dolomite bio

RNAdia kit protocol for nuclei

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Product Information

1. PRODUCT DESCRIPTION

The RNAdia reagent kit enables the processing of biological samples to perform single nucleus RNA-Seq on the Nadia Instrument. Using the RNAdia kit, the Nadia enables the capture of thousands of single nuclei in approximately 30 minutes for subsequent transcriptome analysis by RNA sequencing.

The instructions provided in this document describe the processing of 2 or 8 samples on the Nadia Instrument, along with the description of downstream processing.

2. REAGENTS SUPPLIED IN THE RNADIA REAGENT KIT

Reagent name	Label/abbreviation for reagent	Label/abbreviation for reagent category
Cell Buffer	CB	n/a
1 % BSA	BSA	n/a
Lysis Buffer (-DTT)	LYS (-)	n/a
1 M DTT	DTT	n/a
Barcoded mRNA Capture Beads	BEADS	n/a
Emulsion Oil	OIL	n/a
20X SSC	SSC	n/a
TE-SDS	TE-SDS	n/a
TE-Tween	TE-TW	n/a
20 % w/v Ficoll PM-400	FICL	n/a
20 % PEG	PEG	n/a
5X RT Buffer	RTB	RT
dNTP mix	DNTPs	
RNase Inhibitor	RNAI	
Template Switch Oligo	TSO	
RT Enzyme	RT Enz	
PCR Mix	PCR Mix	PCR
Forward and Reverse PCR Primers	PCR Prim	
Fragmentation Buffer	FB	Lib Prep
Fragmentation Enzyme	F Enz	
Ligation Master Mix	Lig Mix	
Library Amplification Mix	AMP Mix	
Adapter	ADPT	
P5 Primer	P5	
P7 Indexing Primer #1 to #8	P7-1 to P7-8	P7

3. CONSUMABLES SUPPLIED IN THE RNADIA REAGENT KIT

- 5 µm Überstrainer set
- Neubauer Improved haemocytometer C-Chip

4. EQUIPMENT, CONSUMABLES, AND REAGENTS TO BE SUPPLIED BY THE USER

Equipment

- Nadia instrument
- Microcentrifuge with cooling function
- Tube Rotator
- ThermoMixer
- Thermocycler
- Magnetic rack for 0.2ml PCR tubes
- Bioanalyzer (or TapeStation)
- Qubit dsDNA HS assay system
- Dounce tissue grinder set (Sigma, #D8938-1SET)

Consumables

- Eppendorf DNA LoBind 1.5 ml tubes
- 40 µm cell strainer (e.g., Fisher Scientific, # 08-771-1)
- 30 µm cell strainer (e.g., Miltenyi, # 130-098-458)
- 0.2 ml PCR tubes
- 15 ml Falcon tubes
- 50 ml Falcon tubes
- 50 ml or 25 ml Luer-Lok syringe
- Low-retention pipette tips (P1000, P200, P10)
- Low retention gel loading tips (P200, e.g. Gilson, #: F1733481)
- Lint-free wipes
- RNAdia cartridge with the desired number of chips

Reagents

Reagent name	Supplier	Part number
DMEM growth media	Invitrogen	11965092
Fetal Bovine Serum (FBS)	Life Technologies	10437-028
Penicillin/Streptomycin	Life Technologies	15070-063
TrypLE Express Enzyme (1X)	Invitrogen	12604013
0.4 % Trypan Blue stain solution	Gibco	1520061
Nuclease-Free Water	Fisher Scientific	10320995
AMPure XP beads	Beckman Coulter	A63881
Bioanalyzer High Sensitivity DNA Analysis Chip [1]	Agilent	5067-4626
100 % ethanol	Fisher Scientific	10542382
Qubit dsDNA HS assay kit	Invitrogen	Q32851
Nuclei EZ Prep Buffer	Sigma	NUC-101
DNase I (RNase-free, 50U/µl)	Thermo Fisher	EN0523
10X DNase I reaction buffer	Thermo Fisher	EN0523

[1] Or equivalent product for TapeStation system.

02 Buffers and Beads Preparation

1. LYSIS AND WASH BUFFERS

Required equipment and labware

- Eppendorf 1.5 ml tubes
- Microcentrifuge with a cooling function

Required reagents

- Lysis Buffer (-DTT) LYS (-)
- 1 M DTT DTT

Lysis and wash buffers preparation

Lysis Buffer + DTT:

1. Gently defrost the 1 M DTT solution DTT on ice.
2. Spin the tube down to gather all liquid at the bottom of the tube.
3. Add 15 µl of 1 M DTT DTT to the Lysis Buffer tube LYS (-), mix well and store on ice until use.

TE-SDS TE-SDS and TE-Tween TE-TW:

1. Bring both buffers to room temperature before use and vortex vigorously to remove precipitate if necessary.

2. BARCODED BEADS PREPARATION

Required equipment and consumables

- Microcentrifuge with a cooling function
- Low-retention pipette tips (P200, P10)

Required reagents

- Lysis Buffer + DTT
- Barcoded mRNA Capture Beads BEADS

Bead preparation

1. Spin down the tube of Barcoded mRNA Capture Beads BEADS at 1,000 x g, 4 °C for 5 s.
2. Carefully remove supernatant with a P200 tip. Be careful not to disturb the bead pellet.
3. Spin down the barcoded mRNA Capture Beads BEADS again at 1,000 x g, 4 °C for 5 s, and carefully remove remaining supernatant with a P10 tip leaving only pelleted beads.
4. Resuspend the pelleted beads thoroughly in 250 µl of cold Lysis Buffer + DTT and store on ice until use for no longer than 1 h.

IMPORTANT *We recommend using low-retention pipette tips whenever beads are being handled to minimise bead losses.*

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Preparation of Nuclei

The procedure below was developed and tested using mammalian HEK293T (human epithelial kidney) and 3T3 (murine fibroblasts) cell lines. Optimisation may be required for the use of other cell types/tissues that differ significantly from the above.

