scRNA-Seq on the Nadia Instrument

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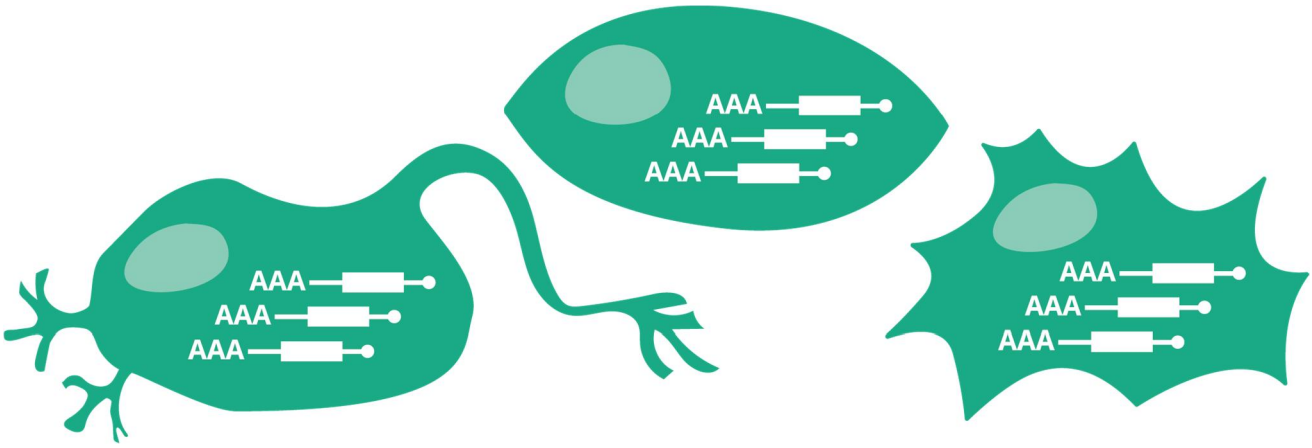

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Chapter 1. Product Information

Product description

Nadia, developed by Dolomite Bio, is a fully automated high-throughput microfluidic droplet system for single cell research such as high-throughput single cell RNA-Seq using the Drop-Seq protocol as described by Macosko *et al.*, Cell 2015. Per cartridge, Nadia enables capture of over 50,000 single cells in under 20 minutes for subsequent transcriptome analysis by RNA-Seq.

Nadia instrument and consumables

Instrument/Consumables	Order Number
Nadia Instrument	3200590
Nadia Training Cartridge	3200605
Nadia Cartridge for scRNA-Seq and sNuc-Seq - 8 Runs (8x1)	3200648
Nadia Cartridge for scRNA-Seq and sNuc-Seq - 8 Runs (2x2 & 1x4)	3200649
Nadia Cartridge for scRNA-Seq and sNuc-Seq - 8 Runs (1x8)	3200650
Nadia Cartridge for scRNA-Seq and sNuc-Seq - 40 Runs (40x1)	3200651
Nadia Cartridge for scRNA-Seq and sNuc-Seq - 40 Runs (10x2 & 5x4)	3200652
Nadia Cartridge for scRNA-Seq and sNuc-Seq - 40 Runs (5x8)	3200653

Required equipment and consumables

Equipment

- Nadia instrument
- Nadia cartridges
- Microcentrifuge with cooling function
- Rotating incubator
- Thermocycler
- Magnetic rack
- Bioanalyzer
- Qubit fluorometer (Thermo Fisher)
- Qubit dsDNA HS assay system

Consumables

- 0.2 µm sterile filter
- Eppendorf DNA LoBind 1.5 ml tubes
- 0.2 ml PCR tubes
- 15 ml Falcon tubes
- 50 ml Falcon tubes
- 5 µm Überstrainer set (Pluriselect, # 43-70005-03)

- 50 ml Luer-Lok Syringe
- 70 µm cell strainer (Fisher Scientific, # 08-771-2)
- 40 µm cell strainer (Fisher Scientific, # 08-771-1)
- Neubauer Improved haemocytometer plastic disposable C-Chip (Nano-EnTek, # DHC-N01)
- Fuchs-Rosenthal haemocytometer plastic disposable C-Chip (Nano-EnTek, # DHC-F01)
- Low-retention pipette tips (P1000, P200, P10)
- Low-retention gel loading tips (P200)
- Lint-free wipes
- Bioanalyzer High Sensitivity chips

Required reagents not supplied

Reagent	Supplier	Part #
Barcoded beads in storage buffer (20-40 µm, pre-filtered)	ChemGenes	Macosko-2011-10 (V+)
Nuclease-free H_2O	User defined	
40 % PEG	Sigma	P1458
Ficoll PM-400	Sigma	F4375
Sarkosyl	Sigma	L7414
EDTA	Fisher Scientific	AM9260G
1M Tris pH 7.5	Sigma	T2319
Tris (1M) pH 8.0	Fisher Scientific	AM9856
DTT	Sigma	10197777001
QX200™ Droplet Generation Oil for EvaGreen	BioRad	1864006
20x SSC	Sigma	S6639
Maxima 5x RT Buffer	Fisher Scientific	EP0751
Maxima H-Rev transcriptase	Fisher Scientific	EP0751
dNTPs	Clontech	639125
RNase inhibitor	Lucigen	30281
Template Switch Oligo Macosko (TSO)	User defined	
Tris-EDTA pH 8.0, 100x concentrate	Sigma	T9285
DMEM growth media	Invitrogen	11965092
10 % Tween 20	Fisher Scientific	15125517
Polyethylene glycol 40 % (w/w) in H_2O	Sigma	P1458
Fetal Bovine Serum (FBS)	Life Technologies	10437-028
Penicillin/Streptomycin	Life Technologies	15070-063
TrypLE Express Enzyme (1X)	Invitrogen	12604013
1x PBS pH 7.4 sterile	Gibco	10010023
10x PBS pH 7.4 sterile	Gibco	70011044
BSA	Sigma	A7906
0.4 % Trypan Blue stain solution	Gibco	15250061

20 % SDS	Sigma	05030
Exonuclease I	NEB	M0293
Exonuclease I buffer	NEB	M0293
KAPA HiFi HotStart ReadyMix Kit	Roche	07958927001
SMART PCR primer	User defined	N/A
10 µM New-P5-SMART PCR hybrid oligo	User defined	N/A
AMPure beads	Agencourt	A63881
BioAnalyzer High Sensitivity Chip	Agilent	5067-4626
Nextera XT DNA Library Preparation Kit	Illumina	FC-131-1024
70 % Ethanol	Fisher Scientific	15420665
100 % Ethanol	Fisher Scientific	10542382
Qubit dsDNA HS assay kit	Thermo Fisher	Q32851

Required primers

Name	Sequence
Macosko template switch oligo (TSO)	5'AAGCAGTGGTATCAACGCAGAGTGAAT rGrGrG [1]
SMART PCR PRIMER (cDNA library amplification)	5'AAGCAGTGGTATCAACGCAGAGT
New-P5-SMART PCR hybrid oligo (Nextera tagmentation amplification)	5'AATGATACGGCGACCACCGAGATCTAC ACGCCTGTCCGCGGAAGCAGTGGTATCA ACGCAGAGT*A*C [2]
Nextera N7XX indexing primers (for example N701 shown here, sequencing index marked in grey)	5'CAAGCAGAAGACGGCATACGAGATTCG CCTTAGTCTCGTGGGCTCGG
Read1CustomSeqB (Sequencing)	5'GCCTGTCCGCGGAAGCAGTGGTATCAA CGCAGAGTAC

[1] rG stands for ribonucleotide G. Ribonucleotide Gs are used to improve binding of the TSO to the cDNA.

