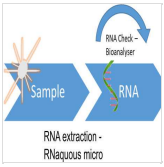

Version 1

Jun 30, 2021

Single-cell total RNA extraction from marine protists (e.g. Acantharia, *Strombidium* cf *basimorphum*, and *Prymnesium parvum*) V.1

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Mixotroph.org

Joost Mansour

ABSTRACT

Many marine protists are not culturable and therefore challenging to study, nonetheless, they are essential in all marine ecosystems. The development of single-cell techniques is allowing for more marine protists to be studied. Such genomic approaches aim to help to disentangle heterotrophic processes such as phagotrophy from osmotrophy and phototrophic-induced anabolic activities. This information will then support cellular and metabolic modeling by better elucidating the physiological mechanisms and quantifying their importance in different scenarios.
However, single-cell protocols and low input RNA kits for transcriptomics are usually made for and tested with mammalian cells, as such the feasibility and efficiency of single-cell transcriptomics on highly diverse mixotrophic protists are not always known. Often single-cell transcriptomics of microbial eukaryotes shows low transcript recovery rates and large variability.

We report on transcriptomic methods that we have successfully performed on single cells of Acantharia, *Strombidium* cf *basimorphum*, and *Prymnesium parvum*.
This first protocol outlines the total RNA extraction step utilizing the
RNAqueous™-Micro Total RNA Isolation Kit **Thermo Fisher Catalog #AM1931**
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DOI

dx.doi.org/10.17504/protocols.io.bp6xmrfn

PROTOCOL CITATION

Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John 2021. Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). **protocols.io** https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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KEYWORDS

Single-cell, Acantharia, RNA, transcriptomics, cDNA library, Strombidium, Radiolaria, ciliates, RNA-seq

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GUIDELINES

- To avoid possible RNA degradation, try to work quickly in all steps and work **On ice** . To help achieve this, do not extract RNA from too many samples simultaneously.
- Always wear clean RNase-free gloves.
- Clean workspace with ethanol and an RNase Decontamination Solution.
- If possible use a dedicated set of pipettes for RNA and use filter tips.

MATERIALS TEXT

- RNAqueous™-Micro Total RNA Isolation Kit **Thermo**
- **Fisher Catalog #AM1931** In 2 steps including:
  Elution Solution (12.5 µL x sample)
  Wash Solution 1
  Wash Solution 2/3
  Micro filter cartridges and Elution tubes
- 100% Molecular grade ethanol **Contributed by users** Step 6
- 1.5 mL microcentrifuge tubes (DNase/RNase free) **Contributed by users** Step 3 (for Elution Solution aliquot)
  PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) **Thermo**
- **Fisher Catalog #AM12230** Step 20 (2x #samples)
- Ice **Contributed by users**
- Micropipettes and filter tips

Centrifuge
Benchtop Centrifuge
Eppendorf **5405000441**
Any benchtop centrifuge will suffice

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

ThermoMixer
Benchtop Incubator

Eppendorf **5382000023**

Any heat block will suffice

Mini-centrifuge
Centrifuge

Fisher **S67601B**

Any standard mini centrifuge with adapters for different tube sizes will suffice

Vortex mixer

Any **xx**

Bioanalyzer 2100 instrument **G2939BA**
with RNA 6,000 Pico LabChip kit

SAFETY WARNINGS

We have tested this for work to acquire transcriptomes from Acantharia, Strombidinium *basimorphum,* and *Prymnesium parvum*.

Adhere to PPE, as dictated under local Health & Safety regulations.

BEFORE STARTING

Be sure to have prepared the Wash Solutions of the
RNAqueous™-Micro Total RNA Isolation Kit **Thermo Fisher Catalog #AM1931** by adding
100% Ethanol **Contributed by users** (see manufacturer manual)

Single-cell samples need to have been previously collected according to good standards in **100 µl**

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

lysis buffer **Contributed by users** from the

RNAqueous™-Micro Total RNA Isolation Kit **Thermo Fisher Catalog #AM1931** . See our sampling protocol here **dx.doi.org/10.17504/protocols.io.bqeamtae**

Also refer to pages 4-7 of the RNAqueous – Micro kit manual (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fcms_057352.pdf&title=UHJvdG9jb2w6IFJOQXF1ZW91cyZyZWc7LU1pY3JvIEtpdCAoRW5nbGlzaCAp).