Required equipment and consumables

- Centrifuge with cooling function
- 50 ml Falcon tubes
- 30 µm cell strainer
- C-Chip Neubauer Improved haemocytometer
- Dounce tissue grinder set

Required reagents

- DMEM growth media
- Fetal Bovine Serum (FBS)
- Penicillin/Streptomycin
- TrypLE Express Enzyme (1X)
- Cell Buffer CB
- 1 % BSA BSA
- RNase inhibitor RNAI
- Nuclei EZ Prep Buffer
- DNase I
- 10X DNase I reaction buffer
- 0.4 % Trypan Blue stain solution
- Nuclease-free water

Required reagents

1. For each sample, gently defrost the 1 % BSA solution BSA on ice.
2. Spin the tube down to gather the liquid at the bottom of the tube.
3. Add 11 µl of 1 % BSA BSA and 1.5 µl of RNase Inhibitor RNAI the Cell Buffer tube CB, mix well and store on ice until use.

Preparation of EZ-DNase_A (EZD-A) and _B (EZD-B)

1. Prepare EZD-A and EZD-B according to the tables below (includes 10 % excess) and keep on ice until use.

EZD-A

Component	n Samples	2 Samples	8 Samples
Nuclei EZ	n x 247 µl	494 µl	1976 µl
10X DNase I reaction buffer	n x 27.5 µl	55 µl	220 µl
DNase I	n x 0.5 µl	1 µl	4 µl
Total	**n x 275 µl**	**550 µl**	**2200 µl**

EZD-B

Component	n Samples	2 Samples	8 Samples
Nuclei EZ	n x 246.5 µl	493 µl	1972 µl
10X DNase I reaction buffer	n x 27.5 µl	55 µl	220 µl
DNase I	n x 1 µl	2 µl	8 µl
Total	**n x 275 µl**	**550 µl**	**2200 µl**

Procedure for preparation of nuclei

1. If cells are adherent: Trypsinise cells using TrypLE, then inactivate with at least 2 volumes of growth-media and spin at 300 x g at room temperature for 1 min. Discard the supernatant and wash the pellet in 10 ml 1X PBS. Spin cells at 300 x g at room temperature for 1 min and discard the supernatant.
2. Resuspend cell pellet in 1 ml PBS, determine the cell density using a Neubauer haemocytometer. Transfer up to 2.5 million cells into a 1.5 ml tube, spin at 300 x g room temperature for 5 mins and discard supernatant.
3. Add 1 ml ice-cold Nuclei EZ to each cell pellet and resuspend thoroughly by pipetting up and down. Transfer cell suspension into glass vessel from the Dounce tissue grinder set.
4. To homogenize cell pellet in cold Nuclei EZ use 20 strokes with pestle “A” followed by 20 strokes with pestle “B” (pestles are provided within the Dounce tissue grinder set).
5. Transfer homogenate into a 1.5 ml DNA LoBind tube. Vortex vigorously 2s “on” 1s “off” x 4 pulses.
6. Pellet nuclei by centrifugation at 500 x g for 5 mins at 4 °C. Discard supernatant then resuspend pellet in 250 µl cold EZD-A. Incubate on ice for 20 mins.
7. Add 250 µl cold Nuclei EZ. Vortex vigorously 2s “on” 1s “off” x 4 pulses.

IMPORTANT *If benchmarking against published data (Habib et al., Nature 2017), nuclei should be prepared from HEK293T and 3T3 cells. Having obtained nuclei, proceed immediately to encapsulation on Nadia. It is NOT recommended to leave nuclei re-suspended in NSB for any extended period of time.*

IMPORTANT *We recommend using Low-Retention Pipette Tips and DNA LoBind tubes whenever nuclei are being handled. This will minimise losses from nuclei being stuck on plastic surfaces.*

IMPORTANT *DNase I treatment and vortexing reduces clumping of nuclei.*

8. Pellet nuclei by centrifugation at 500 x g for 5 mins at 4 °C. Discard supernatant and carefully resuspend nuclei in 250 µl cold EZD-B. Incubate on ice for 20 mins.
9. Add 500 µl cold Nuclei EZ. Vortex vigorously 2s “on” 1s “off” x 4 pulses.
10. Pellet nuclei by centrifugation at 500 x g for 5 mins at 4 °C. Discard supernatant and carefully resuspend nuclei in 1 ml cold Nuclei EZ.
11. Vortex vigorously 2s “on” 1s “off” x 4 pulses.
12. Strain nuclei suspension through a 30 µm cell strainer placed on top of a 15 ml tube.
13. Transfer sieved nuclei into a 1.5 ml DNA LoBind tube. Estimate total volume and vortex vigorously 2s “on” 1s “off” x 8 pulses.
14. Keep aside a small aliquot for Trypan Blue staining (for nuclei counts). To perform Trypan Blue staining and nuclei counts: Mix 1 volume nuclei suspension (in Nuclei EZ) with 1 volume 0.4% Trypan Blue. Visualise on Neubauer haemocytometer and perform counts to determine density of nuclei.
15. Only when ready for encapsulation on Nadia, transfer required number of nuclei into a LoBind tube then pellet nuclei by centrifugation at 500 x g for 5 mins and discard supernatant.
16. Carefully resuspend nuclei in required volume of cold NSB to achieve 300,000 nuclei/ml. If biological material permits, it is recommended to prepare individual suspensions containing at least 300,000 nuclei in NSB. Even if not profiling 300,000 nuclei, a larger volume is more tractable for handling.
17. Proceed immediately to nuclei encapsulation on Nadia.

IMPORTANT *Rinse cell strainer using additional cold Nuclei EZ.*

IMPORTANT *DFor the above, it is crucial to minimise and remove any residual supernatant (Nuclei EZ) before resuspending nuclei in cold NSB. Presence of Nuclei EZ may interfere with successful encapsulation on Nadia.*

IMPORTANT *Be very mindful when handling nuclei in NSB and periodically vortex nuclei suspensions vigorously 2s “on” 1s “off” x 4 pulses. Nuclei resuspended in NSB are prone to clumping.*

IMPORTANT *It is not recommended to keep nuclei in NSB for extended periods of time.*

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Nuclei Encapsulation on Nadia

Required equipment and consumables

- Nadia Instrument
- RNAdia cartridge with the desired number of chips
- Lint-free wipes
- Low retention pipette tips (P1000, P200, P10)
- Low retention gel loading tips (P200)
- Eppendorf DNA LoBind 1.5 ml tubes
- 50 ml Falcon tubes
- 5 µm Überstrainer set
- 50 ml Luer-Lok syringe
- Microcentrifuge with cooling function

