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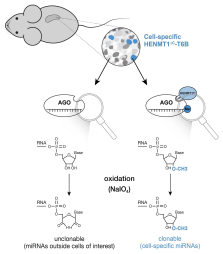
Mime-seq 2.0: a method to sequence microRNAs from specific mouse cell types V.1



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We use this protocol and it's working

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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 for mice (mime-seq 2.0). Mime-seq takes advantage of a chemical small RNA-tagging approach followed by chemo-selective, high-throughput sequencing that enables the identification of tissue- and cell type-specific miRNA profiles in animals in a sensitive and reproducible manner.

Materials

Reagents

	Name	Supplier	Catalogue number
	2-propanol	FisherChemical	C298-500
	Glycogen	Sigma	G1767-1VL
	EtOH abs.	Avantor	20821.321
	H2O, DEPC-treated	Invitrogen	AM9920
	H2O, Rnase free	Invitrogen	AM9935
	Qubit RNA BR Assay Kit	Invitrogen	Q10210
	Ammonium Persulfate	Sigma	A3678
	TEMED	Sigma	T9281
	Sodium Periodate	Sigma	311448-5G
	Sodium Acetate (3M) ph 5.5	Invitrogen	AM9740
	NaOH	Sigma	S5881
	Boric acid	Sigma	B6768
	Sodium tetraborate	Sigma	311448-5G
	Sodium chloride	Sigma	106404
	SequaGel UreaGel System	National Diagnostics	EC-833
	Gel Loading Buffer II	Invitrogen	AM8547
	Hybond-NX membrane	Cytiva	RPN303T
	Methylimidazole	Sigma	M50834
	EDC	Sigma	E7750-25G
	EDTA (0.5M) pH 8	Invitrogen	15575020
	Sodium Phosphate dibasic heptahydrate	Sigma	431478
	Phosphoric Acid	Avantor	02-003-602
	T4 Polynucleotide Kinase	New England Biolabs	M0201L
	g-32P-ATP	Perkin Elmer	BLU002Z250UC
	MicroSpin G-25 Columns	Cytiva	27532501
	Storage Phosphor Screen	Cytiva	28956474

	Name	Supplier	Catalogue number
	Protein G Dynabeads	Invitrogen	10003D
	K227Q truncated T4 RNA ligase 2	New England Biolabs	M0351L
	PEG8000 50%	New England Biolabs	B1004A
	SYBR Gold	Invitrogen	S11494
	TBE buffer, 10x	Quality Biological	351-001-131
	SSC buffer, 20x	Sigma	S6639-1L
	T4 RNA ligase 1	New England Biolabs	M0204L
	Superscript II or III reverse transcriptase	Invitrogen	18064014/18080044
	RNaseOUT	Invitrogen	10777019
	ExoSAP-IT	Applied Biosystems	78201
	Kapa HiFi HotStart Library Amp kit	Roche	KK2612
	Zymoclean Gel DNA recovery kit	Zymo	D4008
	Low Range Agarose	BioRad	1613107
	IGEPAL CA-630	Sigma	I3021
	Sodium Deoxycholate	Sigma	D6750
	Sodium Dodecyl Sulfate	Sigma	L3771
	Sodium Dodecyl Sulfate 20% solution	Sigma	05030
	Tris	Thermo Scientific	75825
	Glycerol	Sigma	15523
	Bromophenol Blue	BioRad	1610404
	2-Mercaptoethanol (BME)	Sigma	M-6250
	Tween	Sigma	P9416
	Magnesium Chloride	Avantor	7791-18-6

Plasticware

- DNA LoBind tubes 1.5 mL (Eppendorf, G172215J)
- Standard transparent heavyweight sheet protector
- Optical Flat 8-Cap strips (BioRad, TCS0803)
- Individual PCR Tubes 8-Tube strip, clear (BioRad, TLS0801)

Equipment

	Name	Company
	Typhoon phosphorimager	Cytiva
	HiSeq V4 sequencer or similar	Illumina
	Qubit flex fluorometer	Invitrogen
	Blue light transilluminator	
	Gel electrophoresis apparatus vertical and horizontal	
	ChemiDoc	BioRad
	(optional) fragment analyzer or similar	Agilent

