DropSeq on the Nadia Instrument

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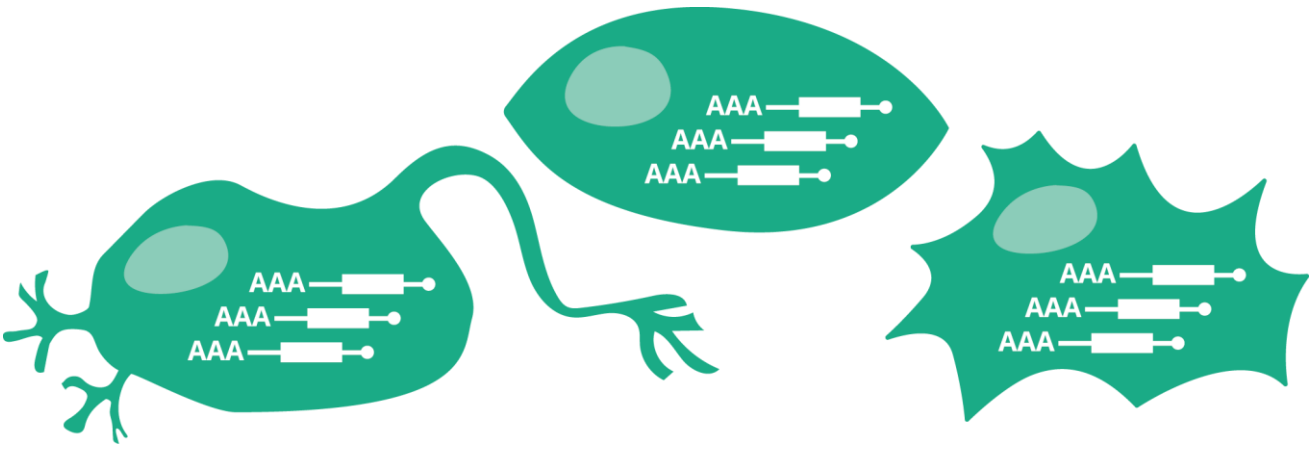

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Chapter 1.
Product Information

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 RNAseq using the DropSeq 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 RNAseq.

Nadia Instrument And Consumables

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

Required Equipment And Consumables

Equipment

- Nadia Instrument
- Nadia Cartridges
- Refrigerated centrifuge capable of spinning 50 ml tubes at min 1000 x g @ 4°C
- Microcentrifuge with cooling function
- Rotating Incubator
- Thermocycler
- Magnetic rack
- Bioanalyzer
- Qubit dsDNA BR System

Consumables

- 0.2 µm sterile filter
- 1.5 ml LoBind Eppendorf tubes
- 0.2 ml PCR tubes
- 15 ml Falcon tubes
- 50 ml Falcon tubes
- 70 µm cell strainer (Fisher Scientific, # 08-771-2)
- 40 µml 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
- Qubit dsDNA BR assay system

- Bioanalyzer High Sensitivity chips

Required Reagents Not Supplied In The Kit

Reagent	Supplier	Part #
Barcoded beads in storage buffer (20-40 µm, pre-filtered)	ChemGenes	Macosko-2011-10 (V+)
Nuclease-free H_2O	User defined	
Ficoll PM-400	Sigma	F4375
Sarkosyl	Sigma	L7414
EDTA	Fisher Scientific	AM9260G
Tris pH 7.5	Sigma	T2319
DTT	Sigma	10197777001
QX200™ Droplet Generation Oil for EvaGreen	BioRad	1864006
Perfluorooctanol (PFO)	Sigma	370533
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 100x	Sigma	T9285
DMEM growth media	Invitrogen	11965092
10 % Tween 20	Fisher Scientific	15125517
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	70022044
BSA	NEB	B9000S
0.4 % Trypan Blue stain solution	Gibco	1520061
20 % SDS	Sigma	05030
Exonuclease I	NEB	M0293
Exonuclease I buffer	NEB	M0293
Kapa HiFi polymerase	Fisher Scientific	NC0580933
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 BR assay system	Invitrogen	Q32851

