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Nova-ST Spatial Transcriptomics protocol

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ABSTRACT

Nova-ST is an open-source, high-resolution sequencing based spatial transcriptomics workflow. This method gives comparable resolution to BGI Stereoseq, SeqScope & PIXEL seq. Nova-ST is derived from dense nano-patterned randomly barcoded Illumina NovaSeq 6000 S4 sequencing flow cells. More details in the [Nova-ST pre-print](#). Nova-ST enables customized, low cost, flexible, and high-resolution spatial profiling of broad range of tissue section sizes (upto 10mm x 8 mm). In this protocol, we provide detailed step-by-step resource for implementing the Nova-ST spatial transcriptomics workflow in your lab. Bioinformatics and data analysis workflows are detailed in:

<https://github.com/aertslab/Nova-ST>. For any protocol related or data analysis clarifications, you can reach out to us via nova.st.aertslab@gmail.com.

GUIDELINES

The Nova-ST workflow is based on fresh frozen tissue samples embedded in cryo-medium.

Following oligonucleotides are used in this protocol:

 TableS1.xlsx 10KB

Protocol status: Working
We use this protocol and it's working

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MATERIALS

Reagents:

- ☒ Pepsin from porcine gastric mucosa **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P7000**
- ☒ Hydrochloric Acid Solution, 0.1N (N/10) (Certified), Fisher Chemical **Fisher Scientific Catalog #7647-01-0**
- ☒ 1x Maxima H- RT buffer **Fisher Scientific Catalog #EP0753**
- ☒ Maxima H-RT enzyme **Fisher Scientific Catalog #EP0753**
- ☒ dNTP Mix (25 mM each) **Thermo Fisher Catalog #R1121**
- ☒ Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #34860-1L-R**
- ☒ Visium Spatial Gene Expression Reagent Kits **10x Genomics Catalog #1000192**
- ☒ 2-Propanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #59304-100ML-F**
- ☒ RNase Inhibitor **Lucigen Catalog #30281-1**
- ☒ Ficoll PM-400 20% in H₂O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F5415-50ML**
- ☒ UltraPure® SSC, 20X **Thermo Fisher Catalog #15557044**
- ☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**
- ☒ dNTP Mix (25 mM each) **Thermo Fisher Catalog #R1121**
- ☒ Exonuclease I (E.coli) - 15,000 units **New England Biolabs Catalog #M0293L**
- ☒ Klenow Fragment (3'-5' exo-) - 1,000 units **New England Biolabs Catalog #M0212L**
- ☒ Tris-HCl, 1M Solution, pH 8.0, Molecular Biology Grade, Ultrapure, Thermo Scientific Chemicals **Thermo Fisher Scientific Catalog #J22638.AE**
- ☒ NaCl (5 M), RNase-free **Thermo Fisher Catalog #AM9760G**
- ☒ SDS, 10% Solution **Life Technologies Catalog #AM9822**
- ☒ EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #AM9260G**
- ☒ Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #P8107S**
- ☒ UltraPure® 1 M Tris-HCl Buffer, pH 7.5 **Thermo Fisher Catalog #15567027**

- ☒ Klenow Fragment (3'-5' exo-) - 1,000 units **New England Biolabs Catalog #M0212L**
- ☒ Tris solution (1M pH 8 RNase-free) **Thermofisher Catalog #AM9851**
- ☒ AMPure XP Beads **Beckman Coulter Catalog #A63882**
- ☒ HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #KK2601**
- ☒ Buffer EB **Qiagen Catalog #19086**

Instruments:**Equipment**

Incubator	NAME
Memmert	BRAND
Incubator IN55	SKU

Equipment

2100 Bioanalyzer Instrument	NAME
Sizing, quantification, and sample quality control of DNA, RNA, and proteins on a single platform	TYP E
Agilent Technologies	BRAND
G2939BA	SKU

Equipment

	NAME
Qubit 2.0 Fluorometer instrument	BRAND
Q33226	SKU
with Qubit RNA HS Assays	SPECIFICATIONS