Buffer recipes

- 5x Borate Buffer 300 mM, pH 8.6

Dissolve 5.49 g Sodium tetraborate decahydrous ($Na_2B_4O_7$:10H2O **[LYC1]** *, MW = 381.37 g/mol) in 80 ml H2O, fill up to 100 ml; dissolve 2.814 g Boric acid (H_3BO_3, MW = 61.83 g/mol) in 80 ml H2O, fill up to 100 ml. Mix equal volumes (i.e. 50 ml + 50 ml) to achieve 300 mM solution, adjust pH to 8.6.*

- PAGE elution buffer (0.3 M NaCl, 0.1% SDS)
- 6x Orange G loading dye (4 g /l orange G, 40% sucrose)
- 10x 3’ ligation buffer (500 mM Tris pH 7.8, 100 mM $MgCl_2$, 100 mM DTT)
- 10x T4 Ligase Buffer (500 mM Tris pH 7.8, 100 mM $MgCl_2$, 10 mM ATP 100 mM DTT)

Sequences

sequences.xlsx

Total RNA isolation

40m

1 Extract total RNA from frozen cells or tissue using TRIzol, depending on sample type follow manufacturer's instructions for proper lysis.

2 Add 200 µl of chloroform per 1 ml of TRIzol, agitate samples vigorously and incubate for 5 min at room temperature (RT).

5m

3 Centrifuge at full speed (12,000 x g) for 15 min at 4°C. The mixture separates into lower, red, phenol-chloroform phase, an interphase, and a colorless aqueous phase.

15m

4 Carefully transfer the upper aqueous phase to a fresh tube.

5 Precipitate RNA by adding 1 µl of glycogen (20 mg/ml) and 500 µl of isopropyl alcohol, vortex.

6 Incubate at 4˚C for 10 minutes.

10m

7 Centrifuge at full speed for 10 minutes at 4˚C; RNA will form a gel-like pellet on the side and bottom of the tube; for low input samples, extend centrifugation time to 60 min.

10m

8 Remove supernatant without losing the RNA pellet.

9 Wash pellet

9.1 Add 300 µl of 80% cold ethanol, vortex.

9.2 Centrifuge 10 minutes at full speed at 4°C.

9.3 Remove supernatant, collect remaining ethanol by quick centrifugation and pipette all remaining liquid using a 10 µl pipette. Air dry pellet for approx. 5 min or until it becomes translucent.

9.4 Air dry pellet for approx. 5 min or until it becomes translucent.

10 Dissolve RNA pellet in 20-50 µl of DEPC-treated or other RNase free water, to obtain a target RNA concentration above 1 µg/µl.

11 Measure concentration (Qubit BR RNA kit); ideally, at least 3.3 µg of total RNA are used for small RNA library preparation per sample (will be split in ox/unox).

NaIO4-mediated oxidation

30m

12 Prepare 3.3 µg of total RNA with a mix of methylated and unmethylated spike-ins in 44 µl RNase-free H2O (includes 10 % extra).

13 For Oxidation reaction, split each sample in half and incubate using following reaction mix 30 min at RT.

30m

	Reagent	Volume
	RNA/Spike-in mix	20 ul
	NaIO4 (50mM) or H2O	5 ul
	5x borate buffer	8 ul
	H2O	7 ul

Prepare a master mix +/- NaIO4 containing everything except for the respective sample. Transfer samples for oxidation and control reaction in 8 strips, distribute master mix last.

14 Add immediately 229 ul H2O, 30 ul NaOAc (pH 5.2) and 1 ul glycogen (20 mg/ml), mix well.

EtOH precipitation

15 Precipitate by adding 900 ul ice-cold EtOH to each sample.

16 Incubate for > 1h at -20˚C (this can be overnight).

1h

17 Centrifuge for 1 h at 4˚C and remove supernatant without disturbing the pellet.

1h

18 Wash

18.1 Add 500 µl of 80% cold ethanol.

18.2 Centrifuge 10 minutes at full speed at 4°C.

18.3 Remove supernatant, collect remaining ethanol by quick centrifugation and pipette all remaining liquid using a 10 µl pipette.

18.4 Air dry pellet for approx. 5 min or until it becomes translucent.

19 Resuspend each pellet in 6 ul H2O (DEPC-treated or RNase free).

3' Ligation

20 Add to each sample 1 µl srBC 3’ adapter (10 µM) and note used barcodes.

Note – oxidized samples should not be run on the same gel as unoxidized samples, due to big differences in ligatable small RNAs. Each sample run on the same gel should have a different srBC adapter.