[2] The stars denote a base modification called phosphorothioate. Such modified bases are available from suppliers such as IDT Technologies.

Ordering, preparation and storage of mRNA capture beads

The mRNA capture beads are available from ChemGenes and can be ordered as a custom part under the product code Macosko-2011-10 (V+). Beads are either available pre-filtered with a size range of 20-40 µm, or unfiltered with a larger size range and can be ordered by contacting info@chemgenes.com. The beads will arrive as dry resin. For bead preparation see Chapter 5.

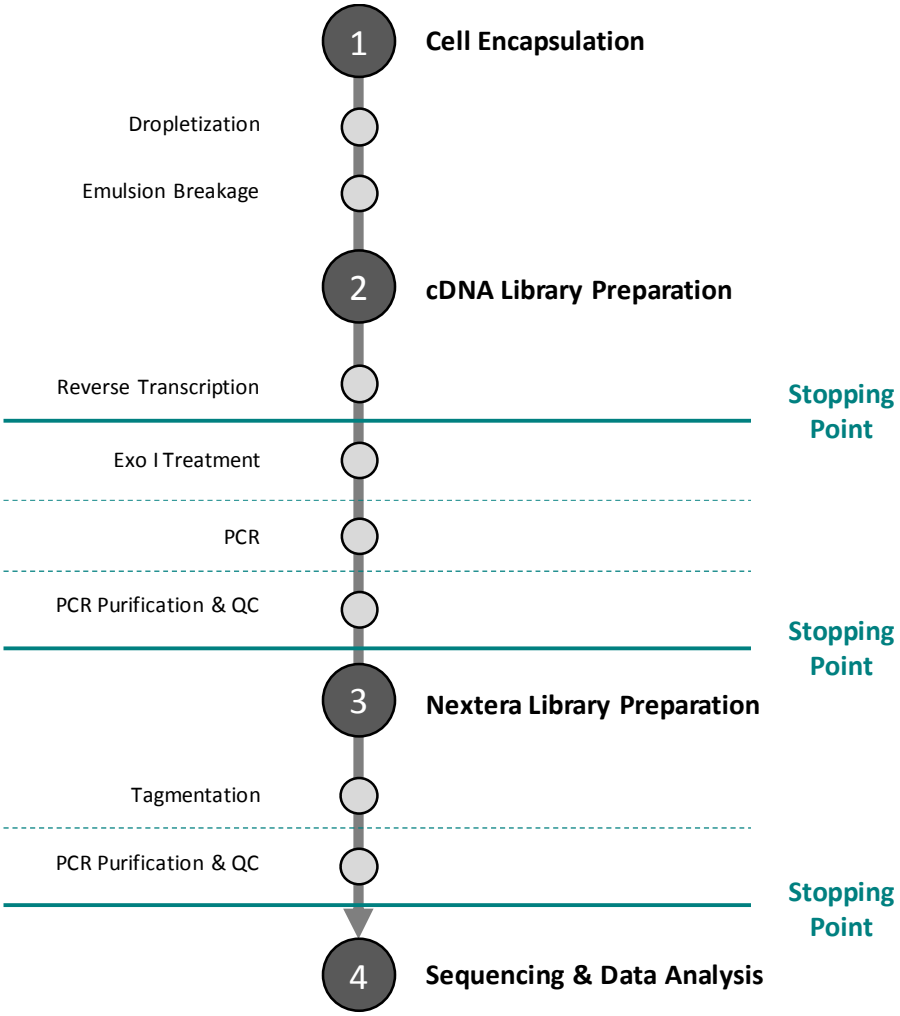
IMPORTANT *For unfiltered beads, an initial filtration through a 70 µm cell strainer followed by a second filtration through a 40 µm cell strainer is required. For pre-filtered beads we still recommend filtering the beads through a 40 µm cell strainer*

for optimal performance. Beads can be stored at 4 °C for about 6 months, longer storage durations have not been tested.

Chapter 2.
Workflow

Workflow overview

The following diagram illustrates the recommended Drop-Seq workflow.



Chapter 3.
Before You Start

Good laboratory practice when working with RNA

When working with RNA, care must be taken to create an RNA-free environment for the entire process. This can include the establishment of “RNA Work Only” areas with dedicated equipment, reagents, and consumables.

The most common sources of RNA contamination include bacteria and other microorganisms derived from airborne dust particles, labware or skin. To prevent contamination, we recommend wearing gloves at all times. Whenever possible, disposable consumables and plastic ware should be considered.

Non-disposable glassware, plastic ware and surfaces should be treated with appropriate RNAse decontamination solutions or procedures prior to use. Nuclease-free water should be used where appropriate.

Chapter 4. Optional Customisation of Protocol

Customisable parameters in Drop-Seq on Nadia protocol

The table below lists parameters that can be adjusted to best suit the biological materials being profiled and improve outcome of the protocol.

Parameter	Default in this guide	Alternative values	Notes
Lysis after dropletization	10 mins	up to an additional 35 mins	Please refer to Chapter 8 **If additional incubation is desired, keep the cartridge on Nadia until the required time has elapsed.**
Number of PCR reactions	4	5-32	Please refer to Chapter 9
Number of beads within single PCR reaction	4000	1000-4000	Please refer to Chapter 9 and Chapter 11
Number of cycles during PCR	9	8–12	Please refer to Chapter 9 and Chapter 11
For a given biological sample, number of Nextera reactions to be pooled as a single library for sequencing	1	up to 4	Please refer to Chapter 10

Chapter 5. Preparation and Aliquoting of Pre-Filtered ChemGenes Beads

Required equipment and consumables

- Refrigerated centrifuge capable of spinning 50 ml tubes at min 1000 g @ 4°C
- 50 ml Falcon tubes
- 100 ml measuring cylinder
- 40 µm cell strainer
- Fuchs-Rosenthal C-Chip haemocytometer

Required reagents

- 100 % Ethanol
- 100x Tris-EDTA
- 10 % Tween 20
- 40 % PEG
- Nuclease-free H_2O

Preparation of ChemGenes beads

1 Prepare 100 ml TE/TW solution (10 mM Tris pH 8.0, 1 mM EDTA, 0.01% Tween) as follows:
   a. A 10 % Tween 20 stock solution in nuclease-free water is required.
   b. Mix 1 ml of 100x Tris-EDTA concentrate with 100 µl of 10 % Tween 20 solution. Adjust final volume to 100 ml using nuclease-free H_2O.

2 Prepare 1 ml of 20 % PEG in nuclease-free water.

3 Resuspend the pellet of pre-filtered ChemGenes beads in 1 ml 100 % Ethanol and 1 ml TE/TW.

4 Transfer bead slurry to a 50-ml Falcon tube.

5 Spin 1 min @ 1000 x g, 4°C and discard the supernatant.

6 Wash beads with 30 ml 100 % Ethanol, spin 1 min @ 1000 x g, 4°C and discard the supernatant.

7 Wash beads once more with 30 ml 100 % Ethanol, spin 1 min @ 1000 x g, 4°C and discard the supernatant.

8 Wash beads with 30 ml TE/TW, spin 1 min @ 1000 x g, 4°C and discard the supernatant.

9 Resuspend beads in 30 ml TE/TW. Keep beads in suspension by pipetting up-and-down or inverting tube several times.

IMPORTANT *For optimal performance of the Nadia instrument we recommend filtering the beads through a 40 µm strainer.*

10 Take a 20 µl aliquot from filtered bead suspension, mix well with 20 µl of 20 % PEG (final concentration of PEG is 10 %) and load 20 µl of the mix into a Fuchs Rosenthal haemocytometer C-Chip. Ensure beads are evenly distributed throughout, otherwise repeat using a new haemocytometer. Count the beads under the microscope.

