentration above 1 µg/µl - At least 20 µg of total RNA are used for small RNA library preparation \(Ghildiyal, M. et al., 2008). **Small RNA library preparation and sodium periodate-mediated oxidation** **Day 1: 18-30 nt RNA size selection** - Prepare a 15% denaturing urea polyacrylamide gel \(SequaGel; 20 cm x 16 cm x 1.5 mm, length x width x thickness): 33 ml concentrate, 16.5 ml diluent, 5.5 ml buffer, 350 µl APS 10% and 35 µl TEMED; use combs for wells that accommodate 40 µl of sample - Pre-run the gel in 0.5x TBE buffer at a constant power of 40-50 W for approximately 30 minutes until the surface temperature of the gel reaches 45–55°C - In the meantime, aliquot samples starting from at least 20 µg total RNA each \(in no more than 20 µl volume) and add an equal volume of gel loading buffer II - In parallel, prepare the size markers by mixing 10 pmoles of each the 18-mer and 30-mer RNA oligos \(2 µl each from 5 µM stock + 30 µl of gel loading buffer II) - Denature all samples and markers by incubating 4 min at 95ºC, spin briefly at room temperature and load into wells pre-washed with running buffer to remove accumulated urea and fragments of gel. IMPORTANT: ideally, we run one sample per gel to avoid cross contamination, otherwise maximize space between two samples - Run gel at a constant power of 45-55 W until the bromophenol blue is about half way down the gel - Remove the top glass plate and with the gel still on the bottom plate, pour 10-15 ml of SYBR gold in 0.5x TBE \(for 50 ml use 5 µl of SYBR gold) and stain for 3-4 minutes - Carefully transfer the gel by holding it at the bottom side onto a transparent sheet protector - Visualize RNA on a UV transilluminator \(take a photograph if desired, without exposing longer than necessary) and cut out small RNAs between 30-mer and 18-mer markers - Cut bigger gel pieces into multiple small parts and collect in 1.5 ml LoBind tubes - Add 800 µl 0.3 M NaCl + 0.1% SDS and allow RNA to elute overnight at room temperature, while rotating. **Day 2: sodium periodate-mediated oxidation and 3´ linker ligation** - Precipitate