Reagent and general preparations 15m

1 Samples need to have been acquired in **100 µl** lysis buffer **Contributed by users** (from the RNAqueous™-Micro Total RNA Isolation Kit **Thermo Fisher Catalog #AM1931**).

See our sampling protocol here:
dx.doi.org/10.17504/protocols.io.bqeamtae

2 Prepare and label two RNase-free tubes per sample, one tube for the total RNA extract and one tube for an aliquot for the Bioanalyzer.

3 Prepare **12.5 µl times number of samples** of Elution Solution (from RNAqueous™-Micro Total RNA Isolation Kit **Thermo Fisher Catalog #AM1931**) in a 1.5 mL microcentrifuge tubes (DNase/RNase free) **Contributed by users** and put the aliquot in a **75 °C** heat block.

Sample preparations 15m

4 20s

For Acantharia samples: thaw and vortex 2 times **00:00:10** all samples (be sure to secure the lids).

For other samples types just thaw **On ice** , and continue with step 6.

4.1 Spin down gently

5 5m

Room temperature for **00:05:00**

RNA extraction 12m 40s

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

6 Add **50 µl** 100% Molecular grade ethanol **Contributed by users** to all samples 1m

6.1 Brief vortex and gentle/short spin down

7 Load the (**150 µl**) samples each onto a separate Microfilter cartridge (use 100 µL pipet twice) 30s

7.1 Centrifuge **10000 x g, 00:00:10** (i.e. at maximum) 10s

8 Add **180 µl** of Wash Solution 1 unto the column (Microfilter cartridge) 30s

8.1 Centrifuge **10000 x g, 00:00:30** (i.e. at maximum) 30s

9 Add **180 µl** of Wash Solution 2/3 unto the column (Microfilter cartridge) 30s

9.1 Centrifuge **10000 x g, 00:00:30** (i.e. at maximum) 30s

10 Repeat addition of **180 µl** of Wash Solution 2/3 unto the column (Microfilter cartridge) 30s

10.1 Centrifuge **10000 x g, 00:00:30** (i.e. at max) 30s

11 Remove and discard all flow-through (pour it out) from the collection/elution tube 5s

12 Centrifuge **10000 x g, 00:01:00** (i.e. at maximum) to remove any liquid still on the filter 1m

13 Replace the collection/elution tube with a clean new one (keeping the top part with filter and RNA!) 5s

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14 Add **6.5 µl** Elution Solution (pre-heated at **75 °C**) 30s

Do this directly to the center of the filter

15 Incubate at **Room temperature** for **00:01:00** 1m

16 Centrifuge **10000 x g, 00:00:30** (i.e. at max) 30s

17 Add **6.0 µl** Elution Solution (pre-heated at **75 °C**) 30s

Do this directly to the center of the filter

18 Incubate at **Room temperature** for **00:01:00** 1m

19 Centrifuge **10000 x g, 00:00:30** (i.e. at max) 30s

20 Transfer your **12.5 µl** RNA from the Elution tube to a (labeled) PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) **Thermo Fisher Catalog #AM12230** (use 100 µL pipet). 15s

20.1 For Bioanalyser take an aliquot of 1.5 µL in a separate (labeled) tube, still **On ice** . Analyze immediately or store. 15s

21 Store RNA at **-80 °C**

Sample verification

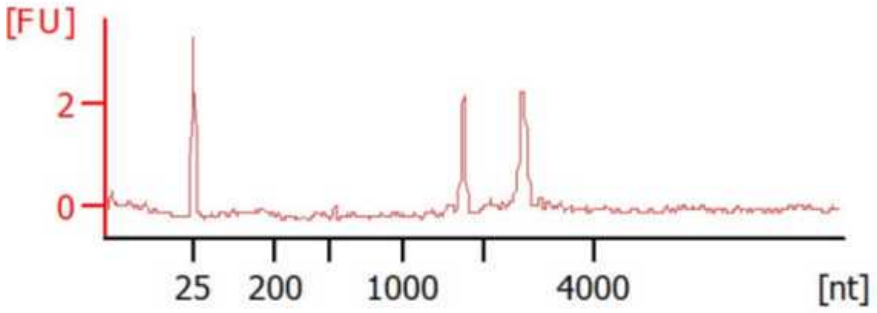
22 Attempt to quantify and assess the quality of total RNA extract using Bioanalyzer.
Follow the manufacture's protocol for the Agilent RNA 6000 Pico Kit.

