Journal of Visualized Experiments

Preparation of small RNA libraries for sequencing from early mouse embryos

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61086R3
Full Title:	Preparation of small RNA libraries for sequencing from early mouse embryos
Section/Category:	JoVE Developmental Biology
Keywords:	miRNA, neural crest, developmental timing, small RNA, pluripotency, differentiation
Corresponding Author:	Ronald J. Parchem, Ph.D. Baylor College of Medicine Houston, Texas UNITED STATES
Corresponding Author's Institution:	Baylor College of Medicine
Corresponding Author E-Mail:	Ronald.Parchem@bcm.edu
Order of Authors:	Rachel A. Keuls
	Ronald J. Parchem, Ph.D.
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Houston, Texas, USA

TITLE:

Preparation of Small RNA Libraries for Sequencing from Early Mouse Embryos

AUTHORS AND AFFILIATIONS:

Rachel A. Keuls[1], Ronald Parchem[2]

[1]Program in Development Disease Models and Therapeutics, Baylor College of Medicine, Houston, TX, USA

[2]Center for Cell and Gene Therapy, Stem Cells and Regenerative Medicine Center, Department of Neuroscience, Baylor College of Medicine, Houston TX, USA

keuls@bcm.edu

Ronald.Parchem@bcm.edu

CORRESPONDING AUTHOR:

Ronald Parchem

KEYWORDS:

neural crest; microRNA; small-RNA sequencing; developmental timing; post-transcriptional regulation; mouse;

SUMMARY:

We describe a technique for profiling microRNAs in early mouse embryos. This protocol overcomes the challenge of low cell input and small RNA enrichment. This assay can be used to analyze changes in miRNA expression over time in different cell lineages of the early mouse embryo.

ABSTRACT:

MicroRNAs (miRNAs) are important for the complex regulation of cell fate decisions and developmental timing. In vivo studies of the contribution of miRNAs during early development are technically challenging due to the limiting cell number. Moreover, many approaches require a miRNA of interest to be defined in assays such as northern blotting, microarray, and qPCR. Therefore, the expression of many miRNAs and their isoforms have not been studied during early development. Here, we demonstrate a protocol for small RNA sequencing of sorted cells from early mouse embryos to enable relatively unbiased profiling of miRNAs in early populations of neural crest cells. We overcome the challenges of low cell input and size selection during library preparation using amplification and gel-based purification. We identify embryonic age as a variable accounting for variation between replicates and stage-matched mouse embryos must be used to accurately profile miRNAs in biological replicates. Our results suggest that this method can be broadly applied to profile the expression of miRNAs from other lineages of cells. In summary, this protocol can be used to study how miRNAs regulate developmental programs in different cell lineages of the early mouse embryo.

INTRODUCTION:

A central question of developmental biology is how a single undifferentiated cell can give rise to an entire organism with numerous complex cell types. During embryogenesis, the developmental potential of cells becomes progressively restricted as the organism develops. One example is the neural crest lineage, which progressively differentiates from a multipotent cell population into various terminal derivatives, such as peripheral neurons, glia, cranial bone, and cartilage. Neural crest cells are specified from the ectoderm during gastrulation and then undergo an epithelial to mesenchymal transition and migrate through the embryo to discrete locations throughout the body where they will terminally differentiate[1]. Decades of work have uncovered a transcriptional gene regulatory network, but far less is known about the mechanisms of post-transcriptional regulation that control the timing of neural crest development.

Previous work suggests that microRNAs (miRNAs) repress gene expression for proper developmental timing and cell fate decisions[2,3,4,5,6]. Studies of miRNAs in neural crest development have largely focused on later stages of craniofacial development. For example, miR-17~92 and miR-140 are critical for palatogenesis during craniofacial development in mouse and zebrafish, respectively[7,8]. The contribution of miRNAs to the earliest neural crest fate decisions of the embryo has not been thoroughly investigated. Studies of miRNAs in early fate decisions have been limited by technical challenges such as the low cell number present in early embryos.

MiRNAs have been profiled in vitro from cell lines using embryoid bodies at different stages of differentiation to model early mouse development[9]. The investigation of small RNAs in vivo during early mammalian development has been relatively limited. Previous methods to profile miRNAs have led to bias as a known sequence is used to analyze expression of a specific miRNA in methods such as qPCR, microarrays, and northern blots[10]. Next generation sequencing and ever improving molecular tools now allow for relatively unbiased analysis of miRNA expression to study their contribution to early mammalian development and cell fate decisions.

Here, we report a technique to harvest and sequence small RNAs expressed in neural crest cells from early mouse embryos spanning gastrulation (E7.5) to the beginning of organogenesis (E9.5). This technique is straightforward and combines lineage tracing, cell sorting, and gel-based size selection to prepare small RNA sequencing libraries from a minimal number of cells for next generation sequencing. We highlight the importance for strict somite stage matching of embryos to resolve 6-hour time intervals to obtain a comprehensive view of miRNAs during the rapid changes of early development. This method can be widely applied to genetic and developmental studies and avoids the pooling of embryos. We describe a way to overcome challenges of current methods such as miRNA enrichment using gel-based purification, library quantification, and minimizing bias introduced from PCR. This method has been used to identify miRNA expression patterns over time to study how miRNAs control developmental timing in the neural crest lineage of mouse embryos.

PROTOCOL:

All research and animal care procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and housed in the Association for Assessment and

Accreditation of Laboratory Animal Care-approved animal facility at Baylor College of Medicine. All strains were maintained on C57BL6 background.

1. Embryo dissection (E7.5-E9.5)

1.1. Remove uterine horns from a pregnant female mouse, sterilizing the abdomen with 70% ethanol where the incision is to be made.

1.2. Clean off the uterus by rinsing with phosphate buffered saline (PBS). Place the uterus into a sterile plastic 10 cm dish.

1.3. Using microdissection scissors, separate each decidua containing region from one another. Peel off the uterine muscle and expose all deciduae. One by one, remove the decidua from each embryo.

1.4. Remove the yolk sac from each embryo and save for genotyping.

1.5. Move all embryos into a new dish of fresh PBS for imaging.

1.6. Take an image of the entire litter. Image each embryo individually and count the number of somites of each embryo. Keep the magnification and exposure (for fluorescence) the same between experiments.

NOTE: We find that 50-200 ms exposure is adequate for fluorescence and that 20 ms is adequate for bright field setting.

2. Embryo dissociation and cell sorting

2.1. For each embryo to be sorted from, do the following.

2.1.1. Decapitate just above the otic placode for embryos older than E8.0 (if only labeled cells of the cranial region are desired). For E7.5 embryos, remove extraembryonic structures (if only the embryo proper is desired).

2.1.2. Move the head in a minimal volume of PBS over to a clean well of a 48-well plate. Add 250 µL of papain (27 U/mL). Pipette up and down gently using a p200.

2.1.3. Check under the microscope and look for clumps and single cells.

2.1.4. Repeat gentle pipetting up and down until a single cell suspension is achieved (usually three rounds of pipetting up and down which should equate to between 30 s to 1 min for E7.5-E9.5).

2.1.5. Quench with 250 µL of fetal bovine serum (FBS).

2.1.6. Repeat steps 2.1.1-2.1.5 for each embryo to be sorted.

2.2. Filter 500 µL of cell suspension through a 35 µm nylon mesh filter cap tube to remove clumps.

2.3. Take the filtrate and move to new 1.5 mL tube and spin at 200 x *g* for 5 min. Remove supernatant carefully and transfer to new tube (save until live cells are known to be in the pellet).

2.4. Resuspend cell pellet in 300 µL of PBS containing 0.5-1% bovine serum albumin and keep on ice until sorting (minimize the time between now and the end of sorting).

2.4.1. If desired, take 10 µL of cell suspension and combine with 10 µL of Trypan blue (0.4%) and count using a hemocytometer to identify approximate cell quantification and viability as Trypan blue only stains dead cells.

2.5. Just before sorting, filter each sample again through a filter cap tube and add DAPI stain for live cells.

2.6. Sort each sample into 500 µL of RNA extraction lysis solution (see **Table of Materials**) on cell sorter with 70 µm nozzle.

2.7. Mix and store at -80 °C until all samples are harvested. Proceed from this point only when all samples needed for an experiment are harvested to reduce technical variation between rounds of library preparation.

3. RNA extraction

NOTE: The protocol is adapted from the RNA isolation kit; see **Table of Materials.**

3.1. Add 500 µL of RNA extraction lysis solution (see **Table of Materials**) to each sample to make the total volume 1000 µL.

3.2. Thaw each sample at room temperature.

3.3. Vortex each sample for 1.5 min, making sure that the cap is securely on each tube before starting homogenization. Incubate the homogenate at room temperature (15–25 °C) for 5 min.

3.4. Add 140 µL of chloroform, cap tube securely and shake vigorously for 15 s. Incubate at room temperature for 3 min.

3.5. Centrifuge for 15 min at 12,000 x *g* at 4 °C. Transfer the upper aqueous phase to a new collection tube on ice. Do not transfer any interphase or any organic phase. When approaching

the pink organic phase, tip the tube to side and remove small aliquots until no clear aqueous phase can be collected.

3.6. Measure the volume of RNA containing aqueous phase collected from each sample for the correct calculation in the next step.

3.7. Add 1.5 volumes (usually 525 µL but may be slightly more or less) of 100% ethanol and mix thoroughly by pipetting.

3.8. Pipette up to 700 µL sample, including any precipitate, into column in a 2 mL collection tube. Close the lid and centrifuge at ≥8,000 x *g* for 15 s at room temperature. Discard the flow-through.

3.9. Repeat step 3.8 using the remainder of the sample.

3.10. Wash the column as per manufacturer instructions. Dry the column by spinning at full speed for 1 min. Transfer the column to a new 1.5 mL collection tube.