each sample by transferring to two fresh tubes \(without transferring gel pieces) and adding to each 400 µl aliquot, 1 µl glycogen \(20 mg/mL) and 1 ml of 100% ethanol; incubate samples for 1.5-2 hours at -20˚C - Centrifuge 40 minutes at full speed \(14000 rpm) at 4˚C, decant supernatant and wash the pellet with 300 µl of 80% cold ethanol as before. Centrifuge again 10 minutes at 4˚C, remove the supernatant completely, perform an extra round of centrifugation for 2 minutes and pipette out all residual ethanol - Without air-drying the pellet, resuspend it in a final volume of 55 µl DEPC-treated water; samples are ready for the oxidation step. _Small RNA oxidation:_ - Set up the reaction for each sample \(final volume = 40 µl) as in "Table 1":http://www.nature.com/protocolexchange/system/uploads/6337/original/Table_1.png. IMPORTANT: this step is key to the success of the experiment. To ensure efficient oxidation, prepare the sodium periodate solution fresh, just before use, by dissolving 10.69 mg sodium periodate in 1 ml water. In addition, the pH of the Borate Buffer is crucial for optimal reaction conditions and to minimize RNA hydrolysis. When optimizing this step, we recommend adding a pre-methylated, spike-in RNA to ensure efficient recovery and enrichment over unmethylated RNA. - Incubate samples at room temperature for 30 minutes \(longer oxidation time should be avoided) - Precipitate samples by adding 229 µl of water, 30 µl of 3 M Sodium acetate \(pH 5.5) and 1 µl glycogen to each tube. Vortex briefly to mix, spin briefly to collect the solution on the bottom of the tube and add 900 µl \(3 volumes) of 100% ethanol to each tube. Again, vortex briefly to mix and incubate 1.5-2 hours at -20ºC - Centrifuge 40 minutes at full speed \(14,000 rpm) at 4˚C, decant supernatant and wash the pellet with 300 µl of 80% cold ethanol as before. Centrifuge again 10 minutes at 4˚C, remove the supernatant completely, perform an extra round of centrifugation for 2 minutes and pipette out all residual ethanol - Without air-drying the pellet, resuspend it in a final volume of 6 µl DEPC-treated water; samples are ready for the 3´ ligation step. _Small RNA 3´ linker ligation:_ - Prepare the master mix \(volumes are calculated for one sample) as in "Table 2":http://www.nature.com/protocolexchange/system/uploads/6339/original/Table_2.png. - Add 4 µl of master mix to each 6 µl of sample, mix and spin - Add 10 µl of PEG \(8000) 50% \(final volume 20 µl) and incubate on ice in the cold room, overnight - In addition to the samples, prepare also a ligation reaction with both the 18-mer and 30-mer markers: 2 µl each from 5 µM stock + 2 µl water \(these will serve as controls that the ligation worked, but also as markers for products of the correct size, as they will shift in size by the same amount as the samples). **Day 3: gel purification of 3´-ligated small RNAs** - To size select 3´-ligated small RNAs, prepare two 15% denaturing urea polyacrylamide gels per sample, as described in Day 1 \(one for the oxidized sample and another one for the unoxidized) - Add an equal volume of gel loading buffer II \(20 µl) to the ligated samples. For the markers, add 40 µl of gel loading buffer II to reach a final volume of 60 µl, and load half of that volume \(30 µl) per gel - After pre-running the gel and sample/marker denaturation at 95ºC, gently load the samples into pre-washed wells and run until the bromophenol blue reaches the end of the gel - Stain the gel as previously described and size select 3´-ligated small RNAs by cutting the area between the ligated 18- and 30-mer markers - Elute RNA overnight in 800 µl 0.3 M NaCl + 0.1% SDS at room temperature, while rotating. **Day 4: 5´ linker ligation** - Precipitate each sample by transferring to two fresh tubes \(without transferring gel pieces) and adding to each 400 µl aliquot, 1 µl glycogen \(20 mg/mL) and 1 ml of 100% ethanol; incubate samples for 1.5-2 hours at -20˚C - Centrifuge 40 minutes at full speed \(14,000 rpm) at 4˚C, decant supernatant and wash the pellet with 300 µl of 80% cold ethanol as before. Centrifuge again 10 minutes at 4˚C, remove the supernatant completely, perform an extra round of centrifugation for 2 minutes and pipette out all residual ethanol - Without air-drying the pellet, resuspend it in a final volume of 7.5 µl DEPC-treated water; samples are ready for the 5´ ligation step. _Small RNA 5´ linker ligation_ - Prepare the master mix \(volumes are calculated for one sample) as in "Table 3":http://www.nature.com/protocolexchange/system/uploads/6341/original/Table_3.png. - Add 7.5