Set up in parallel ligation reaction for 18mer and 30mer as later reference for PAGE purification. Mix 2.5 µl 18mer (10 µM) with 2.5 µl 30mer (10 µM) and 0.5 µl 100 µM 3’ linker and 1.5 µl H_2O.

Thaw 50% PEG8000 at 37˚C, this makes pipetting of viscous solution easier.

21 Incubate RNA + srBC 3’ 5 min at 70˚C, snap-cool on ice.

22 Set up master mix at room temperature:

	Reagent	Volume
	PEG8000 50%	10 ul
	10x 3' Ligation buffer	2 ul
	T4 RNA ligase trunc	2 ul

23 Add 13 µl MM to each sample and incubate o/n at 4˚C.

Note – we normally keep the ligation reactions in a metal cooling rack in the fridge or cold room.

Size selection (PAGE)

24 Prepare a 15% denaturing urea polyacrylamide gel (SequaGel; 20 cm × 16 cm × 1 mm, length × width × thickness).

Note – pre-treat glass plates (one with anti-fog, the other with water repellent) to avoid gel sticking to the plates.

25 Pre-run the gel in 0.5× TBE buffer Tat a constant power of 35 W for 30-60 min until the surface temperature of the gel reaches approximately 45–55°C.

26 In the meantime add an equal volume of form amide loading buffer to the samples and denature by incubating 5 min at 95°C, snap-cool on ice.

Note – include ligated 18 and 30mers, as well as unligated 60mer as size reference.

27 Load samples into pre-washed wells.

Note – IMPORTANT rinse all wells before loading with a syringe to remove all accumulated urea and gel fragments. Rinse again before loading of each sample.

Do not use lanes with bubbles and leave at least one empty lane between each sample for easier handling. Do not run oxidized and unoxidized samples on the same gel.

28 Run gel first at constant power of 10 W until samples are migrated out of their wells. Run at a constant power of 35 – 45 W until the bromophenol blue is about halfway down the gel (1-1.5 h).

29 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.

30 Carefully transfer the gel onto a transparent sheet protector .

Note – open sheet protector on 3 sides, hold gel on one end and transfer between sheets. We normally use tape on the outside to label the individual sample lanes for orientation.

31 Visualize RNA on blue transilluminator (take a photograph if desired) and label area between ligated 18mer and 30mer on each lane on the sheet protector with a marker. Remove from transilluminator and cut out using a razor blade small RNAs between markers, collect in 1.5 ml LoBind tubes.

Note – cutting the gel piece in half and laying them on top of each other makes it easier to transfer gel piece to a tube.

32 Add 800 µl 0.3 M NaCl + 0.1% SDS and allow RNA to elute overnight at room temperature, while rotating.

EtOh precipitation

33 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% EtOH; incubate samples for 1.5-2 hours at -20˚C.

34 Centrifuge 40 minutes at full speed (12,000 x g) 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 EtOH.

35 Without air-drying the pellet, dissolve small RNAs in 6 µl H_2O + 1 µl 5’ adapter (10 µM).

5’ linker ligation

36 Incubate RNA + adapter mix 5 min at 70˚C, snap-cool on ice.

37 Prepare ligation master mix (volumes are calculated for one sample):

	Reagent	Volume
	PEG8000 50%	10 ul
	10x RNA ligae buffer	2 ul
	T4 RNA Ligase 1	2 ul

38 Add to each sample 13 µl of master mix, mix and give it a quick spin.

39 Incubate o/n at 4˚C.

Note – we normally keep the ligation reactions in a metal cooling rack in the fridge or cold room.

Purification using RNA Clean & Concentrator 5 (Zymo)

40 Add 80 µl H2O and mix by pipetting.

41 Purify using the RNA Clean & Concentrator 5 (RCC5) kit

41.1 Add 200 µl RNA binding buffer and mix.

41.2 Add 300 µl EtOH absolute and mix.

41.3 Continue according to manufacturer's instructions and elute in 12 µl H2O.

Reverse transcription

42 Transfer 11.5 µl sample into a PCR strip and add 1 µl RT primer (10 µM).

43 Incubate 5 min at 65˚C, snap-cool on ice.

Note – pre-heat PCR block to 50˚C.

