



## 🌐 cDNA library preparation from total RNA extracts of Single-cell marine protists (e.g. Acantharia, Strombidium basimorphum, and Pymnesium parvum) for transcriptome sequencing V.3

<sup>1</sup>Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, German y;

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Ecology of Marine Plankton (ECOMAP) team - Roscoff  
Tech. support email: [phytopk@sb-roscoff.fr](mailto:phytopk@sb-roscoff.fr)

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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.

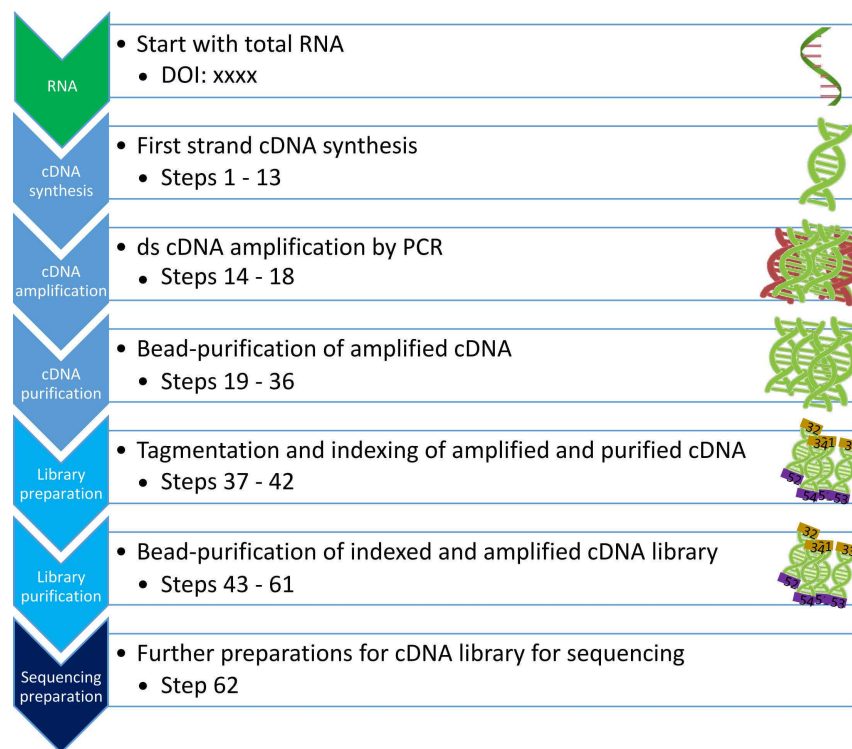
However, single-cell protocols and low input RNA kits for transcriptomics are usually made for and tested with mammalian cells, as such the feasibility and efficiency of single-cell transcriptomics on highly diverse mixotrophic protists is not always known. Often single-cell transcriptomics of microbial 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](https://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 Catalog #FC-131-1096](#)



DOI

[dx.doi.org/10.17504/protocols.io.b3ifqkbn](https://doi.org/10.17504/protocols.io.b3ifqkbn)

Joost S Mansour, Konstantinos Anestis, Fabrice Not, Uwe John 2022. 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.b3ifqkbn>  
Joost Mansour

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

protocol ,

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- 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.

#### cDNA synthesis

- [PCR Tubes & Caps, RNase-free, 0.2 mL \(8-strip format\) Thermo](#)
- **Fisher Catalog #AM12230** In 4 steps (x #samples)
- [1.5 ml reaction tube Eppendorf](#) (x2 for mastermix and reaction buffer)
- [Ice Contributed by users](#)
- [nuclease free water Contributed by users](#) In 3 steps
- [10X Lysis Buffer](#)
- **Takara Catalog #634888** Step 5.1
- [RNase](#)
- **Inhibitor Takara Catalog #2313A** In 2 steps
- [SMART-seq v4 Oligonucleotide \(46](#)
- **μM Takarabio Catalog #634888** Step 9.1 (48 μM, 1 μL per sample)
- [5X Ultra Low First-Strand Buffer](#)
- **Takarabio Catalog #634888** Step 9.1 (4 μL per sample)
- [SMART-seq CDS Primer II A \(12](#)
- **μM Takarabio Catalog #634888** Step 7 (1 μL per sample)
- [SMARTScribe Reverse](#)
- **Transcriptase Takarabio Catalog #634888** In 2 steps (2 μL per sample)