Required reagents

- Barcoded mRNA Capture Beads suspension
- Cell suspension
- Emulsion Oil **OIL**
- 20X SSC **SSC**
- Nuclease-free water
- 20 % Ficoll PM-400 **FICL**
- 5X RT Buffer **RTB**
- Template Switch Oligo **TSO**
- dNTP mix **DNTPs**
- RNase Inhibitor **RNAI**

Step 1: Nadia set-up

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 the corresponding slots in the cartridge as shown in the picture on the right. Press “Next” on the Nadia touch screen.
3. Follow the instructions displayed on-screen and remove gasket from the cartridge. The gasket can either be stored on a clean and dust-free surface or lifted up to rest against the Nadia lid. Press “Next” to proceed.
4. Following the on-screen instructions and using a P1000 pipette, load 3 ml of Emulsion Oil **OIL** into the oil reservoir(s) (Figure 1, A). Press “Next” to proceed to the next step.
5. Place the gasket on the cartridge 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

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.*

Step 2: Nuclei encapsulation

1. Press 'Next' on the Nadia screen to open the lid.
2. Vigorously mix the beads mixture by pipetting up and down 10-20 times with low-retention P200 tips.
3. Load 125 µl of the bead suspension using a gel loading tip into the blue flashing bead well (Figure 1, B). To avoid beads sticking to the side of the well, keep the tip vertical, fully pushed into the well and pipette swiftly and smoothly.
4. Repeat using the remaining 125 µl of the bead suspension to insert a total of 250 µl.
5. Press "Next" on the Nadia screen.
6. Carefully mix the nuclei by pipetting up and down 5 times prior to loading.
7. Load 125 µl of the nuclei suspension into the orange flashing well (Figure 1, C).
8. Repeat with remaining 125 µl of the nuclei suspension.
9. Press "Next" on the Nadia screen.
10. Replace the gasket and close the lid to start the encapsulation process.
11. Whilst the Nadia is running, prepare 45 ml of 6X SSC for each sample, according to the table below and allow it to equilibrate to room temperature before use. 6X SSC can be kept as a stock solution at 4°C.

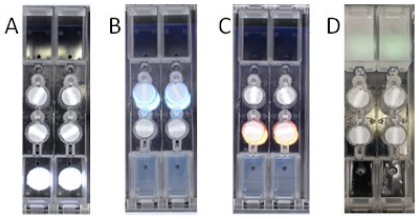


Figure 1: *Guidelights underneath the Nadia cartridge indicate loading positions for the emulsion oil (A), beads (B) and sample (C), and the location of the emulsion (D) to the user after the run is completed.*

Component	n Samples	2 Samples	8 Samples
20X SSC SSC	n x 13.5 ml	27 ml	108 ml
Nuclease-free water	n x 31.5 ml	63 ml	252 ml
Total	**n x 45 ml**	**90 ml**	**360 ml**

12. Also whilst the Nadia is running, prepare the Reverse Transcription (RT) master mix according to the table below (includes 10 % extra). Keep on ice until use.

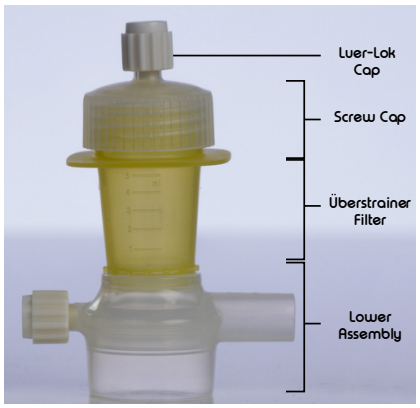
Component	n Samples	2 Samples	8 Samples
Nuclease-Free Water	n x 82.5 µl	165 µl	660 µl
20 % w/v Ficoll PM-400 FICL	n x 44 µl	88 µl	352 µl
5X RT Buffer RTB	n x 44 µl	88 µl	352 µl
Template Switch Oligo TSO	n x 11 µl	22 µl	88 µl
dNTP mix DNTPs	n x 22 µl	44 µl	176 µl
RNase Inhibitor RNAI	n x 5.5 µl	11 µl	44 µl
Total	**n x 209 µl**	**418 µl**	**1672 µl**

13. After 30 mins (20 mins for encapsulation and 10 mins for lysis), the Nadia will prompt the user that the run has completed. Press “Next” to open the lid.
14. Once the Nadia lid has opened, use a P1000 pipette to carefully remove up to 2 ml of the underlying layer of oil within the collection reservoir (Figure 1, D). Be careful not to collect any of the emulsion at this point. Place the oil back in the oil reservoir as it will be used to rinse the collection reservoir to capture as many droplets as possible before proceeding further.
15. Immediately proceed with emulsion breakage.

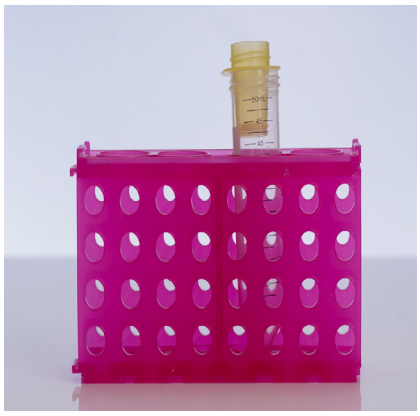
IMPORTANT *The incubation time for the lysis reaction above are optimised for mammalian cell lines HEK293T (human epithelial kidney) and 3T3 (murine fibroblast). Shorter or longer incubation times may be beneficial for other cell types/ tissue.*