3.11. Pipette 11 µL of nuclease free water onto the center of the membrane. Let sit at room temperature for 1 min. Spin max speed for 1 min to elute off the column.

3.12. Measure RNA concentration using a spectrophotometer and a parallel capillary electrophoresis instrument (**Table of Materials**).

4. Library preparation

NOTE: The protocol is adapted from small RNA library preparation kit handbook; see **Table of Materials**.

4.1. Complete the denaturation and 3’ adapter ligation as stated in the small RNA library preparation kit handbook using ¼ dilution of 3’ adapter.

4.2. Continue to excess 3' adapter removal.

4.3. Complete the adapter depletion as stated in the small RNA library preparation kit handbook. Be sure to use freshly prepared 80% ethanol for the bead cleanup and that all excess ethanol is completely removed just prior to the resuspension in nuclease-free water without over-drying the beads (cracking of the bead pellet is observed upon over-drying).

4.4. Proceed directly to excess adapter inactivation.

4.5. Complete the excess adapter inactivation as stated in the small RNA library preparation kit handbook assembling all reagents on ice.

4.6. Continue to 5' 4N adapter ligation.

4.7. Complete 5' adapter ligation as stated in the small RNA library preparation kit handbook being sure to use a ¼ dilution of the 5’ adapter and assembling all reagents on ice.

4.8. Proceed to reverse transcription.

4.9. Complete reverse transcription-first strand synthesis as stated in the small RNA library preparation kit handbook and taking care to assemble all reagents on ice and not keeping the enzyme out of the freezer for extended periods of time.

NOTE: At this point, the protocol may be stopped, with samples stored overnight at 4 °C. Alternatively continue to PCR amplification.

4.10. Complete PCR amplification by assembling the reagents as stated in the small RNA library preparation kit handbook and minimizing the number of PCR cycles. The number of PCR cycles should be experimentally determined for each application and the minimum number of cycles should be used. Here we used 16 cycles to successfully amplify our small RNA libraries.

4.11. Proceed directly to size selection.

5. Size selection

5.1. To each sample, add 5 µL of 6x gel loading dye and pipette to mix.

5.2. Load 10 µL of ladder to the first well of the gel, leaving the well immediately to the right empty. Load all of product from step 5.1 onto a 6% Tris/borate/ethylenediaminetetraacetic acid – polyacrylamide gel (TBE-PAGE) and leave 1 lane in between each sample.

NOTE: Keep the container that the gel came in for subsequent steps.

5.3. Run the gel at 150 V for approximately 30-40 min in 0.5x TBE running buffer (until the lower dye band is near the bottom of the gel, 0.5-1 cm).

5.4. Make 1 L of staining solution while the gel is running: SYBR Gold diluted 1:10,000 in 0.5x TBE.

5.5. When the gel has finished running (i.e., lower dye band is near the bottom of the gel), carefully remove the gel from the glass plates, noting the orientation of the gel.

5.6. Place gel in the tray of its original packaging. Remove the 0.5x TBE and replace with the staining solution from step 5.5. Stain gel for 15 min. Staining time may need to be increased.

5.7. Place the gel on a UV transilluminator, taking care to not rip the gel and maintaining the orientation noted above (re-stain for additional time if bands of ladder cannot be clearly seen).

5.8. Image the gel once staining is complete on UV transilluminator with a camera.

5.8.1. If a better image is required, first use an imaging apparatus other than the UV transilluminator to capture an image of the gel before cutting out the bands on a UV transilluminator as imaging directly on the UV transilluminator causes the variable coloring of the background as shown in **Figure 3A-B**. Do not move the gel too many times as it is delicate and may tear.

5.9. Identify and remove the ~150 bp band using a clean razor blade and place into clean 1.5 mL tube. Do not cut out the ~130 bp band (adapter dimer product).

5.9.1. Image the gel again after removing the slices containing the library product for documentation purposes.

5.10. Spin the microcentrifuge tubes containing the gel slice at max speed for 30 s to collect the slices at the bottom of each tube.

5.11. Use a p200 tip for each sample to crush the slice of gel into the smallest bits possible. Eject each tip into each tube.

5.12. To each tube, add 300 µL of elution buffer, taking care to wash gel bits from side of tube and reattach the p200 tip and wash off the tip for each sample.

5.13. Place the eluting samples in a shaking incubator set to 25 °C. Incubate overnight with 1000 rpm shaking. Remove tubes from incubator and spin at max speed for 10 min at room temperature.

5.14. Transfer the supernatant containing the library product to a 2 mL RNase free 96 well plate. Take care to not transfer any gel.

5.15. Add 50 µL cleanup beads to each sample and mix by pipetting. Immediately, add 350 µL of isopropanol and mix by pipetting. Incubate at room temperature for 10 minutes with rocking.

5.16. Pulse spin plate to pellet beads and then magnetize for 2 min. Carefully remove and discard the supernatant.

5.17. Add 950 µL of 80% ethanol. Incubate for 30 s. Remove all of the supernatant. Repeat for a total of two ethanol washes.

5.18. Dry the sample for 3 min. At this step take care to remove any residual ethanol that collects at the bottom of each well (not more than once) and to not over-dry the beads (over-drying will result in cracking of the bead pellet).

5.19. Remove all residual liquid at the bottom of the tube. Remove the plate from the magnetic stand.

5.21. Resuspend the pellet in 13 µL of resuspension buffer. Mix by pipette until homogeneous. Incubate for 2 min and then magnetize for 3 min.

5.22. Transfer 12 µL of supernatant to a clean tube or well of clean plate for subsequent storage. Store all samples at 4 °C overnight or -20 °C long term.

5.23. Check the size and concentration of fragments present in each library using the capillary electrophoresis high sensitivity DNA assay. Confirm the concentration with more than one method.

NOTE: We have occasionally used the standard sensitivity kit to avoid overloading the capillary electrophoresis.

REPRESENTATIVE RESULTS:

Using the procedure demonstrated here, we have harvested embryos at E7.5, E8.5, and E9.5. Extraembryonic structures were removed from all embryos and then embryos were somite staged to resolve 6-hour time intervals (**Figure 1A-1B**). Using principle component analysis to group samples based on similarity, we find that samples cluster by age, highlighting the variation as a result of embryonic age and the need for careful somite matching for biological replicates (**Figure 1C**). We profiled the pluripotent epiblast at E7.5, lineage traced premigratory and migratory neural crest cells using Wnt1-Cre at E8.5 and migratory neural crest cells using Sox10-Cre at E9.5 (**Figure 1D**). Here we specifically harvest the cranial neural crest by decapitating the embryo just above the otic placode. A comparison of the two Cre-drivers (Wnt1 and Sox10) that are frequently used to label neural crest cells confirms they mark different populations in early mouse embryos (**Figure 1E**). Gating strategies were used to obtain live single RFP positive cells which were sorted directly into RNA extraction lysis solution (see **Table of Materials**) and stored at -80 °C (**Figure 1F**). It is important to keep track of how many cells were harvested from each sample.

RNA isolation was performed using a modified version of the RNA kit protocol. Specifically, we use the mini RNA columns that are to be stored at 4 °C. These columns are useful for eluting in a small volume (11 µL) to obtain the highest possible concentration of RNA from samples where cell input is limiting. For this same reason, it is important to obtain all of the aqueous phase after the phenol chloroform extraction. Slow pipetting and tilting the tube to one side while collecting are critical to maximize yield. In this procedure, we quantify the RNA using both a spectrophotometer, to obtain information on salt/protein contamination, and a capillary electrophoresis to measure concentration (**Figure 2**). The spectrophotometer trace reveals that

RNA was isolated with no contaminating proteins but has high salt content (**Figure 2A-2B**). Ideally the 260/280 ratio should be ~1.8 and the 260/230 ratio should be >2.0. The total RNA yield as measured by the spectrophotometer did not increase with age between E8.5-E9.5. This is due to the resolution of the spectrophotometer not being sensitive enough to detect changes in RNA concentration from the number of cells that we are harvesting, and we recommend using the concentration information obtained from the capillary electrophoresis (**Figure 2C**). The capillary electrophoresis trace can be used to estimate concentration and size of the RNA fragments. Peaks <1000 nucleotides are indicative of degradation. The representative trace is consistent with RNA that is not degraded. Peaks at 2000 nucleotides and 5100 nucleotides are 18s and 28s rRNA, respectively. The small RNA region is located at ~150 nucleotides (**Figure 2D**).

Small RNA sequencing libraries were prepped using the small RNA library prep kit described in the **Table of Materials**. Here we use just less than half of the RNA obtained from each sample (4 µL of the 11 µL elution, ~80-120 ng) that enables RNA-sequencing libraries to be synthesized from the remaining RNA from each sample. Here we dilute the 3' and 5' small RNA adapters ¼ to lower the amount of adapter dimers present and suggest that the adapter ligation, cleanup, and reverse transcription steps are completed as much as possible in a continuous manner. We have used 16 PCR cycles to amplify the libraries and suggest that the minimum number of PCR cycles for any experiment be empirically determined. By completing excess PCR cycles, one could artificially inflate the read count of a lowly expressed miRNA.