#### cDNA amplification

- [1.5 ml reaction tube Eppendorf](#) (for mastermix)
- [nuclease free water Contributed by users](#) In 3 steps (3 µL per sample)
- [2X SeqAmp PCR](#)
- [Buffer Takara Catalog #638526](#) Step 18.1 (25 µL per sample)
- [SeqAmp DNA](#)
- [Polymerase Takara Catalog #638504](#) Step 18.1 (1 µL per sample)
- [PCR Primer II A \(12](#)
- [µM\) Takara Catalog #634888](#) Step 18.1 (1 µL per sample)

#### cDNA purification

- [80% Ethanol Contributed by users](#) In 2 steps (made from
- [100% Molecular grade ethanol Contributed by users](#) and
- [nuclease free water Contributed by users](#) In 3 steps ) (400 µL per sample)
- [50 mL Falcon Tubes Contributed by users](#) (for 80% ethanol)
- [AMPure XP Beads Contributed by users](#) In 3 steps (this can be substituted for a similar product, we use CleanNGS (GC Biotech, CNGS-0050)
- [Magnetic Stand-](#)
- [96 Thermofisher Catalog #AM10027](#) In 2 steps
- [PCR Tubes & Caps, RNase-free, 0.2 mL \(8-strip format\) Thermo](#)
- [Fisher Catalog #AM12230](#) In 4 steps
- [PCR Tubes, 0.2mL, flat cap, natural, PCR Tube; 0.2mL; Natural; w/flat cap; 1000/Pk. Thermo](#)
- [Fisher Catalog #3412](#) Step 6 (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](#) In 4 steps (2x for reagent aliquots)
- [PCR Tubes & Caps, RNase-free, 0.2 mL \(8-strip format\) Thermo](#)
- [Fisher Catalog #AM12230](#) In 4 steps (2x #samples)
- [Nextera XT DNA Library Preparation](#)
- [Kit illumina Catalog #FC-131-1096](#) In 2 steps
- [Nextera XT Index Kit v2 Set A \(96 indexes 384](#)
- [samples\) illumina Catalog #FC-131-2001](#)
- [AMPure XP Beads Contributed by users](#) In 3 steps (This can be substituted for a similar product, we use CleanNGS (GC Biotech, CNGS-0050).
- [80% Ethanol Contributed by users](#) In 2 steps (made from
- [100% Molecular grade ethanol Contributed by users](#) and
- [nuclease free water Contributed by users](#) In 3 steps ) (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](#) In 2 steps

#### General lab equipment

- Micropipettes and filter tips
- [Vortex](#) Contributed by users
- [PCR Thermocycler](#) Contributed by users
- [Ice](#) Contributed by users

#### Mini-centrifuge Centrifuge

Fisher S67601B [↗](#)

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



#### Bioanalyzer Bioanalyzer

Agilent G2991AA [↗](#)

Any bioanalyzer will suffice.



[Agilent High Sensitivity DNA Kit](#) Agilent

[Technologies Catalog #5067-4626](#) In 2 steps

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.

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contributors, administrators, or anyone else associated with [protocols.io](https://dx.doi.org/10.17504/protocols.io), can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Total RNA needs to have been extracted (Protocol: [dx.doi.org/10.17504/protocols.io.bp6xmrfn](https://dx.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](#) **Takarabio 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://dx.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

- [10X Lysis Buffer](#) (from SMART-Seq4 kit)
- **19 µL** **Takara Catalog #634888**
- [RNase](#)
- **1 µL** **Inhibitor Takara 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 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.


## 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).

### 7.1

add  **1 µL** [nuclease free water Contributed by users](#) (total volume 12.5 µL)

### 7.2 Mix gently (vortex) & spin down

## 8

3m

Incubate samples at  **72 °C** for  **00:03:00**

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 [5X Ultra Low First-Strand Buffer](#)
- **4 µL** [Takarabio Catalog #634888](#) (red cap)  
(make sure precipitates are dissolved)
  - **1 µL** [SMART-seq v4 Oligonucleotide \(46 µM\)](#) [Takarabio Catalog #634888](#) (pink cap)
  - **0.5 µL** [RNase Inhibitor](#) [Takara Catalog #2313A](#) (white cap)

10 

2m

**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.

11 Preheat thermocycler to **42 °C**

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):

12.1 [SMARTScribe Reverse](#)  
**2 µL** [Transcriptase Takarabio 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 

1h 40m

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


**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 Catalog #638526**
-  **1 µL**  **PCR Primer II A (12**  
**µM) Takara Catalog #634888** (green cap)
-  **3 µL**  **nuclease free water Contributed by users**
-  **1 µL**  **SeqAmp DNA**  
 **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