11 Prepare 150,000-bead aliquots from the bead suspension and store at 4°C. Beads can be stored for up to 6 months. Beyond 6 months, periodic testing and monitoring by the user is strongly recommended.

Chapter 6. Preparation of Buffers, Master Mixes and Beads for Nadia Run

Cell and lysis buffers

Required equipment and labware

- 0.2 µm sterile filter
- Eppendorf 1.5 ml tubes
- 15 ml Falcon tubes

Required reagents

- Nuclease-free H_2O
- 10x PBS
- 1x PBS
- BSA
- RNase inhibitor
- Ficoll PM-400
- 20 % v/v Sarkosyl
- 0.5 M EDTA
- 1 M Tris pH 7.5
- 1 M DTT

Preparation of stock solutions required for the cell and lysis buffers

1 BSA: Prepare a 1 % stock solution in 1x PBS (Use BSA powder, 10x PBS and nuclease-free water). Filter through 0.2 µm syringe filter, store at -20°C.

2 Ficoll PM-400: Prepare 20 % w/v stock solution in nuclease-free H_2O.

IMPORTANT *Ficoll is slow to dissolve in water, ideally prepare the solution a day in advance. It is recommended to sterilize the Ficoll solution using a 0.2 µm sterile filter to prevent microbial growth. Store at 4°C.*

3 DTT: Prepare 1 M solution in nuclease-free H_2O.

IMPORTANT *DTT is unstable at room temperature, always keep on ice. Store the stock solution at -20°C.*

Preparation of the lysis buffer

Component	1 Sample	2 Samples	4 Samples	8 Samples
Nuclease-free H_2O	110 µl	220 µl	440 µl	880 µl
20 % w/v Ficoll PM-400	82.5 µl	165 µl	330 µl	660 µl
20 % v/v Sarkosyl	2.75 µl	5.5 µl	11 µl	22 µl
0.5 M EDTA	11 µl	22 µl	44 µl	88µl
1 M Tris pH 7.5	55 µl	110 µl	220 µl	440 µl
1 M DTT	13.75 µl	27.5 µl	55 µl	110 µl
Total	**275 µl**	**550 µl**	**1100 µl**	**2200 µl**

IMPORTANT *Lysis buffer without DTT can be prepared in advance, filtered through 0.2 µm syringe filter and stored at 4 °C for 30 days. DTT should only be added immediately prior to use. Leave the prepared buffer + DTT on ice for ~30 mins. Leftover lysis buffer including DTT should be discarded.*

Preparation of the cell buffer (i.e. 1x PBS + 0.01% BSA)

Component	1 Sample	2 Samples	4 Samples	8 Samples
1x PBS	272.25 µl	544.5 µl	1089 µl	2178 µl
1 % BSA in 1x PBS	2.75 µl	5.5 µl	11 µl	22 µl
Total	**275 µl**	**550 µl**	**1100 µl**	**2200 µl**

IMPORTANT *Leftover cell buffer should be discarded.*

Reverse Transcription (RT) master mix

Required equipment and labware

- Eppendorf DNA LoBind 1.5 ml tubes

Required reagents

- Nuclease-free H_2O
- 20 % w/v Ficoll PM-400
- 5x RT Buffer
- 50 µM Macosko TSO
- 10 mM dNTPs
- RNase Inhibitor

Preparation of Reverse Transcription (RT) master mix

For each sample, prepare RT mix according to the table below and keep on ice until use. The volumes below include 10 % extra to allow for pipetting related losses.

Component	1 Sample	2 Samples	4 Samples	8 Samples
Nuclease-free H_2O	82.5 µl	165 µl	330 µl	660 µl
20 % w/v Ficoll PM-400	44 µl	88 µl	176 µl	352 µl
5x RT Buffer	44 µl	88 µl	176 µl	352 µl
50 µM Macosko TSO	11 µl	22 µl	44 µl	88 µl
10 mM dNTPs	22 µl	44µl	88 µl	176 µl
RNase Inhibitor	5.5 µl	11 µl	22 µl	44 µl
Total	**209 µl**	**418 µl**	**836 µl**	**1672 µl**

Bead preparation prior to a Nadia run

Required equipment and consumables

- Refrigerated centrifuge capable of spinning 50 ml tubes at min 1000 x g @ 4°C
- Eppendorf DNA LoBind 1.5 ml tubes
- Low-retention pipette tips (P200, P10)

Required reagents

- Lysis buffer
- Bead aliquot (150,000 beads, see above)

Bead preparation

IMPORTANT *It is recommended to use low-retention pipette tips to handle beads. This will minimise loss through beads being stuck onto plastic surfaces.*

1 Prior to use, prepare the required amount of lysis buffer as detailed above, or add DTT to a pre-prepared buffer solution, and place on ice for 30 min.

2 Once the lysis buffer is chilled, transfer 150,000 beads from the stock suspension into a new tube (or take one of the pre-made 150,000-bead aliquots) and spin down at 1,000 x g, 4°C for no more than 10 s. Make sure not to over-centrifuge the beads, as they will be more difficult to subsequently resuspend and more likely to cause chip blockages.

3 Carefully remove supernatant with a P200 tip, being careful not to disturb the bead pellet.

4 Spin down at 1,000 x g, 4°C for 5 s, carefully remove remaining supernatant with a P10 tip leaving only pelleted beads.

IMPORTANT *If you accidentally aspirate beads, pipette solution back into the tube and spin again. Remove any residual liquids as described.*

4 Resuspend the beads thoroughly in 250 µl of cold lysis buffer (final concentration 600 beads/µl) and store on ice until use.

Chapter 7. Preparation of Cells (HEK/3T3 Cells)

Required equipment and consumables

- Refrigerated lab centrifuge capable of spinning 50 ml tubes at min 1000 x g @ 4 °C
- 50 ml Falcon tubes
- 40 µm cell strainer
- C-Chip Neubauer Improved haemocytometer

Required reagents

- DMEM growth media
- Fetal Bovine Serum (FBS)
- Penicillin/Streptomycin
- TrypLE Express Enzyme (1X)
- Cell buffer
- 1x PBS pH 7.4, sterile
- 1 % BSA solution in 1x PBS
- 0.4 % Trypan Blue stain solution
- Nuclease-free water

Cell preparation

IMPORTANT *Prepare single cell suspensions based on individual cell lines requirements. If benchmarking against published data (Macosko et al., Cell 2015), use a combination of HEK293T and 3T3 cells. Avoid any growth media carryover into the final dilution of the cells with cell buffer as the presence of serum will inhibit the reaction. Prepare cells shortly before the Nadia run. It is generally not recommended to leave the cells suspended in cell buffer on ice for an extended period of time.*

1 Culture cells in DMEM / 10 % FBS / 1x PenStrep until they reach 60-70 % confluency.

2 Gently aspirate the culture media without disturbing the cells.

3 Wash cells once with 10 ml sterile 1x PBS (pre-warmed to 37°C).

4 Add TrypLE (3-5 ml for T25 and 5-10 ml for T75 culture flask) and put cells back in the incubator for 3- 5 mins.

IMPORTANT *Be careful not to overtrypsinise cells. Only allow enough time to detach the cell monolayer from the bottom of the culture flask. Observe the cells under the microscope to verify detachment.*

5 Add an equal volume of culture media to inactivate TrypLE.

6 Collect the cells in a 50 ml Falcon tube and pellet them.

7 Resuspend cell pellet in 1 ml of 1x PBS and spin at 300 x g for 3 min.

8 Aspirate the supernatant and resuspended the cells in 1 ml of 1x PBS, pass through a 40 µm cell strainer and collect a small aliquot of filtered cells.