Size selection is imperative to enrich for libraries of miRNAs and not adapter dimers, which are typically present. The library product size (150 bp) and adapter dimers (130 bp) are very similar in size. Gel extraction is used to isolate the small RNA sequencing libraries away from the adapter dimers (**Figure 3**). An image of a PAGE gel before excision shows that a multitude of product sizes are present in each sample (**Figure 3A**). It is important to leave one lane open between any two samples or a ladder that is loaded onto the gel. The migration front at the bottom of the gel is slightly curved indicating that running at a slower speed may be necessary if the 150 bp band is difficult to distinguish for all samples. This representative image also shows an abundance of 150 bp product from the higher concentration of positive control RNA (total RNA from the brain of a rat) that went into the library prep as compared to that which went into each embryonic sample. The negative control reveals that the reagents were free of contaminating nucleic acid species (**Figure 3A**). Excision of the 150 bp band should be done with a clean razor blade and the area collected is shown in **Figure 3B**. Capillary electrophoresis traces before and after size selection show the dramatic improvement of the purity of the 150 bp library product with gel purification (**Figures 3C-3D**). It is important to note that the traces in **Figures 3C-3D** have sample peaks higher than the markers, indicative of overloading. In these cases, the size of the fragments can be accurate, however the concentration may not. Quantification can be obtained by diluting the libraries into the range of the markers or using alternative methods. We find some variation across alternative methods of library quantification and recommend that multiple methods of quantification be empirically tested. Accurate quantification of concentration is essential when maximizing the number of samples to be sequenced at one time. Libraries will be diluted down to 1.3 pM for sequencing and generally anywhere from 15-25 samples can be sequenced at one

time on a 150 cycle sequencing kit that provides ~140 million reads. This results in about 5 million reads per sample. Generally mapping rates are between 60-80%.

FIGURE AND TABLE LEGENDS:

Figure 1: Harvesting cells from mouse embryos for small RNA sequencing.

(**A**) E8.5 mouse embryo to highlight the removal of the yolk sac and somites used to determine the stage of the embryo (**B**) Somite staging of mouse embryos to capture the stages of neural crest development (**C**) Principle component analysis of libraries prepped from sorted wildtype neural crest cells showing that samples group by age (**D**) Schematic showing how samples were harvested using Wnt1-Cre at E8.5 to label premigratory and migratory neural crest cells and Sox10-Cre at E9.5 to label only migratory neural crest cells (**E**) Principle component analysis of libraries prepped from sorted wildtype neural crest cells showing samples group together by Cre-driver regardless of age (**F**) Gating strategy used to isolate RFP+ neural crest cells from E8.5 and E9.5 embryos using FACS sorting.

Figure 2: RNA isolation from E7.5-E9.5 mouse embryos

(**A**) Representative spectrophotometer trace of an RNA isolation from the youngest stage of embryo that we have applied this protocol. (**B**) Table showing the representative values of RNA quality obtained from the spectrophotometer. (**C**) Example of the RNA harvested from sorted neural crest cells from embryos of each age (**D**) Capillary electrophoresis trace showing good quality RNA.

Figure 3: miRNA libraries before and after size selection

(**A**) TBE-PAGE gel showing representative small RNA libraries for four samples and controls before and (**B**) after 150 bp band excision. The arrows represent the 150 bp band to be excised (**C**) Capillary electrophoresis trace of small RNA library before and (**D**) after size selection.

DISCUSSION:

Developmental processes can proceed rapidly, and cells are undergoing many sequential specifications such that to capture a comprehensive view of the miRNAs contributing to early fate decisions, more specific staging is needed than the widely used half-day increment. A recent study has performed RNA sequencing from Theiler stage 12 embryos which range from having 3 to 6 somites[11]. We find that during this period of time, the neural crest cells are specified (3 somites of age), delaminate (4 somites of age), and migrate (5-6 somites of age). We also find that besides the Cre-driver used to lineage trace cell populations, age is the largest source of variation between biological samples, and only somite matched embryos should be considered as replicates. This should also be taken into consideration when comparing transgenic embryos to wildtype controls.

Previous methods to profile miRNAs during early development have used between 10-100 ng of RNA input for small RNA sequencing library preparation and have pooled multiple embryos into one sample[13,14]. We demonstrate RNA isolation and library preparation from a single E7.5 embryo or from sorted neural crest cells at E8.5 and E9.5 using approximately 100 ng of total RNA as input. When dissociating embryos for sorting, one should take care to watch the

dissociation under a microscope and quench the reaction to observe when single cells are obtained. We find that dissociation of the cranial region of E8.5-E9.5 embryos is almost instant with gentle manual pipetting as described in the protocol. For larger tissues and increasingly older embryos, dissociation time may be longer depending on the portion of the embryo being dissociated. For E7.5-E9.5 embryos, clumps of cells are easily visible under the microscope and the dissociation should continue until no more clumps are visible. Single cells are visible in solution if you adjust the focus through the solution in your well anywhere from 5-10x. Previous methods sort cells directly into lysis buffer for RNA sequencing to prep bulk RNA from a low number of cells[15]. Here we sort directly into RNA extraction lysis solution so that RNA can be isolated before the start of library preparation. Use of mini columns with 11 µL elution volume allowed for a high enough RNA concentration such that a single RNA prep could be split between small RNA and bulk RNA sequencing.

One current limitation of most small RNA sequencing methods is the PCR amplification of converted cDNA. Our method does not overcome this limitation, but we were able to minimize the number of PCR cycles from the 25-maximum recommended down to 16 cycles. This reduction in amplification decreases artificial amplification bias introduced by PCR. Another source of bias is the ligation of adapters, where it is known that specific sequences located at the ends of adapters and miRNAs can ligate together with greater efficiency than other sequences. To avoid this, the adapters used in this protocol have 4 random bases incorporated at the end of each adapter to prevent bias in ligation reactions. Additionally, another common issue is the amount of adapter dimers that form when the RNA input is low. The library preparation kit does include steps to reduce adapter dimer formation such as adapter inactivation and bead cleanups to remove excess adapters after each ligation. We also diluted the 3' and 5' 4N adapters by ¼ to reduce the amount of adapter dimer that can form. We found that when not diluted, the 130 bp band intensity increases making it difficult to distinguish from the 150 bp band containing the desired small RNA libraries on a gel.

Another current challenge of preparing sequencing libraries is the accurate quantification of product prior to sequencing. We have found that different methods give varying results on the same library. We suggest that the researchers use multiple methods of quantification to get an accurate estimation of concentration.

This protocol can be widely applied to genetic, developmental studies, or other applications where RNA is being harvested from a low number of cells. This approach simplifies temporal studies by avoiding the pooling of embryos and can easily be applied to both non-sorted and sorted cells.

ACKNOWLEDGMENTS:

The authors would like to acknowledge the following funding sources: R.J.P. is a CPRIT Scholar in Cancer Research (RR150106), V Scholar in Cancer Research (V Foundation) and was supported by funds from Andrew McDonough B+ Foundation and the NIH (R01-HD099252). The authors would like to acknowledge the Cytometry and Cell Sorting Core at BCM for support.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES: (Instructions)

1. Sauka-Spengler, T., Bronner-Fraser, M. A gene regulatory network orchestrates neural crest formation. *Nature Reviews Molecular Cell Biology*. **9**, 557–568 (2008).

2. Abrahante, J. E. et al. The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. *Developmental Cell*. **4** (5), 625–637 (2003).

3. Lin, S.-Y. et al. The C elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. *Developmental Cell*. **4** (5), 639–650 (2003).

4. 4.Reinhart, B. J. et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature*. **403** (6772), 901 (2000).

5. Slack, F. J. et al. The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Molecular Cell. 5 (4), 659–669 (2000).

6. Vella, M. C., Choi, E.-Y., Lin, S.-Y., Reinert, K., Slack, F. J. The C. elegans microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. *Genes & Development*. **18** (2), 132–137 (2004).

7. Ventura, A. et al. Targeted deletion reveals essential and overlapping functions of the miR-17 92 family of miRNA clusters. *Cell*. **132** (5), 875–886 (2008).

8. Eberhart, J. K. et al. MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. *Nature Genetics*. **40** (3), 290 (2008).

9. Li, Y. et al. Dynamic regulation of small RNAome during the early stage of cardiac differentiation from pluripotent embryonic stem cells. *Genomics Data*. **12**, 136-145 (2017).

10. Chugh, P., Dittmer, D. P. Potential pitfalls in microRNA profiling. *Wiley Interdisciplinary Reviews: RNA*. **3** (5), 601-616 (2012).

11. Werber, M., Wittler, L., Timmermann, B., Grote, P., Herrmann, B. G. The tissue-specific transcriptomic landscape of the mid-gestational mouse embryo. *Development*. **141** (11), 2325-2330 (2014).

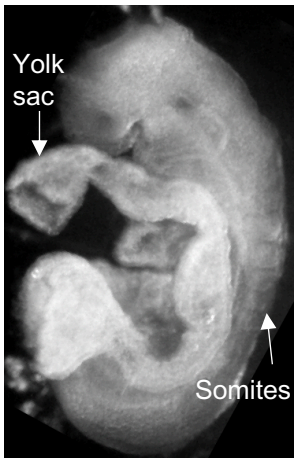
12. Yang, Q. et al. Highly sensitive sequencing reveals dynamic modifications and activities of small RNAs in mouse oocytes and early embryos. *Science Advances*. **2.6** (2016).

13. Ohnishi, Y. et al. Small RNA class transition from siRNA/piRNA to miRNA during pre-implantation mouse development. *Nucleic Acids Research*. **38** (15), 5141-5151 (2010).

14. Loontiens, S. et al. Purification of high-quality RNA from a small number of fluorescence activated cell sorted zebrafish cells for RNA sequencing purposes. *BMC Genomics*. **20** (1), 228 (2019).

