VERSION 2

JUN 30, 2021

OPEN ACCESS

DOI:

dx.doi.org/10.17504/protocols.io.brw3m7gn

Protocol Citation: Joost Mansour, Konstantinos Anestis, Fabrice Not, Uwe John 2021. cDNA library preparation from total RNA extracts of Single-cell marine protists (e.g. Acantharia, Strombidium basimorphum, and Pymnesium parvum) for transcriptome sequencing. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.brw3m7gn> Version created by Joost S Mansour

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Protocol status: Working
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Created: Jan 29, 2021

Last Modified: Jun 30, 2021

PROTOCOL integer ID:
46779

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

cDNA library preparation from total RNA extracts of Single-cell marine protists (e.g. Acantharia, Strombidium basimorphum, and Pymnesium parvum) for transcriptome sequencing V.2

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Symbiosis Model Systems

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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 is 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 basimorphum*, and *Prymnesium parvum*.

This protocol follows up after total RNA extraction (from the protocol at dx.doi.org/10.17504/protocols.io.bp6xmrfn) to prepare cDNA libraries for Illumina sequencing. The described protocol uses the SMART-Seq4 kit (Takara #634891) for cDNA synthesis and amplification, but this can also be successfully performed with the NEBNext kit (NEB #E6421). The NEBNext kit protocol is very similar to the protocol described here and generally the manufacture's protocol can be followed but see the notes at step 4 and step 18 of this protocol, and do the final elution after cDNA purification in 10 mM Tris (pH 8.0).

The subsequent cDNA library is prepared following the

Nextera XT DNA Library Preparation Kit Illumina, Inc. Catalog #FC-131-1096

GUIDELINES

- Always wear clean RNase-free gloves.
- Clean workspace (and thermocyclers) with ethanol and an RNase Decontamination Solution.
- Work On ice
- If possible use a dedicated set of pipettes for RNA and use filter tips.

MATERIALS

cDNA synthesis

- PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) Thermo Fisher Catalog #AM12230 (x #samples)
- 1.5 ml reaction tube Eppendorf (x2 for mastermix and reaction buffer)
- Ice Contributed by users
- nuclease free water Contributed by users
- 10X Lysis Buffer Takara Bio Inc. Catalog #634888
- RNase Inhibitor Takara Bio Inc. Catalog #2313A
- SMART-seq v4 Oligonucleotide (46 μ M) Takara Bio Inc. Catalog #634888 (48 μ M, 1 μ L per sample)
- 5X Ultra Low First-Strand Buffer Takara Bio Inc. Catalog #634888 (4 μ L per sample)
- SMART-seq CDS Primer II A (12 μ M) Takara Bio Inc. Catalog #634888 (1 μ L per sample)
- SMARTScribe Reverse Transcriptase Takara Bio Inc. Catalog #634888 (2 μ L per sample)

cDNA amplification

- 1.5 ml reaction tube Eppendorf (for mastermix)
- nuclease free water Contributed by users (3 μ L per sample)
- 2X SeqAmp PCR Buffer Takara Bio Inc. Catalog #638526 (25 μ L per sample)
- SeqAmp DNA Polymerase Takara Bio Inc. Catalog #638504 (1 μ L per sample)
- PCR Primer II A (12 μ M) Takara Bio Inc. Catalog #634888 (1 μ L per sample)

cDNA purification

- 80% Ethanol Contributed by users (made from 100% Molecular grade ethanol Contributed by users
- and nuclease free water Contributed by users) (400 μ L per sample)
- 50 mL Falcon Tubes Contributed by users (for 80% ethanol)
- AMPure XP Beads Contributed by users (this can be substituted for a similar product, we use CleanNGS (GC Biotech, CNGS-0050)
- Magnetic Stand-96 Thermofisher Catalog #AM10027
- PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) Thermo Fisher Catalog #AM12230

- ✕ PCR Tubes, 0.2mL, flat cap, natural, PCR Tube; 0.2mL; Natural; w/flat cap; 1000/Pk. Thermo Fisher Catalog #3412 (x #samples x2)
- ✕ 1.5 ml reaction tube Eppendorf (for bead aliquot, a 5 mL tube might be preferred)

cDNA library preparation, indexing, and purification

- ✕ PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) Thermo Fisher Catalog #AM12230 (2x for reagent aliquots)
- ✕ PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) Thermo Fisher Catalog #AM12230 (2x #samples)
- ✕ Nextera XT DNA Library Preparation Kit Illumina, Inc. Catalog #FC-131-1096
- ✕ Nextera XT Index Kit v2 Set A (96 indexes 384 samples) Illumina, Inc. Catalog #FC-131-2001
- ✕ AMPure XP Beads Contributed by users (This can be substituted for a similar product, we use CleanNGS (GC Biotech, CNGS-0050).
- ✕ 80% Ethanol Contributed by users (made from ✕ 100% Molecular grade ethanol Contributed by users and ✕ nuclease free water Contributed by users) (400 µL per sample)
- ✕ 1.5 ml reaction tube Eppendorf (for bead aliquot, a 5 mL tube might be preferred)
- ✕ Magnetic Stand-96 Thermofisher Catalog #AM10027

