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Dinoflagellate Single Cell Transcriptome

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Brittany Ott[1]

[1]US FDA

Brittany Ott
US FDA

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Abstract

This protocol provides instruction on how to generate transcriptomes from Dinoflagellates, either from single-cell or small batch cultures.

Before start

NOTE: PLEASE READ THROUGH THE ENTIRE PROTOCOL TO DETERMINE ALL OF THE NECESSARY ITEMS REQUIRED. THERE ARE MANY CONSUMABLE AND NON-CONSUMABLE ITEMS NEEDED, WITH SOME OPTIONS FOR REPLACEMENT.

Step 1. Isolate Cells in RNAlater

4h

1 Utilizing your own way to isolate single dinoflagellate cells, add a cell to 20 µL of RNAlater into a capped PCR tube (either strips or individual tubes).

Note: a PCR plate doesn't really work with this, as the films used allow the RNAlater to evaporate, even while frozen. Capped tubes or strips are best.

1.1 Allow the cells to sit in the RNAlater at room temperature for at least 04:00:00, then freeze (either at -80 °C or -20 °C. The RNAlater is also capable of preserving cells and RNA at Room temperature for up to 2 weeks.

4h

Step 2. RNA Isolation

26m 30s

2 *Note: please also reference the protocol that comes with the extraction kit used in this step*

For downstream methods, RNAlater must be removed from the cells, otherwise it will inactivate sensitive enzymes. So, utilizing a **Macherey Nagel XS RNA extraction kit**, you will remove the RNAlater from the cell.

This technique works with both single cells and batch cells.

2.1 **Gather and prepare the necessary items/reagents:**

5m

a. One, 1.5 mL tube containing 1 mL of 70% ethanol (prepared fresh beforehand with 100% molecular grade ethanol and RNase-free H20; 700 µL *of ethanol +* 300 µL *of water*)

b. Remove the TCEP and thaw On ice. Spin down: 7500 rpm, 4°C, 00:05:00 Remove the supernatant (~ 16 µL) while leaving ~ 4 µL in the tube

c. Pull out samples and place On ice

d. Prepare a working solution of Carrier RNA (thaw on the bench): *add* 1 µL *of stock solution to* 99 µL *of Buffer RA1*. Do ***NOT*** place this working solution

On ice, as it will freeze due to the Buffer EA1. When done with the concentrated Carrier RNA, place this on ice until it can be returned to the freezer.

2.2 **Rupture the Cells:** Add 100 µL of Lysis Buffer RA1 and 2 µL of TCEP, vortex to mix (2 x, 00:00:05 each), then do a quick spin down.

2.3 **Add the Carrier RNA:** Add 5 µL of the Carrier RNA working solution (prepared beforehand) to each tube, vortex to mix (2x, 00:00:05 each), then do a quick spin down.

2.4 **Adjust the RNA binding conditions:** Add 100 µL of the 70% ethanol (prepared fresh beforehand) to the solution from step 2 (adding it to the 1.5 mL tube), vortex to mix (2x, 00:00:05 each), then do a quick spin down.

2.5 **Bind the RNA:** Obtain a nucleospin RNA XS column (light blue ring in individual packets - and should already be in a collection tube), mix the lysate (from Step 2.2) with a pipette and load the liquid into the top of the column (max: 650 µL), centrifuge 11000 x g, Room temperature, 00:00:30 , discard the used collection tube and place into a fresh tube. 30s

2.6 **Desalt the silica membrane:** Add 100 µL of the Membrane Desalting Buffer (MDB) to the top of the column, centrifuge 11000 x g, Room temperature, 00:00:30 , discard the flow-through and place the column back into the collection tube. *Note: if any additional liquid touches the bottom of the column, centrifuge again at* 11000 x g, 00:00:30 . 1m

2.7 **Digest the DNA:** Prepare the DNase mixture in a sterile, RNase-free (DEPC-treated as an option) 1.5 mL microcentrifuge tube. For each isolation, mix 10 µL of the reconstituted DNase to 90 µL of the reaction buffer (example for 2 RNA samples: 20 µL of DNase + 180 µL of buffer), mix by flicking the tube, quickly spin down, apply 95 µL of the solution directly to the center of the membrane (DO NOT PUNCTURE THE MEMBRANE), incubate at Room temperature for 00:15:00 15m