Using small single-celled protists RNA is often not measurable.

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

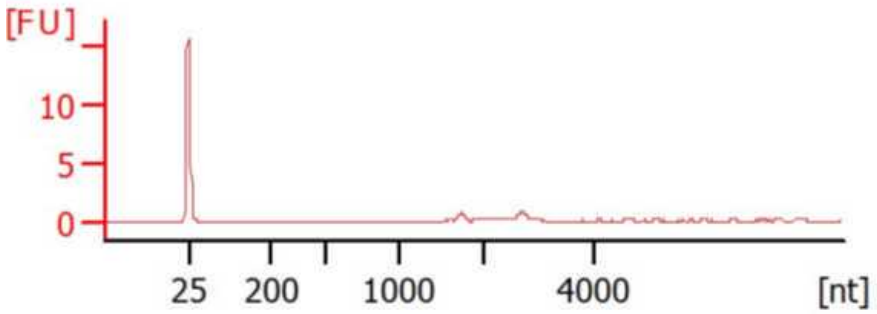
23 Total RNA extracted from single cells are often low in concentration and yield. And could in most cases not be measured (e.g. for *Strombidium and Prymnesium).*
For Acantharia (±80 µm diameter) its was measurable but could still be, as low as < 20 pg/µL, hence quantification of RNA(e.g. by Bioanalyzer) can be difficult. For our purposes, we assessed a subset of each biological replicates and if the subset was consistent, we assumed the other replicates were similar.
With these single-cell low RNA concentrations, the total RNA extract would anyway be used in subsequent protocols without dilution making accurate quantification of concentrations incidental, and this also minimized cost and workload.

24

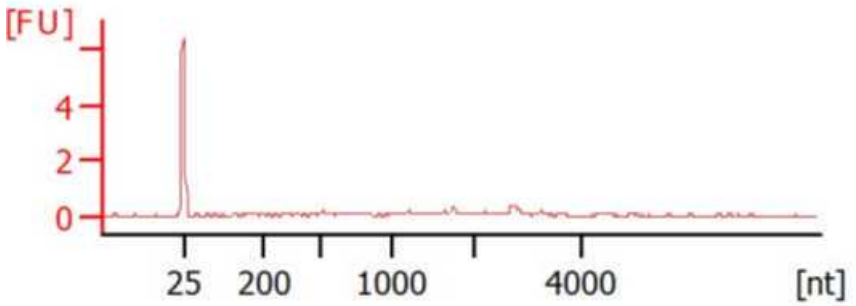


Example of a desirable electrograph showing clear 18S and 28S ribosomal RNA peaks indicating good RNA quality (acantharian sample). The first peak indicates a size and concentration marker of the Bioanalyser chip

25



Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn



Examples of electrographs showing minimal ribosomal RNA peaks. Due to the single-cell approach RNA quantity is generally low (or not measurable) and even in this case, subsequent steps provided quality cDNA product (acantharian sample). The first peak indicates a size and concentration marker of the Bioanalyser chip

Subsequent protocol:

26 Follow up with cDNA synthesis and library preparation for Illumina sequencing

cDNA library preparation from total RNA extracts of Single-cell marine protists (e.g. Acantharia, Strombidium basimorphum, and Prymnesium parvum) for transcriptome sequencing
by Joost Mansour

PREVIEW RUN

26.1 Label for each sample a tube
PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) **Thermo Fisher Catalog #AM12230**

26.2 Prepare a 72°C incubator (e.g. a thermocycler)

26.3 Thaw other reagents **On ice** – except SmartScribe Reverse Transcriptase, take that from the freezer only once needed.