Required Primers

Name	Sequence
Macosko template switch oligo (TSO)	5'AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG
SMART PCR PRIMER (cDNA library amplification)	5'AAGCAGTGGTATCAACGCAGAGT
New-P5-SMART PCR hybrid oligo (Nextera tagmentation amplification)	5'AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGT*A*C
Nextera N7XX indexing primers (for example N701 shown here)	5'CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
Read1CustomSeqB (Sequencing)	5'GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC

Ordering, Preparation And Storage of mRNA Capture Beads

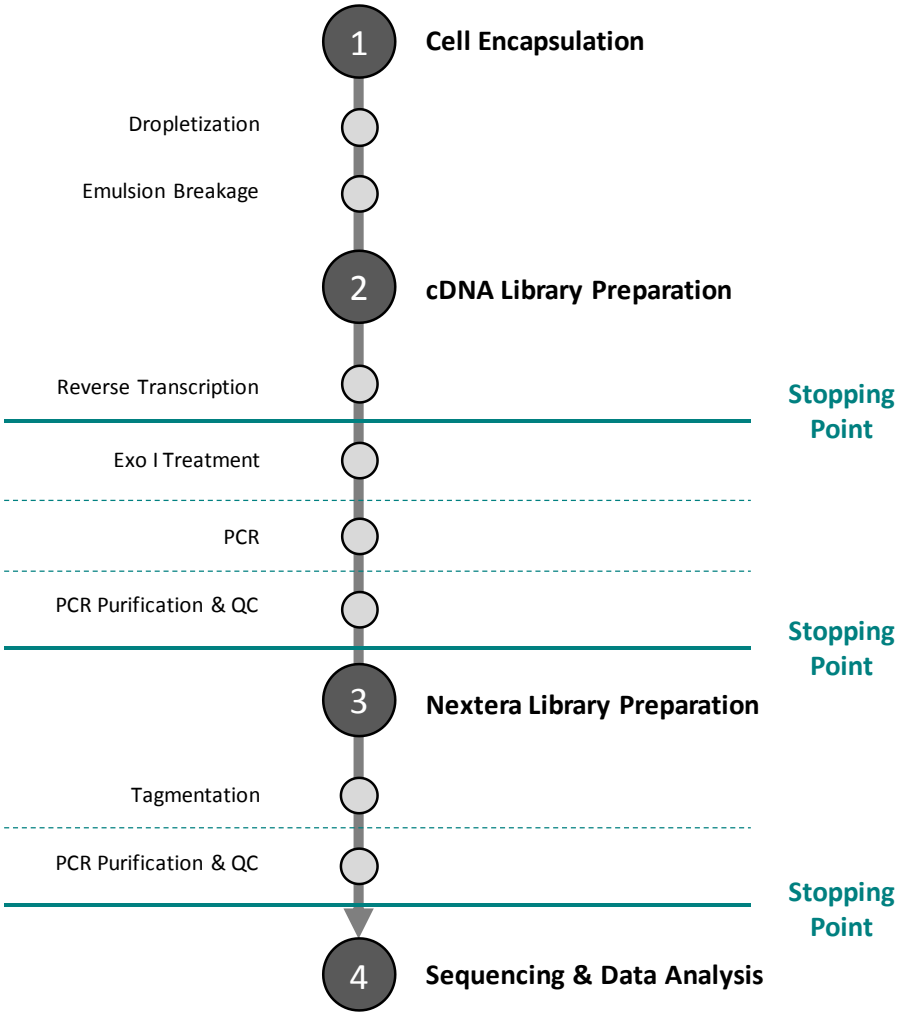
The mRNA capture beads are available from ChemGenes and can be ordered as a custom part under the product code Macosko-2011-10 (V+). Beads are either available pre-filtered with a size range of 20-40 µm, or unfiltered with a larger size range. The beads will arrive as dry resin. For bead preparation see Chapter 4.