2.8 **Wash and dry the silica membrane:** add 100 µL of the Buffer RA2 to the top of the column, Room temperature incubate for 00:02:00 then centrifuge 11000 x g, Room temperature, 00:00:30 then discard the whole collection tube and flow-through and place the column in a new collection tube. Add 400 µL of the Buffer RA3 to the top of the spin column, pipetting around the edges to remove salts from the inner walls, then centrifuge 11000 x g, Room temperature, 00:00:30 then discard the flow-through and place back into the collection tube. Add 200 µL of the Buffer RA3 to the top of the spin column and centrifuge 11000 x g, Room temperature, 00:02:00 to dry the membrane. *Note: if any liquid touches the bottom of the column, then centrifuge again*. Then place the column into a 1.5 mL tube (provided by the kit). 3m

2.9 **Elute the RNA:** Add 5 µL of RNase-free water (provided by the kit). *Note: if you are extracting multiple cells for a total RNA isolation, utilize the maximum elution volume:* 10 µL. Let the tube incubate at room temperature for 00:01:00. Then centrifuge 11000 x g, Room temperature, 00:01:00 2m

Step 3. Check RNA quality and quantity (optional)

3 To check the RNA quantity and quality, we suggest using Agilent's High Sensitivity RNA ScreenTape Analysis for TapeStation Instruments (published 2025; this may become defunct later). Follow the instructions provided by the manufacturer.

Note: this will utilize 2 µL of your total 5-10 µL sample; if you believe that your procedure worked, we suggest that you move straight to Step 4, the SmartSeq v4 RNA kit procedure. However, for a first extraction attempt, it would be good to check to make sure your technique is reliable before moving on.

If you extracted from multiple cells for a total RNA isolation, it is suggested that you check the quantity and quality for downstream applications.

Store the rest of the sample at -80 °C until Step 4 (if Step 4 will not occur directly after the completion of Step 3).

Step 4. SmartSeq v4 RNA Kit

56m

4 *Note: please also reference the original protocol found on the manufacturer's website*

4.1 **Step A: First Strand cDNA synthesis: Set up**

1. Clean and Prep the PCR workstation: wipe down with 10% bleach, followed by 70% ethanol, and RNA-away (or Eliminase, whatever is the preferred Rnase and Dnase remover). Also, clean the vortexer and mini-centrifuge for inside the workstation.

2. Obtain 2 buckets of ice: one for reagents, one for the RNA samples (you want to keep these separate).

3. Preheat your thermal cyclers:

*One at 72 °C

*One with the following program:

i. 42 °C for 01:30:00

ii. 70 °C for 00:10:00

iii. 4 °C for ∞

4. Depending on the number of cells that you are using:

a. More than 1 cell (such as in a total RNA extraction), calculate the amount of RNA needed to make 10 ng total with a volume of 9.5 µL (RNase-free water can be used to make up this volume). If you have the results from the TapeStation (*Step 3: Check RNA quality and quantity*) post RNA isolation, you can use them here to do the calculations. Take the time to analyze these results and consolidate the information before beginning the procedure.

b. If using a single-cell isolation, it is suggested to proceed directly through the protocol so as not to lose sample volume.

5. Mix the 5x Ultra Low First Strand Buffer and SmartSeq V4 Oligonucleotide (hereafter termed Buffer+Oligo) as described in Table 1, and keep On ice.

	A	B	C	D
	Master Mix	**1x (uL)**	**6x (uL) w/o ctrl**	**8x (uL) w/ ctrl**
	5x Ultra Low First Strand	4	26.4	35.2

	A	B	C	D
	Buffer			
	SMARTseq v4 Oligonucleotide (48 uM)	1	6.6	8.8
	Total Volume	5	33	44

Table 1. SmartSeq V4 primer binding PCR.

4.2 **Step A: First Strand cDNA synthesis: Begin Procedure** 5m

1. Thaw the 5x Ultra Low First Strand Buffer at Room temperature . Store at Room temperature after the first thaw, and vortex well before using.

