Chapter 8

RNA Isolation from Ctenophores

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Abstract

RNA-seq or transcriptome analysis of individual cells and small cell populations is essential for virtually any biomedical field. Here, we examine and discuss the different methods of RNA isolation specific to ctenophores. We present a convenient, inexpensive, and reproducible protocol for RNA-seq libraries that are designed for low quantities of samples. We demonstrated these methods on early (one, two, four, eight cells) embryonic and developmental stages, tissues, and even a single aboral organ from the ctenophore *Pleurobrachia bachei* and other ctenophore species (e.g., *Mnemiopsis*, *Bolinopsis*, and *Beroe*).

Key words Ctenophora, Single cells, Transcriptome, RNA-seq, *Pleurobrachia*, Development

1 Introduction

RNA sequencing (RNA-seq) has and will continue to profoundly impact medicine and clinical and basic research. It has become indispensable for virtually any biomedical field, providing an unbiased view of the entire molecular machinery within a biological system of interest. The rapid technological progress has catapulted RNA-seq to be the most sensitive tool for gene expression analysis today. The number of different RNA-seq protocols has grown exponentially from its inception over a decade ago [1–3]. To perform an RNA-seq experiment, a robust sequencing library has to be constructed. Illumina, Inc. provides a diversity of protocols: >50 RNA-seq and >30 single-cell RNA-seq (scRNA-seq) library construction techniques (https://www.illumina.com/techniques/sequencing/rna-sequencing.html). Depending on the amount of starting material, total RNA, message RNA (mRNA), small RNA, targeted RNA, RNA exome-capture, and single-cell, the scope of possible RNA-seq methods is overwhelming.

Here we present a workflow convenient for simpler workplaces and expedition/fieldwork conditions, starting with animal collections, RNA isolation, and high-quality RNA-seq library generation (Fig. 1). We also offer options in the workflow for cloning genes of

Leonid L. Moroz (ed.), *Ctenophores: Methods and Protocols*, Methods in Molecular Biology, vol. 2757,
https://doi.org/10.1007/978-1-0716-3642-8_8,