9 Take 10 µl from the aliquot and add 10 µl of 0.4 % Trypan Blue stain solution (to a final concentration of 0.2 %).

10 Load the mixture onto a Neubauer Improved haemocytometer and count the cells.

11 Adjust the liquid volume to a final concentration of 300,000 cells/ml in cell buffer.

a. If you need to concentrate the cells, pellet them at 300 x g for 3 min, carefully remove supernatant and resuspend the cell pellet in the appropriate volume of cell buffer to reach the final concentration of 300,000 cells/ml.
b. If the cells are too concentrated, you can adjust the volume with 1x PBS and 1 % BSA in 1x PBS. The final concentration should be 300,000 cells/ml in 1x PBS and 0.01 % BSA (i.e. cell buffer).

NOTE *For mixed species libraries prepare both cell types separately as described above and then mix in a 1:1 ratio.*

Chapter 8. Cell Encapsulation on Nadia

Required equipment and labware

- Nadia Instrument
- Low retention pipette tips (P1000, P200, P10)
- Low retention gel loading tips (P200)
- Lint-free wipes
- Eppendorf DNA LoBind 1.5 ml tubes
- Neubauer Improved haemocytometer C-Chip
- 50 ml Falcon tubes
- 5 µm Überstrainer set
- 50 ml Luer-Lok Syringe
- Microcentrifuge with cooling function

Required reagents

- Bead suspension
- Cell suspension
- Emulsion oil (QX200™ Droplet Generation Oil for EvaGreen)
- 6x SSC buffer (prepare 50 ml for each sample; use 20x SSC stock and nuclease-free water)
- 5x RT buffer

Protocol

Step 1: Nadia set-up

IMPORTANT *Before operating the Nadia instrument, ensure all surfaces are clean and free of fibres and dust particles. Use lint-free wipes and 70 % IPA to wipe down the work surfaces and the instrument.*

1 Power up the Nadia instrument, remove the cartridge with the desired number of microfluidic chips from its packaging and place it on the instrument when instructed on-screen.

2 Ensure that locating pins on the instrument fit into corresponding slots in the cartridge as shown in the picture below. Press “Next” on the Nadia touch screen.

3 Follow the instructions displayed on-screen and remove gasket from cartridge. The gasket can either be stored on a clean and dust-free surface or simply folded and placed behind the Nadia clamping mechanism (see below). Press “Next” to proceed.

4 Following the on-screen instructions and using a P1000 pipette or a powered aspirator/dispenser, load 3 ml of emulsion oil into the oil reservoir(s). Press “Next” to proceed to the next step.

5 Re-apply gasket when instructed. Ensure that the 4 holes at each corner of the gasket are securely fitted onto the corresponding retaining pins.

6 Press ‘Next’ to commence the pre-cooling step.

Step 2: Cell encapsulation

1 Press ‘Next’ on the Nadia screen to open the lid.

2 Following on-screen instruction, load beads into the blue flashing wells (A). Use low retention pipette tips to avoid losing bead suspension throughout the process.

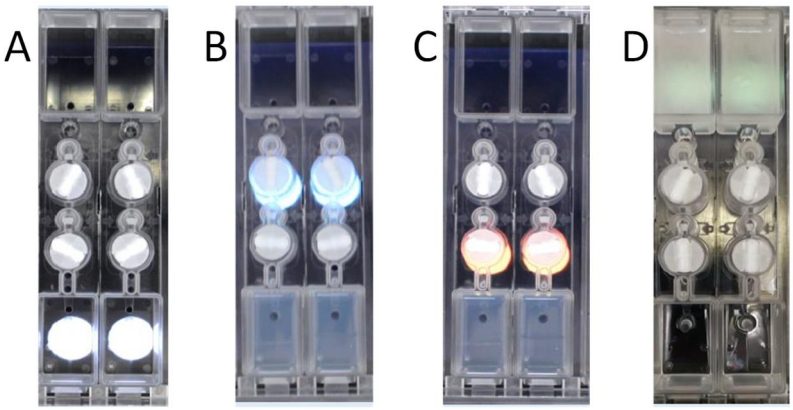


Figure 1: Guidelights underneath the Nadia cartridge indicate loading positions for the emulsion oil (A), beads (B) and sample (C), and indicate the location of the emulsion (D) to the user after the run is completed.

b. Vigorously mix the beads by pipetting up and down 10-20 times with low-retention P200 tips.

c. Load 125 µl of the bead suspension using gel loading tips into the blue flashing bead well (B). Avoid beads sticking to the side of the well by keeping the tip pushed inside the well as deeply as possible and without using excessive force.

d. Repeat using the remaining 125 µl of the bead suspension.

NOTE *Proceed to the encapsulation step as swiftly as possible after bead loading. Beads settle quickly in lysis buffer and prolonged settling can increase the chance of bead blockages.*

3 Press “Next”.

4 When instructed to load sample, transfer cells into the orange flashing wells using standard P200 tips (C).

   a. Carefully mix the cells by pipetting up and down 5 times prior to loading.

   b. Load 125 µl of the cell suspension into the orange flashing cell suspension well.

   c. Repeat with remaining 125 µl of the cell suspension.

5 Replace the gasket and close the lid.

6 Press “Next” to start the encapsulation process. The total processing time includes an incubation step of 10 min at room temperature for improved lysis.

IMPORTANT *This incubation of 10 mins is optimal for mammalian cell lines HEK 293 T (human epithelial kidney) and 3T3 (murine fibroblast). An extended incubation (up to an additional 35 mins) can be beneficial for other cell types/ tissue. In the absence of prior knowledge, some empirical testing may be required to determine the optimal incubation period for a given cell type/ tissue.*

7 When Nadia prompts that the run is completed 10 mins of incubation would have been completed (described above). **If additional incubation is desired**, **leave the cartridge on the Nadia instrument for the required, additional length of time.** There is a timer on screen to facilitate the tracking of this extended incubation.

8 While the Nadia is running, equilibrate 50 ml of 6x SSC buffer to room temperature.

9 Upon completion of this extended incubation (if performed) an emulsion will be present within each chip. This emulsion is creamy white in appearance and will be floating on top of the layer of oil in the output reservoir of the chip (D).

10 If desired, carefully aspirate 8.5 µl of the creamy emulsion from a position within the reservoir and load into a Neubauer Improved haemocytometer to assess monodispersity of droplets.

11 Using a P1000 pipette, carefully remove as much of the underlying layer of oil within the reservoir as possible being extremely careful not to collect any of the emulsion at this point. Place the oil back in the oil reservoir, it will be used to rinse the collection reservoir to capture as many droplets as possible before proceeding further.

12 Immediately proceed with the emulsion breakage.

Step 3: Emulsion breakage

1 Remove the 5 µm Überstrainer set from its packaging. Referring to the diagram below, remove and discard the lower assembly by pulling it out of the coloured filter. Unscrew the screw cap from the filter and keep it aside for later.