General lab equipment

- ✕ Micropipettes and filter tips
- ✕ Vortex Contributed by users
- ✕ PCR Thermocycler Contributed by users
- ✕ Ice Contributed by users

Equipment	
Mini-centrifuge	NAME
Centrifuge	TYPE
Fisher	BRAND
S67601B	SKU
https://www.fishersci.com/shop/products/fisherbrand-standard-mini-centrifuge-standard-mini-centrifuge/s67601b	LINK
Any standard mini centrifuge with adapters for different tube sizes will suffice	SPECIFICATIONS

Equipment

Bioanalyzer

NAME

Bioanalyzer

TYPE

Agilent

BRAND

G2991AA

SKU

<https://www.agilent.com/en/product/bioanalyzer-automated-electrophoresis/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250>

LINK

Any bioanalyzer will suffice.

SPECIFICATIONS

Agilent High Sensitivity DNA Kit Agilent Technologies Catalog #5067-4626

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 START INSTRUCTIONS

Total RNA needs to have been extracted (Protocol: [dx.doi.org/10.17504/protocols.io.bp6xmrfn](https://doi.org/10.17504/protocols.io.bp6xmrfn)) and when possible quantified and quality checked by Bioanalyzer. If Bioanalyzer analysis was possible, only continue with good quality RNA extracts.

- Thaw reagents (except enzymes).
- Allow reagents that need to be at room temperature to incubate at Room temperature (i.e. 5X Ultra Low First-Strand Buffer Takara Bio Inc. Catalog #634888 and GC nucleic acids purification beads.
- Set thermocycler programs and pre-heat thermocyclers.
- For the cDNA purification step Prepare fresh 80% ethanol from 100% Molecular grade ethanol Contributed by users with nuclease free water Contributed by users

cDNA synthesis preparations

- 1 Label for each sample a tube PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) Thermo Fisher Catalog #AM12230
- 2 Prepare a 72°C incubator (e.g. a thermocycler)
- 3 Thaw other reagents On ice – except SmartScribe Reverse Transcriptase, take that from the freezer only once needed.
- 4 Thaw your RNA samples On ice (as prepared in [dx.doi.org/10.17504/protocols.io.bp6xmrfn](https://doi.org/10.17504/protocols.io.bp6xmrfn))

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

- 5.1
 - 19 µL 10X Lysis Buffer Takara Bio Inc. Catalog #634888 (from SMART-Seq4 kit)
 - 1 µL RNase Inhibitor Takara Bio Inc. Catalog #2313A (white cap from SMART-Seq4 kit)
 - Mix/vortex and spin down (avoid bubbles)

cDNA synthesis

1h 45m

- 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 µL of RNA sample & 1 µL of RB

(total 10.5 µL volume, adjust with nuclease free water Contributed by users depending on RNA sample)

Note

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.

- 7 Place samples On ice and add 1 µL of SMART-seq CDS Primer II A (12 µM) Takara Bio Inc. Catalog #634888 (blue cap) to the samples.

Note

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).

7.1

add 1 μL

nuclease free water Contributed by users

(total volume 12.5 μL)

7.2

Mix gently (vortex) & spin down

8

Incubate samples at 72 $^{\circ}\text{C}$ for 00:03:00

3m

Note

Immediately proceed to step 8 after incubation finishes

9

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

9.1

- 4 μL 5X Ultra Low First-Strand Buffer Takara Bio Inc. Catalog #634888 (red cap)

(make sure precipitates are dissolved)

- 1 μL SMART-seq v4 Oligonucleotide (46 μM) Takara Bio Inc. Catalog #634888 (pink cap)

- 5 μL RNase Inhibitor Takara Bio Inc. Catalog #2313A (white cap)

10

Immediately after the 3 min 72 $^{\circ}\text{C}$ incubation from step 8 put samples On ice for 00:02:00

2m

During this incubation time on ice perform steps 11 and 12.