44 RT reaction master mix:

	Reagent	Volume
	5x FS buffer	4 ul
	dNTP mix (10mM)	1 ul
	0.1 M DTT	1 ul
	RNase out	0.5 ul

	Reagent	Volume
	Superscript II or III	1 ul

45 Add on ice 7.5 µl RT master mix, mix well, spin down and immediately transfer to pre-heated PCR block at 50˚C, incubate 30 min.

Note – IMPORTANT, no heat inactivation step after RT.

46 Add 8 µl ExoSAP-IT and incubate 15 min at 37˚C followed by heat inactivation at 80˚C for 15 min.

47 On ice, add 2 µl H2O for a total volume of 30 µl.

qPCR amplification

48 Transfer ½ of each sample (15 µl) to strip tube. Add 10 µl i5/i7 primer mix and 25 µl Kapa HiFi Library Amp Kit mix.

49 Prepare also in duplicates 50 µl of fluorescent standard (STD) 3 and 4.

50 Close strip with an optical flat-cap, vortex, and spin down.

51 Run the following program until samples reach the fluorescence level of standard 3 (assessed from the image acquired by the PCR machine in each cycle).

Note - use qPCR machine (i.e. BioRad CFX96) to monitor library amplification in real-time. Base-line subtraction hould be turned off.

	Temperature	Duration	Repetitions
	95˚C	45 sec	
	95˚C	15 sec	n cycles
	65˚C	15 sec	
	72˚C	20 sec	
		plate read	
	72˚C	10 sec	

When a sample reaches signal between standard 3 and 4, pause the program during the 10 sec step after the plate read and remove carefully the respective tubes from the PCR machine. Repeat until all samples are amplified sufficiently.

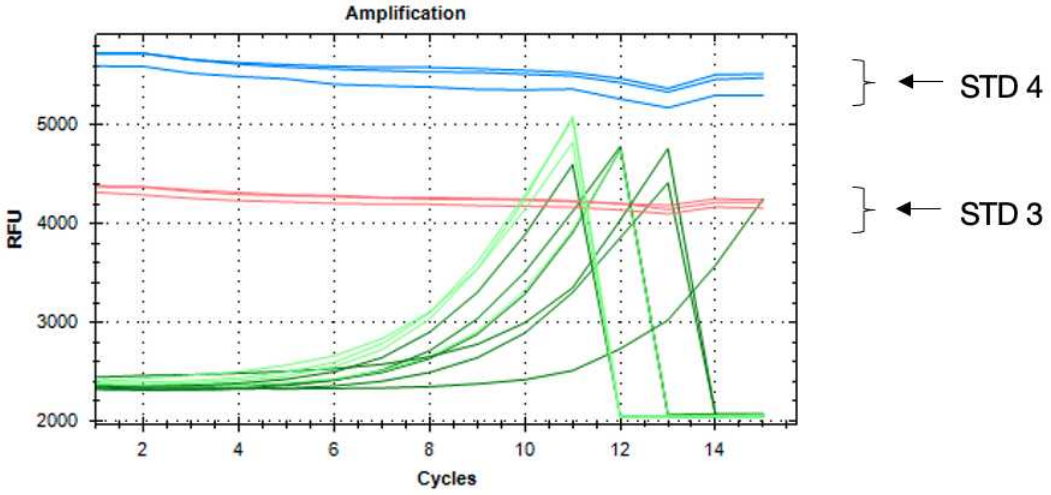

Figure 1: Example of real-time qPCR amplification plot for unoxidized and oxidized samples. Each sample requires a certain number of cycles (x axis) to reach the desired amount (fluorescence level in y axis), depending on the amount of starting material. Unoxidized samples (-) indicated in light green, oxidized samples (+) in dark green. fluorescent STD 3 in pink and STD 4 in blue.

Gel purification of amplified cDNA

52 Prepare a 3% Low-Range Ultra agarose gel according to the manufacturer's instructions (swirl the flask often during heating procedure), with 1.5 µl SYBR Safe per 50 ml of gel.

53 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)

54 Run the gel at constant V (depending on chamber size between 80 and 150V) until the Orange G loading dye band is at 2/3 of the gel length.