Figure 1

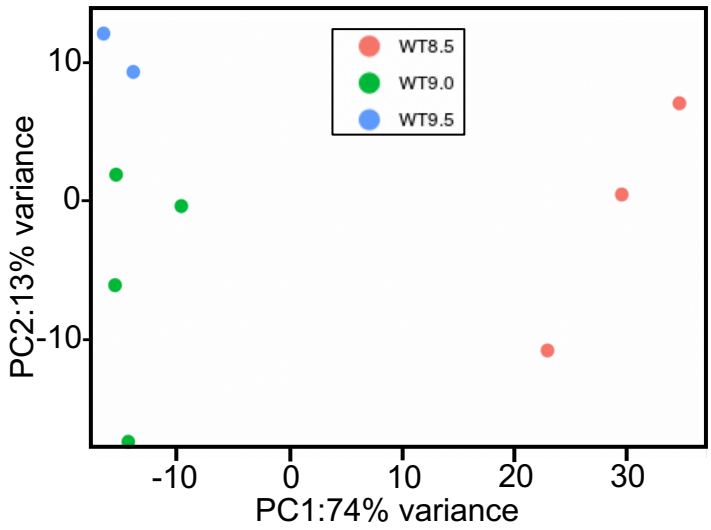
A



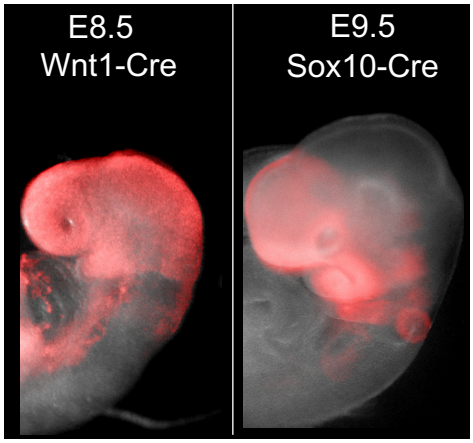
B

Somites	Stage
0-2	E8.0
3-4	E8.25
5-7	E8.5
8-12	E8.75
13-20	E9.0
21-24	E9.5

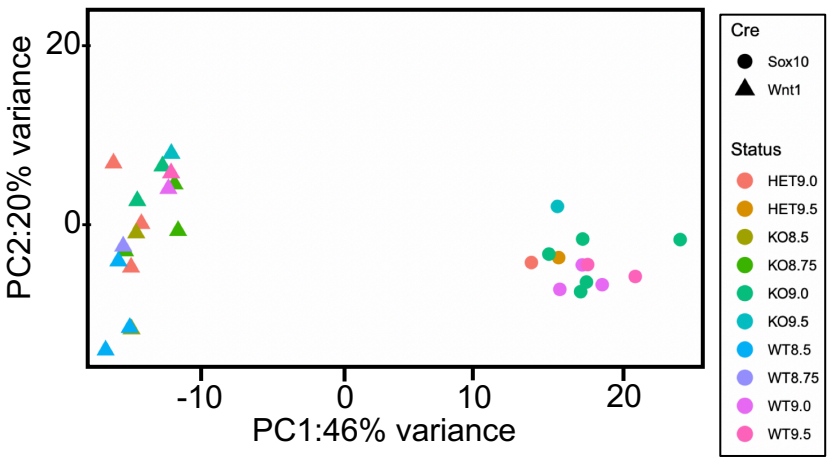
C



D



E



F

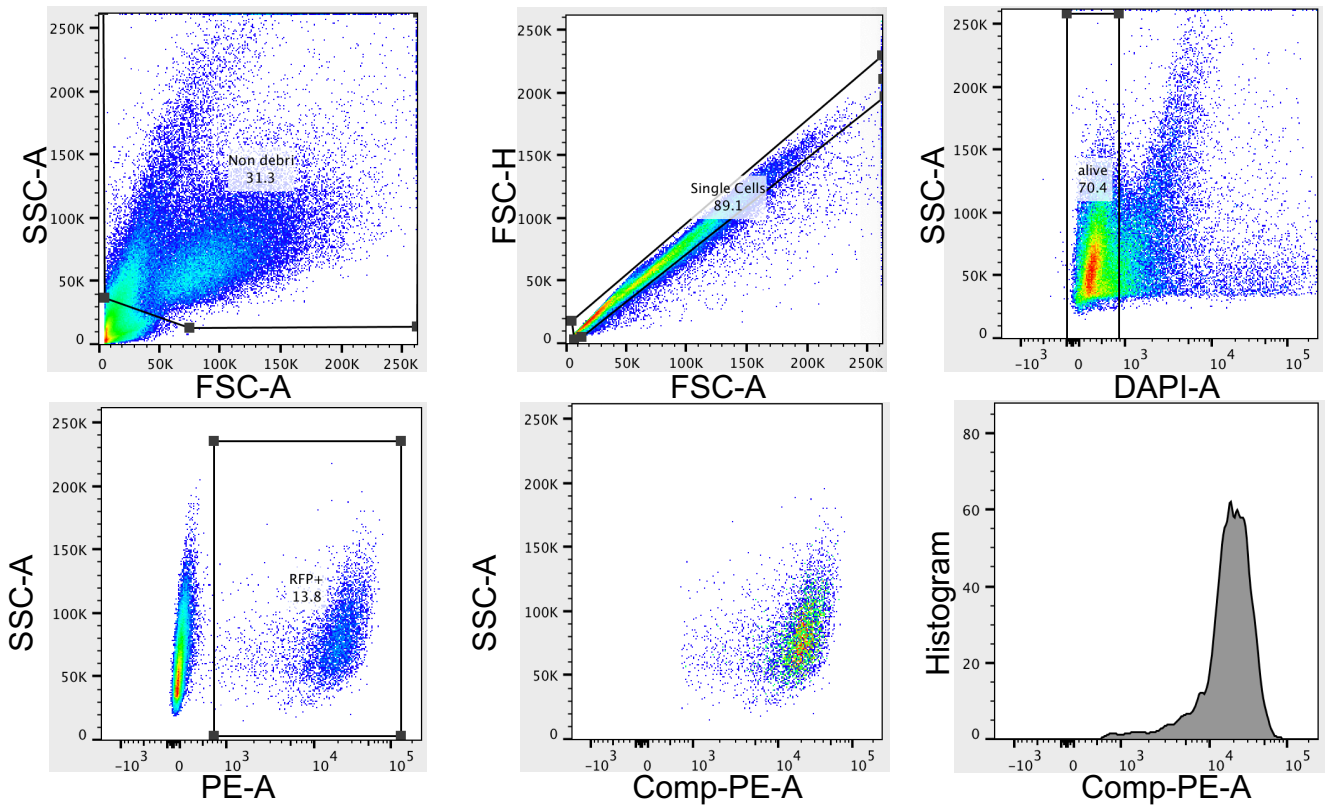
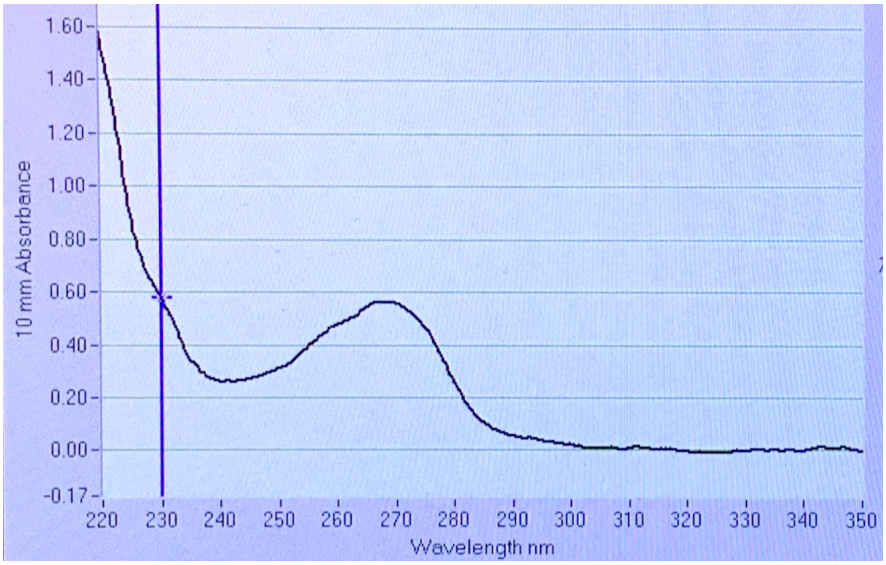


Figure 2

A



B

Absorbance	0.578
A260 10mm path	0.480
A280 10mm path	0.258
260/280	1.86
260/230	0.83
Concentration (ng/µL)	19.2

C

Cre-Driver	Age	Somites	Cell Number	RNA Conc. (ng/µL)	Total RNA yield (ng)
Wnt1	E8.5	5	3023	29.1	320.1
Wnt1	E8.75	10	5224	23.3	256.3
Wnt1	E9.5	21	10972	22.7	249.7