Step 3: Emulsion breakage

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

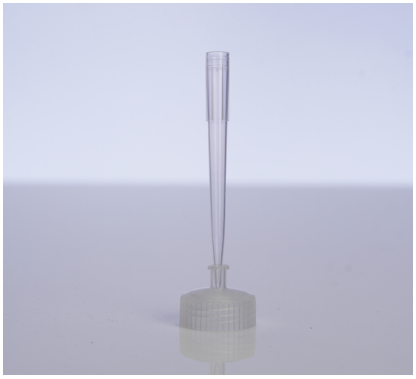


2. Place the coloured Überstrainer filter section inside of an upright 50 ml falcon tube (on the right).
3. Carefully transfer the layer of emulsion from the RNAdia cartridge reservoir onto the Überstrainer filter membrane. Residual oil can be used to wash leftover emulsion from the walls and the bottom of the reservoir. It will accumulate in the far end, making it easy to collect. It is acceptable to transfer some residual oil alongside the emulsion at this point.
4. Replace the Überstrainer screw cap.
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. For each sample, fill a 50 ml Luer-Lok syringe with 40ml of equilibrated 6X SSC buffer. Pull the plunger all the way to the 50 ml graduation to ensure that an air buffer is present. The air buffer is necessary to remove all excess oil from the beads.
7. Attach the syringe to the top of the screw cap (on the right).
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 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 (on the right).
11. By hand, apply downward pressure to the pipette tip until the plastic disk is extruded. Remove pipette tip and discard the disc.
12. Attach the white Luer-Lok cap back onto nozzle of the screw cap. Invert the screw cap and place it nozzle-down into a 1.5 ml microcentrifuge tube rack. Ensure that the assembly is sat firmly on this 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 (on the right).
14. Using a P1000 pipette, add 1 ml of 6X SSC buffer into the Überstrainer. Pipette up and down at least 5 times to resuspend the beads and transfer this bead suspension to a fresh 1.5 ml DNA LoBind microcentrifuge tube.
15. Add another 500 µl of 6X SSC buffer to the Überstrainer. Wash by pipetting up and down and resuspend any residual beads before adding this suspension to the same 1.5 ml DNA LoBind microcentrifuge tube as before.
16. Spin down the tube at 1000 x g at 4°C for 1 min to pellet the beads. Remove the supernatant.
17. Perform a final wash step by adding 300 µl of 5X RT buffer RTB to the bead pellet. Mix by pipetting up and down 5 times.
18. Spin at 1000 x g, 4°C for 1 min and remove as much of the supernatant as possible without disturbing the bead pellet.
19. Immediately proceed with reverse transcription.

NOTE *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 and centrifuge for 1 min at 1000 x g, 4°C.*

Reverse Transcription and PCR Amplification

Required equipment and consumables

- Tube rotator
- Neubauer Improved haemocytometer C-Chip
- 0.2 ml PCR tubes
- Eppendorf DNA LoBind 1.5 ml tubes
- ThermoMixer
- Thermocycler
- Microcentrifuge with cooling function
- Magnetic rack for 0.2 ml PCR tubes
- Bioanalyzer (or TapeStation)
- Qubit dsDNA HS assay system
- Bioanalyzer High Sensitivity DNA Analysis Chip (or TapeStation equivalent)
- Pipette tips P1000, P200, P10

Required reagents

- RT master mix
- RT Enzyme **RT Enz**
- TE-SDS **TE-SDS**
- TE-Tween **TE-TW**
- Nuclease-free water
- 20 % PEG **PEG**
- Forward and Reverse PCR Primers **PCR Prim**
- PCR Mix **PCR Mix**
- AMPure XP beads
- 80 % ethanol
- Qubit dsDNA HS assay kit

Step 1: Reverse Transcription

1. Add the required amount of RT Enzyme **RT Enz** to the previously prepared RT master mix according to the table below (includes 10 % excess). Mix well by pipetting up and down.

Component	n Samples	2 Samples	8 Samples
RT master mix	n x 209 µl	418 µl	1672 µl
RT Enzyme **RT Enz**	n x 11 µl	22 µl	88 µl
Total	**n x 220 µl**	**440 µl**	**1760 µl**

2. Add 200 µl of the RT master mix to the recovered, washed beads and mix by pipetting up and down at least 5 times.
3. On a tube rotator, incubate each sample for 15 mins at room temperature. Then transfer the tube to a ThermoMixer, preheated to 42 °C, and incubate for a further 60 mins at 400 rpm and 42 °C.
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 **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-Tween **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 once.

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

Step 2: PCR

1. Wash the bead pellet in 1 ml of nuclease-free water, spin down at 1000 x g, 4 °C for 1 min and discard the supernatant.
2. Resuspend bead pellet in 1 ml of nuclease-free water and mix well. Take a 20 µl aliquot of bead suspension, mix well with 20 µl of 20% PEG **PEG** in a Lo-Bind 1.5 ml Eppendorf tube. Load 10 µl of the mix into a Neubauer Improved haemocytometer C-Chip. Count the beads under the microscope to determine the bead number per µl.
3. Adjust the bead concentration using nuclease-free water to achieve a final concentration of 800 beads/µl. 8,000 beads should be used in one individual PCR reaction.
4. Prepare the PCR master mix as per the table on the right (includes 10 % extra). Keep the PCR master mix on ice before use.

Component	n PCR reactions	2 PCR reactions	15 PCR reactions
Nuclease-free water	n x 12.1 µl	24.2 µl	181.5 µl
PCR Mix **PCR Mix**	n x 27.5 µl	55 µl	412.5 µl
Fw + Rv Primers **PCR Prim**	n x 4.4 µl	8.8 µl	66 µl
Total	**n x 44 µl**	**88 µl**	**660 µl**

5. For each PCR reaction, mix the beads by pipetting up and down until all beads are evenly resuspended, and pipette 10 µl of the bead suspension (containing 8,000 beads) into 0.2 ml PCR tubes.
6. Add 40 µl of the PCR master mix to each PCR tube and mix well by pipetting up and down.
7. Spin down any remaining beads and resuspend in 1 ml TE-Tween **TE-TW**. Beads in this buffer can be stored at 4°C for up to 1 week.
8. Incubate the reactions in a thermocycler with a heated lid using the following PCR programme:

Cycle numbers	Temperature	Time
1 cycle	95°C	3 min
4 cycles	98°C 60°C 72°C	20 s 45 s 3 min
9 cycles (possible range from 8 to 12 cycles*)	98°C 62°C 72°C	20 s 20 s 3 min
1 cycle	72°C	3 min
	4°C	Hold

** The default recommendation of 9 PCR cycles was established using mammalian cell lines with high transcriptional activity. PCR cycle number may need to be optimised based on specific sample sources. In general, we recommend the addition of +1 PCR cycle for primary tissue.*

***NOTE** Ensure that the beads are evenly distributed within the haemocytometer slide by pipetting them into the aperture swiftly and smoothly with the chip aligned flat on a benchtop. Count the number of beads occupying each of the 4 large corner squares which are divided into 16 smaller squares and calculate the bead concentration according to the following equation:*

$$\text{Beads per µl} = \frac{\text{(Beads in 4 large squares)}}{4} \times 2\ \text{(dilution factor)} \times 10\ \text{(volume factor)}$$

If all steps have been followed accurately up to this point, users can expect a total bead yield of 60,000 – 120,000 beads.