2. Thaw the 10x Lysis buffer, RNase Inhibitor, Diluted Control RNA (optional, only if this is being used as a control), nuclease-free water, sample RNA, 3' SmartSeq CDS Primer IIA, and SmartSeq V4 Oligonucleotide ON ICE. *Note: remember that the sample RNA goes into its own bucket*. Vortex to mix each reagent (briefly and gently).

3. Prepare a stock solution of the 10x Reaction Buffer (keep On ice until 3' CDS Primer IIA is thawed). Mix briefly and spin down (to avoid bubbles).

19 µL 10x Lysis Buffer
1 µL RNase inhibitor
_____
20 µL total volume 10x reaction buffer

4. Prepare each sample (10.5 µL total volume) in individual 0.2 mL RNase-free PCR tubes. If using control RNA as a positive, dilute to 10 ng/uL with nuclease-free water and RNase inhibitor (1 µL of inhibitor + 49 µL of water to create a stock solution of the RNase inhibitor).

5. Place the samples On ice and add 2 µL of 3' SMARTSeq CDS Primer IIA. Mix well by vortexing, followed by a quick spin.

10.5 µL total RNA in reaction buffer

2 µL 3' SMARTSeq CDS Primer IIA

12.5 µL total volume

6. Incubate the tubes at 72 °C in a pre-heated, hot-lid thermal cycler for 00:03:00 . During this time, begin the next step (7).

7. Prepare enough Master Mix for all reactions + 10% of the total reaction volume as shown in Table 2. Mix the previously made Buffer+Oligo with RNase Inhibitor:

	A	B	C	D
	Master Mix	**1x (uL)**	**6x (uL) w/o ctrl**	**8x (uL) w/ ctrl**
	Buffer+Oligo	5	33	44
	RNase Inhibitor (40U/uL)	0.5	3.36	4.4
	Total Volume	5.5	36.3	48.4

Table 2. Buffer+Oligo and RNase Inhibitor Master Mix.

8. Immediately place the tubes from Step 5 On ice for 00:02:00 . Ensure that the thermal cycler lid of another block is preheated to 42 °C .

9. Add 2 µL per reaction (plus 10%) of the SmartSeqScribe Reverse Transcriptase to the Master Mix from Step 7:

6x reaction (w/o ctrl): 13.2 µL
8x reaction (w/ ctrl): 17.6 µL

Note: do NOT vortex the master mix after the enzyme is added; swirl gently with the pipette tip.

IMMEDIATELY PROCEED TO THE NEXT STEP (10).

10. Add 7.5 µL of the Master Mix to each reaction tube. Mix the contents of the tube by gently pipetting followed by a brief spin down.

11. Place the tubes into the second thermocycler block (where the lid was preheated to 42 °C) and run the following program (preprogrammed in step 4.1.3):

42 °C for 01:30:00
70 °C for 00:10:00
4 °C for ∞

Note: the reaction from Step 11 can be stored at 4 °C overnight.

4.3 **Step B: cDNA Amplification by LD PCR: Set up**

Note: Steps 1-3 below can be done in the PCR workstation, but the remaining steps must be done on the bench space afterward to avoid contamination of the PCR station.

1. Program a thermocycler block to run the following PCR program:

	A	B
	Temp	**Time**
	95°C	1 min
	98°C	10 sec
	65°C	30 sec
	68°C	3 min
	Go to 2	nX
	72°C	10 min
	4°C	∞

Table 3. cDNA amplification PCR

Note: Regarding cycle number (nX in Step 5), you will want to optimize your PCR depending on the total RNA or number of cells being used in the beginning. *For large RNA extractions (such as hundreds of thousands of cells), the maximum RNA volume and concentration should be used with the minimum number of cycles.*

	A	B	C
	Input (total RNA)	**Input (# of cells)**	**Typical # of cycles**
	10 ng	1,000	7-8
	1 ng	100	10-11
	100 pg	10	14-15
	10 pg	1	17-18