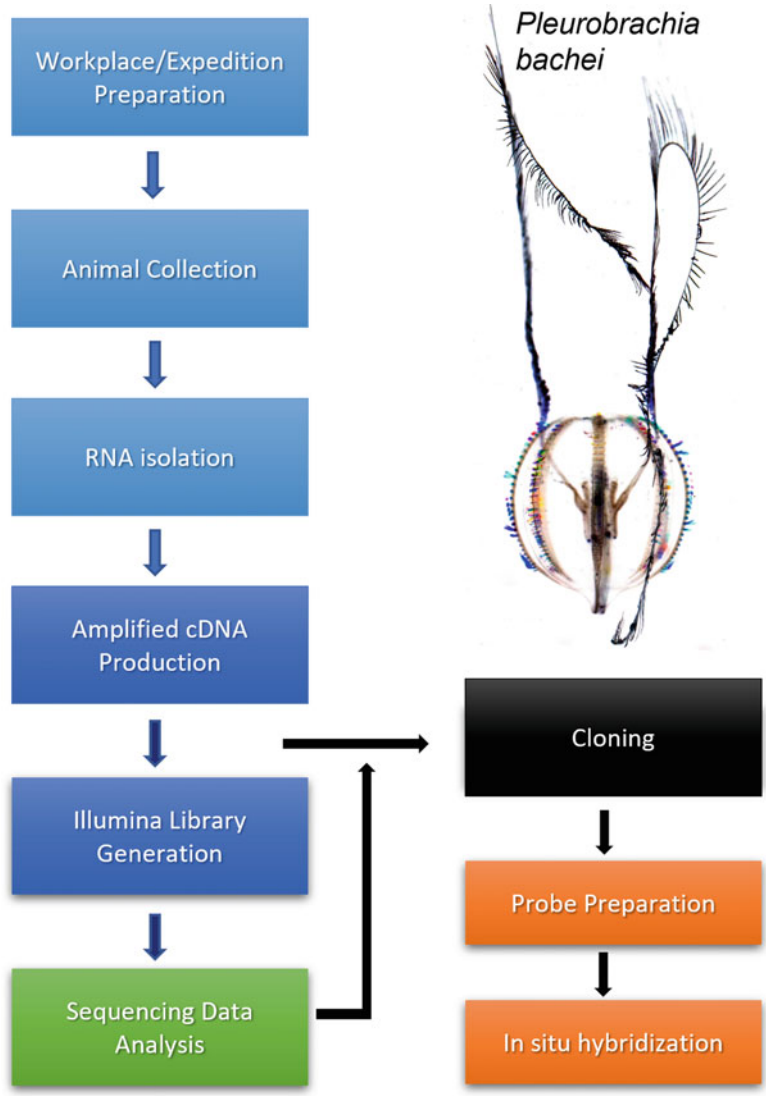

Fig. 1 Workflow for RNA isolation and possible downstream applications. Amplified cDNA can be used as a template for cloning (*see* **Note 3**). Gene sequences are obtained from the analyzed sequencing data

interest and probe production for validation with in situ hybridization (Fig. 1); see also the respective chapter in this book. Libraries can be constructed from very low input <40 pg of total RNA, and our protocol produces high-quality sequencing data. As illustrative examples, the starting material included early (one, two, four, eight cells) embryonic and developmental stages, tissues, and even a single aboral organ from the ctenophore *Pleurobrachia bachei* [4] as well as other ctenophores and mollusks [5–9]. We validated these results with hundreds of in situ hybridizations using probes designed from many of these sequencing projects.

2 Materials

2.1 RNA Isolation

RNeasy Micro Kit (Cat # 74104, QIAGEN).

RNeasy Mini Kit (Cat # 74004, QIAGEN).

QIAshredder (Cat # 79654, QIAGEN).

gDNA Eliminator Spin Columns (Cat # 74134, QIAGEN).

RNase-free DNase Set (Cat # 79254, QIAGEN).

Nuclease-free Water (not DEPC-treated) (Cat # AM9937, ThermoFisher Scientific).

RNaseZap™ RNase Decontamination Solution (Cat # AM9780, ThermoFisher Scientific).

MagaZorb® total RNA mini-prep kit (Cat # MB2004, Promega).

Maxwell® RSC simply RNA tissue kit (Cat # AS1340, Promega).

NUCLEOSPIN RNA II from Macherey-Nagel (Fisher Cat # NC9581114).

E-Gel® SizeSelect™ 2% Agarose (Cat # G6610-02, ThermoFisher Scientific).

Qubit™ RNA BR Assay Kit (Cat # Q10210, ThermoFisher Scientific).

Qubit™ RNA HS Assay Kit (Cat # Q32852, ThermoFisher Scientific).

Qubit™ dsDNA BR Assay Kit (Cat # Q32850, ThermoFisher Scientific).

RNA ScreenTape (Cat # 5067-5576, Agilent Technologies).

RNA ScreenTape Sample Buffer (Cat # 5067-5577, Agilent Technologies).

RNA ScreenTape Ladder (Cat # 5067-5578, Agilent Technologies).

High Sensitivity RNA ScreenTape (Cat # 5067-5579, Agilent Technologies) total.

High Sensitivity RNA ScreenTape Sample Buffer (Cat # 5067-5580, Agilent Technologies).

High Sensitivity RNA ScreenTape Ladder (Cat # 5067-5581, Agilent Technologies).

2.2 Library Construction

Advantage® UltraPure PCR dNTP Mix (Cat # NC9287432, ThermoFisher Scientific).

SMARTScribe Reverse Transcriptase (Cat # 639536, Takara Bio).

Advantage 2 PCR Kit (Cat # 639207, Takara Bio).

Rnase Inhibitor (Cat # N8080119, Takara Bio).

Table 1
Adaptors and primers for library construction

Primer name	Primer sequence
CapTS oligonucleotide	5′-AGCAGTGGTATCAACGCAGAGTACrGrGrG-3′[a]
CapT30 primer	5′-AAGCAGTGGTATCAACGCAGAGTACT(30)-3′
Cap PCR primer	5′-AAGCAGTGGTATCAACGCAGAGT-3′

[a]The r is designated as a ribonucleotide

SMART® cDNA Library Construction Kit (Cat # 63490, Takara Bio).