µl of master mix to each sample, mix and spin - Add 5 µl of PEG \(8000) 50%, to a final volume of 20 µl, and incubate on ice in the cold room, overnight. **Day 5: gel purification of 5´-ligated \(and 3´-ligated) small RNAs** - To size select small RNAs with both adaptors, prepare 10% denaturing urea polyacrylamide gels \(SequaGel; 20 cm x 16 cm x 1.5 mm, length x width x thickness): 22 ml concentrate, 27.5 ml diluent, 5.5 ml buffer, 350 µl APS 10% and 35 µl TEMED. - Pre-run the gel in 0.5x TBE buffer at a constant power of 40 W for approximately 30 minutes until the surface temperature of the gel reaches 45–55°C - Meanwhile, prepare the marker by mixing 1µl \(10 pmol) of the 60-mer, _in vitro_ transcribed marker, with 0.5 µl of Century RNA Marker \(1mg/ml) in 20 µl of gel loading buffer II. This is sufficient for one gel - After sample/marker denaturation at 95ºC, gently load samples into pre-washed wells and run the gel until the bromophenol blue reaches the end of the gel - Stain the gel as previously described and size select 3´- and 5´- ligated small RNAs by cutting the gel above the 60-mer band and below the lowest Century marker band \(100 nt) - Elute RNA overnight in 800 µl 0.3 M NaCl + 0.1% SDS at room temperature, while rotating. **Day 6: Reverse Transcription and qPCR amplification** - Precipitate each sample by transferring to two fresh tubes \(without transferring gel pieces) and adding to each 400 µl aliquot, 1 µl glycogen \(20 mg/mL) and 1 ml of 100% ethanol; incubate samples for 1.5-2 hours at -20˚C - Centrifuge 40 minutes at full speed \(14,000 rpm) at 4˚C, decant supernatant and wash the pellet with 300 µl of 80% cold ethanol as before. Centrifuge again 10 minutes at 4˚C, remove the supernatant completely, perform an extra round of centrifugation for 2 minutes and pipette out all residual ethanol - Without air-drying the pellet, resuspend it in a final volume of 17 µl DEPC-treated water. Samples are ready for the Reverse Transcription reaction. _Reverse Transcription \(RT)_ - Prepare the RT mix \(volumes are calculated for one sample but we recommend calculating two reactions per sample to perform reactions with and without reverse transcriptase) as in "Table 4":http://www.nature.com/protocolexchange/system/uploads/6343/original/Table_4.png. - Add 1 µl \(100 µM) small RNA seq RT primer to each sample \(17 µl 5´ ligation product) and incubate at 72˚C for 2 minutes. Spin at 20,000 x g for 1 minute at room temperature and cool on ice for 2 minutes - Add 18 µl of RT mix to each sample, for a total 36 µl reaction and split them into two tubes \(18 µl each), in order to generate for each sample the “+RT” tube and the control “-RT” tube. For that, add either 2 µl Superscript III reverse transcriptase \(+RT) or water \(-RT); final volume per sample is 20 µl - Incubate each tube at 50˚C for 1 hour, heat inactivate RT enzyme for 15 minutes at 70˚C and store samples at -20˚C; they are ready to undergo qPCR amplification step, either directly or on the following day. _qPCR amplification_ - To amplify the cDNA libraries, use the KAPA Real Time Library Amplification Kit - PCRs are performed using the TruSeq Universal Adapter primer \(Solexa_PCR_fwd; see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf) and TruSeq Index reverse primers \(Solexa_IDX_rev; see "Table 7":https://www.nature.com/protocolexchange/system/uploads/6349/original/Table_7.pdf). The latter include barcodes that can be assigned to different samples for multiplexing - Mix 10 µl of each RT reaction \(half of the volume) with 1 µl of 25 µM TruSeq Index reverse primer \(Solexa_IDX_rev) to each one; assign a unique barcode per sample IMPORTANT: work in individual PCR tubes with optical flat caps suitable for qPCR - Set up a master mix \(add the 25 µl of 2x KAPA HiFi HS RM last and mix gently) as in "Table 5":http://www.nature.com/protocolexchange/system/uploads/6345/original/Table_5.png. - Add 39 µl to each sample for a final volume of 50 µl, mix and collect by centrifugation - Prepare also tubes with 50 µl of each fluorescent standard \(standards 3 and 4) in triplicate - Run the qPCR program as in "Table 6":http://www.nature.com/protocolexchange/system/uploads/6347/original/Table_6.png, until the libraries reach the fluorescence level of standards 3-4 \(assessed from the image acquired by the PCR machine in each