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 DropSeq 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, lab ware 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. Buffer Preparation Prior To Starting A Nadia Run

Lysis And Cell Buffer

Required equipment and lab ware

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

Required reagents

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

Prepare stock solutions for Cell and Lysis Buffer preparation

- PBS: Prepare a 10x stock solution with nuclease-free H_2O. Store at room temperature.
- BSA: Prepare a 1 % stock solution with nuclease-free H_2O. Store at -20°C.
- Ficoll PM-400: Prepare 20 % stock solution in nuclease-free H_2O.

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

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

Prepare Lysis Buffer

Component	1 Sample	2 Samples	4 Samples	8 Samples
Nuclease -free H_2O	110.00 µl	220.0 µl	440 µl	880 µl
20 % w/v Ficoll PM-400	82.50 µl	165.0 µl	330 µl	660 µl
20 % v/v Sarkosyl	2.75 µl	5.5 µl	11 µl	22 µl
0.5 M EDTA	11.00 µl	22.0 µl	44 µl	88µl
1 M Tris pH 7.5	55.00 µl	110.0 µl	220 µl	440 µl
*1 M DTT	13.75 µl	27.5 µl	55 µl	110 µl
Total	**275.00 µl**	**550.0 µl**	**1100 µl**	**2200 µl**

NOTE: *The 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 on ice for ~30 min. Left-over Lysis Buffer including DTT should be discarded.*

Prepare Cell Buffer

Component	1 Sample	2 Samples	4 Samples	8 Samples
Nuclease-free H_2O	244.75 µl	489.5 µl	979 µl	1958 µl
10x PBS	27.50 µl	55.0 µl	110 µl	220 µl
1 % BSA	2.75 µl	5.5 µl	11 µl	22 µl
Total	**275.00 µl**	**550.0 µl**	**1100 µl**	**2200 µl**

NOTE: *Once prepared, the Cell Buffer should be filtered through 0.2 µm syringe filter. Left-over Cell Buffer should be discarded.*

Reverse Transcription (RT) Master Mix

Required equipment and lab ware

- 1.5 ml LoBind Eppendorf tubes

Required reagents

- Nuclease-free H_2O
- 20 % Ficoll PM400
- 5x RT Buffer
- 50 µM TSO-Macosko
- 10 mM dNTPs
- RNase Inhibitor

Prepare Reverse Transcription (RT) Master Mix

For each sample, prepare a 200 µl sized RT-mix and keep on ice until use. The volumes assume 10 % extra volume for pipetting losses.

Component	1 Sample	2 Samples	4 Samples	8 Samples
Nuclease-free H_2O	82.5 µl	165 µl	330 µl	660 µl
20 % Ficoll PM400	44 µl	88 µl	176 µl	352 µl
5x RT Buffer	44 µl	88 µl	176 µl	352 µl
50 µM TSO-Macosko	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 Counting

Bead Preparation

Required equipment and consumables

- Refrigerated lab centrifuge capable of spinning 50 ml tubes at min 1000 x g @ 4°C
- 1.5 ml Lo-Bind Eppendorf tubes
- Low-Retention Pipette Tips (P200, P10)

Required reagents

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

Bead Preparation

Important: We recommend using of Low-Retention Pipette Tips whenever beads are being handled. This will minimise loss by beads sticking to the pipette tip.

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 1 min, carefully remove supernatant with a P200 tip. Be careful not to disturb the bead pellet.

3 Spin down at 1,000 x g, 4°C for 30 s, carefully remove remaining supernatant with a P10 tip leaving only pelleted beads.

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

4 Resuspend the beads in 250 µl of cold Lysis Buffer (final concentration 620 beads/µl) and store on ice until use.

Required equipment and consumables

- Refrigerated lab 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
- C-Chip haemocytometer (Fuchs-Rosenthal)

Required reagents

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

Bead Counting

5 Prepare 100 ml TE/TW solution (10 mM Tris pH 8.0, 1 mM EDTA, 0.01% Tween) as follows:
   - Prepare a 10 % Tween 20 stock solution in nuclease-free water.
   - In a measuring cylinder mix 10 ml of 100x Tris-EDTA concentrate with 100 µl of 10 % Tween 20 solution.
   - Add nuclease-free H_2O water for a total volume 100 ml

6 Float the beads in 1 ml 100 % Ethanol + 1 ml TE/TW.

7 Transfer bead suspension to a 50-ml Falcon tube.

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

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

10 Repeat step 4.

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

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

NOTE: *For optimal performance of the Nadia instrument we recommend filtering the beads through a 40 µm sieve.*

13 Take a 20 µl aliquot and load it into the Fuchs Rosenthal C-Chip haemocytometer and count the beads under the microscope.

