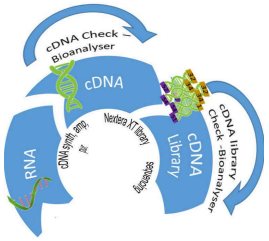
Jan 05, 2022 Version 3

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



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We use this protocol and it's working

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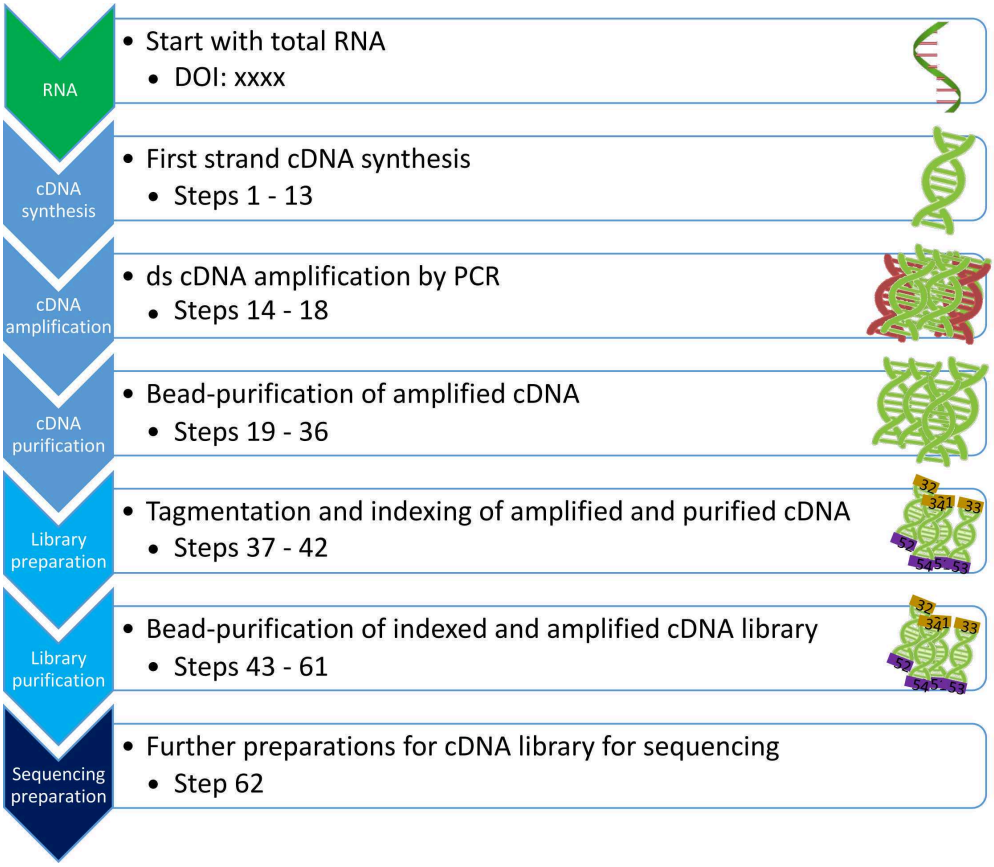
Abstract

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
- nuclease free water
- 10X Lysis Buffer **Takara Bio Inc. Catalog #**634888
- RNase Inhibitor **Takara Bio Inc. Catalog #**2313A
- SMART-seq v4 Oligonucleotide (46 µM) **Takara Bio Inc. Catalog #**634888 (48 µM, 1 µL per sample)
- 5X Ultra Low First-Strand Buffer **Takara Bio Inc. Catalog #**634888 (4 µL per sample)
- SMART-seq CDS Primer II A (12 µM) **Takara Bio Inc. Catalog #**634888 (1 µL per sample)
- SMARTScribe Reverse Transcriptase **Takara Bio Inc. Catalog #**634888 (2 µL per sample)

cDNA amplification

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

cDNA purification

- 80% Ethanol (made from 100% Molecular grade ethanol and nuclease free water) (400 µL per sample)
- 50 mL Falcon Tubes (for 80% ethanol)
- AMPure XP Beads **(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 **(This can be substituted for a similar product, we use CleanNGS (GC Biotech, CNGS-0050).**
- 80% Ethanol (made from 100% Molecular grade ethanol and nuclease free water) (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
- PCR Thermocycler
- Ice