SPRIselect Reagent (Cat # B23318, Beckman Coulter).

NEBNext® Ultra II DNA Library Prep Kit for Illumina® (Cat # E7645S, New England Biolabs).

NEBNext® Ultra II multiplex oligos (Cat # E7335S, New England Biolabs).

NEBNext® Ultra II multiplex oligos Dual Index Primers (Cat # E7600S, New England Biolabs).

High Sensitivity D1000 Screen tape (Cat # 5067-5584, Agilent Technologies).

High Sensitivity D1000 Reagents (Cat # 5067-5585, Agilent Technologies).

High Sensitivity D1000 Ladder (Cat # 5067-5587, Agilent Technologies).

High Sensitivity D1000 Sample Buffer (Cat # 5067-5603, Agilent Technologies).

TOPO-TA cloning kit (Cat # K203001, ThermoFisher Scientific).

1 kb DNA ladder (Cat # N3232L, New England Biolabs)

Primers 0.2 uM scale HPLC purified, IDT, Integrated DNA Technologies, Inc. (*see* Table 1 for all primer and adaptor sequences).

2.3 Equipment

LoBind tubes 0.5 uL, 1.5 uL (Cat # 80077-236, 80077-230 Eppendorf, VWR International).

DynaMag™-2 magnet (Cat # 123-21D, ThermoFisher Scientific).

MicroTUBE AFA fiber Screw-Cap 6 × 16 mm (Cat # NC0380760/520096, Covaris).

Agilent TapeStation™ 4200 or Bioanalyzer (Cat # G2991AA, Agilent Technologies).

Galaxy Ministar Centrifuge (120 V, 50/60 Hz) (Cat # 93000-196, V.W.R. International).

Sonicating bath (2- to 3-L tank; 80 W, 40 kHz transducer) (Cat # 15-335-20, Fisher).

Qubit® 2.0-4.0 Fluorometer (Cat # Q33238, ThermoFisher Scientific).

PCR machine, MJ Research Thermo Cycler (Cat # PTC-100, MJ Research).

Covaris M220 Focused-ultrasonicator (Cat # 4482277, Covaris).

E-Gel® iBase™ and E-Gel® Safe Imager™ Kit (Cat # G8152ST, ThermoFisher Scientific).

3 Methods

3.1 Sample Preparation

3.1.1 Workplace Preparation

Being aware of the risk of Ribonuclease (RNase) contamination is critical before any animal is collected and RNA isolated. These enzymes degrade RNA and can have detrimental effects on any downstream processes. RNases are found in practically every cell type for both prokaryotes and eukaryotes. They are the toughest enzymes to inactivate and can retain activity after freeze-thaw cycles and even autoclaving. For more details on working with RNA, *see* **Note 1**.

Fieldwork is often required for collecting many novel and rare species of ctenophores and is a challenging environment for every demand of molecular biology laboratories. Decontaminating and managing RNases in these environments is of utmost importance. All surfaces, including benchtops, equipment, and even pipettes, should be assumed to be contaminated with RNases. Decontaminate your work area with 70% ethanol, 4% bleach or RNAaseZap to control RNase contamination, but make sure none of these reagents come in contact with your isolated RNA sample. For more details on setting up an appropriate workstation for fieldwork, (*see* **Note 1**).

3.1.2 Specimen Preparation

Before RNA isolation, all ctenophores should be microscopically examined for potential ectoparasites or food (such as copepods) and then washed three times for at least 30 min in filtered seawater (FSW) obtained from the same location the animals are collected. Animals are anesthetized in 60% (volume/body weight) isotonic $MgCl_2$ (337 mM). Specific tissues are surgically removed with sterile fine forceps and scissors and processed for RNA isolations (*see* **Note 1** for more details on dealing with RNase contamination).

3.2 RNA Isolation

High-quality RNA is the first and often the most critical step in performing many molecular techniques. Here, we will focus on total RNA isolations because it is the most reliable method when working with ultralow amounts of starting material and obtaining

dedicated RNA-seq libraries sufficient for deep transcriptome sequencing (e.g., [4, 5, 8, 10, 11]). When considering the total RNA isolation process, several technologies are available including organic extraction methods, centrifuge column-based, magnetic particle based, and direct lysis. Given the fragile nature of ctenophores, column-based technology has worked best.