D

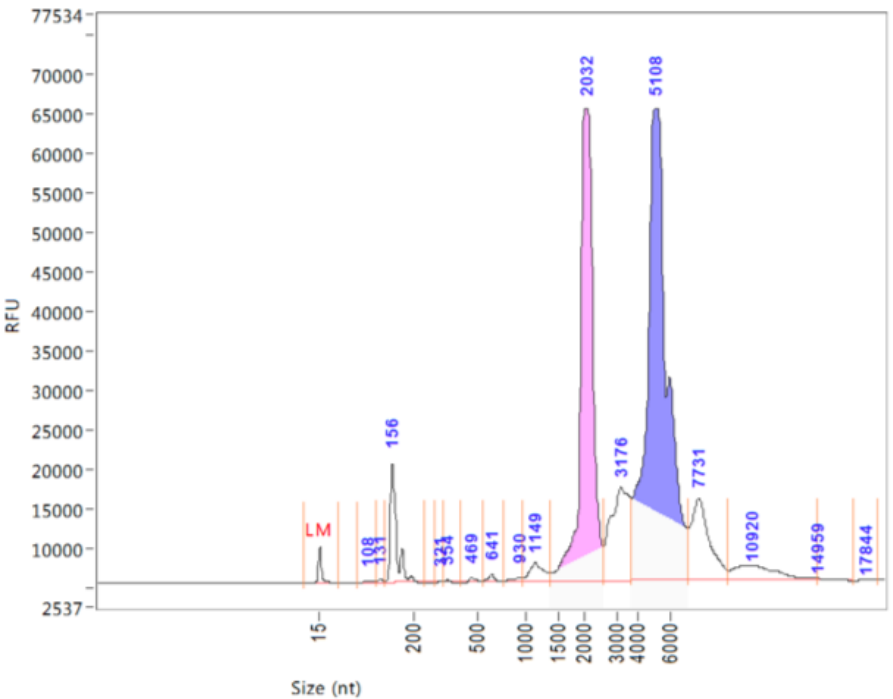


Figure 3

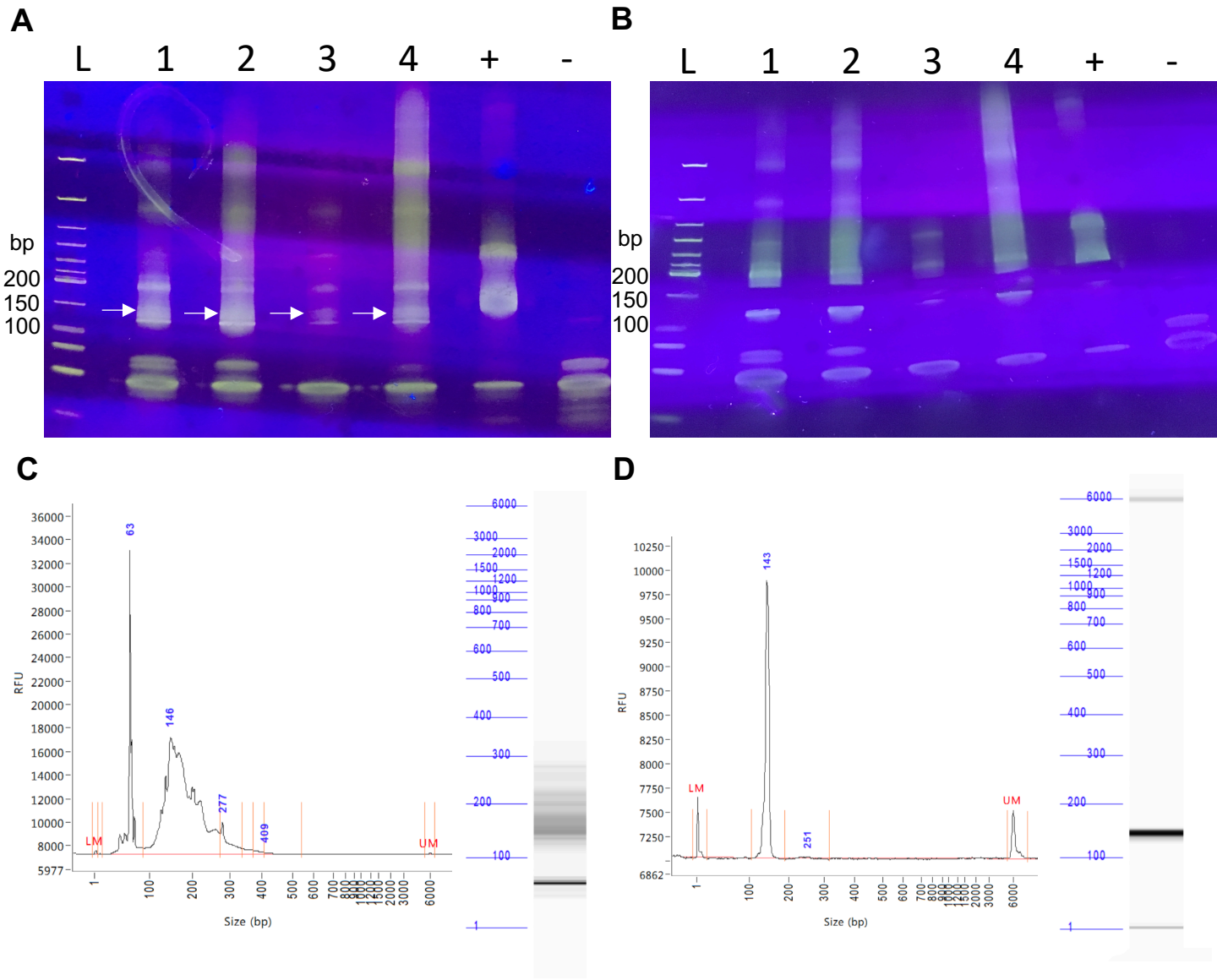


Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_1.9.20.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
#5 Forceps Fine Science Tools	Fisher Scientific	NC9277114	
0.4% Trypan blue	ThermoFisher	15250061	
10 cm Petri dishes	Fisher Scientific	07-202-011	
2-Propanol	Miilapore Sigma	I9516-500ML	
5 % Criterion TBE Polyacrylamide Gel, 12+2 well, 45 µL	Bio-Rad	3450047	
5200 Fragment Analyzer	Agilent	M5310AA	
96 well PCR plates high profile semi skirted clear/clear	Bio-Rad	HSS9601	
BD FACSAria II	bdbiosciences		
Bovine Serum Albumin, fraction V	Fisher Scientific	BP1600100	
Dulbecco's Phosphate-Buffered Saline (DPBS)	Gendepot	CA008-300	
Eppendorf tubes	Fisher Scientific	05-408-129	
Ethanol Pure, 200 proof anhydrous	Sigma Aldrich	E7023-500ml	
FC-404-2001	Illumina	FC-404-2001	
HS NGS Fragment kit	Agilent	DNF-474-0500	
HS RNA Kit	Agilent	DNF-472-0500	
miRNeasy Mini Kit (50)	Qiagen	217004	
NEXTflex Small RNA-Seq Kit v3 (48 barcodes)	Fisher Scientific	NC1289113	
NextSeq 500/550 Mid Output Kit v2 (150 cycles)	Illumina	FC-404-2001	
NGS Fragment kit	Agilent	DNF-473-1000	
Papain	Worthington	LK003176	resuspend in 5 mL of Ca/Mg free PBS for a final concentra
SYBR Gold nucleic acid gel stain	ThermoFisher	S11494	
Trizol LS	ThermoFisher	10296028	

UVP Benchtop UV Transilluminators: Single UV	Fisher Scientific	UVP95044701
Vannas Scissors Straight Fine Science Tools #91500-09	Fisher Scientific	NC9609583

ation of 27 U/mL

We thank the reviewers for their critical feedback. To summarize this response, we have added more detail to the protocol, figure legends, and the results section. Additionally, we have more clearly labeled the figures. We have also added an animal ethics statement and removed commercial language. A detailed response to all reviewer comments can be found below.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples (this is not an exhaustive list):
1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.
2) Line 81: Mention animal strain.

We have now added an ethics statement and mouse strain on lines 77-80:

"All research and animal care procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility at Baylor College of Medicine. All strains were maintained on C57BL6 background."

3) Line 90: Mention imaging settings including fluorescence settings.

We have added the settings for imaging on lines 96-97:

"We find that 50-200 ms exposure is adequate for fluorescence and that 20 ms is adequate for bright field setting."

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow

from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

We have highlighted ~2.5 pages of text.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have included this information in the discussion on lines 439-486:

"Developmental processes can proceed rapidly, and cells are undergoing many sequential specifications such that to capture a comprehensive view of the miRNAs contributing to early fate decisions, more specific staging is needed than the widely used half-day increment. A recent study has performed RNA sequencing from Theiler stage 12 embryos which range from having 3 to 6 somites11. We find that during this period of time, neural crest are specified (3s), delaminate (4s), and migrate (5-6s)12. We also find that besides the Cre-driver used to lineage trace cell populations, age is the largest source of variation between biological samples, and only somite matched embryos should be considered as replicates. This should also be taken into consideration when comparing transgenic embryos to wildtype controls.

Previous methods to profile miRNAs during early development have used between 10-100ng of RNA input for small RNA sequencing library preparation and have pooled multiple embryos into one sample13,14. We demonstrate RNA isolation and library preparation from a single E7.5 embryo or from sorted neural crest cells at E8.5 and E9.5 using approximately 100ng of total RNA as input. When dissociating embryos for sorting, one should take care to watch the dissociation under a microscope and quench the reaction to observe when single cells are obtained. We find that dissociation of the cranial region of E8.5-E9.5 embryos is almost instant with gentle manual pipetting as described in the protocol. For larger tissues and increasingly older embryos, dissociation time may be longer depending on the portion of the embryo being dissociated. For E7.5-E9.5 embryos, clumps of cells are easily visible under the microscope and the dissociation should continue until no more clumps are visible. Single cells are visible in solution if you adjust the focus through the solution in your well anywhere from 5-10x. Previous methods sort cells directly into lysis buffer for RNA sequencing to prep bulk RNA from a low number of cells15. Here we sort directly into RNA extraction lysis solution so that RNA can be isolated before the start of library preparation. Use of mini columns with 11 µl elution volume allowed for a high enough RNA concentration such that a single RNA prep could be split between small RNA and bulk RNA sequencing.

One current limitation of most small RNA sequencing methods is the PCR amplification of converted cDNA. Our method does not overcome this limitation, but we were

able to minimize the number of PCR cycles from the 25-maximum recommended down to 16 cycles. This reduction in amplification decreases artificial amplification bias introduced by PCR. Another source of bias is the ligation of adapters, where it is known that specific sequences located at the ends of adapters and miRNAs can ligate together with greater efficiency than other sequences. To avoid this, the adapters used in this protocol have 4 random bases incorporated at the end of each adapter to prevent bias in ligation reactions. Additionally, another common issue is the amount of adapter dimers that form when the RNA input is low. The library preparation kit does include steps to reduce adapter dimer formation such as adapter inactivation and bead cleanups to remove excess adapters after each ligation. We also diluted the 3' and 5' 4N adapters by ¼ to reduce the amount of adapter dimer that can form. We found that when not diluted, the 130 bp band intensity increases making it difficult to distinguish from the 150 bp band containing the desired small RNA libraries on a gel.