26.4 Thaw your RNA samples **On ice** (as prepared in dx.doi.org/10.17504/protocols.io.bp6xmrfn)

26.5 Prepare 10X Reaction Buffer (**RB**), **On ice** as follows (1 µL is used per sample (adjust as needed, & write down exact volumes):

26.5.1 10X Lysis Buffer
- **19 µl** **Takara Catalog #634888** (from SMART-Seq4 kit)

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

RNase

- **1 µl** Inhibitor **Takara Catalog #2313A** (white cap from SMART-Seq4 kit)
- Mix/vortex and spin down (avoid bubbles)

26.6 Take into clean (labeled)
PCR Tubes, 0.2mL, flat cap, natural, PCR Tube; 0.2mL; Natural; w/flat cap; 1000/Pk. **Thermo Fisher Catalog #3412**
1 µl to 9.5 ul of RNA sample & **1 µl** of **RB**
(total 10.5 µL volume, adjust with nuclease free water **Contributed by users** depending on RNA sample)

For single-cells we recommend **5 µl** total RNA. In essence either all total RNA sample can be used, or it is safer to use <50% to allow redo when needed and [RNA] permitting. The total amplification cycles would also be affected by the volume used here.

26.7 Place samples **On ice** and add **1 µl** of
SMART-seq CDS Primer II A (12 µM) **Takarabio Catalog #634888** (blue cap) to the samples.

We are performing 17+ PCR cycles. If fewer cycles are envisioned **2 µl** of
SMART-seq CDS Primer II A (12 µM) **Takarabio Catalog #634888** should be used instead, though keeping the total volume the same by disregarding step 7.1).

26.7.1 add **1 µl** nuclease free water **Contributed by users** (total volume 12.5 µL)

26.7.2 Mix gently (vortex) & spin down

26.8 Incubate samples at **72 °C** for **00:03:00** 3m

Immediately proceed to step 8 after incubation finishes

26.9 While samples are incubating prepare Master Mix (MM) as below for each sample (+10%; write down exact volumes)
On ice

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

26.9.1 5X Ultra Low First-Strand Buffer

- 4 µl **Takarabio Catalog #634888** (red cap)

(make sure precipitates are dissolved)

SMART-seq v4 Oligonucleotide (46 µM) **Takarabio Catalog #634888**

- 1 µl (pink cap)

RNase

- 5 µl Inhibitor **Takara Catalog #2313A** (white cap)

2m

26.10 **Immediately** after the 3 min 72°C incubation from step 8 put samples **On ice** for **00:02:00**
During this incubation time on ice perform steps 11 and 12.

26.11 Preheat thermocycler to **42 °C**

26.12 SMARTScribe Reverse
Take the Transcriptase **Takarabio Catalog #634888** (purple cap), gently mix it without vortexing and add to the prepared Master Mix (from step 9):

26.12.1 SMARTScribe Reverse

2 µl Transcriptase **Takarabio Catalog #634888** for each sample

(x #samples +10%)

26.12.2 Mix MM by gentle vortex and spin down

26.13 Add **7.5 µl** of the MM to the samples (total volume now 20 µL)

26.13.1 Mix by pipetting and follow with short spindown

1h 40m

26.14 Incubate samples in pre-heated Thermocyler with heated lid and the following program:
42 °C **01:30:00** ,
70 °C **00:10:00** ;
4 °C forever

26.15

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

STOPPING POINT - 4°C overnight

26.16 Thaw all the reagents (see step 18) **On ice** except the enzyme
(Vortex and spin down reagents except for enzyme)

26.17 Preheat thermocycler to **95 °C**

26.18 Prepare Mastermix (+10%), one sample is as below:

26.18.1

- 2X SeqAmp PCR
- **25 µl** Buffer **Takara Catalog #638526**
- PCR Primer II A (12
- **1 µl** µM) **Takara Catalog #634888** (green cap)
- **3 µl** nuclease free water **Contributed by users**
- SeqAmp DNA
- **1 µl** Polymerase **Takara Catalog #638504** (take out last minute and mix without vortexing, spin down)
- Mix Master Mix well and gently (finger flick) and spin down

26.19 Add **30 µl** of Mastermix to each sample from cDNA synthesis.
Mix well (pipetting) and spin down gently.

26.20 Run samples on pre-heated thermocycler with the program:

A	B	C
95°C	1 min	
98°C	10 sec	repeat step 2, 18 times
65°C	30 sec	
68°C	3 min	
72°C	10 min	
4°C	forever	

This thermocycler program is run with 18 cycles and works for us. Nonetheless, it is recommended to test this beforehand. Over-amplification can result in ahigher yield of cDNA, however, it introduces a bias towards more abundant transcripts. We settled on the following number of amplification cycles.