NOTE: *Beads can be difficult to load evenly into the counting chamber. Hold the haemocytometer vertically while loading and inspect the loading evenness under the microscope. If the beads are not loaded evenly, take a new haemocytometer and repeat the process.*

14 Prepare 150,000-bead aliquots from the stock and store at 4°C for subsequent use.

Cell Preparation (HEK/3T3 Cells)

Required equipment and consumables

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

Required reagents

- DMEM growth media
- Fetal Bovine Serum (FBS)
- Penicillin/Streptomycin
- TrypLE Express Enzyme (1X)
- 1x PBS pH 7.4 sterile
- 10x PBS pH 7.4
- 1 % BSA solution
- 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 Drop-Seq Cell Buffer as the presence of serum inhibits the reaction. Prepare cells shortly before the dropletisation run. It is generally NOT recommended to leave the cells suspended in Drop-Seq 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.

NOTE: *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 + 0.01 % BSA 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 10x PBS and 1 % BSA to achieve 300,000 cells/ml in 1x PBS and 0.01 % BSA.

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

Chapter 5.
Cell Encapsulation Using Nadia

Required equipment and lab ware

- Nadia Instrument
- Low retention pipette tips (P1000, P200, P10)
- Low retention gel loading tips (P200)
- Lint-free Wipes
- Eppendorf DNA Lo-Bind, 1.5 ml
- Neubauer Haemocytometer
- 50 ml Falcon Tubes
- Refrigerated lab centrifuge capable of spinning 50 ml tubes at min 1000 x g @ 4°C
- Microcentrifuge with cooling function

Required reagents

- Bead Suspension
- Cell Suspension
- Emulsion Oil (QX200™ Droplet Generation Oil for EvaGreen)
- Perfluorooctanol (PFO)
- 5x RT buffer

Protocol

Step 1: Nadia Set-up

IMPORTANT: Before operating Nadia 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 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 front screen.

3 Follow the instructions displayed on-screen and remove gasket from chip. It 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”.

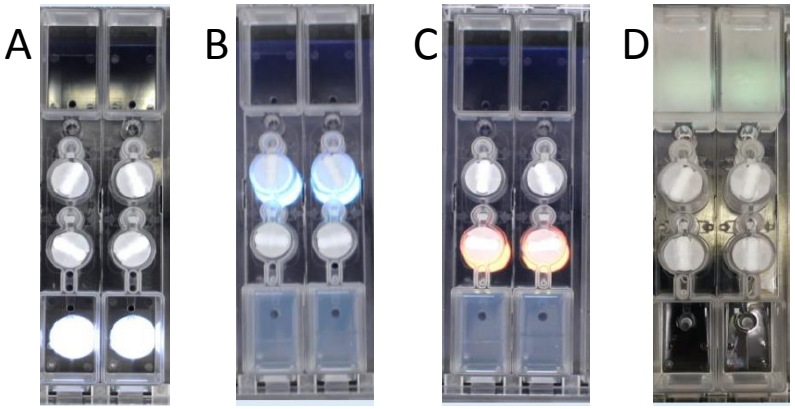
5 Re-apply gasket when instructed. Ensure that the 4 holes at each corner of the gasket are securely fitted onto the corresponding retaining pins.

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.