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
65°C	30 sec	step 2,
68°C	3 min	18
		times
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 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

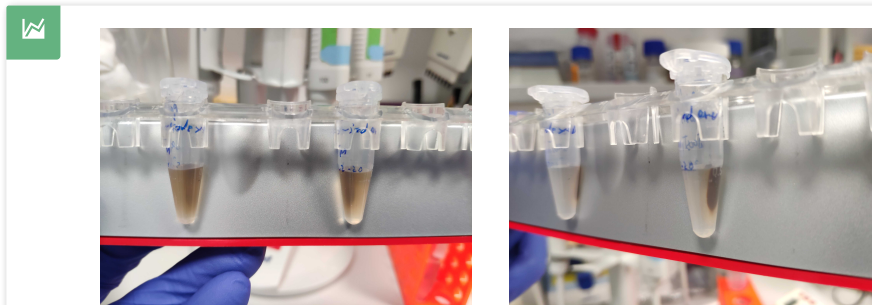
25 [Magnetic Stand](#)

5m

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.



Not yet clear, beads have not yet all pelleted

clear, all beads have pelleted

26 

Pipet and discard the supernatant (72.5  $\mu$ L), keeping the samples in the magnetic device

27 Keeping the samples in the magnetic device, add [200  \$\mu\$ L](#) fresh [80% Ethanol Contributed by users](#) to each sample.

Do not disturb the beads

27.1 Wait [00:00:30](#)

30s

27.2 

Pipet and discard supernatant containing contaminants (use 100  $\mu$ L)

28 Repeat the EtOH washing step for a total of 2 washing steps [↻](#)

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).

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

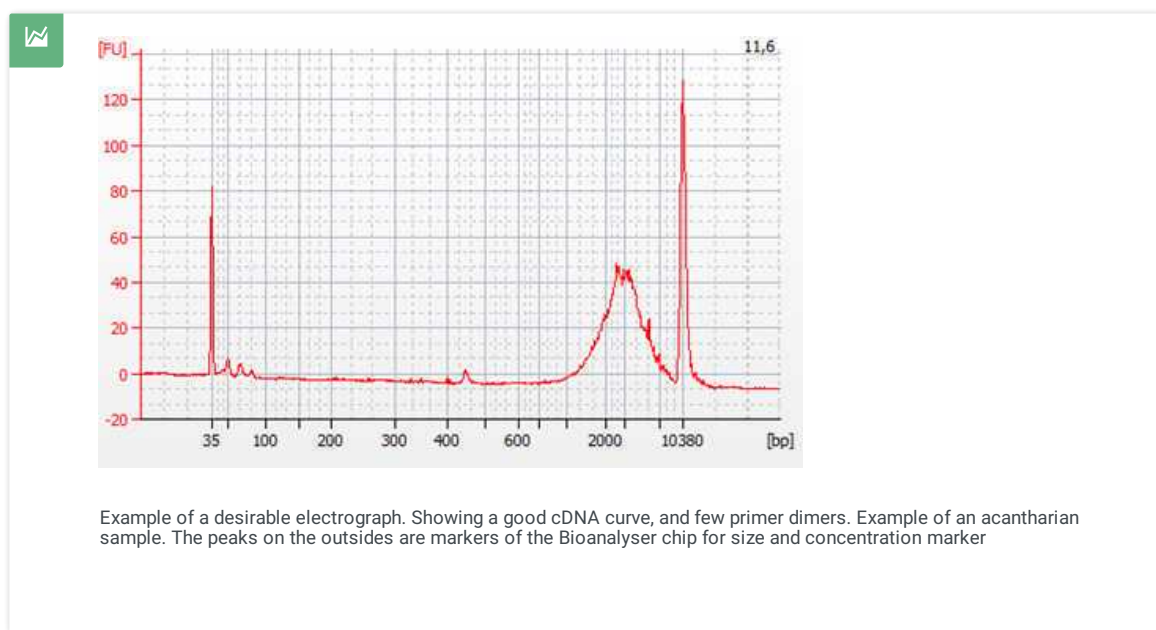
37.1 Make immediately an aliquot for Bioanalyzer analysis to prevent unnecessary freeze-thawing cycles.

STOPPING POINT - Label and store at  $\delta$  -20 °C

#### cDNA Sample verification

 [Agilent High Sensitivity DNA Kit](#) **Agilent**

Check the quality of cDNA by **Technologies Catalog #5067-4626** following the manufacture's protocol.



