Single-cell RNA extraction and cDNA library preparation from challenging marine protists (e.g. Acantharea)

Margaret Brisbin,Lisa Mesrop

Abstract

Marine protists are important in all marine ecosystems, but many are not culturable and therefore difficult to study. The increasing development of single-cell techniques is allowing for more marine protists to be studied, but many single-cell protocols are only optimized for model organisms and do not perform well with marine plankton. The SMART-seq v4 manual specifically states that cells entering the pipeline should be washed and resuspended in cell culture-grade PBS, but most marine protists do not tolerate PBS. Additionally, many marine protists have mineral skeletons, the components of which may interfere with the chemistry of the SMART-seq kit. Calcium and Strontium both dissolve to form divalent cations which could saturate the kit's RNase inhibitor. When we synthesized cDNA directly from acantharians ($SrSO_4$ skeletons) without first extracting RNA, the cDNA was highly degraded with a 300 bp average fragment length. Therefore, it is desirable to perform RNA extractions from single marine protists before cDNA synthesis and amplification, but the amount of RNA in single marine protists is too low to be extracted with column-based kits (even the MN Nucleospin RNA XS kit suggested in the SMART-seq manual). To overcome these problems, we developed a column-free method for extracting RNA from single marine protists by making use of Agencourt RNAClean XP magnetic beads. When this protocol is followed, the synthesized cDNA is higher quality with a 2,000 bp average fragment length.

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Guidelines

- Always wear clean RNase-free gloves. Clean gloves and work space with ethanol and RNase zap.
- Perform single-cell RNA extractions and cDNA synthesis in a PCR-clean workstation, if possible.
- It is good practice to have a separate set of pipettes for RNA and to use filter tips.
- After cDNA synthesis, perform remaining steps in the general lab space.

Before start

- Clean PCR-clean workstation and lab area with ethanol and RNase Zap.
- Clean thermocyclers with RNase Zap.
- Thaw reagents (except enzymes).

- Allow reagents that need to be room temperature to incubate at room temperature for 30 min (RNAClean XP magnetic beads, AMPure XP magnetic beads, Bioanalyzer reagents)
- Set thermocylcer programs and pre-heat thermocyclers.
- Prepare fresh 80% ethanol.

Materials

- Reagent Reservoirs 8600A58 by Contributed by users
- P200 micropipets and 200 µl filter tips by Contributed by users
- P20 micropipet and filter tips by Contributed by users
- Thermocycler by Contributed by users

PCR-clean Workstation 36-101-8894 by Fisher Scientific

Magnetic Stand-96 AM10027 by Thermofisher

Vortex-Genie 2 SI-0236 by Scientific Industries, Inc.

Protocol

Marine Protist Isolation

Step 1.

Collect your favorite marine protists with the plankton net of your choice. We use a 100 µm-mesh-size Rigo simple net (20 cm diameter).

Observe plankton samples under a dissecting microscope.

Isolate individual protists with a pulled-glass micropipette connected to an aspirator (Sigma) with silicone tubing and a 0.45 µm filter. Transfer protists to small, clean petri dishes while including the smallest volume of seawater possible.

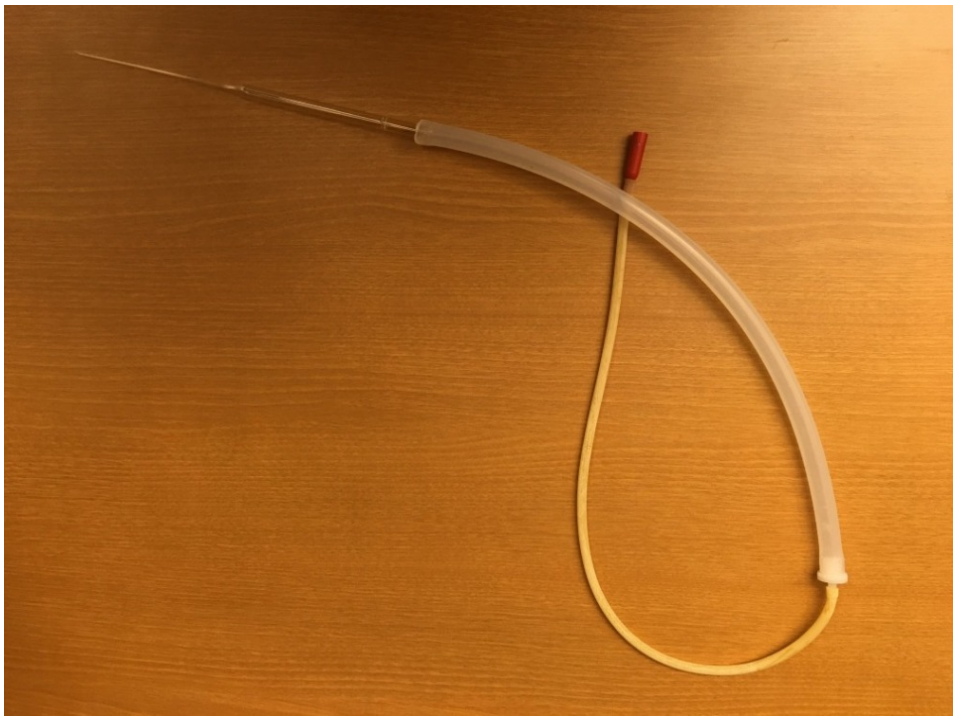
Use a sterile dropper to add 0.2 µm-filter sterilized seawater to rinse the isolated protists.