Guide lights underneath the Nadia cartridge chips indicate loading positions for the emulsion oil (A), beads (B) and cells (C), and direct the user to the emulsion (D) after the run is complete.

a Carefully mix the beads by pipetting up and down 10 times with low-retention P200 tips.

b 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 keeping the tip pushed inside the well as deeply as possible and without using excessive force.

c Repeat using the remaining 125 µl of the bead suspension

3 Press “Next”

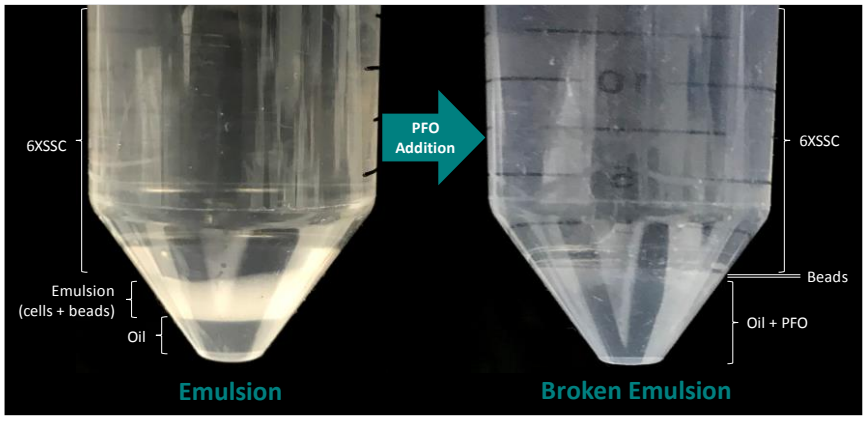
4 When instructed, load cells into the orange flashing wells using standard P200 tips (C).
   a Carefully mix the cells by pipetting up and down 5 times prior to loading.
   b Load 125 µl of the cell suspension into the orange flashing cell suspension well.
   c Repeat with remaining 125 µl of the cell 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.
7 When Nadia prompts that the Run is completed, 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).
8 If desired, carefully aspirate 8.5 µl of the creamy emulsion from a position within the reservoir and load into Neubauer Haemocytometer to assess monodispersity of droplets.
9 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. Discard the oil.

   NOTE: *If small amounts of the emulsion have been collected unintentionally, wait until the emulsion inside the pipette tip has risen to the top, then carefully discard the bottom phase (clear oil) and transfer the remaining emulsion (milky) to an Eppendorf DNA Lo-Bind Tube.*
10 Carefully transfer the remaining emulsion into an Eppendorf DNA Lo-Bind 1.5 ml Tube or combine with the previously collected emulsion if appropriate.

   NOTE: *It is acceptable to transfer some residual oil alongside the emulsion.*
11 Immediately proceed with the emulsion breakage.

Step 3: Emulsion breakage

1 Prepare 30 ml of room temperature 6X SSC in a 50 ml Falcon tube. Gently and carefully add to the emulsion.
2 Add 1 ml of PFO. Shake tube vigorously 3-4 times to break the droplets.
3 Spin down at 1000 x g, 4°C for 1 min.
4 Carefully remove the tube from the centrifuge without disturbing the beads in the interface (see figure below, red arrow) and place on ice.



5 Use a powered aspirator or a P1000 pipette and remove supernatant, leave 2-3 ml of residual liquid above the interface.
6 Add 30 ml of 6X SSC by ejecting the fluid forcefully to mix the beads. This will float the beads back into suspension and ensure the oil sinks to the bottom of the tube.
7 Leave the tube standing for 1 min to allow oil to settle
8 Transfer the bead-suspension located on top of the oil layer into a new 50 ml Falcon tube.
9 Spin at 1000 x g, 4°C for 1 min. The beads are now pelleted at the bottom of the Falcon tube but may not be visible.
10 Carefully remove and discard supernatant leave ~1 ml of liquid in the Falcon Tube.
11 With a P1000 pipette, mix the bead suspension and transfer to a 1.5 ml Lo-Bind Eppendorf tube.

12 Spin down at 1000 x g, 4°C for 1 min in a microcentrifuge. Remove and discard the supernatant.

NOTE: *We recommend leaving a small amount of supernatant rather than accidentally aspirating and losing beads. If any residual oil is visible, add 1 ml of 6X SSC to the tube and let the oil accumulate at the bottom before transferring the oil-free bead suspension into a new Eppendorf tube.*

13 Wash by adding 1 ml of 6X SSC, mix by pipetting up and down 5 times.

14 Spin at 1000 x g, 4°C for 1 min, and remove the supernatant.

15 Repeat step 12.

16 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.