cycle; no more than 16-17 cycles should be required, even for low concentrated samples). - When a sample reaches a level between standards 3 and 4 \(close to standard 4), pause the program during the 10 sec step and remove respective tubes from the PCR machine; continue cycling until all samples have reached the desired amount. "Figure 2":http://www.nature.com/protocolexchange/system/uploads/6333/original/Figure_2.png is an example of few samples that were taken out from the qPCR machine as soon as they reached the desired target. Unoxidized samples contain more material than oxidized ones and will reach the target in fewer cycles \(typically 7-10 for unoxidized and 9-16 for oxidized). _Gel purification of amplified cDNA_ - Prepare a 2% Low-Range Ultra agarose gel according to the manufacturer's instructions \(swirl the flask often during heating procedure), with 1x SYBR safe - Load 6 µl of GeneRuler 50 bp DNA Ladder and the amplified cDNA after addition of 10 µl of Orange G loading dye 6x \(total volume 60 µl) - Run the gel at constant 80-100 V until the Orange G loading dye band is at 3/4 of the gel length - Visualize the gel on a long wave UV transilluminator and excise the DNA band between 150-200 bp with a clean scalpel or razor blade and place in a clean tube, as shown in "Figure 3":http://www.nature.com/protocolexchange/system/uploads/6335/original/Figure_3.png IMPORTANT: there will be a strong lower band of ~120 bp that corresponds to adaptor dimers, avoid cutting into it - Dissolve gel by adding 3 volumes \(~800-900 µl) of ADB buffer \(Zymoclean^{TM} Gel DNA Recovery Kit) and incubating samples at 55ºC for 10 minutes or until the gel slice is completely dissolved; mix and collect by centrifugation - Transfer the melted agarose solution to a Zymo-Spin^{TM} Column in a collection tube, centrifuge 1 minute at 10,000 rpm and discard the flow-through - Add 200 µl of DNA Wash Buffer to the column, centrifuge 1 minute and discard the flow-through; repeat the wash step once more - Add 15-20 µl of water directly to the column matrix, place the column into a 1.5 ml tube and centrifuge for 1 minute to elute DNA; purified DNA is now ready for quality control and deep sequencing \(HiSeqV4 SR50). **Bioinformatics and data analysis** Small RNA-library reads were recovered by adapter clipping: The adapter was cut once with cutadapt v1.12.0 \(Martin, M., 2011). Adapter-derived random 4mers on the 5´ and 3´ ends of recovered reads were removed with custom scripts. Processed reads were size-selected \(i.e. 18-30-nt). Mapping of sequencing libraries to _Drosophila_ genome \(dm3), the _mus musculus_ genome \(mm10), or the _C. elegans_ genome \(WBcel235) was performed as described \(Reimão-Pinto, M. M. et al., 2015). Annotations were derived from FlyBase \(r5.57) and mirBase \(v21). Reads were assigned to miRNA arms with htseq-count \(v0.6.1p1, Anders, S. et al., 2015). Multimapping reads were counted only as fraction \(1/number of mapped locations). The Nextflow \(Di Tommaso, P. et al., 2017) pipeline developed for this analysis, as well as the R script used for further analysis \(see below) can be found at: https://gitlab.com/tburk/smallRNA-meth To identify the tissue- or cell-type- specific miRNAs for both _C. elegans_ and _Drosophila_, we calculated the enrichment for every miRNA by comparing the oxidation treated with untreated samples using DESeq2 \(v1.16.1). For all _C. elegans_ samples, we first removed spuriously expressed miRNAs by requiring an average cpm \(counts per millions) >10 over all untreated samples analyzed in this study. All miRNA arms above that cutoff were used for a robust estimation of the dispersion parameters of the negative binomial distributions employed by DESeq2. However, beyond this step, the miRNA arms that showed higher expression in more than half of the samples without oxidation treatment were retained, and the lower expressed miRNA* arms were discarded. By comparing the respective pairs of treated and untreated samples, we obtained the fold changes \(in log2 scale) and enrichment scores \(i.e. p-values) from DESeq2. For experiments conducted in the wild-type background, where the endogenous methyltransferases \(Cel-HENN-1 or Dme-HEN1) are still present, we had to subtract the background methylation that occurs on some miRNAs. In order to do this, we included an interaction term between genotypes \(e.g. ASE::Ath-HEN1 and non-transgenic N2, and similarly for the muscle experiment done in both

backgrounds) and treatments in the design formula of DESeq2 to obtain miRNAs that are not only enriched due to treatment but also show higher enrichment than in the background methylation.