Repeat transfers and rinses until the protists of interest are clean and free of contaminants. Image the protist as desired.

Allow protists to self-clean for 0.5–2 hr.

Use the glass micropipette to transfer the protist to an Axygen maxymum recovery PCR tube including the smallest volume of seawater possible.

Aspirator and glass micropipette set-up:

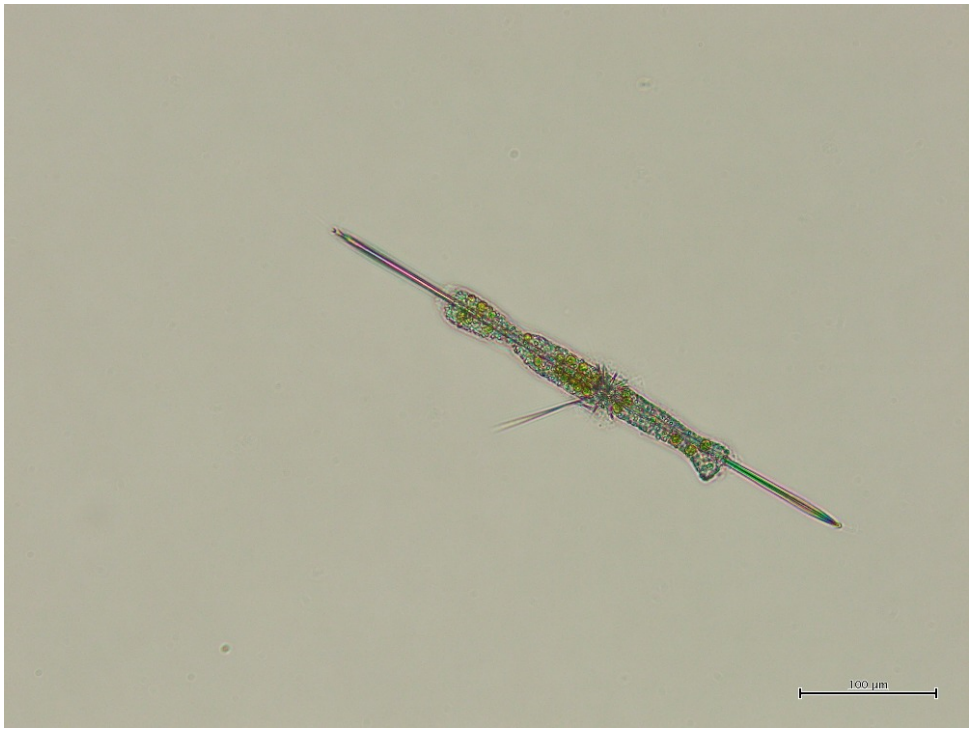


Place the PCR tube under the dissecting microscope to ensure that the protist of interest is indeed in the tube.