Table 4. Cycling guidelines based on the amount of starting material

4.4 Step B: cDNA Amplification by LD PCR: Begin Procedure

1. Thaw all reagents (except enzymes) On ice then vortex gently:

   2x SeqAmp PCR Buffer
   PCR Primer IIA (12 uM)
   Nuclease-free Water

2. Prepare the PCR Master Mix (+10% of the total volume of each reagent) as shown in Table 5.

	A	B	C	D
	Master MIx	**1x (uL)**	**6x (uL) w/o ctrl**	**8x (uL) w/ ctrl**
	2x SeqAmp PCR Buffer	25	165	220
	PCR Primer IIA (12 uM)	1	6.6	8.8
	SeqAmp DNA Polymerase	1	6.6	8.8
	Nuclease-Free Water	3	19.8	26.4
	Total Volume	30	198	264

Table 5. PCR Master Mix.

Note: vortex the Master Mix before adding the polymerase (enzyme). Add the enzyme right before and swirl gently with the pipette tip to mix, followed by a quick spin down.

3. Add 30 µL of PCR Master Mix to each tube, which contains 20 µL of the first-strand cDNA product from Step A (First Strand cDNA synthesis); for a total volume of 50 µL. Mix well and spin down quickly.

4. Place the tubes into a preheated thermocycler with a heated lid. Run the PCR protocol step in Table 3. *Note: for Step 5, nX cycles, consult Table 4.*

4.5 **Step C: Purification of Amplified cDNA using Agentcourt AMPure XP Kit: Set up** 30m

Note: all of the steps in this section should be done on a lab bench, not in a PCR hood. Amplified DNA should never be introduced to a PCR hood or clean station.

1. Pull out the AMPure XP beads and let sit at Room temperature for 00:30:00. If you are proceeding directly from Step B, this can be done while the PCR runs. Depending on the number of samples that you are working with, you will need to pull out one or more tubes (generally, the large bottle of beads is aliquoted to 500 µL to prevent constant temperature changes of removing the larger bottle from cold storage).

2. Prepare fresh 80% ethanol; you will need 400 µL per sample, plus a little extra for pipetting error.

3. Thaw the Elution Buffer and maintain at Room temperature.

4. Fit a 96 well plate (semi-skirted) to hold the PCR tubes in the magnetic stand (depending on what magnetic stand you have).

4.6 **Step C: Purification of Amplified cDNA using Agentcourt Ampure XP Kit: Begin Procedure** 21m

1. Add 1 µL of 10X lysis buffer to each PCR product from Step B (the ENTIRE product).

2. Vortex the AMPure XP beads until they are evenly mixed, then add 50 µL of beads to each PCR tube/sample (*note: the beads are viscous, so work slowly*).

3. Mix by vortexing OR pipetting the entire volume up and down 10 times to mix (ensure that the PCR product is SATURATED with the bead mixture).

4. Incubate at Room temperature for 00:08:00 to let the cDNA bind to the beads.

5. Briefly spin the samples down, the place onto the magnetic separation device for ~ 00:05:00 (or longer) until the supernatant becomes COMPLETELY clear (no beads are left in the supernatant). *To check the supernatant for beads, pipette the liquid out and hold the tip up to the light. If you see any specs or brownish/red coloring, the supernatant still contains beads.*

6. With the samples STILL ON THE STAND: pipette out the supernatant and discard.

7. Then, again with the samples STILL ON THE STAND, add 200 µL of the fresh 80% ethanol to each sample WITHOUT disturbing the beads. Wait 00:00:30, then carefully pipet off the supernatant (which contains contaminants). The cDNA will stay bound to the beads. ***DO NOT DISTURB THE BEADS***.

8. Repeat step 7 again.

9. Briefly spin the samples down and replace to the magnetic stand. Leave on the stand for 00:00:30 then remove the remaining ethanol. (*Note: if the beads are seen in the ethanol, carefully pipette liquid back into the tube and let it sit a little longer.*)

10. Then, with the samples still on the stand, leave the tubes open and allow them to sit at room temperature to allow the ethanol to evaporate for 00:02:00. Ensure the pellet is dry, but only just. The pellet will look matte (without a shine), but it shouldn't be cracked. Keep checking the samples; some will dry faster than others.