QIAGEN's RNeasy kits, which are column-based, have produced high-quality total RNA from ctenophores for all downstream processes. The RNeasy kits come in two varieties depending on the amount of RNA input: the mini-kit which is designed to isolate up to 100 μg of total RNA and the micro kit designed to isolate up to 45 μg total RNA. The key to both kits is not to overload the columns and clog them. An option with the RNeasy kits is the QIAshredder that can be used for simple and rapid homogenization of cell and tissue lysates but is usually not needed for ctenophores given their fragile bodies. The gEliminator spin column is another option for removing genomic DNA (gDNA) contamination and is also an excellent alternative. See QIAGEN's webpage for a list of all RNease kits (https://www.qiagen.com).

Before beginning any protocol, read all the manufacturer's manuals. All procedures presented follow the manufacturer's recommendation except where noted. All solution preparations must be adhered including adding β-mercaptoethanol (β-ME) to Buffer RLT (*see* **Note 2**), adding four volumes of ethanol to the RPE, and preparing DNase I stock solution if the gEliminator spin column is not used. Here we present the general scheme for isolating total RNA from ctenophores with the RNeasy kits.

3.2.1 RNA Isolation Protocol (QIAGEN's RNeasy Kits)

1. Sample lysis is performed in prepared RLT buffer and loaded onto a RNeasy column membrane.
2. Genomic DNA contamination is removed either by a DNase 1 step on the column or a gEliminator spin column.
3. Ethanol is added to the lysate, producing conditions that promote selective binding of RNA to the RNeasy column membrane.
4. The total RNA binds to the column, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water.
5. This can also be a pause point, and samples can be stored at −80 °C without degradation.

It should be noted that the Promega MagaZorb® total RNA mini-prep kit also produced high-quality RNA from ctenophores. And for automation, the Maxwell® RSC simplyRNA tissue kit also gave very good quality RNA. Nevertheless, the kits described above cannot be used effectively with pigmented tissue like retina or

chromatophores. For these purposes, we employed Nucleospin RNA II from Macherey-Nagel. Many RNA isolation/extraction kits are available, and all need to be tested for your species of interest. Here, we present the kits that worked well to isolate total RNA from ctenophores.

3.2.2 Quality Control for Isolated RNA

All molecular biology reactions require high-quality pure (free of all contaminants including gDNA) and precise amounts of RNA for optimal downstream application performance. RNA samples are run on an Agilent® Technologies TapeStation™ or Bioanalyzer™ according to the manufacturer's recommendation. The Agilent® TapeStation™ instrument automatically analyzes RNA concentration and integrity through a combination of microfluidics, capillary electrophoresis, and fluorescent dye that binds to nucleic acid (see https://www.agilent.com for more details). The TapeStation™ has two different tapes depending on the amount of input RNA: the RNA ScreenTape for the sensitivity of 5 ng/μL and a high sensitivity RNA ScreenTape for measured concentrations down to 100 pg/μL. The analysis algorithm for both Agilent® Technologies TapeStation™ and Bioanalyzer™ can provide information about RNA integrity (the RNA Integrity Number, or RIN) with a maximum value of 10 when analyzing total RNA. Significant decreases in the RIN are indicative of degraded total RNA. High-quality RNA will have a minimal amount of degradation. A typical ctenophore sample has both 18 s and 28 s ribosomal peaks, compared to the hidden break in the *Aplysia* ribosomal RNA. A high sensitivity RNA ScreenTape was used to analyze isolated total RNA from the combs rows in *Mnemiopsis leidyi*, (*see* Fig. 2).