**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  $\delta$  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

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](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:

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

47

Spin down your indexed cDNA samples (total 50 µL)

48



2m

Add **30 µL** of [AMPure XP Beads Contributed by users](#) to each sample

- 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

[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.

51



Pipet and discard the supernatant (80  $\mu$ L), keeping the samples in the magnetic device

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

Do not disturb the beads

- 52.1 Wait  00:00:30

- 52.2 



Pipet and discard supernatant containing contaminants (use 100  $\mu$ L pipet)

- 53 Repeat the EtOH washing step for a total of 2 washing steps 



- 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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ L

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

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**

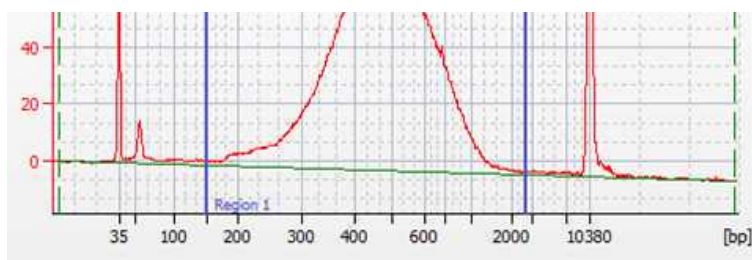
**Technologies Catalog #5067-4626**

following the manufacture's

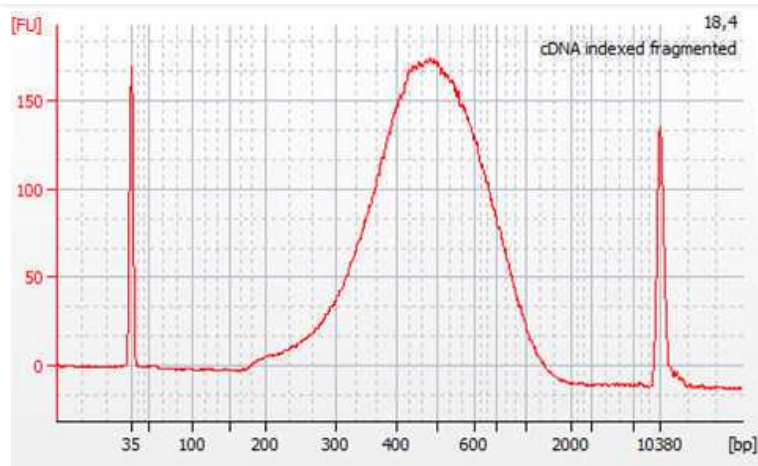
protocol. Alternatively, a Bioanalyser DNA 7500 Kit (Agilent #5067-1506) could be used as a more cost-efficient alternative and if sample concentration permitting. See for example the third graph.



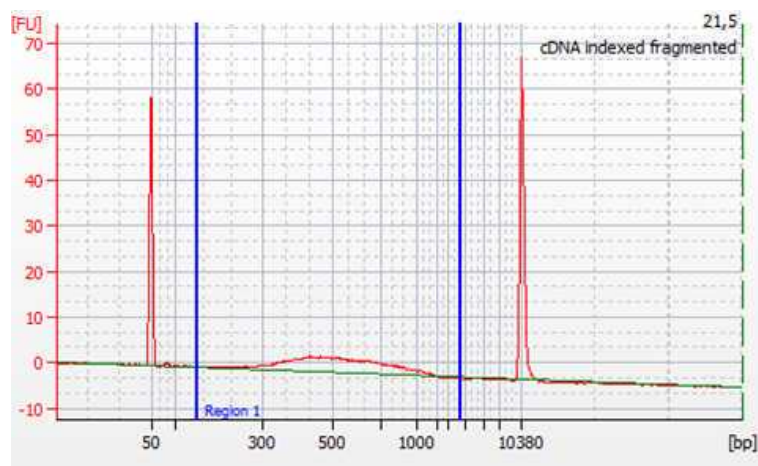




Example electropherogram for a good cDNA library run with a Bioanalyser High Sensitivity DNA Kit (Agilent #5067-4626). Though primer-dimers are here still present and a follow up (repeat) cleaning is recommended. The region for smear analysis is indicated between blue lines. Example of an acantharian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



Example electropherogram 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 electropherogram 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 pooling for sequencing

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/ $\mu\text{L}$  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.