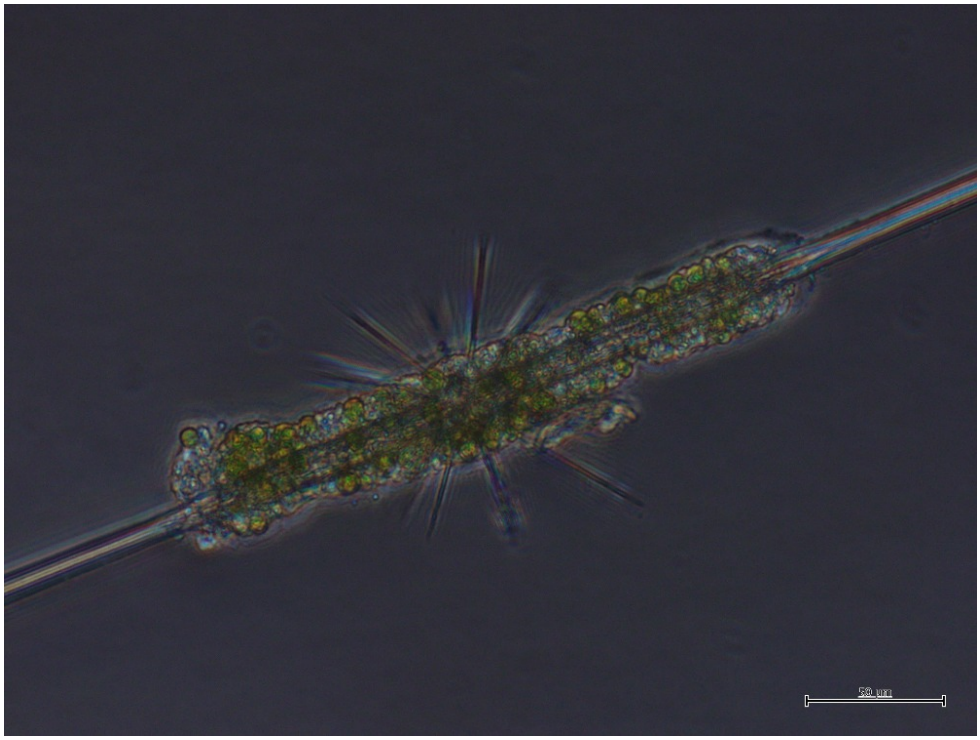
Add 30 µL of Qiagen RLT+ Buffer (with 1% β-mercaptoethanol) to the sample tube. Place sample in small plastic zip-locked bag along with a sample label (pencil on water-proof paper), and submerge sample in liquid nitrogen.

Store samples at −80 °C until ready to proceed with RNA extraction.

Representative acantharians used in this protocol:



(scale bar is 100 µm)



(scale bar is 50 µm)

REAGENTS

Buffer RLT Plus 1053393 by Qiagen

2-mercaptoethanol M6250 by Sigma Aldrich

Cell-lysis and RNA extraction

Step 2.

Allow Agencourt RNAClean XP beads to reach room temperature by removing from the refridgerator at least 30 minutes before use.

REAGENTS

Agencourt RNAClean XP Magnetic Beads A63987 by Beckman Coulter

RNase Zap R2020-250ML by Sigma Aldrich

NOTES

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It is recommended to perform single-cell RNA extractions in a PCR-clean workstation. Whether using a PCR-clean workstation or not, remember to clean all surfaces and equipment with 100% etOH and RNase Zap.

Cell-lysis and RNA extraction

Step 3.

Allow frozen samples to thaw over ice.

Cell-lysis and RNA extraction

Step 4.

Vortex each sample 2x for 10 sec at speed 7 on a Vortex-Genie 2. Gently spin each sample to collect liquid at the bottom of the tube.

Cell-lysis and RNA extraction

Step 5.

Incubate each sample at room temperature for 5 min.

Cell-lysis and RNA extraction

Step 6.

Determine the volume of each sample using a micropipette (set the pipette to 50 µL and draw up the entire sample, decrease the volume of the pipette until there is no air remaining between the sample and the opening of the pipette tip and record the final volume).

Cell-lysis and RNA extraction

Step 7.

Vortex room temperature Agencourt RNAClean XP magnetic beads. Add beads to each sample at a 2.2:1 v:v ratio (multiply the volume of each sample by 2.2 to determine the volume of magnetic beads to add).

Thoroughly mix each sample by pipetting the entire volume (sample + beads) up and down at least 10 times.

REAGENTS

Agencourt RNAClean XP Magnetic Beads A63987 by Beckman Coulter

Cell-lysis and RNA extraction

Step 8.

Incubate samples with RNAClean XP beads at room temperature for 30 min to fully bind the RNA to the beads.

Cell-lysis and RNA extraction

Step 9.

Place samples on magnetic stand until sample appears clear (2–5 min).

Cell-lysis and RNA extraction

Step 10.

Using a multichannel pipette, remove the clear supernatant from all samples without disturbing the beads. Discard the supernatant.

Cell-lysis and RNA extraction

Step 11.

Fill a sterile, nuclease-free disposable reservoir with 80% ethanol (etOH) in nuclease free water. With a multichannel pipette, add 200 µL of 80% etOH to each sample without disturbing the beads.

REAGENTS

- Ultrapure Distilled, Nuclease Free Water by Contributed by users
- Ethanol by Contributed by users

Cell-lysis and RNA extraction

Step 12.

With a multichannel pipette, remove the 80% etOH from all samples and discard. With a multichannel pipette, again add 200 µL of 80% etOH to each sample.

Cell-lysis and RNA extraction

Step 13.