Another current challenge of preparing sequencing libraries is the accurate quantification of product prior to sequencing. We have found that different methods give varying results on the same library. We suggest that the researchers use multiple methods of quantification to get an accurate estimation of concentration.

This protocol can be widely applied to genetic, developmental studies, or other applications where RNA is being harvested from a low number of cells. This approach simplifies temporal studies by avoiding the pooling of embryos and can easily be applied to both non-sorted and sorted cells."

• **References:**

1) Please use superscripted in-text citations numbering.
2) Please ensure that references are numbered in the manuscript in chronological order, that is, the first reference that appears in the manuscript should be numbered 1.
3) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

The references have been reformatted and have been numbered in the text.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are E-tube, BD FACSAria™, Trizol-LS, Qiagen miRNeasy, Bioo Scientific NEXTflex V3

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

We have now made sure not to include any commercial language throughout the manuscript.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We have removed figures from other sources.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
This manuscript describes the procedure to obtain small RNA from sorted cells of mice embryos and prepared it for sequencing. The authors document proper sample concentration and quality. Besides, they prepared the RNA library as well as described the selection of samples by their size. Overall this is an interesting protocol but in parts it read a bit vague and not methodologically detailed. Editing of the abstract, protocol, representative results, and figures to have a more directed correlation are needed. In order to improve this manuscript for publication, I have a few specific comments as well as some minor suggestions:

Major Concerns:
Title and abstract:
*In my opinion, the title "Temporal profiling of miRNA expression during early mouse development" is not reflecting the procedure described in the manuscript.

We agree and have changed the title to “Preparation of small RNA libraries for sequencing from early mouse embryos”.

*The phrase: "Experimentally, we demonstrate that stage-matched mouse embryos must be used to accurately profile miRNAs in biological replicates." is does not deal adequately with the specific protocol developed in the study (small RNA preparation from mice embryos for sequencing), since neither miRNA elucidation nor comparison are performed. Rephrasing it should be considered.

We have rephrased this sentence on lines 30-32:

"We identify embryonic age as a variable accounting for variation between replicates and stage-matched mouse embryos must be used to accurately profile miRNAs in biological replicates."

Introduction:
*Some references are omitted, especially in the first paragraph.

We have added references to the introduction on lines 38-74:

"A central question of developmental biology is how a single undifferentiated cell can give rise to an entire organism with numerous complex cell types. During embryogenesis the developmental potential of cells becomes progressively restricted as the organism develops. One example is the neural crest lineage, which progressively differentiates from a multipotent cell population into various terminal derivatives, such as peripheral neurons, glia, cranial bone, and cartilage. Neural crest cells are specified from the ectoderm during gastrulation and then undergo an epithelial to mesenchymal transition and migrate through the embryo to discrete locations throughout the body where they will terminally differentiate[1]. Decades of work has uncovered a transcriptional gene regulatory network, but far less is known about mechanisms of post-transcriptional regulation that control the timing of neural crest development.

Previous work suggests that miRNAs repress gene expression for proper developmental timing and cell fate decisions[2,3,4,5,6]. Studies of miRNAs in neural crest development have largely focused on later stages of craniofacial development. For example, miR-17~92 and miR-140 are critical for palatogenesis during craniofacial development in mouse and zebrafish, respectively[7,8]. The contribution of miRNAs to the earliest neural crest fate decisions of the embryo has not been thoroughly investigated. Studies of miRNAs in early fate decisions have been limited by technical challenges such as the low cell number present in early embryos.

MiRNAs have been profiled *in vitro* from cell lines using embryoid bodies at different stages of differentiation to model early mouse development[9]. The investigation of small RNAs *in vivo* during early mammalian development has been relatively limited. Previous methods to profile miRNAs have led to bias as a known sequence is used to analyze expression of a specific miRNA in methods such as qPCR, microarrays, and northern blots[10]. Next generation sequencing and ever improving molecular tools now allow for relatively unbiased analysis of miRNA expression to study their contribution to early mammalian development and cell fate decisions.

Here we report a technique to harvest and sequence small RNAs expressed in neural crest cells from early mouse embryos spanning gastrulation (E7.5) to the beginning of organogenesis (E9.5). This technique is straightforward and combines lineage tracing, cell sorting, and gel-based size selection to prepare small RNA sequencing libraries from a minimal number of cells for Next Generation Sequencing on an Illumina platform. We highlight the importance for strict somite stage matching of embryos to resolve 6-hour time intervals to obtain a comprehensive view of miRNAs during the rapid changes of early development. This method can be widely applied to genetic and developmental studies and avoids the pooling of embryos. We describe a way to overcome challenges of current methods such as miRNA enrichment using gel-based purification, library quantification, and minimizing bias introduced from PCR. This method has been used to identify miRNA expression patterns over time to study how miRNAs control developmental timing in the neural crest lineage of mouse embryos."

*It is not clear what authors say in lines 60-62, this sentence needs to be rephrased.

We have reworded the sentence to clarify that we are identifying a knowledge gap of in-vivo information as to how miRNAs are contributing to early mammalian development on lines 55-57:

"MiRNAs have been profiled *in vitro* from cell lines using embryoid bodies at different stages of differentiation to model early mouse development[9]. The investigation of small RNAs *in vivo* during early mammalian development has been relatively limited."

Protocol:
*In the "II. Embryo dissociation and cell sorting" protocol, neither the concentration nor the time of papain incubation is detailed (line 100). From my experience, this a critical step and both variables should be stated for all the developmental stages analyzed. Besides, this enzyme does not appear in the table of materials.

We thank you for pointing out this oversight. We have added the concentration of papain to the protocol and the enzyme/dilution instructions has been added to the table of resources. We have adjusted the text on lines 99-129 to reflect these changes, which is quoted below:

1. "For each embryo to be sorted from do the following:
    a. Decapitate just above the otic placode for embryos older than E8.0 (if only labeled cells of the cranial region are desired). For E7.5 embryos, remove extraembryonic structures (if only the embryo proper is desired).
    b. Move the head in a minimal volume of PBS over to a clean well of a 48-well plate.
    c. Add 250 µl of papain (27U/ml).
    d. Pipette up and down gently using a p200.
    e. Check under the microscope and look for clumps and single cells.
    f. Repeat gentle pipetting up and down until a single cell suspension is achieved (usually three rounds of pipetting up and down which should equate to between 30 seconds to 1 minute for E7.5-E9.5).
    g. Quench with 250 µl of fetal bovine serum (FBS).
    h. Repeat steps 1a-g for each embryo to be sorted.
2. Filter 500 µl of cell suspension through a 35 µm nylon mesh filter cap tube to remove clumps.
3. Take filtrate and move to new 1.5ml tube and spin at 200g for 5min.
4. Remove supernatant carefully and transfer to new tube (save until you know that you have live cells in your pellet).
5. Resuspend cell pellet in 300 µl of PBS containing 0.5-1% bovine serum albumin and keep on ice until sorting (minimize the time between now and the end of sorting). If desired, take 10 µl of cell suspension and combine with 10 µl of

trypan blue (0.4%) and count using a hemocytometer to identify approximate cell quantification and viability as trypan blue only stains dead cells.
6. Just before sorting, filter each sample again through a filter cap tube and add DAPI stain for live cells.
7. Sort each sample into 500 µl of RNA extraction lysis solution (see table of materials) on cell sorter with 70 µm nozzle.
8. Mix and Store at -80 until all samples are harvested. We recommend proceeding from this point only when all samples needed for an experiment are harvested to reduce technical variation between rounds of library preparation."

*The description between brackets in lines 109-110 is not clear. How could I know that I have live cells in the pellet?

We have now included this on lines 118-122:

5. "Resuspend cell pellet in 300 µl of PBS containing 0.5-1% bovine serum albumin and keep on ice until sorting (minimize the time between now and the end of sorting). If desired, take 10 µl of cell suspension and combine with 10 µl of trypan blue (0.4%) and count using a hemocytometer to identify approximate cell quantification and viability as trypan blue only stains dead cells."

*In line 133, authors should suggest which type of RNA measurement is the appropriate one.

We have now included this on line 163-164:

21. "Measure the RNA concentration using both a spectrophotometer and a parallel capillary electrophoresis instrument, see table of materials (high sensitivity RNA kit)."

Representative Results:
*Sentence in lines 262-264 is confused and should be rephrased.

We have now rephrased this sentence to make the purpose of PCA analysis and the variation that results from embryonic age on lines 344-347:

"Using principle component analysis to group samples based on similarity, we find that samples cluster by age, highlighting the variation as a result of embryonic age and the need for careful somite matching for biological replicates (Figure 1C)."

*Why do you think that the total RNA yield did not increase with an increase in cell number harvested from older embryos (comment in lines 281-282)?

We have now included this information on line 365-370:

"The total RNA yield as measured by the spectrophotometer did not increase with age between E8.5-E9.5 and we expect that this is due to the resolution of the spectrophotometer not being sensitive enough to detect changes in RNA concentration from the number of cells that we are harvesting and we recommend using the concentration information obtained from the capillary electrophoresis (Figure 2C)."

*The PCR steps mentioned in lines 293-294 are not described within the protocol.

We thank you for pointing out this oversight, we have included this step on lines 208-212:

> 11."Complete PCR amplification by assembling the reagents as stated in the small RNA library preparation kit handbook and minimizing the number of PCR cycles. The number of PCR cycles should be experimentally determined for each application and the minimum number of cycles should be used. Here we used 16 cycles to successfully amplify our small RNA libraries."

*Better pictures of gels should be presented. The slightly curved at the bottom of the gel is indicating that running at a slower speed may be necessary. Besides, there is a variable background (as lights and shadows) in both pictures. Molecular weights of markers should be indicated, as well as the sample information. The white box should be thinner or discontinuous.