2 Place the coloured Überstrainer filter section inside of an upright falcon tube.

3 Carefully transfer the layer of emulsion from the Nadia cartridge reservoir onto the Überstrainer filter membrane. Residual oil can be used to wash leftover emulsion from the walls and the bottom of the reservoir. It will accumulate in the far end, making it easy to collect. It is acceptable to transfer residual oil alongside the emulsion at this point.

4 Replace the Überstrainer screw cap, making sure that it is tight.

5 Remove the white Luer-Lok cap from the top of the screw cap. Do not discard this, as it will be required again later.

6 Fill a 50 ml luer-lok syringe with 45 ml of equilibrated 6x SSC buffer. Pull the plunger all the way to the 50 ml graduation to ensure that an air buffer is present.

IMPORTANT *Do not overfill the syringe with 6x SSC buffer. The ~5 ml of air buffer space is important to adequately remove all excess oil from the beads.*

7 Attach the syringe to the top of the screw cap, ensuring that it is sealed tightly.

8 Press down on the plunger, applying consistent force until all the liquid has passed through the strainer and the remaining air has been expelled from the syringe.

9 Detach the 50 ml syringe and unscrew the screw cap.

10 Inside the screw cap, there is a plastic disk which needs to be removed. To accomplish this, place the screw cap thread-down on a clean flat surface and insert a clean P1000 pipette tip into the small aperture.

11 By hand, apply downward pressure to the pipette tip until the plastic disk is extruded. Remove and discard the disc.

12 Screw the white Luer-Lok cap back on to the screw cap. Invert the screw cap and place it nozzle-down into a standard well in a 1.5 ml microcentrifuge tube holding rack.

13 Insert the coloured Überstrainer filter containing washed beads into the screw cap. Push down lightly on the filter until a seal is formed.

14 Using a P1000 pipette, add 1 ml of 6x SSC buffer into the Überstrainer. Pipette up and down 5-6 times to resuspend the beads and transfer this bead solution to a fresh 1.5 ml DNA LoBind microcentrifuge tube.

15 Add another 500 µl of 6x SSC buffer to the Überstrainer. Wash out any remaining beads and add the solution to the same tube as before.

16 Spin down the tube at 1000 x g at 4 °C for 1 minute to pellet the beads. Remove the supernatant.

IMPORTANT *If the beads stick to the wall of the tube instead of settling down at the bottom, put the tube back in the centrifuge rotated by 180 degrees (hinge-down instead of the usual hinge-up orientation) and centrifuge for 1 min at 1000 x g, 4 °C. Rotate the tubes and spin again for 1 min at 1000 x g, 4 °C. Repeat until beads sit at the bottom.*

5 Perform a final wash step by adding 300 µl of 5x Maxima RT buffer to the bead pellet. Mix by pipetting up and down 5 times.

6 Spin at 1000 x g, 4°C for 1 min and remove as much of the supernatant as possible without disturbing the bead pellet.

7 Immediately proceed with reverse transcription.

Chapter 9. Reverse Transcription and PCR Amplification

Required equipment and labware

- Rotating incubator
- Fuchs-Rosenthal haemocytometer C-Chip
- PCR tubes 0.2 ml
- Eppendorf DNA LoBind 1.5 ml tubes
- Thermocycler
- Microcentrifuge
- Magnetic rack
- Bioanalyzer
- Qubit dsDNA HS assay system
- Bioanalyzer High Sensitivity chips
- Pipette tips P1000, P200, P10

Required reagents

- 10x Maxima H- Enzyme
- RT master mix
- TE/SDS (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 0.5 % SDS)
- TE/TW (10 mM Tris pH 8.0, 1 mM EDTA pH8.0, 0.01 % Tween 20)
- Exonuclease I
- 10x Exonuclease I Buffer
- Nuclease-free H_2O
- 10 mM Tris pH 8.0
- 20 % PEG
- 100 µM SMART PCR PRIMER
- 2x Kapa HiFi Hotstart Ready mix
- AMPure XP beads
- 80 % Ethanol
- Qubit dsDNA HS assay kit

Protocol

Step 1: Reverse Transcription

1 Add the required amount of 10x Maxima H- Enzyme to the previously prepared RT master mix as of the table below and mix the sample by pipetting up and down 5 times. The volumes assume 10 % extra volume for pipetting losses.

Component	1 Sample	2 Samples	4 Samples	8 Samples
RT master mix	209 µl	418 µl	836 µl	1672 µl
10x Maxima H- Enzyme	11 µl	22 µl	44 µl	88 µl
Total	**220 µl**	**440 µl**	**880 µl**	**1760 µl**

2 Add 200 µl of the RT master mix to each tube and mix by pipetting up and down 5 times.

3 In a rotator, incubate at room temperature for 30 mins followed by a second incubation step at 42 °C for 90 min.

4 Pellet the beads at 1000 x g, 4 °C for 1 min. Remove supernatant and discard.

5 Wash the beads by adding 1 ml TE/SDS. Mix by pipetting up and down 5 times. Spin at 1000 x g, 4°C for 1 min. Remove supernatant and discard.

6 Wash the beads by adding 1 ml TE/TW. Mix by pipetting up and down 5 times. Spin at 1000 x g, 4°C for 1 min. Remove supernatant and discard.

7 Repeat previous step.

STOPPING POINT. Beads can be stored overnight at 4 °C in 1 ml TE/TW. If proceeding further, spin down at 1000 x g, 4°C for 1 min and remove supernatant.

Step 2: Exonuclease I treatment

NOTE *This step removes excess bead primers that did not capture an RNA molecule.*

1 Prepare exonuclease mix as per the table below. The volumes assume 10 % extra volume for pipetting losses.

Component	1 Sample	2 Samples	4 Samples	8 Samples
10x Exonuclease I Buffer	22 µl	44 µl	88 µl	176 µl
Nuclease-free H_2O	187 µl	374 µl	748 µl	1496 µl
Exonuclease I	11 µl	22 µl	44 µl	88 µl
Total	**220 µl**	**440 µl**	**880 µl**	**1760 µl**

2 Add 1 ml of 10 mM Tris pH 8.0 to the bead pellet and mix by pipetting up and down 5 times. Spin at 1000 x g, 4 °C for 1 min. Remove supernatant and discard.

3 Resuspend the pellet in 200 µl of exonuclease mix.

4 In a rotator, incubate at 37°C for 45 min.

5 Pellet the beads at 1000 x g, 4 °C for 1 min. Remove supernatant and discard.

6 Wash the beads by adding 1 ml TE/SDS. Mix by pipetting up and down 5 times. Spin at 1000 x g, 4 °C for 1 min. Discard supernatant.

7 Wash the beads by adding 1 ml TE/TW. Mix by pipetting up and down 5 times. Spin at 1000 x g, 4 °C for 1 min. Discard supernatant.

8 Repeat previous step

STOPPING POINT. Beads can be stored at 4 °C overnight in 1 ml of TE/TW. When ready to proceed with PCR, spin down at 1000 x g, 4 °C for 1 min and remove supernatant.

Step 3: PCR

1 Wash the bead pellet in 1 ml of nuclease-free H_2O, spin down at 1000 x g, 4 °C for 1 min and discard supernatant.

2 Resuspend bead pellet in 1 ml nuclease-free H_2O and mix well. Take a 20 µl aliquot of bead suspension, mix well with 20 µl of 20 % PEG and load 20 µl of the mix into a Fuchs Rosenthal haemocytometer C-Chip. Hold the haemocytometer vertically while loading the beads and ensure beads are evenly distributed throughout. If not, repeat using a new haemocytometer. Count the beads under the microscope to determine bead number per µl.