***NOTE** 8000 beads will yield ~280 STAMPs (Single-cell Transcriptome Attached to MicroParticles).*

***NOTE** The RNAdia kit contains enough reagents to perform up to 15 PCR reactions per sample, each reaction containing 8,000 beads.*

***STOPPING POINT.** PCR reactions can be stored overnight at -20 °C. PCR purification must be performed the next day.*

Step 3: PCR purification

1. Remove AMPure XP beads from the fridge and equilibrate to room temperature (this takes ~30 mins).
2. Prepare 500 µl of 80 % ethanol for each PCR reaction. Do not store unused 80 % ethanol.
3. Vortex AMPure XP beads thoroughly to mix suspension before use. Use a combination of pipetting and vortexing to resuspend any pellet before use.
4. Add the appropriate volume of room temperature AMPure XP beads to each tube resulting in a **0.6:1** ratio of beads to sample. In this case, to purify 50 µl of sample, 30 µl of beads are required.
5. Mix well by pipetting 40 µl up and down at least 30 times. Avoid producing bubbles. Inadequate mixing can compromise binding of DNA onto the beads.
6. Incubate for 5 mins at room temperature.
7. Place the 0.2 ml PCR tubes onto a magnetic rack and leave standing for 5 mins at room temperature, or until a solid AMPure bead pellet has formed.
8. Whilst keeping the tubes on the magnetic rack, carefully remove the supernatant without disturbing the brown AMPure bead pellet.
9. On the magnetic rack, wash each AMPure bead pellet with 200 µl of 80 % ethanol. Remove the supernatant and discard.
10. Repeat wash on the magnetic rack by adding 200 µl of 80 % ethanol to each tube. Remove the supernatant and discard.
11. Leave the tubes with lids open on the magnetic rack for 3 mins to air dry. Do not over-dry the pellets (tell-tale cracks form in the pellets).
12. Take the tubes off the magnetic rack and carefully resuspend each pellet with 12 µl of nuclease-free water.
13. Incubate the suspensions for 2 mins at room temperature.
14. Place the tubes back on the magnetic rack and leave until a solid AMPure bead pellet has formed (~2-3 mins).
15. Carefully remove 10 µl of the supernatant and pipette into new 0.2 ml PCR tubes.

IMPORTANT *Ethanol is hygroscopic. Washing AMPure beads with overdiluted ethanol may compromise library yield. Always prepare 80 % ethanol fresh from a stock of ethanol absolute.*

IMPORTANT *Employ maximum precision with the volumes of AMPure XP beads added. Any deviation can compromise the final outcome.*

IMPORTANT *Always keep the beads on the magnetic rack when removing the supernatant. Avoid dislodging the bead pellet.*

NOTE *Use a P10 pipette to remove and discard any remaining 80% ethanol. Residual ethanol can be detrimental to success.*

NOTE *If storing for an extended period of time, eluting cDNA with nuclease-free 10 mM Tris (pH 7.5), instead of nuclease-free water, can be beneficial.*

Step 4: Quantification

1. Determine the cDNA concentration of each sample using 1 µl of purified product and a fluorometric-based system such as the Qubit dsDNA HS system.

STOPPING POINT. *Purified PCR products can be stored at -20 °C for several days.*

IMPORTANT *Ideally, a total of at least 1 ng of purified DNA is required to proceed further. However, the user can still perform library preparation despite the cDNA library being non-quantifiable on the Qubit system. The additional amplification step during library preparation is likely to generate a quantifiable product. In this case, use the maximum possible volume as a reaction input in the subsequent step, i.e., 40 µl.*

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

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Sequencing Library Preparation