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

Protocol materials

- 5X Ultra Low First-Strand Buffer **Takara Bio Inc. Catalog #**634888
- 100% Molecular grade ethanol
- nuclease free water
- Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #**FC-131-1096
- 2X SeqAmp PCR Buffer **Takara Bio Inc. Catalog #**638526
- 80% Ethanol
- Ice
- PCR Primer II A (12 µM) **Takara Bio Inc. Catalog #**634888
- PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format) **Thermo Fisher Catalog #**AM12230
- AMPure XP Beads
- Magnetic Stand-96 **Thermofisher Catalog #**AM10027
- Vortex
- RNase Inhibitor **Takara Bio Inc. Catalog #**2313A
- SMARTScribe Reverse Transcriptase **Takara Bio Inc. Catalog #**634888
- 1.5 ml reaction tube **Eppendorf**
- SeqAmp DNA Polymerase **Takara Bio Inc. Catalog #**638504
- 50 mL Falcon Tubes
- SMART-seq v4 Oligonucleotide (46 µM) **Takara Bio Inc. Catalog #**634888
- SMART-seq CDS Primer II A (12 µM) **Takara Bio Inc. Catalog #**634888
- PCR Thermocycler
- Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #**5067-4626
- Ice
- PCR Tubes, 0.2mL, flat cap, natural, PCR Tube; 0.2mL; Natural; w/flat cap; 1000/Pk. **Thermo Fisher Catalog #**3412
- 10X Lysis Buffer **Takara Bio Inc. Catalog #**634888
- Nextera XT Index Kit v2 Set A (96 indexes 384 samples) **Illumina, Inc. Catalog #**FC-131-2001
- Resuspension Buffer

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

Total RNA needs to have been extracted (Protocol: **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 **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 with nuclease free water

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)

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 ul of RNA sample & 1 µL of **RB**

(total 10.5 µL volume, adjust with nuclease free water 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) **Takara Bio Inc. 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 (total volume 12.5 µL)

7.2 Mix gently (vortex) & spin down

8 Incubate samples at 72 °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)
- 0.5 µL RNase Inhibitor **Takara Bio Inc. Catalog #**2313A (white cap)

10 **Immediately** after the 3 min 72°C incubation from step 8 put samples On ice for 2m
00:02:00
During this incubation time on ice perform steps 11 and 12.

11 Preheat thermocycler to 42 °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 Thermocyler 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
- 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°	3 min	

	A	B	C
	C		
	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 ahigher 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
	Strombidium basimorphum	SMARTseq-v4	18
	Prymnesium parvum	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), 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

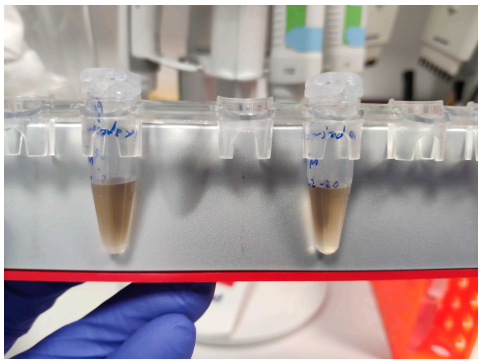
23 Add 22.5 µL of beads to each sample (amplified cDNA from the previous section)

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

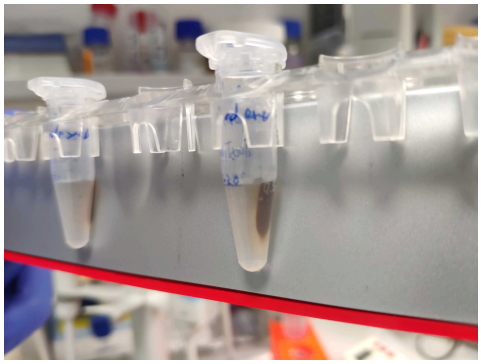
24 Incubate at Room temperature 00:08:00 to let cDNA bind to the beads 8m

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 µL), keeping the samples in the magnetic device

27 Keeping the samples in the magnetic device, **add** 200 µL **fresh** 80% Ethanol 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 µL)

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 Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #**5067-4626 following the manufacture's protocol.

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: 30m

- Vortex the bead stock well (AMPure XP Beads), 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 **Add 30 µL** of AMPure XP Beads 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 µL), keeping the samples in the magnetic device

52 Keeping the samples in the magnetic device, **add** 200 µL **fresh** 80% Ethanol 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 µL 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 µL 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 µL **of** Resuspension Buffer (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 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).