3 Adjust the bead concentration using nuclease-free H_2O to achieve a final concentration of 400 beads/µl. Up to 4000 beads can be used in an individual PCR reaction. If a different number of beads are to be used, adjust the concentration accordingly to obtain the required number of beads in 10 µl.

4 For each PCR preparation, mix the beads by pipetting up and down until beads are evenly resuspended in nuclease-free water and pipette 10 µl of the bead suspension equating to 2000 beads (or more) into a PCR tube.

IMPORTANT *4000 beads will yield ~200 STAMPs (Single-cell Transcriptome Attached to MicroParticles). Using 2000 beads will yield ~100 STAMPs. The use of a knee-plot to identify the accurate number of STAMPs for further analysis is highly recommended. Please refer to Chapter 12.*

5 Spin down any remaining beads and resuspend in 1 ml TE/TW. Beads in this buffer can be stored at 4 °C for up to 2 weeks.

6 Prepare the PCR Master mix as per the table below. Each sample may require between 4 - 32 PCRs. To perform more than 4 PCRs (i.e. n PCRs) determine the quantities required as a multiple of a single PCR.

The volumes include 10 % extra to allow for pipetting losses. Keep the PCR master mix on ice prior to use.

Component	**1 PCR**	**4 PCRs**	**n PCRs**
Nuclease free H_2O	12.1 µl	48.4 µl	12.1 * n
10 µM SMART PCR PRIMER	4.4 µl	17.6 µl	4.4 * n
2x Kapa HiFi Hotstart Ready mix	27.5 µl	110 µl	27.5 * n
Total	44 µl	176 µl	44 * n

7 Add 40 µl of the PCR Master mix to each sample and mix well. Run the PCR program as described in the table below.

Cycle numbers	**Temperature**	**Time**
1 cycle	95°C	3 min
4 cycles	98°C	20 s
	65°C	45 s
	72°C	3 min
9 cycles	98°C	20 s
(possible range from 8 to 12 cycles)	67°C	20 s
	72°C	3 min
1 cycle	72°C	3 min
	4°C	Hold

Step 4: PCR purification and QC

1 Remove AMPure XP beads from the fridge and equilibrate to room temperature (it takes ~30 min to equilibrate a 60-ml bottle).

2 Prepare appropriate volume of 80% ethanol.

IMPORTANT *Ethanol is hygroscopic and gets progressively diluted as it absorbs moisture from the air. Washing AMPure beads with overdiluted ethanol may compromise the library yield. Always prepare 80% ethanol fresh from a stock of ethanol absolute. You will need ~1.8 ml of 8 0% ethanol / sample (this volume includes extra 20% for pipetting losses).*

3 Vortex the bottle of AMPure XP beads to thoroughly mix suspension before use.

4 If there are <u>more than one PCR reaction</u> to be purified, apply the below procedure to process each PCR-reaction separately:

a. Add 30 µl of room temperature AMPure XP beads to each PCR tube resulting in a 0.6:1 beads-to-sample ratio. Mix well by pipetting up and

down 10 times. Transfer each sample to a fresh Eppendorf DNA LoBind 1.5 ml tube.

5 Incubate for 5 min at room temperature.

6 Place the Eppendorf DNA LoBind 1.5 ml tubes onto a magnetic rack and leave standing for 5 min at room temperature, or until a solid AMPure bead pellet has formed.

7 Carefully remove the supernatant together with the RNA capture beads (white pellet at the bottom of the tube) without disturbing the brown AMPure bead pellet.

IMPORTANT *Always keep the beads on the magnetic rack when removing the supernatant. AMPure bead pellets will become unstable as soon as the tube is moved away from the magnet.*

8 Wash each AMPure bead pellet with 1 ml of 80 % ethanol while keeping tubes on the magnetic rack then remove the supernatant and discard.

9 Repeat wash by adding 500 µl of 80 % ethanol to each tube, then remove the supernatant and discard. Spin tube briefly in microcentrifuge, then put tubes back onto magnet to facilitate complete removal of any residual ethanol. If required, use a P10 pipette to do this. Leave the tubes on the magnetic rack for 3-5 min to air dry. Open the lid, remember not to over-dry the pellets (tell-tale cracks form in the pellets).

10 Take the tubes off the rack and resuspend each pellet with 12 µl of nuclease-free H_2O.

IMPORTANT *Take the tubes off the magnetic rack for this step and inspect them carefully. There might be small clumps of AMPure bead pellet scattered on the wall of the tube. Be mindful to resuspend <u>all</u> visible clumps. Mix the beads by pipetting until evenly suspended.*

11 Incubate the suspensions for 5 min at room temperature.

12 Place the tubes back on the magnetic rack and leave for 5 min, or until a solid AMPure bead pellet has formed.

13 Carefully remove the supernatant and pipette into fresh tubes.

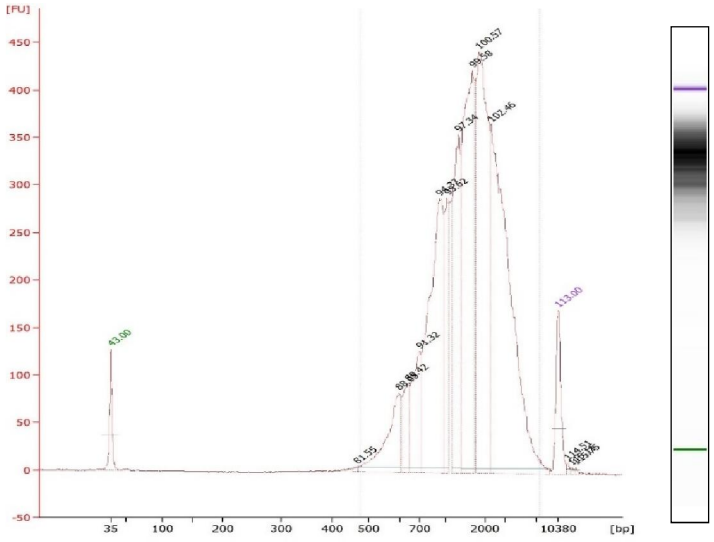
14 Determine each sample concentration using a fluorometric based system such as the Qubit dsDNA HS system.

IMPORTANT *A total of 600 pg of purified DNA is required to proceed. Less than 600 pg may compromise the sequencing results. When generating standard mixed species libraries (3T3 and HEK293T cells mixed at a 1:1 ratio) you can expect a yield of 1-3 ng/µl.*

15 Analyse 1 µl of the purified cDNA sample on a BioAnalyzer High Sensitivity Chip.

IMPORTANT *The use of a highly sensitive analytical system is crucial at this point.*

NOTE *The desired cDNA library should have a smooth profile with an average size of 1300-2000 bp.*



STOPPING POINT. The purified cDNA library can be stored at -20 °C for several weeks.

Chapter 10. Tagmentation of cDNA with Nextera XT Kit

Required equipment and labware

- Thermocycler
- Microcentrifuge
- 0.2 ml PCR tubes
- Eppendorf DNA LoBind 1.5 ml tubes
- Magnetic rack
- Bioanalyzer
- Qubit dsDNA HS assay system
- Bioanalyzer High Sensitivity chips
- Pipette tips P1000, P200, P10

Required reagents

- Nextera XT Kit
- Nuclease-free H_2O
- 10 µM New-P5-SMART PCR hybrid oligo
- 10 µM Nextera N7XX oligo
- AMPure XP beads
- 80 % Ethanol
- Qubit dsDNA HS assay kit

Step 1: Tagmentation

IMPORTANT *Single Nextera reaction. If creating a library comprised of more than a single PCR reaction, pool equal quantities of purified cDNA from each PCR reaction to achieve the final 600 pg before commencing the Nextera XT process.*

For example, if 2 PCR reactions are to be processed as a single library for sequencing, pool 300 pg of cDNA from each individual PCR reaction to achieve a final 600 pg.