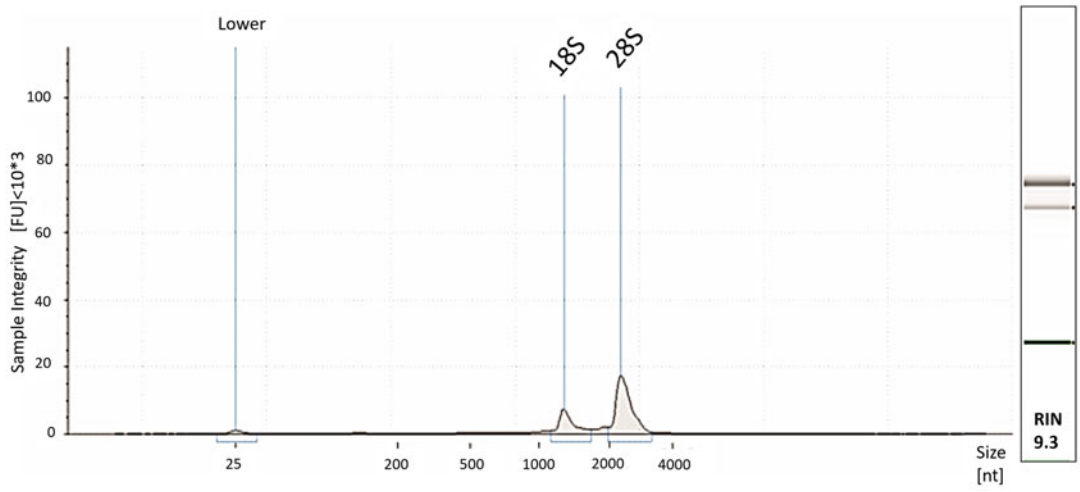

Fig. 2 *Mnemiopsis leidyi* total RNA isolated from a small piece of a comb row run on a high sensitivity RNA ScreenTape. This is high-quality total RNA with a RIN of 9.3

The concentration of RNA is determined on a Qubit® 2.0 fluorometer. Concentration can be obtained for the Agilent® RNA. ScreenTape is not as accurate as the Qubit® measurement. The Qubit assays use dyes that bind selectively to DNA, RNA, or protein, thus making the assay more sensitive than any UV absorbance methodology; see https://www.thermofisher.com for more information. There are two types of assays depending on the concentration of input RNA, a broad range kit for measuring a quantification range of 20–1000 ng and a high sensitivity kit for the 5–100 ng range. The sample in Fig. 2 had a Qubit RNA concentration of 12.9 ng/μL.

Once the quality and quantity are satisfactory, this material is used to construct a sequencing library. The ultimate test of high-quality RNA is the quality of the sequencing data produced. Lower indicates the marker provided in the kit.

3.3 Library Construction for RNA-seq

RNA-seq is a precise tool to measure the gene expression profile of the transcriptome (all expressed RNAs) in a single cell, a population of cells, a tissue, or a whole organism. There are numerous commercially and noncommercially available kits or methods for performing RNA-seq available. However, many kits are designed for sequencing cores. These kits are usually for high-throughput production in 96- or 384-well formats. Most importantly, they are costly. We present a convenient, inexpensive, and reproducible protocol for RNA-seq that is designed for a small number of samples, even one sample. Our protocol is applicable for single cells and ultralow amounts of starting material.

Today direct sequencing of RNA is possible with technologies like Pacific Biosystems and Oxford Nanopores Technologies, Inc. However, these methods are not applicable yet to single cells or small amounts of starting material. Since we are not sequencing RNA directly, we go through the production of a stable intermediate, complementary DNA (cDNA). The cDNA is synthesized from total RNA with an oligo dT primer that targets message RNA (mRNA) or all RNA with a polyadenylated 3′ tail. This method has a 3′ bias, but we feel these libraries are highly informative about the transcriptional landscape of all samples tested and an excellent starting point. Additional libraries with more stringent requirements can be constructed based on the initial analysis of these RNA-seq libraries.

Our library construction method is divided into two parts, production of cDNA from total RNA and then generation of an Illumina sequencing library through adaptor-ligated fragmented cDNA. There are entire kits available for cDNA synthesis at added cost; we make our own primers and oligos (Table 1) and purchase the individual components at a fraction of the cost of a kit. We use a commercial kit to make the sequencing library NEBNext® Ultra II DNA Library Prep Kit for Illumina® for convenience.