With a multichannel pipette, remove the 80% etOH from all samples and discard. Remove samples from the magnetic stand and gently spin to collect all remainig etOH. Place samples back on the magnetic stand and use a 10 µL pipette tip to remove all remaining etOH from each sample.

Cell-lysis and RNA extraction

Step 14.

Dry the magnetic beads by leaving the sample caps open and watching the bead pellets carefully. As

the bead pellets lose their sheen and begin to appear matte, close the sample lids to prevent over drying.

Cell-lysis and RNA extraction

Step 15.

Elute the RNA from the magnetic beads. As soon as beads are dry (before cracks appear in the bead pellet), add 11 µL of elution buffer to samples. The elution buffer contains 10.72 µL of nuclease-free water and 0.28 µL of RNase inhibitor per sample.

Pipette the elution buffer over bead pellets until the pellet breaks up in the elution buffer. After elution buffer has been added to all samples, mix the samples by pipetting up and down 10 times to further resuspend beads (they do not need to be fully resuspended).

Gently spin samples to collect the eluate.

Place the samples back on the magnetic stand until the samples appear clear.

REAGENTS

- Ultrapure Distilled, Nuclease Free Water by Contributed by users
- RNase Inhibitor (40 U/µL) 634888 by Takara

Cell-lysis and RNA extraction

Step 16.

Collect eluted RNA by aspirating 10.5 µL of each sample, without disturbing the beads, and tranfer eluted RNA to clean low-bind PCR tubes.

cDNA synthesis

Step 17.

Prepare 10X Reaction Buffer: 15 µL 10X lysis buffer, 5 µL RNase inhibitor, mix without causing bubbles, spin

Add 1 µL of 10X Reaction Buffer to each sample. Pipette to mix and gently spin.

REAGENTS

- 10X Lysis Buffer 634888 by Takara
- RNase Inhibitor (40 U/µL) 634888 by Takara

cDNA synthesis

Step 18.

Add 1 µL of 3' SMART-sew CDS Primer II A (12 µM) to each sample. Pipette to mix. Gently spin.

REAGENTS

SMART-seq CDS Primer II A (12 µM) 634888 by Takarabio

cDNA synthesis

Step 19.

Anneal CDS primer to RNA. Incubate samples at 72 °C for 3 min in a preheated thermocycler with a heated lid.

TEMPERATURE

72 °C Additional info:

cDNA synthesis

Step 20.

Prepare PCR master mix WHILE samples are incubating in the thermocycler.

Per reaction (includes 5% margin):

4.2 µL 5X Ultra Low First-Strand Buffer

1.05 µL SMART-seq v4 Oligonucleotide (46 µM)

0.5025 µL RNase Inhibitor

REAGENTS

5X Ultra Low First-Strand Buffer 634888 by Takarabio

SMART-seq v4 Oligonucleotide (46 µM) 634888 by Takarabio

RNase Inhibitor (40 U/µL) 634888 by Takara

cDNA synthesis

Step 21.

After the 3 min annealing step, immediately place samples on ice for 2 min.

During this time, mix SMARTScribe Reverse Transcriptase by pipetting and then add 2.1 µL per sample to the PCR master mix. Gently vortex PCR master mix and spin down.

REAGENTS

SMARTScribe Reverse Transcriptase 634888 by Takarabio

cDNA synthesis

Step 22.

Add 7.5 µL of PCR master mix to each sample. Mix by pipetting and gently spin.

cDNA synthesis

Step 23.

Transer samples to a preated thermocycler with heated lid with the following program:

42 °C for 90 mins,
70 °C for 10 mins
4 °C forever

Samples may stay at 4 °C overnight

cDNA amplification

Step 24.

Prepare master mix:

Per sample (5% margin included):

26.25 µL SeqAmp PCR Buffer

1.05 µL PCR Primer IIA

1.05 µL SeqAmp DNA Polymerase

Gently vortex master mix and gently spin down.

REAGENTS

2X SeqAmp PCR Buffer 638526 by Takara

SeqAmp DNA Polymerase 638504 by Takara

PCR Primer II A (12 µM) 634888 by Takara

cDNA amplification

Step 25.

Add 30 µL of master mix to each sample. Mix by pipetting. Gently spin down.

cDNA amplification

Step 26.

Place samples in preheated thermocycler with heated lid for the following program:

95 °C for 1 min

17 x (98 °C for 10 sec, 65 °C for 30 sec, 68 °C for 3 min)

72 °C for 10 min

4 °C forever

Samples may stay at 4 °C overnight.

cDNA purification (Agencourt AMPure XP)
Step 27.

Allow AMPure beads to reach room temperature (30 min).