Required equipment and consumables

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

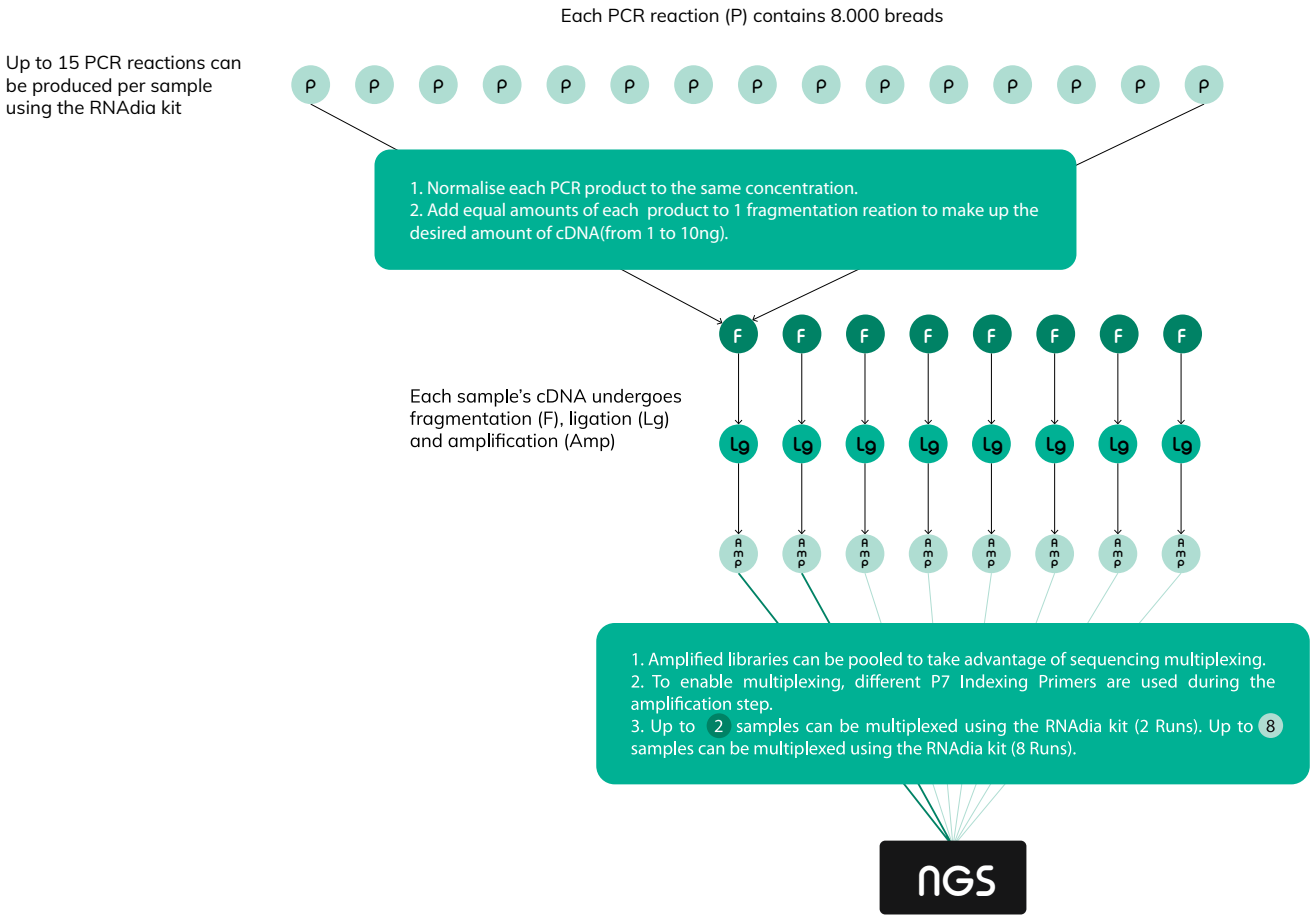
Required reagents

- Fragmentation Buffer **FB**
- Fragmentation Enzyme **F Enz**
- Adapter **ADPT**
- Nuclease-free water
- Ligation Master Mix **Lig Mix**
- Library Amplification Mix **AMP Mix**
- P5 Primer **P5**
- P7 Indexing Primer(s) **P7**
- AMPure XP beads
- 80 % Ethanol
- Qubit dsDNA HS assay kit
- The RNAdia kit (2 Runs) contains 2 different P7 Indexing Primers, allowing for 2 samples to be multiplexed for subsequent sequencing. The RNAdia kit (8 Runs) contains 8 different P7 Indexing Primers, allowing for up to 8 samples to be multiplexed.
- The following procedure assumes usage of 0.2 ml PCR strips.
- The RNAdia kit contains sufficient reagents for one library preparation reaction per sample, and a minimum of 1 ng of cDNA is the recommended total input for one library prep reaction.
- The user must pool all PCR reactions from the same sample to fit into one library prep reaction. This is done by first normalising the concentration of each purified PCR product and then adding equal amounts of each product to the fragmentation reaction to make up the desired total amount of cDNA (see illustration below).

  For example, to prepare a total input of 1 ng for library preparation when starting off with 10 PCR reactions, normalise concentrations to e.g., 100 pg/µl and add 1 µl from each reaction to make up the 1 ng of input.

IMPORTANT *Illumina's Nextera XT kit is* **NOT** *compatible with cDNA libraries produced with the RNAdia kit. Always use the sequencing library preparation reagents provided by Dolomite Bio to process the RNAdia cDNA libraries.*

NOTE *A DNA input quantity of up to 10 ng can be used, within the constraint of the 40 µl input volume (see below). Additional library prep reagents are provided to enable the user to test 2 different input quantities (e.g., 1 then 10 ng).*



Step 1: Enzymatic fragmentation, blunt repair and A-tailing

1. Based on the fluorometric quantification measurement after PCR purification, for each sample, normalise the concentration of each PCR reaction and add equal amounts of each PCR reaction product in a new 0.2 ml PCR tube up to 1 ng or more of purified cDNA, as described above.
2. Add nuclease-free water to make up a final volume of 40 µl.
3. Prepare the fragmentation master mix as per the table below (includes 10% extra). Prepare 1 fragmentation reaction per sample.

Component	n Reactions	2 Reactions	8 Reactions
Fragmentation Buffer FB	n * 4.4 µl	8.8 µl	35.2 µl
Fragmentation Enzyme F Enz	n * 6.6 µl	13.2 µl	52.8 µl
Total	**n * 11 µl**	**22 µl**	**88 µl**

4. Mix well by pipetting up and down, spin down to collect liquid at bottom of tube, keep the tube on ice before use but for no longer than 2 h.
5. To each 40 µl of purified cDNA, add 10 µl of master mix for a total volume of 50 µl.
6. Spin down briefly to collect all liquid within the tube.

IMPORTANT *Mix well by pipetting 30 µl up and down at least 30 times. Avoid producing bubbles.*

7. Incubate in a thermocycler with heated lid using the temperatures and durations in the sequence shown below:

Temperature	Time
30°C	10 mins
65°C	30 mins
4°C	**Hold**

Step 2: Adapter ligation

1. Prepare the required quantity of 1.5 µM Adapter according to the table below. Mix well and keep on ice for no longer than 2 h.

Component	2 Reactions (minimum)	n Reactions
Adapter ADPT	1.1 µl	n x 0.55 µl
Nuclease free water	9.9 µl	n x 4.95 µl
Total	**11 µl**	**n x 5.5 µl**

2. When the fragmentation, repair and A-tailing reaction is finished, pipette 20 µl of the Ligation Master Mix Lig Mix into each sample reaction for a total volume of 70 µl.
3. Add 5 µl of 1.5 µM Adapter for a total volume of 75 µl.
4. Incubate in thermocycler 20°C for 20 mins. This is best done without heated lid to given that the working temperature is relatively low.
5. Once the incubation is finished, immediately proceed with post-ligation clean-up.