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

Species	cDNA kit	Number of cycles
Strombidium basimorphum	SMARTseq-v4	18
Prymnesium parvum	NEBNext	25
Acantharia	SMARTseq-v4	18
Acantharia	NEBNext	16

26.21

STOPPING POINT 4°C overnight

6.22 30m

Preparations:

- Label for each sample two tubes
  PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) **Thermo Fisher Catalog #AM12230** . One tube is used for the cDNA after purification, and one is for an aliquot of the purified cDNA for Bioanalyzer.
- Vortex the bead stock well (AMPure XP Beads **Contributed by users**), this needs to be very well and evenly mixed
- Aliquot beads, **22.5 µl** x samples (plus extra)
- Bring the bead aliquot to **Room temperature** for at least **00:30:00**
- Vortex the bead aliquot until evenly mixed
- Prepare fresh 80% EtOH, 400 µL x samples

6.23 Add **22.5 µl** of beads to each sample (amplified cDNA from the previous section)

26.23.1 Mix by pipetting up and down at least 10 times, and vortex

6.24 8m

Incubate at **Room temperature** **00:08:00** to let cDNA bind to the beads

6.25 5m

Magnetic Stand-
Briefly spin down and place the samples **on a** 96 **Thermofisher Catalog #AM10027** **for** **00:05:00** or longer. Until the liquid appears completely clear and there are no beads in the supernatant.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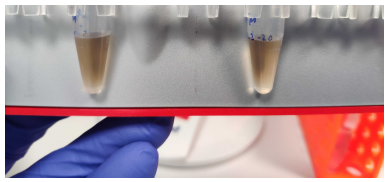
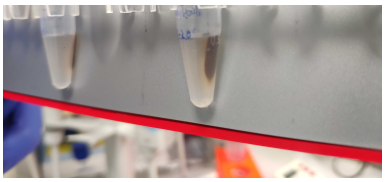


Not yet clear, beads have not yet all pelleted



clear, all beads have pelleted

6.26 Pipet and discard the supernatant (72.5 µL), keeping the samples in the magnetic device

6.27 Keeping the samples in the magnetic device, **add** **200 µl fresh** 80% Ethanol **Contributed by users** to each sample.

Do not disturb the beads

26.27.1 Wait **00:00:30** 30s

26.27.2 Pipet and discard supernatant containing contaminants (use 100 µL)

6.28 Repeat the EtOH washing step for a total of 2 washing steps

6.29 Briefly spin the samples to collect liquid off the sides

6.30 Place samples back in the **magnetic device for** **00:00:30** , beads will again be collected on the side 30s

6.31 Remove all remaining ethanol/supernatant with a pipet (use 10 µL pipet)

6.32 Place samples at **Room temperature for** **00:02:00 minutes.** (it might take a bit longer) 2m

Until the pellet is no longer shiny, but before a crack appears. It needs to be 'just' dry, matte with no shine.

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6.33 Once the beads are dry add **15 µl of Elution buffer to all samples** to cover the bead pellet

26.33.1 Remove samples from the magnetic device

26.33.2 Mix to re-suspend the beads by (multi)pipetting (can scrap of beads from the side)

6.34 Incubate at **Room temperature for 00:02:00 (longer)** to rehydrate 2m

6.35 Briefly spin the samples to collect liquid off the sides

6.36 Place the samples back in the **magnetic device for 00:01:00** , until the solution is completely clear 1m

6.37 **Transfer the clear supernatant** containing purified cDNA to PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) **Thermo Fisher Catalog #AM12230** tube (use 10 µL pipet).