REAGENTS
Agencourt AmPure XP beads A63880 by Contributed by users

cDNA purification (Agencourt AMPure XP)
Step 28.

Add 1 µL of 10X lysis buffer to each sample.

REAGENTS
10X Lysis Buffer 634888 by Takara

cDNA purification (Agencourt AMPure XP)
Step 29.

Vortex AMPure beads. Add 50 µL of beads to each sample. Mix by pipetting the entire voume (101 µL) up and down 10 times.

cDNA purification (Agencourt AMPure XP)
Step 30.

Incubate for 8 min.

cDNA purification (Agencourt AMPure XP)
Step 31.

Gently spin the samples and then place them on the magnet stand until they appear clear.

cDNA purification (Agencourt AMPure XP)
Step 32.

Using a multichannel pipette, remove the clear supernatant from all samples without disturbing the beads. Discard the supernatant.

cDNA purification (Agencourt AMPure XP)

Step 33.

Fill a sterile, nuclease-free disposable reservoir with 80% ethanol (etOH) in nuclease free water. With a multichannel pipette, add 200 µL of 80% etOH to each sample without disturbing the beads.

REAGENTS

- Ultrapure Distilled, Nuclease Free Water by Contributed by users
- Ethanol by Contributed by users

cDNA purification (Agencourt AMPure XP)

Step 34.

With a multichannel pipette, remove the 80% etOH from all samples and discard. With a multichannel pipette, again add 200 µL of 80% etOH to each sample.

cDNA purification (Agencourt AMPure XP)

Step 35.

With a multichannel pipette, remove the 80% etOH from all samples and discard. Remove samples from the magnetic stand and gently spin to collect all remainig etOH. Place samples back on the magnetic stand and use a 10 µL pipette tip to remove all remaining etOH from each sample.

cDNA purification (Agencourt AMPure XP)

Step 36.

Dry the magnetic beads by leaving the sample caps open and watching carefully. As the beads lose their sheen and begin to appear matte, close their lids to prevent over drying.

cDNA purification (Agencourt AMPure XP)

Step 37.

Elute the RNA from the magnetic beads. As soon as beads are dry (before cracks appear in the bead pellet), add 20 µL of Elution Buffer (10 mM Tris-Cl, pH 8.5) to samples.

Pipette the elution buffer over bead pellets until the pellet breaks up in the elution buffer. After elution buffer has been added to all samples, mix the samples by pipetting up and down 10 times to further resuspend beads.

REAGENTS

Elution Buffer 634888 by Takara

cDNA purification (Agencourt AMPure XP)

Step 38.

Incubate beads in Elution Buffer for 10 min (longer if the beads were over dried).

cDNA purification (Agencourt AMPure XP)

Step 39.

Gently spin samples and place on magnetic stand until the samples beocme clear. Transfer 18 µL of clear supernatant to new low-bind PCR tubes.

cDNA Quantification (Qubit 3.0, dsDNA High Sensitivity assay)

Step 40.

Prepare Qubit working solution.

Per sample (plus 2 standards):

200 µL dsDNA HS Buffer

1 µL dsDNA HS Reagent

REAGENTS

Qubit™ dsDNA HS Assay Kit Q32851 by Invitrogen - Thermo Fisher

cDNA Quantification (Qubit 3.0, dsDNA High Sensitivity assay)

Step 41.

Add 190 µL Qubit working solution to 2 Qubit assay tubes. Add 10 µL Qubit dsDNA HS Standard #1 to one and 10 µL dsDNA Standard #2 to the second.

For each sample, add 199 µL Qubit working solution and 1 µL sample to a Qubit assay tube.

Briefly vortex each standard and sample.

cDNA Quantification (Qubit 3.0, dsDNA High Sensitivity assay)

Step 42.

Incubate standards and samples for 2 min.

cDNA Quantification (Qubit 3.0, dsDNA High Sensitivity assay)

Step 43.

Read samples on the Qubit 3.0 Fluorometer.

EXPECTED RESULTS

Single acantharians typically yielded 1–3 ng/µL cDNA with this protocol.

cDNA Qualification (Agilent Bioanalyzer HS DNA assay)

Step 44.

Typically, the cDNA concentration is always less than 10 ng/µL which is the maximum input for the High Sensitivity DNA assay. If the cDNA concentration is more than 0.5 ng/µL, you may dilute samples by 1/2 or 1/10.

Follow the manufacturer's protocol for the Agilent Bioanalyzer High Sensitivity DNA assay.