We have labeled the molecular weight markers, changed the box to arrows to designate what should be cut out and mentioned in the results that the gel should be run at a slower speed on lines 393-395:

"The migration front at the bottom of the gel is slightly curved indicating that running at a slower speed may be necessary if the 150bp band is difficult to distinguish for all samples."

We have now also discussed the limitations of our imaging within the protocol on lines 237-289:

> 11. "Image the gel once staining is complete on UV transilluminator with a camera. If a better image is required, we recommend first using an imaging apparatus other than the UV transilluminator to capture an image of the gel before cutting out the bands on a UV transilluminator as imaging directly on the UV transilluminator causes the variable coloring of the background as shown in figure 3A-B. We want to caution against moving the gel too many times as it is delicate and may tear."

Discussion:
*In line 366-367, the cell dissociation process should have been optimized and detailed (time and enzyme concentration) unless for the three developmental stages analyzed in the present protocol.

We thank you for highlighting the need for more detail on the dissociation. We now include more detail in the discussion on lines 454-459:

"We find that dissociation of the cranial region of E8.5-E9.5 embryos is almost instant with gentle manual pipetting as described in the protocol. For larger tissues and increasingly older embryos, dissociation time may be longer depending on the portion of the embryo being dissociated. This must be determined empirically. For E7.5-E9.5 embryos, clumps of cells are easily visible under the microscope and the dissociation should continue until no more clumps are visible. Single cells are visible in solution if you adjust the focus through the solution in your well anywhere from 5-10x."

*If the lowering in the PCR steps performed in this protocol overcome the problem of less bias introduced by this technique, results should be compared and demonstrated.

Lowering the number of PCR cycles to reduce bias has been established previously (van Dijk et al. 2014).

Minor Concerns:
*In line 43, the word "cells" after neural crest is absent.

We have now added the word "cells" after neural crest on line 43.

*I wondered if each section protocol (roman numbers) should start with a new Arabic number counter or not. Since both options are coexisting.

We have also seen examples of both and chose the roman numbers.

*The micro unit (µ instead of u) should be properly specified all throughout the protocol. Besides, the temperature in which the different procedures are performed should be detailed.

We thank you for pointing this out we have replaced with the proper symbol and added more details regarding temperature throughout the protocol.

*In line 106, the "4a-g" expression does not correlate with the protocol numbers.

We thank the reviewer for pointing out this oversight and we have now changed this to match the protocol on line 112:

"h. Repeat steps 1a-g for each embryo to be sorted."

*In line 107, the filter specification of the "filter cap tube" should be stated.

We have now included the 35 µm size of the filters

*In line 117, the verb "be sequenced" should be changed for "proceed with the protocol" or similar, since much more steps come before the sequencing.

We have changed the wording to clarify that we only mean to not proceed until all samples for an experiment are harvested. Since embryos of the preferred genotype may not all come out of a single dissection, it is better to wait until you have all the samples needed for the experiment to reduce technical variation. We have changed this text on lines 127-129:

8. "Mix and Store at -80 until all samples are harvested. We recommend proceeding from this point only when all samples needed for an experiment are harvested to reduce technical variation between rounds of library preparation. "

*In line 135, the explanation between brackets should be avoided.

We have clarified the wording as to why this is listed as two steps in the protocol instead of a single step. We find that is important to first measure the volume of RNA containing aqueous phase obtained and then add the appropriate amount of ethanol. We have changed this on lines 149-152:

9. "Measure the volume of RNA containing aqueous phase collected from each sample for the correct calculation in the next step.
10. Add 1.5 volumes (usually 525 µl but may be slightly more or less) of 100% ethanol and mix thoroughly by pipetting. "

*In line 140, it is not clear which step should be repeated (since this is step 12).

We thank you for pointing this out and we have now changed line 156 so the correct step 11 (not 12) is stated to be repeated.

*Information stated on lines 157 to 161 is duplicated and should be unified.

We have now removed this duplication and have replaced the text on lines 166-169 to read:

I. "Library preparation (Protocol adapted from small RNA library preparation kit handbook, see table of materials)
    1. Complete the denaturation and 3' adapter ligation as stated in the small RNA library preparation kit handbook using ¼ dilution of 3' adapter."

*Line 188 should be removed.

We thank the reviewer for pointing this out. We have changed the text on lines 206-213 to read:

10. At this point, the protocol may be stopped, with samples stored overnight at 4°C. Alternatively continue to PCR amplification.
11. Complete PCR amplification by assembling the reagents as stated in the small RNA library preparation kit handbook and minimizing the number of PCR cycles. The number of PCR cycles should be experimentally determined for each application and the minimum number of cycles should be used. Here we used 16 cycles to successfully amplify our small RNA libraries.
12. Proceed directly to size selection.

*Information between brackets in lines 211-212 should be excluded.

We have now changed this step on lines 237-289:

> 11.“Image the gel once staining is complete on UV transilluminator with a camera. If a better image is required, we recommend first using an imaging apparatus other than the UV transilluminator to capture an image of the gel before cutting out the bands on a UV transilluminator as imaging directly on the UV transilluminator causes the variable coloring of the background as shown in figure 3A-B. We want to caution against moving the gel too many times as it is delicate and may tear.”

*Sentence in lines 248-251 is confused and should be rewritten.

We have now changed the text on lines 325-327 to read:

> 37.“Transfer 12 µL of supernatant to a clean tube or well of clean plate for subsequent storage. We recommend storing all samples at 4°C overnight or -20°C long term.”

Figure and Table legends:
*In Figure 1A should be indicated with arrows or asterisk the yolk sac and somites.

We have now added arrows and labels to the yolk sac and somites

*Title of Figure 2 should be changed, since it mentioned embryos at stage E7.5 but the Figure shows results from different stages.

We have now changed the figure title to:

"Figure 2: RNA isolation from E7.5-E9.5 embryos"

*In Figure legend 2, A and B are switched.

We thank the reviewer for pointing this out and we have now fixed the legend on lines 428-431:

"Figure 2: RNA isolation from E7.5 mouse embryo
(A) Representative spectrophotometer trace of an RNA isolation from the youngest stage of embryo that we have applied this protocol, E7.5.and (B) table showing the representative readings. (C) Example of the RNA harvested from sorted neural crest cells from embryos of each age. (D) Capillary electrophoresis, trace showing good quality RNA."

*In Figure 3, pictures A and B should be replaced for better ones.

We have now better labeled the photos and we have chosen these photos to demonstrate the imaging on a UV transilluminator and to suggest the optimization of voltage for running the gel.

We have now changed this step on lines 237-289:

> 11."Image the gel once staining is complete on UV transilluminator with a camera. If a better image is required, we recommend first using an imaging apparatus other than the UV transilluminator to capture an image of the gel before cutting out the bands on a UV transilluminator as imaging directly on the UV transilluminator causes the variable coloring of the background as shown in figure 3A-B. We want to caution against moving the gel too many times as it is delicate and may tear."

We have also added within the results clarification on lines 391-402:

"An image of a PAGE gel before excision shows that a multitude of product sizes are present in each sample (Figure 3A). It is important to leave one lane open between any two samples or a ladder that is loaded onto the gel. The migration front at the bottom of the gel is slightly curved indicating that running at a slower speed may be necessary if the 150bp band is difficult to distinguish for all samples. This representative image also shows an abundance of 150bp product from the higher concentration of positive control RNA (total RNA from the brain of a rat) that went into the library prep as compared to that which went into each embryonic sample. The negative control reveals that the reagents were free of contaminating nucleic acid species (Figure 3A). Excision of the 150bp band should be done with a clean razor blade and the area collected is shown in Figure 3B. Capillary electrophoresis traces before and after size selection show the dramatic improvement of the purity of the 150bp library product with gel purification (Figures 3D-E)."

Reviewer #2:
This manuscript described a protocol for profiling microRNAs in early mouse embryos. The authors state that this protocol overcomes the challenge of low cell input and small RNA enrichment and can be used to analyze changes in miRNA expression over time in different cell lineages of the early mouse embryos. While it is true that profiling microRNAs in early mouse embryos is limited by some challenges, including the low cell number and a technique that can overcome these challenges is needed for elucidating the role of microRNAs in the early mouse development, there are some concerns with the described protocol.

One of the major concerns is that the objective of this protocol is not clear. As indicated in the title of this manuscript "Temporal profiling of miRNA expression during early mouse development", this protocol should focus on different developmental stages. However, this protocol has a more detailed description of cell sorting and focuses on "Cell-specific profiling" rather than "Temporal profiling". Another concern is that the described protocol only includes three steps of the miRNA profiling: cell sorting, RNA extraction and library preparation. If, as described in the title and throughout the protocol, the protocol is used for profiling of miRNA expression during early mouse development, the methods for small RNA sequencing and related bioinformatics analysis should also be included in this protocol. In addition, all procedures in this protocol should be described in more detailed.

We have now changed the title to better reflect the content of the protocol:

“Preparation of small RNA libraries for sequencing from early mouse embryos”

In addition, we have added more detail to steps in the protocol such as within the embryo dissection and dissociation sections on lines 82-129:

“

Protocol

II. “Embryo Dissection (E7.5-E9.5)

1. Remove uterine horns from a pregnant female mouse sterilizing the abdomen with 70% ethanol where the incision is to be made.
2. Clean off the uterus by rinsing with phosphate buffered saline (PBS).
3. Place uterus into a sterile plastic 10 cm dish.
4. Using microdissection scissors separate each decidua containing region from one another.
5. Peel off the uterine muscle and expose all deciduae.
6. One by one, remove the decidua from each embryo.
7. Remove the yolk sac from each embryo and save for genotyping.
8. Move all embryos into a new dish of fresh PBS for imaging.
9. Take an image of the entire litter.