55 Visualize on a UV transilluminator and excise upper DNA band between 160-200 bp with a clean scalpel or razor blade and place in a clean tube, as shown in Figure 2 below.

Note - IMPORTANT in case 2 bands are visible – avoid cutting into lower band as this corresponds to adapter dimers; this is especially common with oxidized library samples.

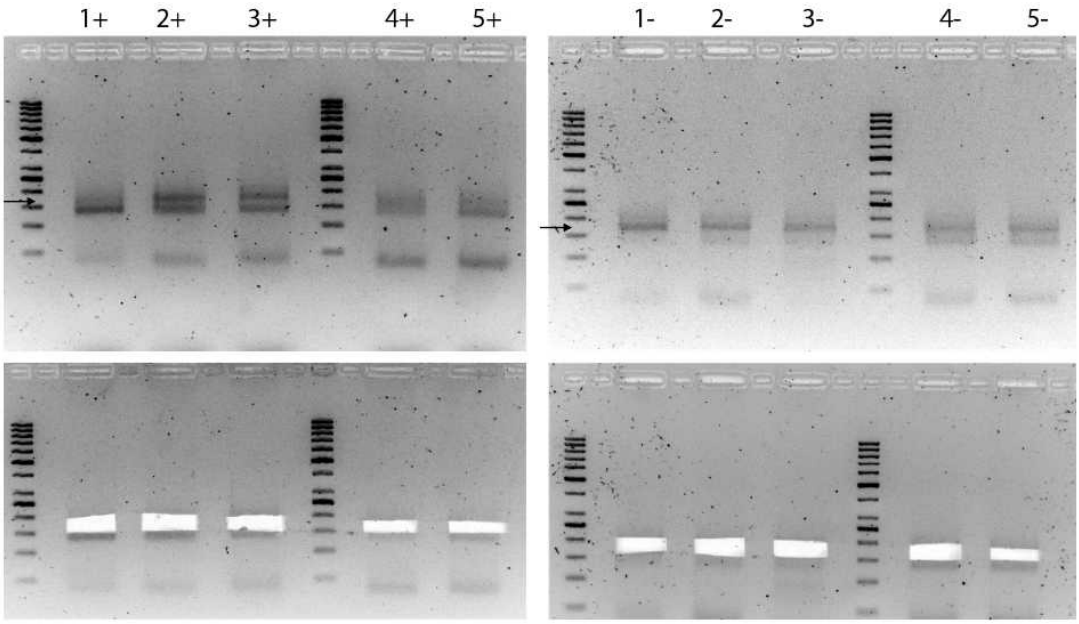

Figure 2: Example of 3% low-range agarose gel, before and after DNA band excision. Oxidized samples 1-4 were loaded on the left gel using the GeneRuler 50 bp DNA Ladder as reference, unoxidized samples are loaded on the right gel. Please, notice that the desired upper band can be very faint in case of oxidized sample (i.e. 1+). Even in those cases, cut above the strong band of adaptor dimers and proceed with DNA extraction (see below).

56 Dissolve gel by adding 3 volumes (~800-900 µl) of ADB buffer (Zymoclean™ 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.

57 Transfer the melted agarose solution to a Zymo-Spin™ Column in a collection tube, centrifuge 1 minute at 10,000 rpm and discard the flow-through.

58 Add 200 µl of DNA Wash Buffer to the column, centrifuge 1 minute and discard the flow-through; repeat the wash step once more.

59 Add 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.

Note - we routinely run libraries on fragment analyzer or tape station to make sure adapter dimers are sufficiently removed.

Bioinformatics and data analysis

60 Libraries are sequenced using an Illumina short read sequencer with a minimum read length of 50 nucleotides as for example NovaSeq SP in SR100 XP mode.

61 Custom NextFlow pipelines for processing can be found on GitHub under https://github.com/popitsch/pysrna. In short:

61.1 Sequencing quality control with fastqc v 0.11.8.

61.2 Raw reads are demultiplexed, parsed and filtered.

61.3 Spike-in reads are filtered and counted allowing for alignment 1 mismatch.

61.4 Remaining small RNA reads are mapped to a miRNA transcriptome obtained from MirGeneDB v2.0 or miRbase annotations.

61.5 Small RNAs were counted and normalized to reads obtained from methylated spike-ins.