The use of the knee-plot to identify the optimal number of STAMPs for further analysis is highly recommended. Please refer to Chapter 12.

2 to 4 Nextera reactions. For any given biological sample, to increase the quantity of an individual library that would be available for sequencing, 2 to 4 Nextera reactions can be subsequently pooled to create such a library. This pooling of Nextera reactions for individual samples can facilitate the obtention of more concentrated libraries that meet Illumina's recommendations (please check with your NGS provider).

For example, to pool 2 Nextera reactions starting from 2 PCR reactions that would eventually be processed into a single library for sequencing you should initially pool 600 pg of cDNA from each individual PCR reaction to obtain the required 1200 pg. 600 pg should subsequently be used in each Nextera reaction for a total of 2 reactions thus the requirement for 1200 pg.

It is NOT recommended to perform a Nextera reaction starting out with more than 600 pg, hence the recommendation of performing 2 separate Nextera reactions and pooling afterwards.

1 Preheat the thermocycler to 55°C.

2 For each Nextera reaction, in a fresh 0.2 ml PCR tube add 600 pg of purified cDNA to nuclease-free H_2O for a total volume of 5 µl.

3 To each Nextera reaction add 10 µl of Tagment DNA buffer (TD) and 5 µl of Amplicon Tagment Mix (ATM) for a total volume of 20 µl.

4 Mix by pipetting up and down 5 times. Spin down briefly in a microcentrifuge to collect all liquid within the tube.

5 Incubate at 55 °C for 5 min.

6 Add 5 µl of Neutralization Buffer (NT) to each Nextera reaction. Mix by pipetting up and down 5 times and spin down.

7 Incubate at room temperature for 5 min.

8 Prepare the Tagmentation PCR master mix as per the table below. The volumes assume 10 % extra volume for pipetting losses. Keep the Tagmentation PCR Master mix on ice prior to use.

IMPORTANT *The table below assumes no multiplexing. If you want to multiplex samples using different indexing oligos, prepare the Tagmentation PCR master mix without the Nextera N7XX indexing oligos.*

Component	1 Nextera reaction	2 Nextera reactions	4 Nextera reactions	8 Nextera reactions
Nextera PCR Master Mix (NPM)	16.5 µl	33 µl	66 µl	132 µl
Nuclease-free H_2O	8.8 µl	17.6 µl	35.2 µl	70.4 µl
10 µM New-P5-SMART PCR hybrid oligo	1.1 µl	2.2 µl	4.4 µl	8.8 µl
10 µM Nextera N7XX indexing oligo	1.1 µl	2.2 µl	4.4 µl	8.8 µl
Total	**27.5 µl**	**55 µl**	**110 µl**	**220 µl**

IMPORTANT *We are aware that some customers might want to take advantage of the Illumina indexing kit that contains all the N7XX indexing oligos (N701-N712). Unfortunately, Illumina does not state the concentration of the oligo supplied in their kit. Moreover, the Illumina protocol calls for adding 5 µl of the indexing oligo into the post-tagmentation PCR reaction. Our internal tests show that using 1 µl Illumina-supplied N7XX indexing oligo per PCR reaction works well.*

9 Add 25 µl of the Tagmentation PCR master mix to each reaction. The final volume in each tube is 50 µl.

IMPORTANT *If sample multiplexing is desired, use the Tagmentation PCR master mix without the Nextera N7XX. Add 24 µl of master mix to each reaction and then add 1 µl of different Nextera 7XX indexing oligos into each sample to enable multiplexed sequencing.*

10 Run the following PCR programme.

Cycle numbers	Temperature	Time
1 cycle	95°C	30 s
12 cycles	95°C	10 s
	55°C	30 s
	72°C	30 s
1 cycle	72°C	5 min
	4°C	Hold

Step 2: Purification of Nextera reactions and QC

1 Remove AMPure XP beads from the fridge and equilibrate to room temperature (it takes ~30 min to equilibrate a 60-ml bottle).

IMPORTANT *If pooling 2-4 individual Nextera reactions to create a single library, pool these reactions (refer to page 24) into a single Eppendorf DNA LoBind 1.5 ml tube before proceeding.*

2 Prepare appropriate volume of 80 % ethanol.

IMPORTANT *Ethanol is hygroscopic and gets progressively diluted as it absorbs moisture from the air. Washing AMPure beads with overdiluted ethanol may compromise the library yield. Always prepare 80 % ethanol fresh from a stock of ethanol absolute. You will need ~1.8 ml of 80 % ethanol / sample (this volume includes extra 20% for pipetting losses).*

3 Vortex the bottle of AMPure XP beads to thoroughly mix suspension before use.

4 Add appropriate volume of room temperature AMPure XP beads to each tube resulting in a 0.6:1 ratio of beads to sample. For example, to purify 50 µl of sample, 30 µl of beads are required whereas to purify 150 µl of sample, 90 µl of beads are required. Mix well by pipetting up and down 10 times and transfer each sample to a fresh Eppendorf DNA LoBind 1.5 ml tube.

5 Incubate for 5 min at room temperature.

6 Place the tubes onto a magnetic rack and leave standing for 5 min at room temperature, or until a solid AMPure bead pellet has formed.

7 Keeping the tubes on the rack, carefully remove the supernatant without disturbing the brown AMPure bead pellet.

IMPORTANT *Always keep the beads on the magnetic rack when removing the supernatant. AMPure bead pellets will become unstable as soon as the tube is moved away from the magnet.*

8 On the magnetic rack wash each AMPure bead pellet with 1 ml of 80 % ethanol. Remove the supernatant and discard.

9 Repeat wash by adding 500 µl of 80 % ethanol to each tube. Remove the supernatant and discard. If required, use a P10 pipette to remove any remaining 80 % ethanol. Leave the tubes on the magnetic rack for 5-10 min to air dry. Open the lid, remember not to overdry the pellets (tell-tale cracks form in the pellets).

10 Take the tubes off the magnetic rack and resuspend each pellet in 12 µl of nuclease-free H_2O.

IMPORTANT *Take the tubes out of the magnetic rack for this step and inspect them carefully. There might be small bits of AMPure beads pellet scattered on the wall of the tube; Be careful to resuspend all visible pellet. Mix the beads by pipetting until evenly suspended.*

11 Incubate the suspensions for 5 min at room temperature.

12 Place the tubes back on the magnetic rack and leave for 5 min, or until a solid AMPure bead pellet has formed.

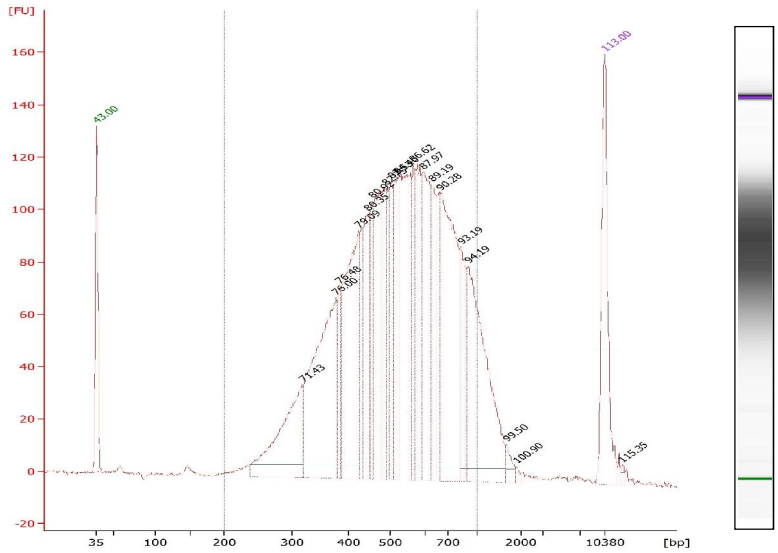
13 Carefully remove the supernatant and pipette into fresh Eppendorf DNA LoBind 1.5 ml tubes.