Library construction starts with total RNA isolation (*see* Subheading 3.2) that is reverse transcribed by a specific reverse transcriptase enzyme to first-strand cDNA with an oligo dT primer. The second strand of the cDNA is produced through a template-switch method as described [8, 12, 13]. The cDNA is amplified through a polymerase chain reaction (PCR) and then purified. At this point, a small amount of the cDNA is reserved for downstream applications like cloning (*see* **Note 3**). The resultant amplified cDNA is then converted to an Illumina sequencing library with NEBNext® Ultra II DNA Library Prep Kit for Illumina® and the NEBNext® Multiplex Oligos for Illumina according to manufacturer's recommendations. We present this protocol for one sample, but if more than one library is being made, it is best to make master mixes; *see* **Note 4** for master mix setup.

3.3.1 Library Construction Protocol

cDNA Synthesis

1. For isolated RNA, combine the following in a 0.5- or 0.2-mL LoBind tube:

   3.5 μL RNA (2 ng–1 μg total RNA)

   1 μL 5X first-strand buffer

   1 μL of 3′ CapT30 Primer (10 μM) (Table 1)

   5.5 μL total volume

2. For single cells, obtain single cell and add to 4.5 uL of 1X first-strand buffer.

   Sonicate for 20 s and add the following:

   1 μL of 3′ CapT30 primer (10 μM)

   5.5 μL total volume

   Mix contents and spin the tubes briefly in a microcentrifuge.

3. Incubate the tubes at 72 °C in a hot-lid thermal cycler for 3 min.

4. Add the following reagents in the order shown:

   1.0 μL 5X first-strand buffer

   0.25 μL DTT (20 mM)

   1.0 μL dNTP Mix (10 mM)

   1.0 μL CapTS oligonucleotide (12 μM) (Table 1)

   0.25 μL RNase inhibitor

   1.0 μL SMARTScribe reverse transcriptase (100 U)

   10.0 μL total volume

   Add the reverse transcriptase just prior to use. Mix well by pipetting, and spin the tube briefly in a microcentrifuge.

5. Incubate the tubes at 42 °C for 1.5 h (not to exceed 2 h).

6. Prepare the PCR reagents in the order shown and add them to the above cDNA sample:

   74.0 μL RNase- and DNase-free H_2O

   10.0 μL 10X Advantage 2 PCR buffer

   2.0 μL dNTP Mix (10 mM)

   2.0 μL Cap PCR primer (10 μM) (Table 1)

   2.0 μL 50X Advantage 2 polymerase mix

   90 μL total volume

7. Amplify in thermal cycling using the following program:

   95 °C 1 min, one cycle

   The following steps perform 12–18 cycles, depending on the amount of starting material (usually 12–16 cycles for tissue, 16–18 cycles for single cells)

   95 °C 15 s

   65 °C 30 s

   68 °C 3 min

   Hold 4 °C ∞*

8. Run an E-Gel® SizeSelect™ 2% agarose with 5 uL of the amplified cDNA.
9. Save 10 uL of this cDNA for further use; *see* **Note 3**.

Purification of Amplified cDNA

SPRIselect bead purification is a proprietary paramagnetic bead-based chemistry for purifying DNA fragments to the desired size for library preparation.

1. Make fresh 80% ethanol with RNase- and DNase-free H_2O.
2. Place amplified cDNA sample (85 mL) in 1.5 mL LoBind tube.
3. Add 51 μL of SPRIselect® reagent (0.6 × sample volume).
4. Pipet up and down five times to thoroughly mix the bead suspension with the DNA.
5. Incubate the mixture at room temperature for 5 min.
6. Place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 min or until the solution is clear of brown tint when viewed at an angle.
7. Carefully remove and discard the supernatant without disturbing the bead pellet.
8. Without removing the tube from the magnet, add 500 μL of freshly prepared 80% ethanol.
9. Incubate for 30 s on the magnet. After the solution clears, remove and discard the supernatant without disturbing the pellet.

10. Repeat **steps 7–9** for a second wash.
11. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-μL pipettor without disturbing the pellet.
12. Keeping the tube on the magnet, air-dry the beads at room temperature for 2 min or until the pellet appears dry.
13. Remove the tube from the magnetic rack and add 50 μL of RNase- and DNase-free H_2O directly to the pellet to disperse the beads. Pipet the suspension up and down five times, then vortex the sample for 10 s, mix thoroughly, and incubate at room temperature for 5 min.
14. Pulse-spin and place the tube in the magnetic rack for at least 1 min until the solution clears. Transfer the supernatant containing the eluted DNA to a tube (50 μL) microTUBE AFA Fiber Screw-Cap.