IMPORTANT *Ensure samples have been cooled on ice before continuing.*

NOTE *The minimum number of Adapter reactions that should be prepared is 2.*

IMPORTANT *Prepare only as much as required. The diluted 1.5 µM Adapter should not be stored for future use.*

IMPORTANT *Mix well by pipetting 40 µl up and down at least 30 times. Avoid creating bubbles. Inadequate mixing can produce variability in the final result.*

Step 3: Post-ligation clean-up

1. Remove AMPure XP beads from the fridge and equilibrate to room temperature.
2. Prepare 500 µl of 80 % ethanol for each PCR reaction. Do not store unused 80 % Ethanol.
3. Vortex AMPure XP beads thoroughly to mix suspension before use. Use a combination of pipetting and vortexing to resuspend any pellet before use.
4. Add appropriate volume of room temperature AMPure XP beads to each sample tube resulting in a **0.8:1** ratio of beads to sample. In this case, to purify 75 µl of sample, 60 µl of beads are required.
5. Mix well by pipetting 70 µl up and down at least 30 times. Avoid producing bubbles. Inadequate mixing can compromise binding of DNA onto the beads.
6. Incubate for 5 mins at room temperature.
7. Place the 0.2 ml PCR tubes onto a magnetic rack and leave standing for 5 min at room temperature, or until a solid AMPure bead pellet has formed.

IMPORTANT *Ethanol is hygroscopic. Washing AMPure beads with overdiluted ethanol may compromise the library yield. Always prepare 80 % ethanol fresh from a stock of ethanol absolute.*

IMPORTANT *Employ maximum precision with volumes of AMPure XP beads added. Any deviation can compromise the final outcome.*

8. Keeping the tubes on the magnetic rack, carefully remove the supernatant without disturbing the brown AMPure bead pellet.
9. On the magnetic rack wash each AMPure bead pellet with 200 µl of 80 % ethanol. Remove the supernatant and discard.
10. Repeat wash on the magnetic rack by adding 200 µl of 80 % ethanol to each tube. Remove the supernatant and discard.
11. Leave the tubes with lids open on the magnetic rack for 3 mins to air dry. Do not over-dry the pellets (tell-tale cracks form in the pellets).
12. Take the tubes off the magnetic rack and carefully resuspend each pellet with 22 µl of nuclease-free water.
13. Incubate the suspensions for 2 mins at room temperature.
14. Place the tubes back on the magnetic rack and leave until a solid AMPure bead pellet has formed (~2-3 mins).
15. Carefully remove 20 µl of the supernatant and pipette into new 0.2 ml PCR tubes. Immediately proceed with library amplification.

IMPORTANT *Always keep the beads on the magnetic rack when removing the supernatant. Avoid dislodging the bead pellet.*

NOTE *Use a P10 pipette to remove and discard any remaining 80 % ethanol. Residual ethanol can be detrimental to success.*

Step 4: Library amplification

When multiplexing various numbers of samples, the user must use the following P7 index combinations:

NOTE *For multiplexing, each sample reaction must be tagged with a different P7 Indexing Primer* **P7**. *In this case, first prepare the amplification master mix without the P7 Indexing Primer* **P7**. *Next, divide the master mix into tubes and add the relevant P7 Index to individual reactions.*

Number of samples to multiplex	P7 index combinations (RNAdia 8 Runs)	P7 index combinations (RNAdia 2 Runs)
2	1+3 / 2+3 / 2+4 / 4+5 / 1+6 / 7+8	1+3 (as provided)
4	1+2+3+4 / 5+6+7+8	n/a
8	1+2+3+4+5+6+7+8	n/a

1. Prepare the amplification master mix as per the table below (includes 10 % extra). Mix well and keep on ice for no longer than 1 h before use.

Component	n Reactions	2 Reactions	8 Reactions
Library Amplification Mix **AMP Mix**	n * 27.5 µl	55 µl	220 µl
P5 Primer **P5**	n * 2.75 µl	5.5 µl	22 µl
P7 Indexing Primer **P7**	n * 2.75 µl	5.5 µl	22 µl
Total	**n * 33 µl**	**66 µl**	**264 µl**

2. To each 20 µl of purified DNA from the previous step, add 30 µl of the above prepared amplification master mix. The final volume in each tube is 50 µl.
3. Incubate the library amplification reactions in a thermocycler with a heated lid and run the appropriate PCR program detailed below. Depending on the amount of input DNA used, the number of cycles should be varied as indicated:

IMPORTANT *Mix very well by pipetting a 30 µl volume of liquid at least 30 times. Avoid producing bubbles. Inadequate mixing can lead to variability in the end result.*

Cycle numbers	Temperature	Time
1 cycle	98°C	45 s
4 cycles	98°C 53°C 72°C	15 s 30 s 30 s
See details below	98°C 60°C 72°C	15 s 30 s 30 s
1 cycle	72°C	1 min
	12°C	Hold

Input DNA	Cycle numbers
1 ng (or not quantifiable after PCR)	8 cycles
2-4 ng	4 cycles
5-10 ng	3 cycles

Step 5: Clean-up of amplified library and QC

1. Remove AMPure XP beads from the fridge and equilibrate to room temperature.
2. Prepare 500 µl of 80 % ethanol for each PCR reaction. Do not store unused 80 % Ethanol.
3. Vortex AMPure XP beads thoroughly to mix suspension before use. Use a combination of pipetting and vortexing to resuspend any pellet before use.
4. Add appropriate volume of room temperature AMPure XP beads to each tube resulting in a **0.8:1** ratio of beads to sample. In this case, add 40 µl of beads to 50 µl of sample.
5. Mix well by pipetting 45 µl up and down at least 30 times. Avoid producing bubbles. Inadequate mixing can compromise binding of DNA onto the beads.
6. Incubate for 5 mins at room temperature.
7. Place the 0.2 ml PCR tubes onto a magnetic rack and leave standing for 5 mins at room temperature, or until a solid AMPure bead pellet has formed.
8. Keeping the tubes on the magnetic rack, carefully remove the supernatant without disturbing the brown AMPure bead pellet.
9. On the magnetic rack wash each AMPure bead pellet with 200 µl of 80 % ethanol. Remove the supernatant and discard.
10. Repeat wash on the magnetic rack by adding 200 µl of 80 % ethanol to each tube. Remove the supernatant and discard.
11. Leave the tubes with lids open on the magnetic rack for 3 mins to air dry. Do not over-dry the pellets (tell-tale cracks form in the pellets).
12. Take the tubes off the magnetic rack and carefully resuspend each pellet with 14 µl of nuclease-free water. Avoid producing bubbles.
13. Incubate the suspension for 2 mins at room temperature.
14. Place the tubes back on the magnetic rack and leave until a solid AMPure bead pellet has formed (~2-3 mins).