11

Preheat thermocycler to 42 $^{\circ}\text{C}$

12

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

12.1

 2 μL

SMARTScribe Reverse Transcriptase Takara Bio Inc. Catalog #634888

for each sample (x #samples +10%)

12.2

Mix MM by gentle vortex and spin down

13

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

13.1

Mix by pipetting and follow with short spindown

14

Incubate samples in pre-heated Thermocycler with heated lid and the following program:

1h 40m

42 °C	01:30:00
70 °C	00:10:00
4 °C	forever

15

STOPPING POINT - 4°C overnight

cDNA Amplification

16

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

17

Preheat thermocycler to 95 °C

18

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

18.1

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

- 19 Add 30 µL of Mastermix to each sample from cDNA synthesis. Mix well (pipetting) and spin down gently.

- 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	

Note

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 a higher yield of cDNA, however, it introduces a bias towards more abundant transcripts. We settled on the following number of amplification cycles.

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

21 STOPPING POINT 4°C overnight

cDNA cleanup/bead purification

43m

22 Preparations:

30m

- 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

23 Add 22.5 µL of beads to each sample (amplified cDNA from the previous section)

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

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

8m

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

5m

Expected result

Not yet clear, beads have not yet all pelleted

clear, all beads have pelleted

26 Pipet and discard the supernatant (72.5 μ L), keeping the samples in the magnetic device

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

Note

Do not disturb the beads

27.1 Wait 00:00:30

30s

27.2 Pipet and discard supernatant containing contaminants (use 100 μ L)

- 28 Repeat the EtOH washing step for a total of 2 washing steps go to step #27
- 29 Briefly spin the samples to collect liquid off the sides
- 30 Place samples back in the **magnetic device** for 00:00:30 , beads will again be collected on the side 30s
- 31 Remove all remaining ethanol/supernatant with a pipet (use 10 µL pipet)
-
- 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.
- 33 Once the beads are dry add 15 µL of **Elution buffer to all samples** to cover the bead pellet
- 33.1 Remove samples from the magnetic device
- 33.2 Mix to re-suspend the beads by (multi)pipetting (can scrap of beads from the side)
- 34 Incubate at Room temperature for 00:02:00 (**longer**) to rehydrate 2m
- 35 Briefly spin the samples to collect liquid off the sides
- 36 Place the samples back in the **magnetic device** for 00:01:00 , until the solution is completely clear 1m

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).

Note

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

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

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

cDNA Sample verification

39

Check the quality of cDNA by
manufacture's protocol.

Agilent High Sensitivity DNA Kit Agilent Technologies Catalog #5067-4626

following the

Expected result

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

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

cDNA library preparation and indexing - Nextera XT

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

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.

42 Prepare to work very timely for this protocol

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

- Prepare from the Nextera XT DNA Library Preparation Kit Illumina, Inc. 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

43 Follow the Nextera XT DNA Library Preparation Kit Illumina, Inc. 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).

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.

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

cDNA library purification

46m

46 Preparations:

- 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

30m

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

48 Add 30 μ L of AMPure XP Beads Contributed by users to each sample

2m

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

49 Incubate at Room temperature 00:05:00 to let cDNA bind to the beads

5m

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

51 Pipet and discard the supernatant (80 μ L), keeping the samples in the magnetic device

52 Keeping the samples in the magnetic device, add 200 μ L fresh 80% Ethanol Contributed by users to each sample.

Note

Do not disturb the beads

52.1 Wait 00:00:30

52.2 Pipet and discard supernatant containing contaminants (use 100 μ L pipet)

53 Repeat the EtOH washing step for a total of 2 washing steps go to step #52

54 Briefly spin the samples to collect liquid off the sides

- 55 Place samples back in the **magnetic device** for 00:00:30 , beads will again be collected on the side
- 56 Remove all remaining ethanol/supernatant with a pipet (use 10 μ L pipet)
- 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.
- 58 Once the beads are dry add 52.5 μ L of Resuspension Buffer Contributed by users (NexteraXT kit) to all samples to cover the bead pellet
- 58.1 Remove samples from the magnetic device
- 58.2 Mix to re-suspend the beads by (multi)pipetting (can scrap of beads from the side)
- 58.3 Vortex for 00:02:00 followed by a very short spindown 2m
- 59 Incubate at Room temperature for 00:02:00 to rehydrate
- 60 Briefly spin the samples to collect liquid off the sides
- 61 Place the samples back in the **magnetic device** for 00:02:00 , until the solution is completely clear 2m
- 62 **Transfer the clear supernatant (50 μ L)** 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).

Note

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

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

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

cDNA library verification

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.

Expected result

Example electropherogram 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.

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.

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

4.4.3 Follow up steps: library quality control; sample normalization/dilution and..

- 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: $\text{nM DNA} = [\text{ng}/\mu\text{L}] \times 10^6 / (660 \times \text{fragment length bp})$. 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 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.