Fragmentation of cDNA

Shear purified cDNA with a Covaris M220 Focused-ultrasonicator.

1. Place tube in M220 Focused-ultrasonicator, and sonicate to 400 bp size.
2. Fragmented cDNA is ready for library preparation for Illumina sequencing.

Library Preparation for Illumina Sequencing

The next steps of this protocol use the NEBNext® Ultra DNA Library Prep Kit for Illumina® because it can be scaled down to produce one library, and the high sensitivity is sufficient for even a single cell. A new kit has been introduced but not tested by us with a fragmentation buffer, so the Covaris sonication step would not be necessary; see https://www.neb.com/products/ for a list of all products available. Ultralow RNA input is an idea with this kit because the end-repair, dA-tailing, and linker-ligation are performed sequentially without purification between the steps, thus minimizing material loss. To avoid index hopping or index switching, a specific type of misassignment of the indexed library, we recommend the dual index primer also set by NEB.

We run an E-Gel® SizeSelect™ 2% agarose with 2 uL of the amplified library before the final purification step to see if more cycles of PCR are needed (*see* Fig. 3a). If nothing is seen on the gel, a couple of cycles are added.

Quality Control of Sequencing Libraries

1. The competed libraries are run on a high sensitivity D1000 ScreenTape (*see* Fig. 3b).
2. Qubit assay is also performed to determine the concentration of the libraries.
3. Further quality control is performed by our sequencing core before the libraries are run on the sequencer.

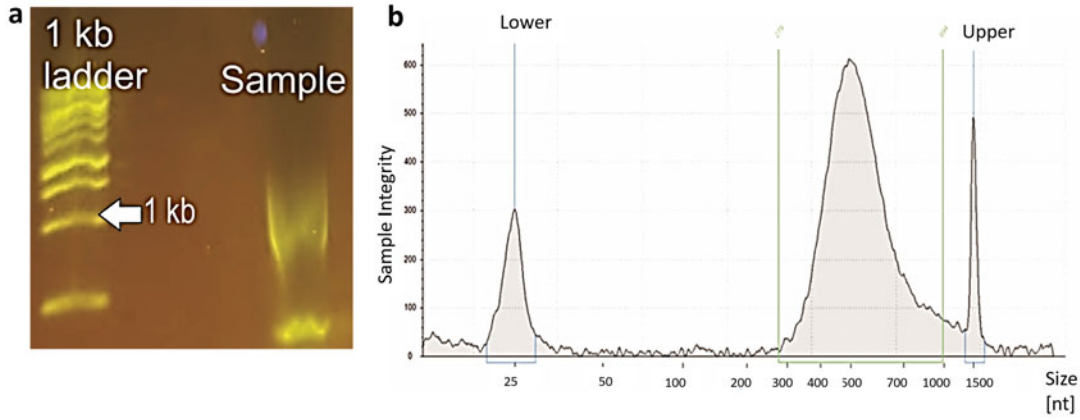

Fig. 3 (**a**) *Bolinopsis infundibulum* skin RNA-seq library run on an E-gel before SPRIselect bead purification after 16 cycles of amplification and before bead purification. The size of smear is between 500 bp and 1 kb. (**b**) Same RNA-seq library after bead purification run with a D1000 ScreenTape and Agilent 2200 TapeStation system. The average size is 542 bp with a concentration of 85 nM. Lower and upper refer to the markers provided in the kit

3.4 Conclusions

High-quality RNA can be isolated from ctenophores despite their fragile body plan and unique mesoglea challenges. Described RNA-seq libraries generate high-quality data, and we validated our libraries with hundreds of in situ hybridizations based on analysis of the RNA-seq data.