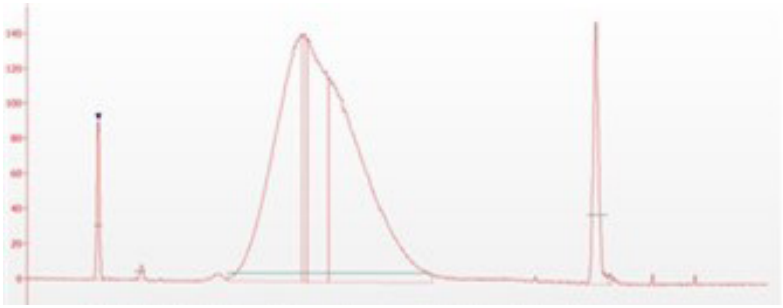
IMPORTANT *Ethanol is hygroscopic. Washing AMPure beads with overdiluted ethanol may compromise the library yield. Always prepare 80 % ethanol freshly from a stock of ethanol absolute.*

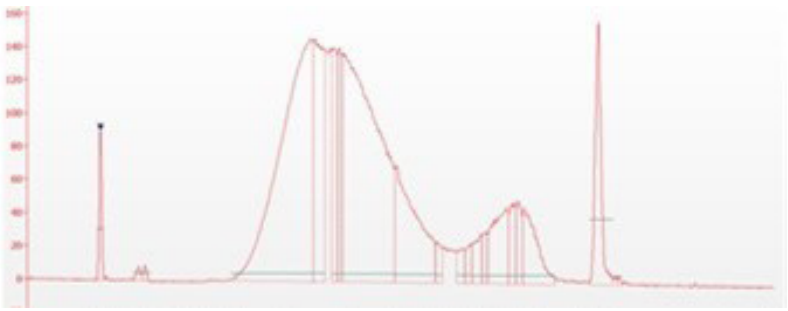
NOTE *Employ maximum precision with volumes of beads added. Any deviation can compromise the final outcome.*

IMPORTANT *Always keep the beads on the magnetic rack when removing the supernatant. Avoid dislodging the bead pellet.*

IMPORTANT *Use a P10 pipette to remove and discard any remaining 80 % ethanol. Residual ethanol can be detrimental to success.*

15. Carefully remove 12 µl of the supernatant and pipette into new 0.2 ml PCR tubes.
16. Determine each sample cDNA concentration using 1 µl of purified product and a fluorometric based system such as the Qubit dsDNA HS system.
17. Analyse 1 µl of the purified product on a Bioanalyzer High Sensitivity DNA Analysis Chip (or TapeStation equivalent).





NOTE *If storing for an extended period of time, eluting DNA with nuclease-free 10 mM Tris (pH 7.5) instead of nuclease-free water can be beneficial.*

IMPORTANT *The fragmented library should have a profile resembling on the left shown examples with an average size of 400-500 bp. Both types of profile are suitable for NGS.*

STOPPING POINT. *The fragmented library can be stored at -20°C for several months. Libraries are now ready for NGS.*

07

Additional Information

GUIDELINES FOR NGS (ILLUMINA)

Volume and concentration requirements

A provider of NGS will typically require 30 µl of a library that is at least 2 nM in concentration. It is recommended to exceed 2 nM as much as possible. This affords the provider of NGS versatility when clustering on the sequencer's flow cell and increases the likelihood of a good outcome from NGS.

Molecular architecture of DNA insert produced with RNAdia kit

The RNAdia workflow culminates in libraries that are compatible with Illumina's single indexed Paired End sequencing. The molecular architecture of a DNA insert following library preparation is illustrated below:

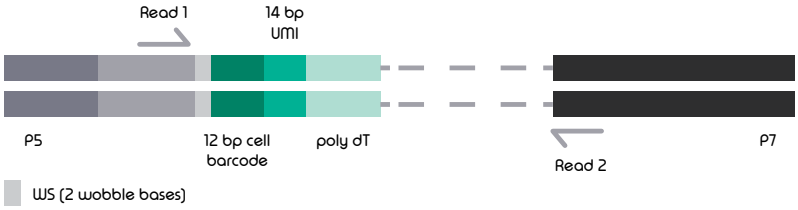


Figure 2: *Molecular architecture of the DNA-insert after library preparation. Read 1 includes the WS, cell barcode and UMI whilst Read 2 has transcript information.*

Multiplexing, recommended read lengths and sequencing depth

Read lengths for Read 1 and Index Read, if applicable, are mandatory. Read length for Read 2 is recommended. Consult your NGS provider to determine the most cost-effective options available.

Read	Length
Read 1	28 bp
Index Read	8 bp
Read 2	>91 bp

With above read lengths, use the standard Illumina P5 Sequencing Primer for Read 1 and the standard Illumina P7 Sequencing Primer for Read 2.
The inclusion of 1 % PhiX is recommended to improve overall read qualities.
The recommended sequencing depth on Illumina machines is 25k read pairs per cell.

Indexes available in the RNAdia kit

Give the relevant 'bases for sample sheet' sequence to your NGS provider.

Name of P7 Indexing Primer	Bases for sample sheet
P7-1	CTGATCGT
P7-2	ACTCTCGA
P7-3	TGAGCTAG
P7-4	GAGACGAT
P7-5	CTTGTCGA
P7-6	TTCCAAGG
P7-7	CGCATGAT
P7-8	ACGGAACA

RNADIA PARTS LIST

RNAdia kits

Part number	Part name
3200971	RNAdia Kit (8 Runs)
3200972	RNAdia Kit (2 Runs)

RNAdia cartridges

Part number	Part name
3200973	Nadia Cartridge for RNAdia 8 Runs (8x1)
3200974	Nadia Cartridge for RNAdia 8 Runs (2x4)
3200975	Nadia Cartridge for RNAdia 8 Runs (4x2)
3200976	Nadia Cartridge for RNAdia 8 Runs (2x2 & 1x4)
3200977	Nadia Cartridge for RNAdia 8 Runs (1x8)
3200978	Nadia Cartridge for RNAdia 40 Runs (40x1)
3200979	Nadia Cartridge for RNAdia 40 Runs (10x2 & 5x4)
3200980	Nadia Cartridge for RNAdia 40 Runs (5x8)
3200985	Nadia Cartridge for RNAdia 2 Runs (1x2)

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