References

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Figures

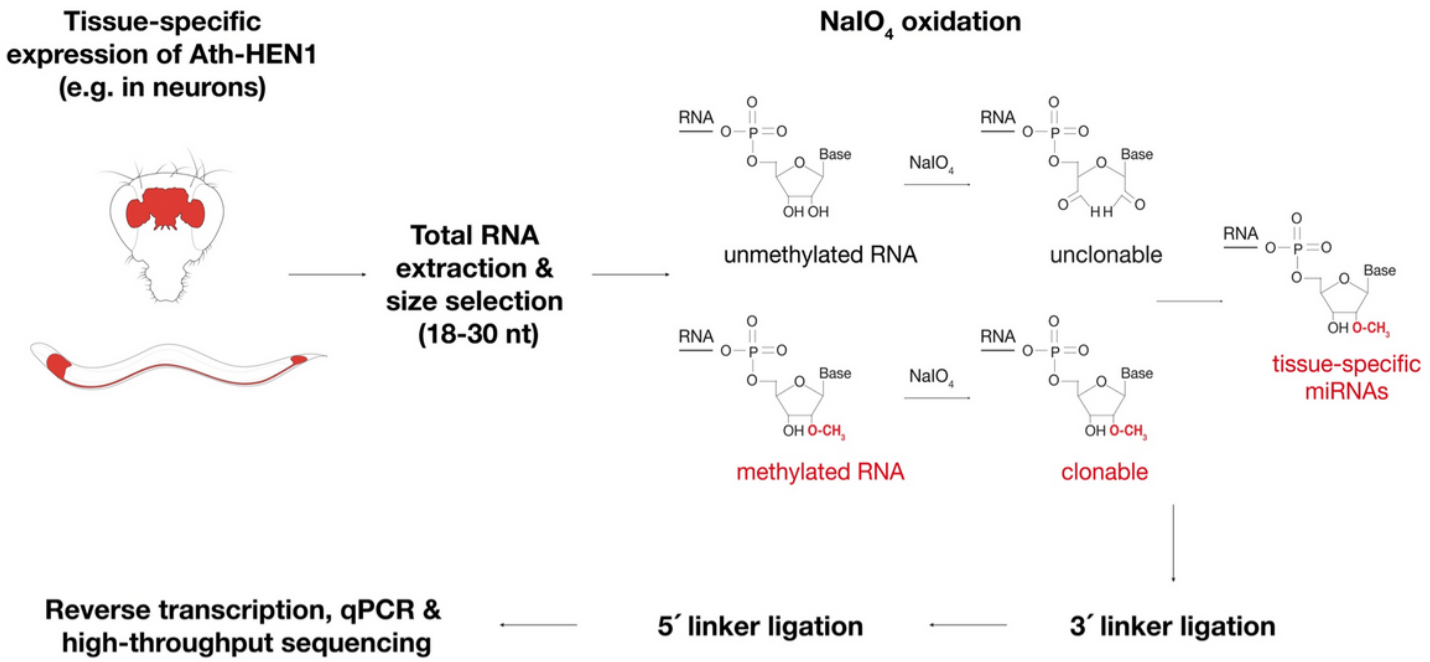


Figure 1

mime-seq Workflow associated with miRNome by methylation-dependent sequencing (mime-seq). The protocol relies on a well-established small RNA cloning protocol combined with cell-specific miRNA methylation by the genetically-encoded HEN1 from _Arabidopsis thaliana_ (Ath-HEN1) and a chemoselective step where unmethylated RNAs are rendered unclonable by periodate-mediated oxidation.