10. Image each embryo individually and count the number of somites of each embryo. It is best to keep the magnification and exposure (for fluorescence) the same between experiments. We find that 50-200 ms exposure is adequate for fluorescence and that 20 ms is adequate for bright field setting.

III. Embryo dissociation and cell sorting

9. For each embryo to be sorted from do the following:
    a. Decapitate just above the otic placode for embryos older than E8.0 (if only labeled cells of the cranial region are desired). For E7.5 embryos, remove extraembryonic structures (if only the embryo proper is desired).
    b. Move the head in a minimal volume of PBS over to a clean well of a 48-well plate.
    c. Add 250 µl of papain (27U/ml).
    d. Pipette up and down gently using a p200.
    e. Check under the microscope and look for clumps and single cells.
    f. Repeat gentle pipetting up and down until a single cell suspension is achieved (usually three rounds of pipetting up and down which should equate to between 30 seconds to 1 minute for E7.5-E9.5).
    g. Quench with 250 µl of fetal bovine serum (FBS).
    h. Repeat steps 1a-g for each embryo to be sorted.
10. Filter 500 µl of cell suspension through a 35 µm nylon mesh filter cap tube to remove clumps.
11. Take filtrate and move to new 1.5ml tube and spin at 200g for 5min.
12. Remove supernatant carefully and transfer to new tube (save until you know that you have live cells in your pellet).
13. Resuspend cell pellet in 300 µl of PBS containing 0.5-1% bovine serum albumin and keep on ice until sorting (minimize the time between now and the end of sorting). If desired, take 10 µl of cell suspension and combine with 10 µl of trypan blue (0.4%) and count using a hemocytometer to identify approximate cell quantification and viability as trypan blue only stains dead cells.
14. Just before sorting, filter each sample again through a filter cap tube and add DAPI stain for live cells.
15. Sort each sample into 500 µl of RNA extraction lysis solution (see table of materials) on cell sorter with 70 µm nozzle.
16. Mix and Store at -80 until all samples are harvested. We recommend proceeding from this point only when all samples needed for an experiment are harvested to reduce technical variation between rounds of library preparation."

We have also added detail within the library preparation section to include more detail about the number of PCR cycles on lines 208-212:

11. "Complete PCR amplification by assembling the reagents as stated in the small RNA library preparation kit handbook and minimizing the number of PCR cycles. The number of PCR cycles should be experimentally determined for each application and the minimum number of cycles should be used. Here we used 16 cycles to successfully amplify our small RNA libraries."

Other concerns:
1. Before the embryos dissection section, a section for animals, including the information for wnt1-Cre and Sox10-Cre mice, should be added.

We have now added information regarding our animal procedures to lines 77-80:

"All research and animal care procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility at Baylor College of Medicine. All strains were maintained on C57BL6 background."

2. In "embryo dissection", Step 2. "Clean off the uterus by spraying off quickly with 70% ethanol". Instead of spraying uterus with ethanol, ethanol should be sprayed to the caudal dorsal area of the pregnant female mouse before an incision in the body wall is made.

We have now changed lines 83-85 to read:

1. "Remove uterine horns from a pregnant female mouse sterilizing the abdomen with 70% ethanol where the incision is to be made.
2. Clean off the uterus by rinsing with phosphate buffered saline (PBS)."

3. In the "embryo dissociation and cell sorting" section, 11. a. "Decapitate just above the otic placode" and 11b "Move the head". These two steps should be stage-specific. If whole embryos are collected from E7.5, these steps should be deleted.

We have now clarified this on lines 100-102:

a. "Decapitate just above the otic placode for embryos older than E8.0 (if only labeled cells of the cranial region are desired). For E7.5 embryos, remove extraembryonic structures (if only the embryo proper is desired)."

4. Throughout the manuscript, a space between number and unit is needed, e.g. "10cm" should be "10 cm". In addition, ul should be µl

We thank the reviewer for pointing this out and we have now made these changes throughout the manuscript.

5. Fig. 2 C. Example of the RNA harvested from sorted neural crest cells from embryos of each age. As indicated on page 8 line 265, neural crest cells will be traced using Sox1-Cre at E9.5. It is unclear why, in this figure, Wnt1-Cre was used for sorting neural crest cells at E9.5.

We thank the reviewer for pointing this out and we have now clarified this on lines 347-352:

"We profiled the pluripotent epiblast at E7.5, lineage traced premigratory and migratory neural crest cells using Wnt1-Cre at E8.5 and migratory neural crest using Sox10-Cre at E9.5 (Figure 1D). Here we specifically harvest the cranial neural crest by decapitating the embryo just above the otic placode. A comparison of the two Cre-drivers (Wnt1 and Sox10) that are frequently used to label neural crest confirms they mark different populations in early mouse embryos (Figure 1E)."

6. Fig. 2 C. While the number of the Wnt1 positive cells is significantly higher in E8.75 than E8.5 and in E9.5 than E8.75, it is unclear why the total RNA (not Wnt1 mRNA) in E8.75 is less than that in E8.5 and the total RNA in E9.5 is less than that in E8.75.

We have included this information as a rough guide for researchers and have clarified this on lines 365-370:

"The total RNA yield as measured by the spectrophotometer did not increase with age between E8.5-E9.5 and we expect that this is due to the resolution of the spectrophotometer not being sensitive enough to detect changes in RNA concentration from the number of cells that we are harvesting and we recommend using the concentration information obtained from the capillary electrophoresis (Figure 2C)."

Reviewer #3:
Manuscript Summary:
This is a nicely written method article providing a protocol for profiling miRNAs of low cell input.

Major Concerns:
none

Minor Concerns:
1. For RNA extraction step 9, page 5 line 133 "Measure the 'amount' of RNA containing aqueous phase", does 'amount' mean the 'volume'?

We thank the reviewer for pointing out the confusion caused by our wording and we have now clarified these steps on lines 149-152 to read:

3. "Measure the volume of RNA containing aqueous phase collected from each sample for the correct calculation in the next step.
4. Add 1.5 volumes (usually 525 µl but may be slightly more or less) of 100% ethanol and mix thoroughly by pipetting. "

2. For RNA extraction step 12, page 5 line 140 "Repeat step '12' using the remainder of the sample", should be step '11'.

We thank the reviewer for pointing out this oversight and we have changed this to step 11 instead of step 12 on line 156.

3. Figure legend should be more descriptive. Figure 1C: is the PCA analysis based on wild type embryos? Is the RNA from sorted neural crest cells? Figure 1D: why compare two neural crest cell cre-drivers at different stages but not the same (wnt1-cre at E8.5 and Sox10-cre at E9.5)? Figure 1E: the genotypes for "WT, heter, KO" should be stated.

We thank the reviewer for pointing out our lack of detail and have changed the figure 1 legend on lines 415-424:

"Figure 1: Harvesting cells from mouse embryos for small RNA sequencing
(A) E8.5 mouse embryo to highlight the removal of the yolk sac and somites used to determine the stage of the embryo. (B) Somite staging of mouse embryos to capture the stages of neural crest development. (C) Principle component analysis of libraries prepped from sorted wildtype neural crest cells showing that samples group by age. (D) Schematic showing how samples were harvested using Wnt1-Cre at E8.5 to label premigratory and migratory neural crest cells and Sox10-Cre at E9.5 to label only migratory neural crest cells. (E) Principle component analysis of libraries prepped from sorted wildtype neural crest cells showing samples group together by Cre-driver regardless of age. (F) Gating strategy used to isolate RFP+ neural crest cells from E8.5 and E9.5 embryos using FACS sorting."

Figure 2C: It looks like the total RNA yield decreased with more cell number from older embryos, any explanations? Is the RNA isolation method only good for younger embryos?

We have now commented on this within the representative results section on lines 365-370:

"The total RNA yield as measured by the spectrophotometer did not increase with age between E8.5-E9.5 and we expect that this is due to the resolution of the spectrophotometer not being sensitive enough to detect changes in RNA concentration from the number of cells that we are harvesting and we recommend using the concentration information obtained from the capillary electrophoresis analyzer (Figure 2C)."

4. For Results part, page 8 line 285: "Peaks at 2000 nucleotides and 5100 nucleotides are 18s and '26s' rRNA", '26s' should be '28s' rRNA if the RNA is from mouse.

We thank the reviewer for pointing out this obvious mistake and we have changed line 372-374 to read:

"Peaks at 2000 nucleotides and 5100 nucleotides are 18s and 28s rRNA, respectively. The small RNA region is located at ~150 nucleotides (Figure 2D)."

1. Please upload each figure separately.

We have now removed the titles from the figures and uploaded each as its own file.

2. Additionally, there are some discrepancies with the figures and the figure captions that need to be addressed. Comments on this are in the attached manuscript.

There was a discrepancy between the Figure 2 title in the figure versus the manuscript and we have changed the Figure 2 title within the manuscript on line 407:

"Figure 2: RNA isolation from E7.5-E9.5 mouse embryos"

Commented [A1]: The Figure says E7.5-E9.5. Which is it?

The description for panel B was lacking in detail and we have now changed line 409-410 to read:

"Table showing the representative values of RNA quality obtained from the spectrophotometer."

Commented [A2]: Please use the Greek symbol mu for microliter abbreviations. Please capitalize the L in the microliter abbreviation. Similarly for Panel C.

We have replaced the images used in Figure 1D with images of two different embryos. These embryos are of the same genotype and developmental stage, however we want to avoid potential overlap with images used in another submitted manuscript. We believe that this change has no scientific impact on the manuscript.

3. Additional comments are in the attached manuscript.

There was confusion regarding the definition of "s" on line 424-425 and we have now clarified that we are talking about embryonic age within this sentence:

"We find that during this period of time, the neural crest cells are specified (3 somites of age), delaminate (4 somites of age), and migrate (5-6 somites of age)."

Commented [A3]: Neural crest cells?

We have also removed the reference to our recent work that is under review and is not technically accepted yet.