14 Determine each sample concentration using a fluorometric based system such as the Qubit dsDNA HS assay system.

15 Analyse 1 µl of the purified Nextera product on a BioAnalyzer High Sensitivity Chip. The tagmented library should have a smooth profile with an average size of 500-680 bp.

NOTE *Dolomite Bio's tagmented DNA libraries were sequenced on the Illumina NextSeq 500 instrument using a 2x75bp paired-end run. Read lengths were: 26 bp for read 1, 8 bp for the index read and 116 bp for read 2. Users are advised to enquire with their sequencing facility to find a sequencing setup that will serve their specific needs.*

NOTE *Smaller-sized libraries will have more polyA reads; larger-sized libraries may have lower sequence cluster density and cluster quality.*



STOPPING POINT. The purified tagmented library can be stored at -20 °C for several weeks. Libraries are ready for sequencing according to the Illumina NextSeq or HiSeq user guide instructions.

Chapter 11. Additional Guidance

Parameter	Guidance
Number of beads within single PCR reaction	The use of 4000 beads within a single PCR reaction will yield approximately 200 STAMPs whilst the use of additional beads will yield correspondingly more STAMPs. An increased number of STAMPs will require additional sequencing to achieve the recommended "sequencing depth per STAMP". Be aware that using more than 4000 beads per PCR reaction can result in decreased library complexity.
Number of cycles during PCR	In this Guide, the default of 9 cycles had been established using mammalian cell lines 293T and 3T3. The use of 10-12 cycles (additional amplification) may be necessary with other cell types/ tissues. The use of additional amplification can result in a higher extent of undesirable amplicon-duplication and some distortion of the fidelity of a transcriptome. The guidance here would be to balance an increased number of beads used in a PCR reaction against the minimum number of PCR cycles required to obtain the desired amplified cDNA product. In the absence of prior knowledge, it is recommended to optimise through empirical testing for a given cell type/ tissue.

Chapter 12. Guidelines for NGS (Illumina)

Molecular architecture of DNA inserts produced by Drop-Seq on Nadia

Drop-Seq on Nadia culminates in libraries that are compatible with Illumina's single indexed Paired-End sequencing. The molecular architecture of a DNA insert following Nextera XT tagmentation is illustrated in Figure 2.

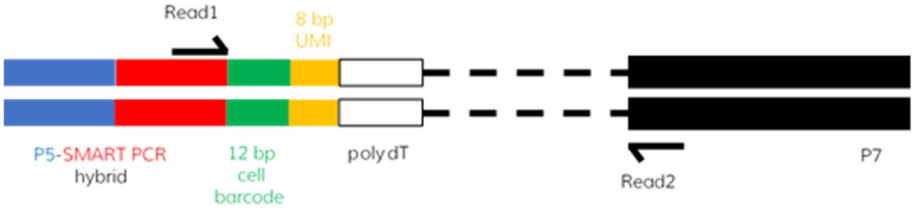


Figure 2: Molecular architecture of a DNA insert after Nextera library preparation. Read 1 includes the cell barcode and UMI whilst Read 2 has transcript information.

Number of STAMPs to be analysed

This will inevitably be determined by the complexity of the tissue and the objectives of the biological study. For example, researchers studying retinal tissue [1] were better able to distinguish between different clusters of amacrine cells when they analysed 9000 STAMPs compared to when they considered only 500 STAMPs.

However, budgetary constraints may dictate a compromise between the number of STAMPs and sequencing depth per STAMP. As continually increasing numbers of STAMPs are being profiled, this has resulted in a correlated decrease in the sequencing depth per STAMP [2]. A lower sequencing depth per STAMP may result in the detection of fewer transcripts and gene per transcriptome. A compromise between quantity of STAMPs profiled and sequencing depth per STAMP may be required. We recommend that the user be guided by prevailing studies in his or her scientific field.

Multiplexing and recommended read length

The N701 – N712 oligos from the Nextera XT Index Kit (Illumina #FC-131-1002) can be used to create libraries suitable for multiplexing during sequencing.

NOTE *Do not use the S5XX oligos and do not use dual indexing during library preparation/sequencing.*

Table 1: Recommended read lengths with 2x75 bp Paired-End chemistry

Read	Length
Read 1	26 bp
I7 index read*	8 bp
Read 2	116 bp

*only relevant if multiplexing

With above read lengths, use the standard Nextera P7 Sequencing Primer for Read 2 and "Read1CustomSeqB" for Read 1.

Guidelines for computational tools and data analysis

Computational tools

For data analysis, Partek Flow (Partek) offers a powerful solution to computationally process, analyse and visualise data. Please contact Dolomite Bio for additional details.

Alternatively, the "dropSeqPipe (https://github.com/Hoohm/dropSeqPipe), a computational pipeline developed by the McCarroll Lab at Harvard, can be used. Additional guidance on usage of this tool can be obtained from:

http://mccarrolllab.org/wp-content/uploads/2016/03/Drop-seqAlignmentCookbookv1.2Jan2016.pdf.

Data analysis: use of the knee-plot to identify high quality STAMPs

The knee-plot allows the user to identify a subset of STAMPs that will be used for analysis. Use of the knee-plot is highly recommended during data analysis and is instrumental in producing good data (see Figure 3).

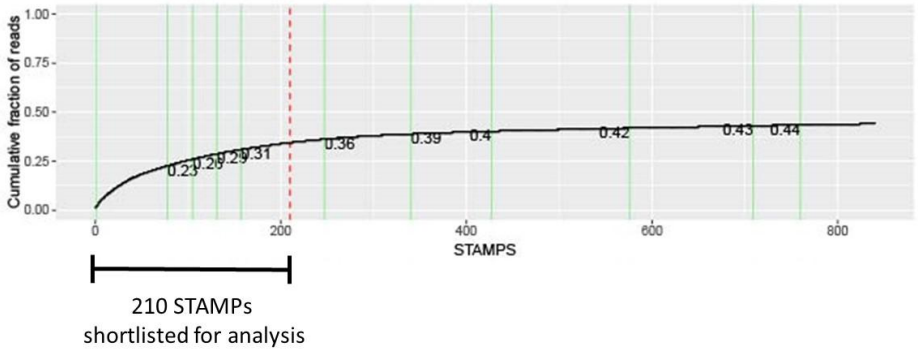


Figure 3: The knee-plot arranges barcodes in descending number of sequenced reads associated with each individual barcode. It allows the user to identify a cut-off threshold (dashed red line) and shortlist data-rich barcodes that represent high-quality STAMPs. Beyond the point along the plot at which the cut-off is placed, barcodes are associated with decreasing numbers of reads that approach zero.

[1] Massively-parallel single nucleus RNA-seq with DroNc-seq (Habib et *al*., 2017)

[2] Single cells make big data: New challenges and opportunities in transcriptomics (Angerer et *al*., 2017)