REAGENTS

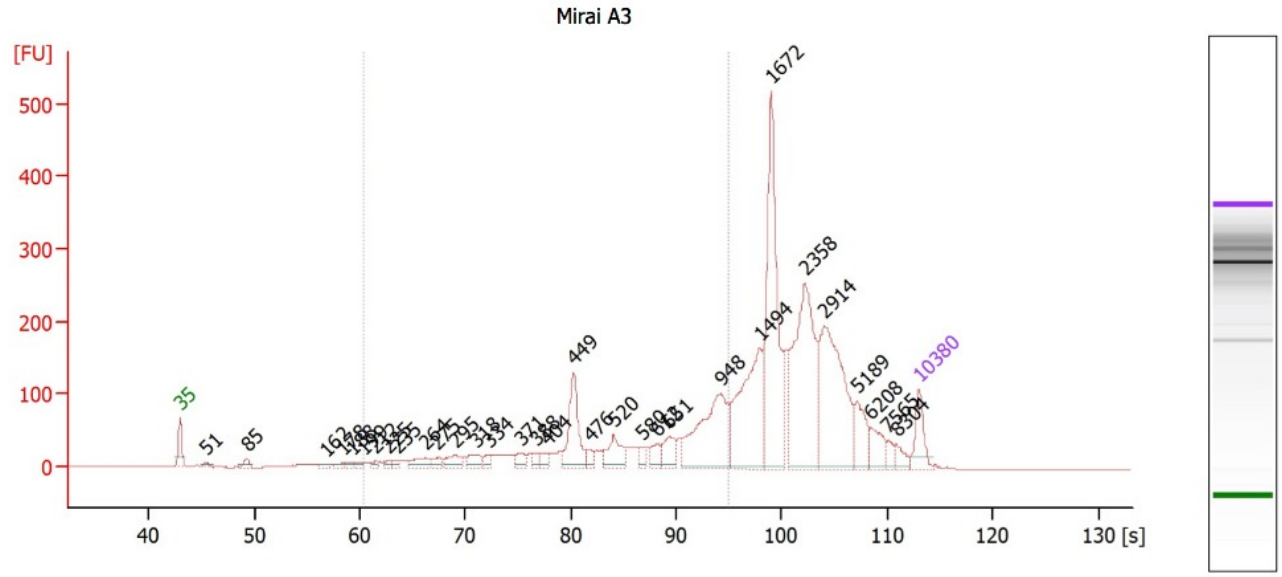
Agilent High Sensitivity DNA Kit 5067-4626 by Agilent Technologies

LINK:

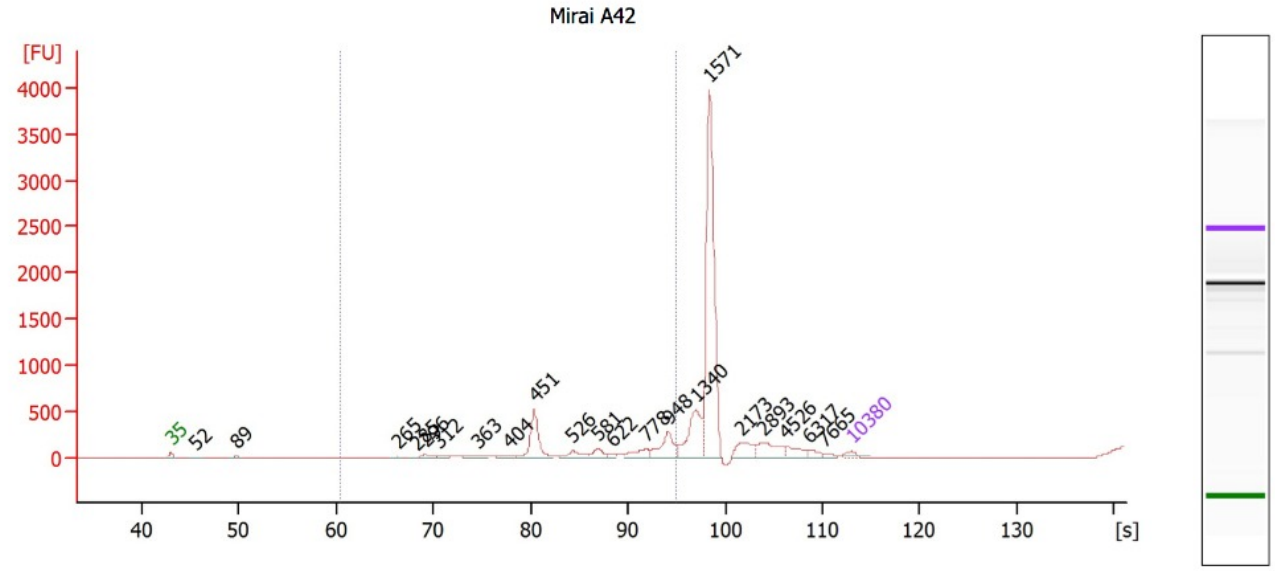
https://www.agilent.com/cs/library/usermanuals/Public/G2938-90322_HighSensitivityDNA_QSG.pdf