RNA-seq data analysis is challenging and not the scope of discussion here. One of the examples is https://neurobase.rc.ufl.edu/Pleurobrachia which has been described elsewhere [4]. This webpage has multiple RNA-seq datasets that have been assembled and annotated. All projects are searchable by keywords, PFAM domains, KEGG pathways, GO ontology, and BLAST. All data is also downloadable. This is the publicly available database for ctenophore transcriptomic data.

4 Notes

1. Proper technique when working with RNA is one of the most critical and challenging tasks in this process because the ramifications will considerably impact the quality of your data. First, always wear gloves and change them often because of skin RNases coined "fingerases." Besides ensuring the workplace is contamination-free of RNases, care needs to be given to instruments and all equipment used. Metal and glass are extremely difficult to decontaminate. For metal instruments, wipe with ethanol, and then flame if possible. New plastic containers should be used instead of glass. It is best to use unopened,

petri dish sleeves, prepackages, plastic pestles for dissolving tissue if needed, unopened transfer pipettes, unopened pipette tips, and unopened bagged RNase-free LoBind Eppendorf tubes of 0.5 uL and 1.5 uL. Use reagents and nuclease-free supplies, including water and reserved chemicals for RNA work only. Designate a "low-traffic" area of the lab away from air vents or open windows as an "RNase-free zone," and place all your instruments, equipment, and supplies in this area.

2. During field trips and expeditions, we prepare QIAGEN RNA isolation kits (RNeasy) lysis buffer RLT with β-ME in RNase-free LoBind 1.5 mL Eppendorf tubes, and freeze in bags that get shipped on dry ice to locations. Both of QIAGEN's RNeasy kits, mini and micro, use the same buffer and volume, so the type of isolation can be determined in the lab. We will isolate tissue or animals for future RNA isolation on-site. Most critical in this process is the tissue for the RNA isolation must be completely dissolved in the RLT buffer before freezing and shipping. Small disposable on-time use petals can be used to help dissolve tissue. Or sonication in short bursts of 5–10 s alternated with placement on ice until the tissue is dissolved is also an alternative. Once the tissue is dissolved, freeze, and then ship samples on dry ice. If time and conditions are appropriate, RNA isolation may be performed on-site.
3. Validation of sequencing data is critical. We reserved some of the amplified cDNA for cloning genes of interest. The amplified cDNA is highly concentrated and diluted at least 1:50 and used as a template in a PCR reaction to cloning. Primers are designed from the RNA-seq data. After bands are amplified, they are purified and cloned into a cloning vector such as TOPO-TA (see https://www.thermofisher.com/us/en/home/life-science/cloning/ta-cloning-kits.html for details). The TOPO vector is ideal for probes made for in situ hybridization.
4. Master mixes save time and money. There is always a 0.5 μL overage in the volumes to accommodate pipette error. Also, it is often inaccurate to pipette very small amounts of reagent, so a master mix circumvents this issue. An example of a setup of a master mix with two, four, and six samples for the cDNA synthesis is shown here in Table 2.

Acknowledgments

We would like to thank Dr. David A. Amador Moraga and the UF/ICBR core staff for their dedication, support, and hard work to accommodate challenging sequencing demands. This work was supported in part by the Human Frontiers Science Program

Table 2
Master mix setup

1×	Reagent	2.5×	4.5×	6.5×
1.0 μL	5X first-strand buffer	5.0 μL	9.0 μL	13 μL
0.25 μL	DTT (20 mM)	0.625 μL	1.0 μL	1.625 μL
1.0 μL	dNTP mix (10 mM)	2.5 μL	4.5 μL	6.5 μL
1.0 μL	CapTS oligonucleotide (12 μM)	2.5 μL	4.5 μL	6.5 μL
0.25 μL	RNase inhibitor	0.625 μL	1.0 μL	1.625 μL
1.0 μL	SMARTScribe reverse transcriptase (100 U)	2.5 μL	4.5 μL	6.5 μL
	Add 4.5 μL of master mix per reaction			

(RGP0060/2017) and National Science Foundation (IOS-1557923) grants to L.L.M. Research reported in this publication was also supported in part by the National Institute of Neurological Disorders and Stroke of the National Institutes of Health under Award Number R01NS114491 (to L.L.M). The content is solely the authors' responsibility and does not necessarily represent the official views of the National Institutes of Health.

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