*If you **under** dry the pellet, there will still be ethanol in the sample, which will reduce your cDNA recovery; if you **over** dry the pellet, it will take longer than 00:02:00 to rehydrate and will reduce your cDNA recovery*.

11. After the beads dry, with the samples still on the stand, add 17 µL of Elution Buffer to cover the pellet. Then remove the samples from the magnetic stand, and mix thoroughly to resuspend the beads (light vortex or swirl with the pipette tip).

12. Incubate at Room temperature (with the tubes closed) for 00:02:00.

13. Quickly spin down the tubs. Place the samples back on the magnetic stand for 00:01:00 or longer. Ensure the supernatant is completely clear. *If the beads don't*

pellet, pipette the supernatant and beads together, then pipette down towards the magnet. Continue until no beads remain in the supernatant. This can take a while for some samples (the beads can sometimes have difficulty pelleting).

14. Transfer the completely clear supernatant containing the purified cDNA from each tube into a nuclease-free, low-adhesion tube (1.5 mL). Ensure that the tubes are properly labeled. Aliquot out 2 µL of the cDNA for bioanalysis (place this sample into a 0.2 mL , nuclease-free PCR tube). Samples can then be stored at -20 °C indefinitely.

Step 5. Bioanalyzer

5 Using a bioanalyzer of your choice, determine the size and quantity of the cDNA produced. If the samples are of high enough quality following the previous procedures, proceed with Step 6: Nextera Library Preparation Kit.

Step 6. Nextera Library Preparation Kit

33m 30s

6 *Note: Please also reference the original protocol, as well as the pooling guidelines that can be found on Nextera's website.*

6.1 **Step A: Tagment Genomic cDNA: Set up**

1. Clean your mini-centrifuge and ensure that there is a microplate adapter on your vortexer (replacing the standard vortexing head).

2. Consumables for this step:

a. ATM (Amplicon Tagment Mix): Thaw On ice , gently invert the tube 3-5 times, then briefly centrifuge

b. TD (Tagment DNA buffer): Thaw On ice , invert 3-5 times, then briefly centrifuge

c. NT (Neutralize Tagment buffer): Check for precipitates, vortex until resuspended

d. cDNA from Step 4 (SmartSeq V4 kit): 0.2 ng/uL per sample max concentration; thaw On ice , invert 3-5 times or vortex lightly; note: some samples will not make this maximum.

e. Hardshell 96-well plate (skirted)

f. Microseal B adhesive seals

3. Enter and save the following protocol ("Tagmentation Protocol") into a thermocycler (make sure it can hold a 96 well plate):
1-Preheat Lid
2- 55 °C for 00:05:00
3-Hold at 10 °C

4. Pull out the Index Primers you plan to use (these need to be thawed at Room temperature for 00:20:00).

6.2 Step A: Tagment Genomic cDNA: Begin Procedure 6m

1. Prepare your cDNA: your target volume is 5 µL with 1 ng total or 0.2 ng/uL. *If your concentration is higher than 0.2 ng/uL, you will need to dilute with RNase-free water or Tris Buffer. If using Tris, it needs to be 10 mM with a pH of 7.5-8.5.*

Then add the following reagents to a hard-shell PCR plate in the order listed:
1- TD: 10 µL
2-cDNA: 5 µL
This ensures that the most valuable item is added last, as well as to reduce potential contamination of the TD reagent.

2. Add 2 µL of ATM to each well. Pipette to mix. Seal with a Microseal B adhesive seal.

3. Centrifuge at 280 x g, 20°C, 00:01:00 (in a plate centrifuge).

4. Place the plate into the pre-programmed thermocycler (Section 6.1.3). Run the Tagmentation Protocol. *Once the program reaches 10 °C , immediately proceed to the next step (step 5), as the transposome is still active.*

5. Quickly spin down the plate. At Room temperature , carefully peel up the seal then add 5 µL of NT to each well. Pipette to mix. Replace the seal (use a new one if necessary).