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

18 Immediately proceed with reverse transcription.

Chapter 6. Reverse Transcription

Required equipment and lab ware

- Rotating incubator
- Fuchs-Rosenthal haemocytometer
- PCR tubes 0.2 ml
- 1.5 ml LoBind Eppendorf tubes
- Thermocycler
- Microcentrifuge
- Magnetic rack
- Bioanalyzer
- Qubit dsDNA BR 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
- 100 µM SMART PCR PRIMER
- 2x Kapa HiFi Hotstart Ready mix
- AMPure XP beads
- 80% Ethanol
- Qubit dsDNA BR assay system

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 min followed by a second incubation step at 42 °C for 90 min.

4 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.

5 Repeat step 4 twice.

STOPPING POINT. Beads can be stored overnight at 4 °C in 1 ml TE/TW. Spin down at 1000 x g, 4°C for 1 min and remove supernatant prior to proceeding with Exo I treatment.

Step 2: Exonuclease I treatment

Important: This step removes excess bead primers that did not capture an RNA molecule.

1 Prepare Exonuclease mix as of 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 H2O	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 step 6.

STOPPING POINT. Beads can be stored at 4 °C overnight in 1 ml TE/TW. Spin down at 1000 x g, 4°C for 1 min and remove supernatant prior to proceeding with PCR.

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, mix well to evenly resuspend the beads, and pipette 20 µl of the bead suspension into a Fuchs-Rosenthal haemocytometer to determine bead number per µl.

3 Adjust the bead concentration using nuclease-free water to achieve a final concentration of 200 beads / µl.

4 For each experiment, 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 into a PCR tube.

NOTE: *2000 beads will yield ~100 STAMPs (Single-cell Transcriptome Attached to MicroParticles). Should you be left with less than 2000 beads, we recommend repeating the procedure from the beginning. Spin down the remaining beads and resuspend beads in 1 ml TE/TW. Store at 4°C until use.*

5 Prepare the PCR Master mix according to the table below. The volumes assume 10% extra volume for pipetting losses. Remember to keep the PCR Master mix on ice prior to use.

Component	1 Sample	2 Samples	4 Samples	8 Samples
Nuclease free H_2O	16.06 µl	32.12 µl	64.24 µl	128.48 µl
100 µM SMART PCR PRIMER	0.44 µl	0.88 µl	1.76 µl	3.52 µl
2x Kapa HiFi Hotstart Ready mix	27.5 µl	55.0 µl	110.0 µl	220.0 µl
Total	44.0 µl	88.0 µl	176.0 µl	352.0 µl

6 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
	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.

NOTE: *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 30 µl of room temperature AMPure XP beads to each PCR tube resulting in a 0.6:1 beads to sample ratio. Transfer each sample to a fresh 1.5 ml DNA LoBind Eppendorf tube.

5 Incubate on a rotator for 5 min at room temperature.

6 Place the PCR 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.

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. Do not disturb the pellet. 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 lid, remember not to overdry the pellets (tell-tale cracks form in the pellets).

10 Resuspend each pellet in 12 µl of nuclease-free H_2O.

11 Incubate the suspensions for 5 min at room temperature.

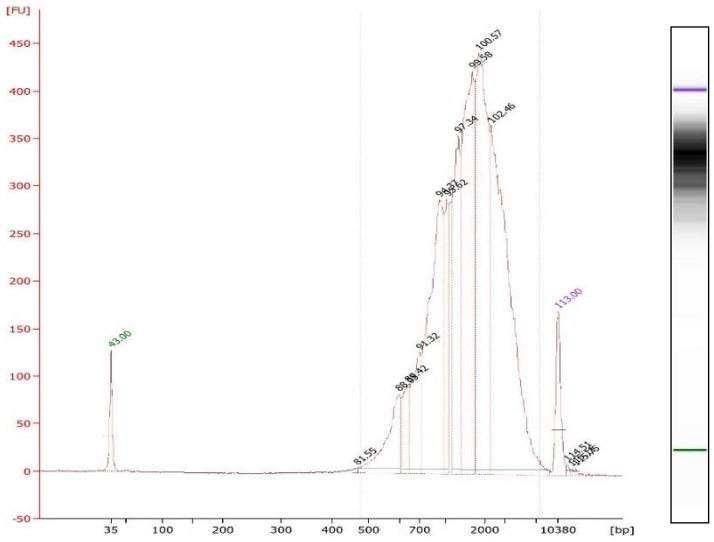
12 Place the tubes back on the magnetic rack and leave for 5 min, or until a solid AMPure bead pellet has formed.