EXPECTED RESULTS

One of our best results:

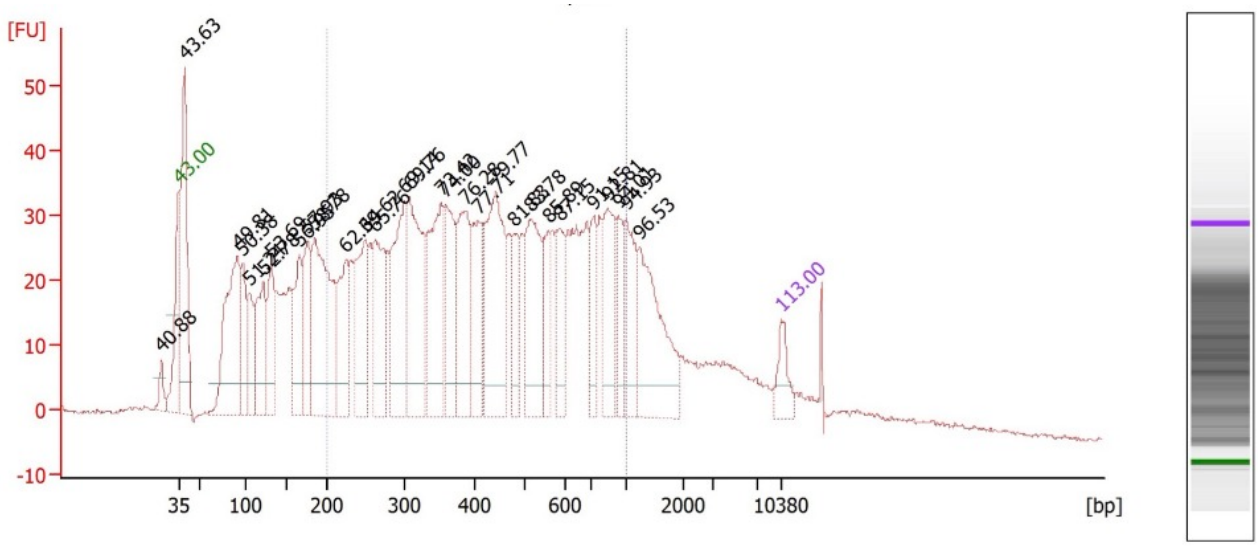


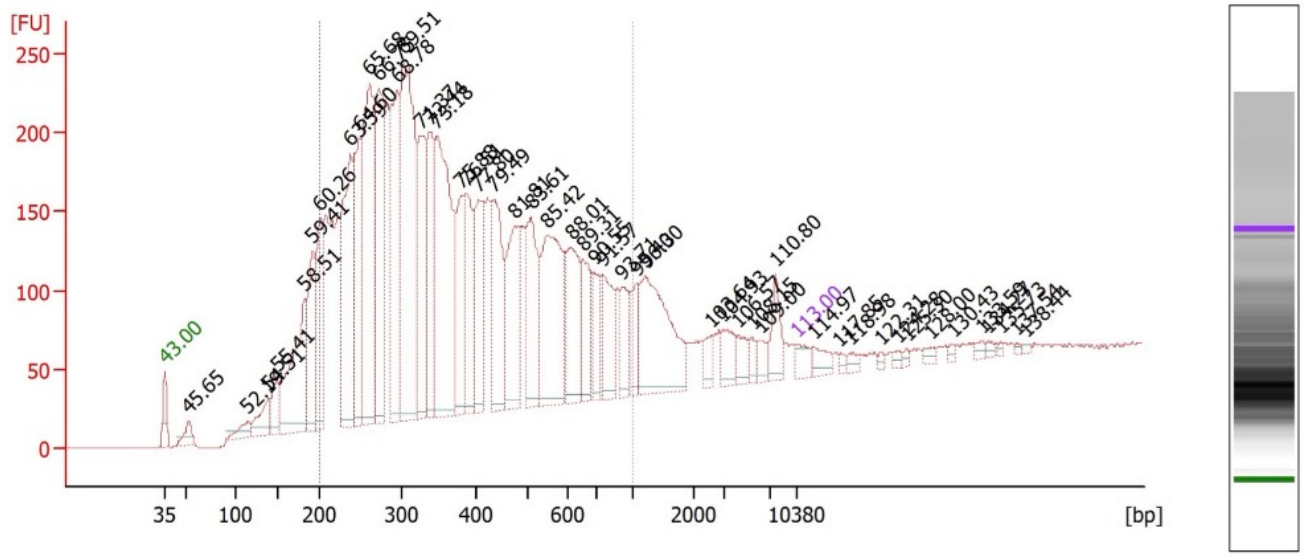
One of our worst results:



For comparison:

Examples of cDNA directly synthesized from single acantharians without RNA extraction:





Nextera XT DNA Library Preparation

Step 45.