6. Incubate at Room temperature for 00:05:00 . Prepare for Step B (Amplify Libraries) during this incubation time. Then proceed with Step B.

6.3 **Step B: Amplify Libraries: Set up**

1. Consumables for this step:
    a. NPM (Nextera PCR MasterMix): Thaw On ice
    b. Index Adapters/Primers: Thaw at Room temperature for 00:20:00 (see above); spin down in a mini centrifuge; you will need i7 primers and i5 primers
    c. TruSeq plate fixture
    d. Microseal A film
    e. Replacement caps for the Index primers

2. Save the PCR program found in Table 6 (below) into a thermocyler that can hold a 96 well plate and has a heated lid

	A	B
	Temp.	**Time**
	72°C	3 min
	95°C	30 sec
	95°C	10 sec
	55°C	30 sec
	72°C	30 sec
	Go to 3	12x
	72°C	5 min
	10°C	∞

Table 6. Index Adapter PCR protocol

6.4 Step B: Amplify Libraries: Begin Procedure

1. Arrange the Index Primers in the TruSeq Index plate fixture with your S5 (i5) primers in the A-H rows, and N7 (i7) primers in the 1-12 columns.

2. Using a pipette, add 5 µL of each index 1 (i7) adapter down each column. Replace the cap on each i7 primer with a new ***orange cap.*** IT IS ALSO CRITICAL THAT YOU CHANGE YOUR PIPETTE TIP EVERY TIME YOU DISPENSE THE 5 µL. THIS WILL PREVENT CONTAMINATION ISSUES.

3. Using a pipette, add 5 µL of each index 2 (i5) adapter to each row. Replace the cap on each i5 primer with a new ***white cap.*** IT IS ALSO CRITICAL THAT YOU CHANGE YOUR PIPETTE TIP EVERY TIME YOU DISPENSE THE 5 µL. THIS WILL PREVENT CONTAMINATION ISSUES.

4. Add 15 µL of NPM to each well containing the index adapters. Pipette to mix. AGAIN, ALWAYS ENSURE TO CHANGE YOUR PIPETTE TIPS BETWEEN WELLS.

5. Place a new Microseal A film on the plate: remove the purple backing, place on the plate over the wells using gloved fingers and a Kim wipe; make sure the silicone is pressed down tight - this is done by pushing on each well containing a sample; leave the see-through instruction sheet ON. Centrifuge at 280 x g, 00:01:00.

6. Place the plate into the pre-programmed thermocycler (Table 6). Ensure that the lid of the PCR machine is moderately tight.

Note: you can stop here and store the plate at 2-8 °C for up to 2 days; if you do so, replace the "A" film with the more resilient "B" film.

If you decide to continue in the same day, pull out tubes of AMPure XP beads and let sit at Room temperature for 00:30:00 minimum.

6.5 Step C: Clean Up Libraries: Set up

Consumables:

a. RSB (Resuspension buffer): Thaw at Room temperature. Store at 4 °C after the initial thaw.

b. AMPure beads (aliquoted): Thaw at Room temperature for 00:30:00 minimum; pull these out during Step B while the thermocycler runs (last step) if continuing in the same day. Depending on the volume you are adding to each sample and the number of samples, you may need to pull out multiple tubes (each should be aliquoted at 500 µL); the AMPure beads should not be reused after leaving at room temperature.

c. Prepare fresh 80% ethanol: 400 µL per sample is required, plus some extra for pipetting error.

d. V-bottomed or round-bottomed midi plate (v-bottomed is preferred).

6.6 Step C: Clean Up Libraries: Begin Procedure

27m 30s

1. Centrifuge the 96 well plate, taken either directly from the thermocycler or from the refrigerator, at 280 x g, Room temperature, 00:01:00 .

2. Transfer 50 µL of the PCR product from each well to the corresponding well of a v-bottomed (or round-bottomed) midi plate.

3. Add 30 µL or 90 µL of the AMPure XP beads to each well: this is a size selection step. If you wish to grab smaller fragments, use 30 µL ; if you wish to grab larger fragments, use 90 µL . Using 30 µL is best when working with sheared cDNA (see the Nextera Manual). Cover with a Microseal "B" film.