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 BR assay system. A total of 600 pg of purified DNA is required to proceed. Less than 600 pg may compromise the sequencing results.

Note: *When generating standard mixed species libraries (3T3 and HEK293T cells mixed 1:1) you can reasonably expect a yield of 1-3 ng/µl.*

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

Chapter 7. Tagmentation of cDNA with Nextera XT

Required equipment and lab ware

- Thermocycler
- Microcentrifuge
- 0.2 ml PCR tubes
- 1.5 ml DNA LoBind Eppendorf tubes
- Magnetic rack
- Bioanalyzer
- Qubit dsDNA BR 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 BR assay system

Step 1: Tagmentation

1 Preheat the thermocycler to 55°C.

2 For each sample, 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 sample 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 sample. 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. Remember to keep the Tagmentation PCR Master mix on ice prior to use.

Component	1 Sample	2 Samples	4 Samples	8 Samples
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**

Note: *We are aware that some of the 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.

10 Run the following PCR program:

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: PCR Purification and QC

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

12 Prepare appropriate volume of 80% ethanol.

NOTE: *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).*

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

14 Add 30 µl of room temperature AMPure XP beads to each PCR tube resulting in a 0.6:1 beads to sample ratio. Transfer each sample to a fresh 1.5 ml DNA LoBind Eppendorf tube.

15 Incubate on a rotator for 5 min at room temperature.

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

17 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.

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

19 Repeat wash by adding 500 µl of 80% ethanol to each tube. Do not disturb the pellet. 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 lid, remember not to overdry the pellets (tell-tale cracks form in the pellets).

20 Resuspend each pellet in 12 µl of nuclease-free H_2O.

21 Incubate the suspensions for 5 min at room temperature.

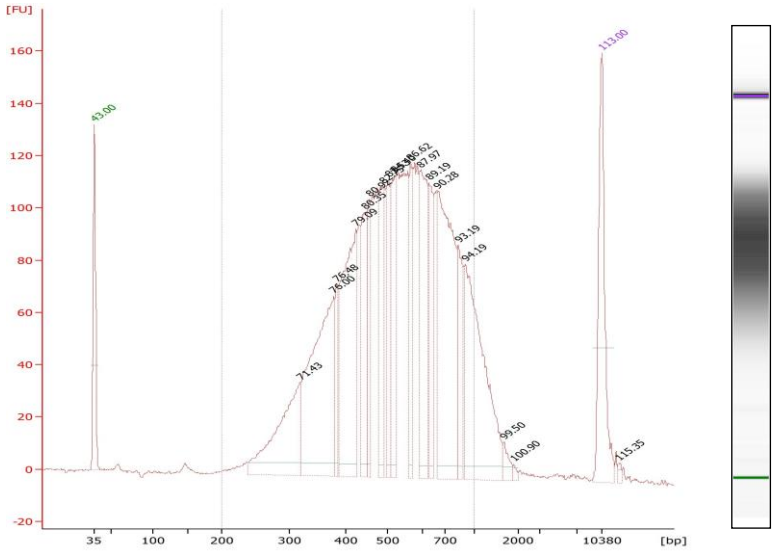
22 Place the tubes back on the magnetic rack and leave for 5 min, or until a solid AMPure bead pellet has formed.

23 Carefully remove the supernatant and pipette into fresh tubes.

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

25 Analyse 1 µl of the purified cDNA sample on a BioAnalyzer High Sensitivity Chip. The cDNA library should have a smooth profile with an average size of 500-680 bp.

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 cDNA library can be stored at -20 for several weeks. Libraries are ready for sequencing according to the Illumina NextSeq or HiSeq Userguide instructions.