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

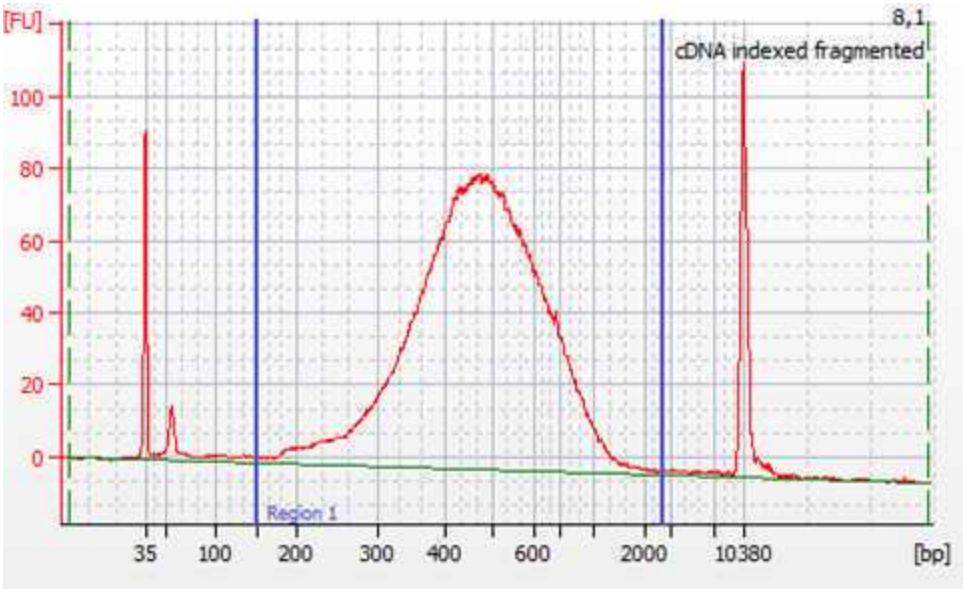
cDNA library verification

64 Check the quality of the cDNA libraries by

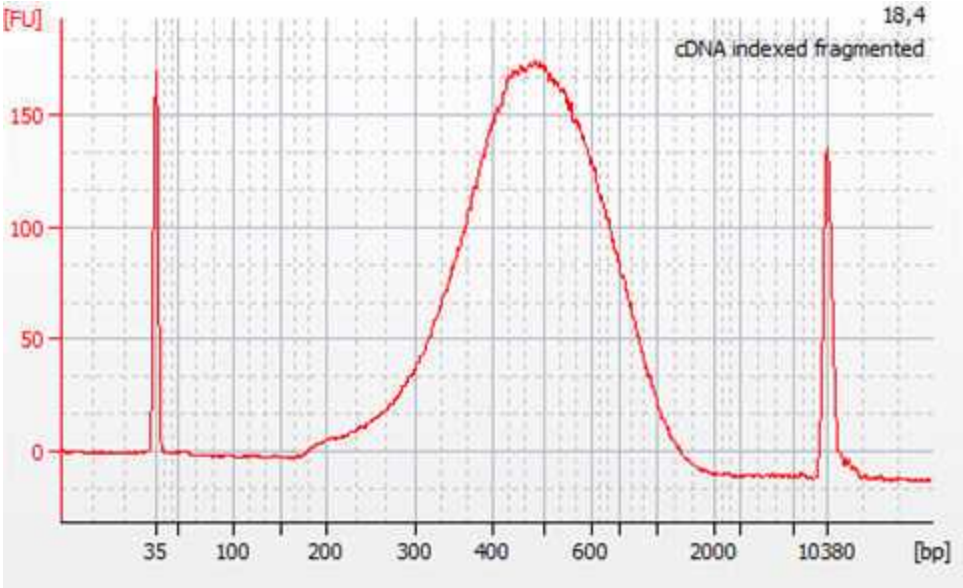
Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #**5067-4626

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.

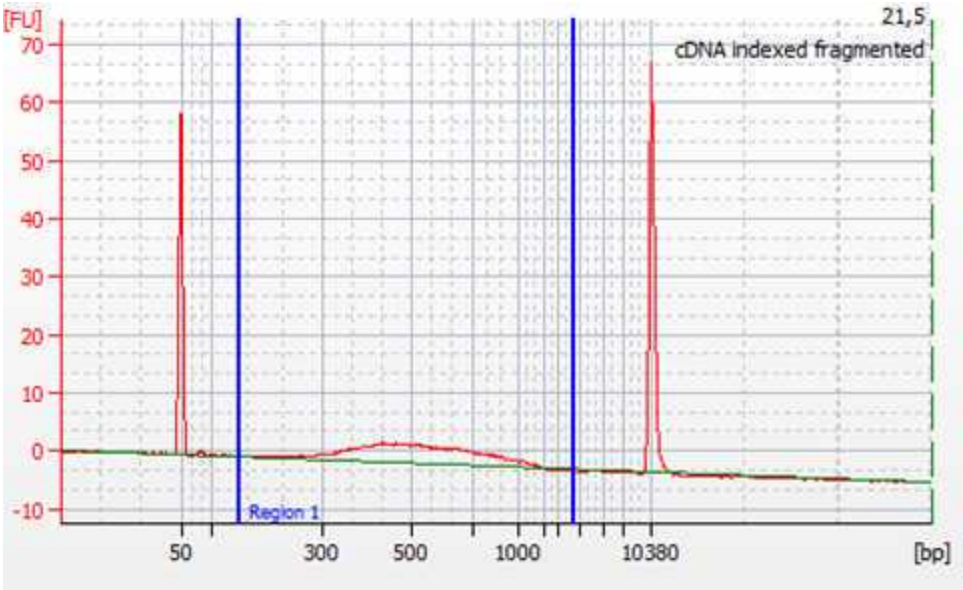
Expected result



Example 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.



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 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: 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 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.