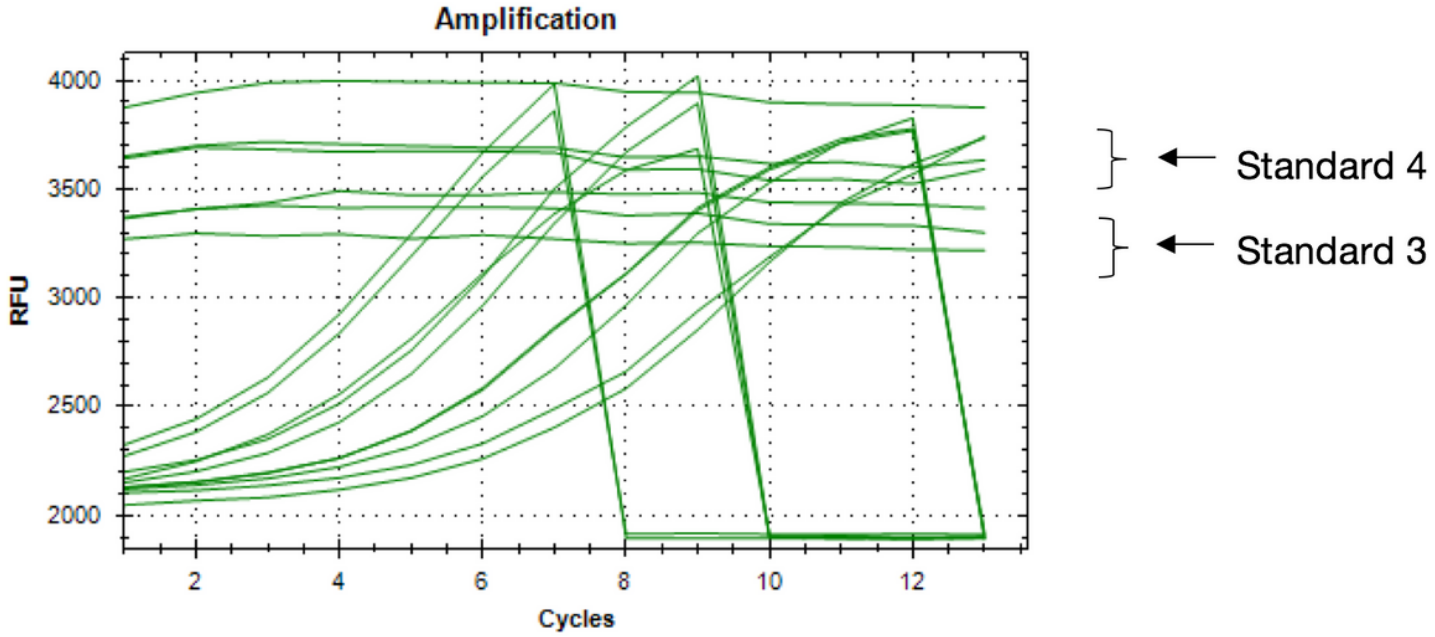


Figure 2

Example of qPCR amplification plot for unoxidized and oxidized samples Each sample requires a certain number of cycles (x axis) to reach the desired amount (fluorescence level in y axis), depending on the amount of starting material.