4. Shake the plate at 1800 rpm, Room temperature , 00:02:00 (you can use a 96 well plate adapter, and the vortexer can be set to an approximate RPM; check your vortexer specs).

5. Incubate at room temperature for 00:05:00 . Centrifuge at 280 x g, 00:01:00 , and then briefly at a higher speed.

6. Place on the magnetic stand and wait until the liquid is clear (~ 00:02:00).

7. Remove and discard all supernatant from each well. Ensure the liquid is clear by holding the pipette tip containing the liquid up to the light (but ensure no liquid is dripped out of the end or flows back into the pipetter).

8. Wash the samples 2 times:

a. Add 200 µL of fresh 80% ethanol to each well

b. Incubate on the magnetic stand for 00:00:30

c. Remove and discard all supernatant from each well (use the same technique as described above)

9. Using a small pipette tip (20 µL), remove any remaining ethanol.

10. Air dry the pellets on the magnetic stand for 00:15:00 (film removed).

Note: you are looking for the pellet to lose its shine (becoming matte), but you do not want the pellet to crack. Over-drying and under-drying can reduce your yield and cause issues downstream. So watch
the pellet for the entire time frame.

11. Remove the plate from the magnetic stand.

12. Add 52 µL of the RSB to each well (change tips each time to avoid contamination).

13. Add a new Microseal "B" film to the plate top and secure. Shake at 1800 rpm, Room temperature , 00:02:00

14. Incubate at room temperature for 00:02:00 . Centrifuge at 280 x g, Room temperature, 00:01:00 with a very quick spin at a higher speed to get the excess liquid off of the film.

15. Place the plate back on the magnetic stand and wait until the liquid is clear.

16. Transfer the supernatant to a fresh PCR plate (with wells corresponding to each other to keep track of your samples and indices). Seal with Microseal "B" film. Check the supernatant for any transfer of beads. Aliquot out 2 µL of each sample into individual PCR tubes. Place these tubes and the plate at -20 °C for storage.

17. Submit the PCR tubes containing the 2 µL aliquots for Bioanalysis and qPCR. This will allow for normalization of the samples manually in Section 7 (below) for an example spreadsheet for normalizing samples. Once the samples are normalized and pooled (see the pooling protocol from Nextera), samples can then be submitted for sequencing.

7. Example Normalization Spreadsheet

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	A	B	C	D	E	F	G	H	I
	Sample	**ng/ul**	**avg bp size**	**dsDNA mw**	**nM**	**Final nM**	**Final volume**	**Initial volume**	**RSB to add**
	Gpacif_sh	0.487	249	660	2.96336863	10	5	16.87268994	N/A

	A	B	C	D	E	F	G	H	I
	GspRII_sh	0.739	278	660	4.02768694	10	5	12.41407307	N/A
	Gsilvae_sh	0.329	255	660	1.95484254	10	5	25.5775076	N/A
	Gruetz_sh	0.811	272	660	4.5176025	10	5	11.06781751	N/A
	Gcarib_sh	0.751	343	660	3.31743087	10	5	15.07190413	N/A
	Gaust_sh	1.553	327	660	7.19581132	10	5	6.9484868	N/A
	Gpacif	5.829	467	660	18.9118162	10	5	2.643849717	2.35615028
	GspRII	5.941	519	660	17.3439598	10	5	2.882848005	2.11715199
	Gsilvae	10.73	524	660	31.0259079	10	5	1.611556384	3.38844362
	Gruetz	10.93	532	660	31.1289588	10	5	1.606221409	3.39377859
	Gcarib	6.964	497	660	21.2304128	10	5	2.355112005	2.644888
	Gaust	3.466	433	660	12.1282105	10	5	4.122619735	0.87738027
	qPCR results								

	A	B	C	D	E	F	G	H	I
	P1N	1.916	494	660	5.87657956	10	30	51.05010438	
	P2S	0.908	299	660	4.60119591	10	30	65.20044053	58.1252725
		2.33	494	660	7.14636241				
		1.3	299	660	6.58761528				

Table 7. Example Normalization Spreadsheet