Beads that do not pellet can be pipetted for resuspension and then towards the magnet, and incubation continued until there are no more beads in the supernatant

26.37.1 Make immediately an aliquot for Bioanalyzer analysis to prevent unnecessary freeze-thawing cycles.

26.38

STOPPING POINT - Label and store at **-20 °C**

26.39

Check the quality of cDNA by Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626** following the manufacture's protocol.

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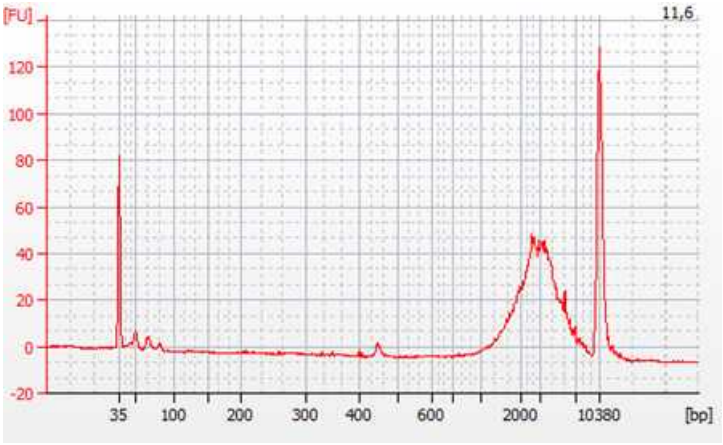

Example of a desirable electrograph. Showing a good cDNA curve, and few primer dimers. Example of an acantharian sample. The peaks on the outsides are markers of the Bioanalyser chip for size and concentration marker

26.39.1 Quantify and calculate the concentration of cDNA. This is needed for the next cDNA library procedure.

6.40 Proceed with cDNA library preparation only for good quality samples from the previous step.

6.41 Normalize cDNA samples to 30pg/ul

Dilute each sample of amplified and purified cDNA to 30 pg/µL in either Elution buffer or as per the final step of the used protocol for cDNA purification. Work with a minimum of 1 µL amplified cDNA and a total volume of 5 µL.

6.42 Prepare to work very timely for this protocol

- Preheat a PCR thermocycler to **55 °C** , with preheat lid at 100 °C

  Nextera XT DNA Library Preparation
- Prepare from the Kit **illumina Catalog #FC-131-1096** the **ATM** and **NT reagents** in sufficient quantity (i.e. 5 ul per sample for each) separated over multiple tubes to facilitate multi-pipetting

6.43 Nextera XT DNA Library Preparation

Follow the Kit **illumina Catalog #FC-131-1096** manufacturer's protocol for " Tagment genomic DNA", and "Amplify Libraries", with the changes listed below.

Refer to pages 7-9 of the Nextera XT manual (https://emea.support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-05.pdf).

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

6.44 Changes to manufacturer' s protocol:

- Start the tagmentation with **5 µl** of 30 pg/µl amplified cDNA sample (from step 37)
- all steps indicated as "centrifuge at 280 x g at 20 °C for 1 minute" can be substituted short spindown in a tabletop mini-centrifuge.

6.45 Store samples at **4 °C** for up to 2 days or proceed immediately with purification

6.46 Preparations: 30m

- Vortex the bead stock well (AMPure XP Beads **Contributed by users**), this needs to be very well and evenly mixed
- Aliquot beads, **30 µl** x samples (plus extra)
- Bring the bead aliquot to **Room temperature** for at least **00:30:00**
- Vortex the bead aliquot until evenly mixed
- Prepare fresh 80% EtOH, 400 µL x #samples

6.47 Spin down your indexed cDNA samples (total 50 µL)

6.48 **Add 30 µL** of AMPure XP Beads **Contributed by users** to each sample 2m

- Mix by pipetting up and down
- Shake/vortex for **00:02:00**

6.49 Incubate at **Room temperature** **00:05:00** to let cDNA bind to the beads 5m

6.50 Magnetic Stand-
Briefly spin down and place the samples **on a** 96 **Thermofisher Catalog #AM10027** **for** **00:05:00** or longer. Until the liquid appears completely clear and there are no beads in the supernatant.