Dilute cDNA to 0.2 ng/µL per sample using the formula $C_1V_1 = C_2V_2$ with C_1 equal to the concentration measured with Qubit and, C_2 equals 0.2 ng/µL, and V_2 is 6 µL. Solve for V_1 and add V_1 of sample to $V_2 - V_1$ µL of nuclease free water. Use 1 µL to check the concentration of the diluted cDNA with the Qubit 3.0 HS DNA assay.

Follow the manufacturer's protocol for the Nextera XT DNA Library Prep Kit to prepare sequencing libraries.

Perform manual normalization of sequencing libraries (do not use bead normalization). Quantify libraries with Qubit 3.0 and determine the average library size (fragment length) with the Bioanalyzer High Sensitivity DNA assay. Use both the library concentration and average library size to calculate the molarity of libraries in nM and then dilute accordingly before pooling the sequencing libraries.

$$\frac{(\text{concentration in ng/µl})}{(660\ \text{g/mol} \times \text{average library size in bp})} \times 10^6 = \text{concentration in nM}$$

REAGENTS

Nextera XT DNA Library Prep Kit FC-131-1024 by illumina

Qubit™ dsDNA HS Assay Kit Q32851 by Invitrogen - Thermo Fisher

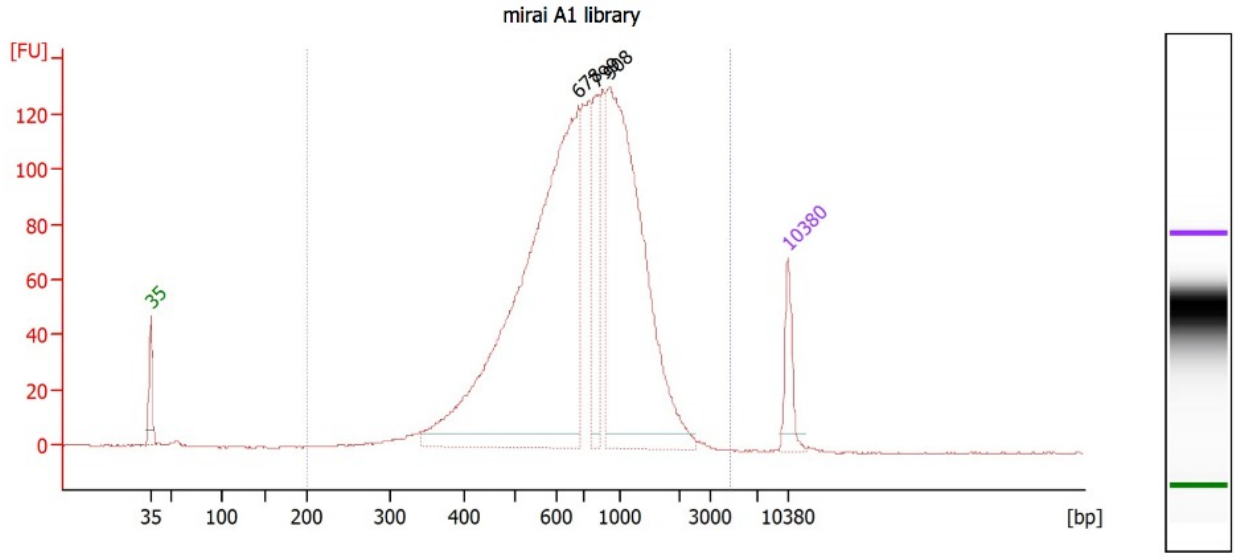
Ultrapure Distilled, Nuclease Free Water by Contributed by users

Agilent High Sensitivity DNA Kit 5067-4626 by Agilent Technologies

EXPECTED RESULTS

Sequencing library concentrations were typically 3–6 ng/µL.

Example single acantharian sequencing library bioanalyzer trace:



Average library size from single acantharians prepared with this protocol was 850 bp.

Warnings

- Prepare 1% β-mercaptoethanol in Qiagen RLT+ buffer under a fume hood. β-mercaptoethanol has a strong odor and can be toxic.
- Practice care when flaming and pulling glass micro-pipettes. Be careful not to stab yourself, burn yourself, or light the lab on fire.
- Always use liquid nitrogen in a well-ventilated space to avoid nitrogen asphyxiation.