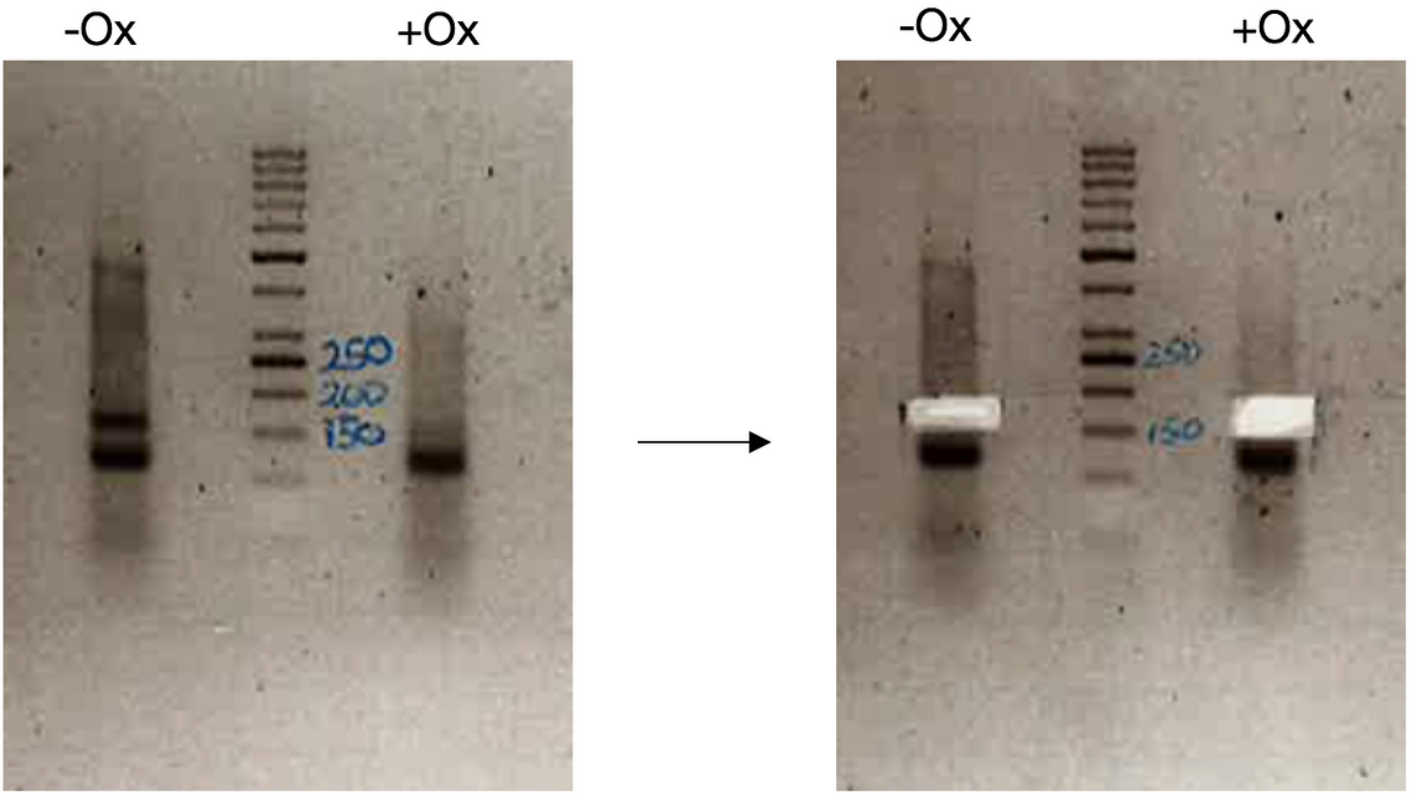


Figure 3

Example of 2% low-melt agarose gel, before and after DNA band excision An unoxidized (-Ox) sample was loaded on the left side of the GeneRuler 50 bp DNA Ladder, while an oxidized (+Ox) sample can be seen on the right side. Please, notice that the desired upper band can be very faint in case of oxidized sample. Even in those cases, cut above the strong band of adaptor dimers and proceed with DNA extraction.

	- Oxidation	+ Oxidation
small (19-29 nt) RNAs	27µl	27 µl
5x Borate Buffer (pH 8.6)	8 µl	8 µl
50 mM Sodium periodate	0 µl	5 µl
Water	5 µl	0 µl

Figure 4

Table 1 Reaction setup for small RNA oxidation

10x NEB Ligase Buffer	2 µl
3´ linker [50 µM]	1 µl
T4 RNA Ligase 2, truncated K227Q (NEB)	1 µl

Figure 5

Table 2 Reaction setup for small RNA 3´ linker ligation

10x NEB Ligase Buffer	2.4 µl
5´ linker [100 µM]	0.5 µl
ATP 10mM	4 µl
T4 RNA Ligase 1 (NEB)	1 µl

Figure 6

Table 3 Reaction setup for small RNA 5´ linker ligation

10x RT Buffer	2 µl
10 mM dNTP mix	1 µl
0.1 M DTT	2 µl
25 mM $MgCl_2$	4 µl

Figure 7

Table 4 Reaction setup for reverse transcription

2x KAPA HiFi HS RM 1x	25 µl
25 µM Solexa_PCR_ fwd	1 µl
DEPC-treated H_2O	13 µl

Figure 8

Table 5 Reaction setup for qPCR amplification

Denaturation	98℃	45 sec
Cycling	98℃	15 sec
	65℃	30 sec
Extension	72℃	30 sec (fluorescence is measured at the end)
	72℃	10 sec (pause to remove amplified samples)
Final extension	72℃	1 min

Figure 9

Table 6 Cycling conditions for qPCR amplification

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement0.pdf