6.51 Pipet and discard the supernatant (80 µL), keeping the samples in the magnetic device

6.52 Keeping the samples in the magnetic device, **add** **200 µl fresh** 80% Ethanol **Contributed by users** to each sample.

Do not disturb the beads

26.52.1 Wait **00:00:30**

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn

26.52.2 Pipet and discard supernatant containing contaminants (use 100 µL pipet)

6.53 Repeat the EtOH washing step for a total of 2 washing steps

6.54 Briefly spin the samples to collect liquid off the sides

6.55 Place samples back in the **magnetic device for** **00:00:30** , beads will again be collected on the side

6.56 Remove all remaining ethanol/supernatant with a pipet (use 10 µL pipet)

6.57 Place samples at **Room temperature for** **00:05:00 minutes.** 5m
Until the pellet is no longer shiny, but before a crack appears. It needs to be 'just' dry, matte with no shine.

6.58 Once the beads are dry add **52.5 µl** **of** Resuspension Buffer **Contributed by users** (NexteraXT kit) to all samples to cover the bead pellet

26.58.1 Remove samples from the magnetic device

26.58.2 Mix to re-suspend the beads by (multi)pipetting (can scrap of beads from the side)

26.58.3 Vortex for **00:02:00** followed by a very short spindown 2m

6.59 Incubate at **Room temperature for** **00:02:00** to rehydrate

6.60 Briefly spin the samples to collect liquid off the sides

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2m

6.61 Place the samples back in the **magnetic device for** **00:02:00** , until the solution is completely clear

6.62 **Transfer the clear supernatant (50 uL)** containing your purified cDNA library to PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) **Thermo Fisher Catalog #AM12230** tube (use 10 µL pipet).

Beads that do not pellet can be pipetted for resuspension and then towards the magnet, and incubation continued until there are no more beads in the supernatant

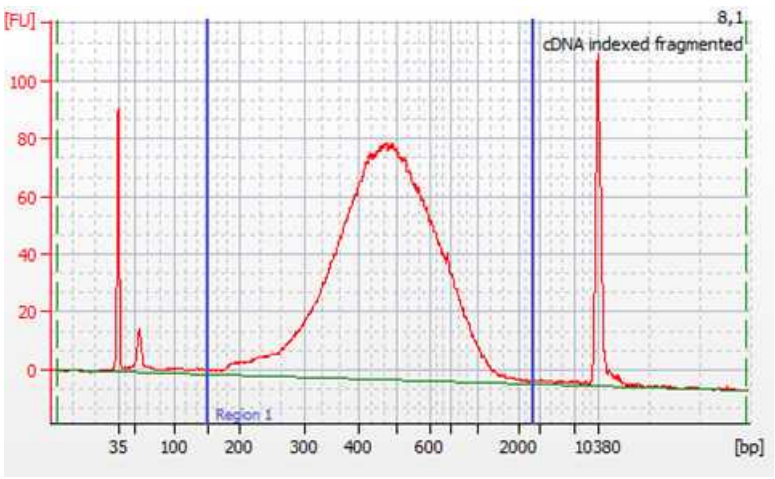
26.62.1 Make immediately an aliquot for Bioanalyser analysis to prevent unnecessary freeze-thawing cycles.

26.63

STOPPING POINT - Label and store at **-20 °C** for sequencing

26.64

Check the quality of the cDNA libraries by Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626** following the manufacture's protocol. Alternatively, a Bioanalyser DNA 7500 Kit (Agilent #5067-1506) could be used as a more cost-efficient alternative and if sample concentration permitting. See for example the third graph.



Example electrograph for a good cDNA library run with a Bioanalyser High Sensitivity DNA Kit (Agilent #5067-4626). Though primer-dimers are here still present and a follow up (repeat) cleaning is recommended. The region for smear analysis is indicated between blue lines. Example of an acantharian sample. The peaks on the outsides are markers.

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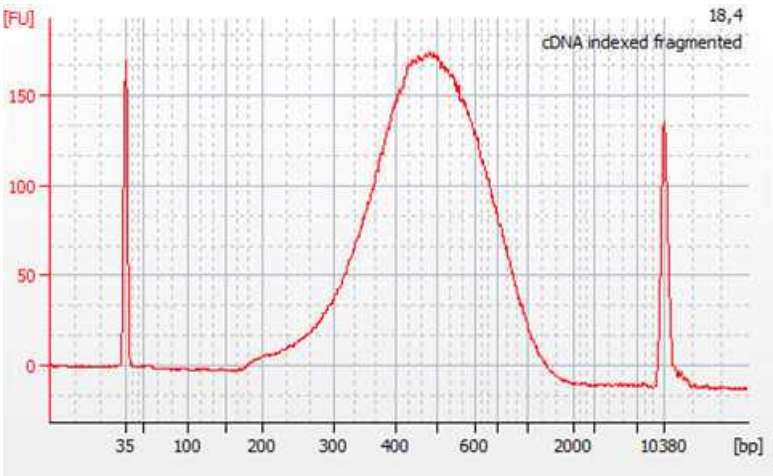
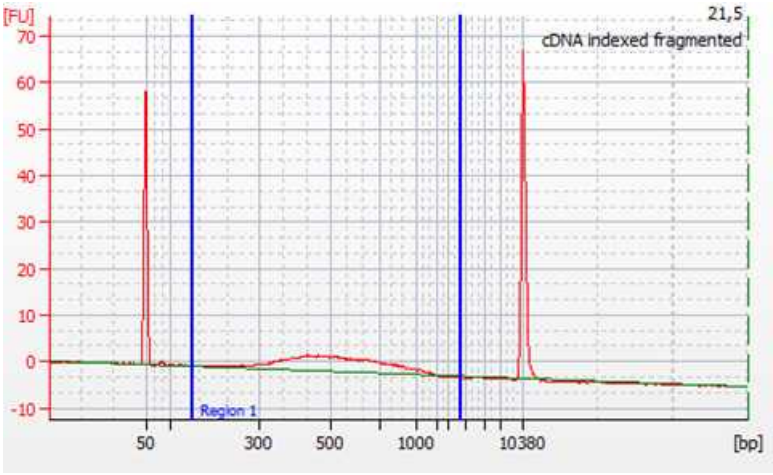


Example electrograph for a desirable cDNA library run with a Bioanalyser High Sensitivity DNA Kit (Agilent #5067-4626). Example of an acantharian sample. The peaks on the outsides are markers.



Example electrograph for a desirable cDNA library run with a Bioanalyser DNA 7500 Kit (Agilent #5067-1506) instead of a Bioanalyser High Sensitivity DNA Kit (Agilent #5067-4626). This still allows for smear analysis though the concave parabola is less clear. This is more cost-effective than using a high sensitivity kit. Example of an acantharian sample The peaks on the outsides are markers.

26.64.1 Quantify and calculate the concentration of cDNA by smear analysis. This is needed for the normalization of samples for sequencing.

6.65 The quality and quantity control of the generated cDNA libraries is performed using the Agilent High Sensitivity DNA kit (Agilent #5067-4626). In case primer-dimers or adapters are still present, an additional step of cleaning with magnetic beads is to be performed. A bead to sample ratio of 0.7:1 was found to be efficient in eliminating both primer dimers and remaining adapters.

The cDNA libraries are normalized to equal molarity, as well as fragment size before the final pooling and subsequent sequencing. Calculate nM cDNA of each sample as: nM DNA = [ng/µL] x 10^6 / (660 x fragment length bp). Where the concentration in ng/µL and the average fragment length in base pairs are obtained from Bioanalyzer smear analysis.

The molarity upon which the cDNA libraries are normalized is determined based on the yield of cDNA, as well as the

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requirements for the subsequent sequencing (e.g. >0.5 nM). The final pool of all the samples should again be checked using the Bioanalyzer in order to verify that the normalization process was successful.

The pools are ready for Illumina sequencing.

Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John (06/30/2021). Single-cell total RNA extraction from marine protists (e.g. Acantharia, Strombidium cf basimorphum, and Prymnesium parvum). https://dx.doi.org/10.17504/protocols.io.bp6xmrfn