Tissue preparation for spatial transcriptomics profiling

- 1 The first steps of the Nova-ST workflow starts with preparation of tissue for cryo-embedding. Follow the tissue preparation guidelines from [10X Genomics Visium Tissue Preparation guide](#)

Other useful video resources available from 10X Genomics:

1. [Flash Freezing of Tissue.](#)
2. [Embedding Frozen Tissue in OCT.](#)
3. [Simultaneous Flash Freezing and Embedding in OCT.](#)
4. [Cryosectioning of OCT Embedded Tissue Block](#)

- 2 Assess the RNA quality of the OCT embedded tissue using the guidelines in the [10X Genomics Visium Tissue Preparation guide](#). Refer the section: **RNA Quality Assessment**.



Note

It is recommended to use samples with RIN quality value of atleast >7. Sample with lower RIN value can be used but this can negatively impact the Nova-ST sequencing library quality.

Assessment of tissue permeabilization time

- 3 Follow the [10X Genomics Visium Tissue Preparation guide](#), to place 8 consecutive tissue section in the capture area slots of the Visium Spatial Tissue Optimization Slide (PN: 3000394). The optimization slide



with sectioned tissue can be preserved at -80°C as recommended by the manufacturer.

- 4 Perform H&E staining of the tissue sections on the Visium Spatial Tissue Optimization Slide using [Methanol Fixation, H&E Staining & Imaging for Visium Spatial Protocols](#).
- 5 To perform tissue permeabilization: the protocol in [Visium Spatial Tissue Optimization Reagents Kits User Guide](#) is followed with the following key differences:
 - 5.1 Prepare 100mg/ml of pepsin stock by dissolving the pepsin powder with 0.1N HCl (pH 2). Mix the solution with P1000 pipette, till the enzyme is homogenized. DONOT vortex the enzyme stock. Prepare aliquots of the enzyme stock for single use and store them in -20 °C for long term storage. On the day of the experiment thaw the 100mg/ml of pepsin stock on ice.

 - 5.2 Prepare 0.65U/ul working stock of Pepsin enzyme by diluting the 100 mg/ml enzyme stock with 0.1N HCl (pH 2) warmed to 37 °C .
 - 5.3 In the [Visium Spatial Tissue Optimization Reagents Kits User Guide](#) replace the permeabilization enzyme (PN: 2000214) form the 10X Genomics Visium Spatial Tissue Optimization kit with the pepsin permeabilization reagent prepared above.
 - 5.4 Choose a time course with 8 different time point to perform the tissue permeabilization experiment. The time points for the time course may be adjusted depending on the tissue types being handled.

Preparation for Nova-ST profiling

6 Preparation of Nova-ST chips for Spatial Transcriptomics:

Things to prepare:

- Put the PCR block at 37°C and place the 10X Genomics thermocycler adapter (PN:3000380) into the block and let the adapter equilibrate to 37°C.

6.1 Retrieve the 24-well plate with Nova-ST chip stored in the 1X IDT TE () buffer from the  fridge. Remove the parafilm seal.

6.2 Choose the desired chip number/s from the storage plate. Register the corresponding Nova-ST chip ID/s (this will be needed for later ST data analysis).

6.3  Remove and discard the storage TE buffer from the corresponding well/s. Using a sharp forceps retrieve the Nova-ST chip/s and place it into a fresh 24 well plate (the functional surface of the chip must face the top). Ensure not to disturb the functional surface of the Nova-ST while handling the chip

6.4 Add 3 ml of NFW, drop by drop on to the surface of the chip to wash the chip. Discard the wash and repeat this step for a total of 3X times.

6.5 Transfer the chip to fresh well in the 24 well plate. Using either an air gun or pressurized air canister, blow away excess liquid on the surface of the chips. With the help of forceps, transfer the chip to the thermocycler adapter at 37°C. Incubate for 1 min for the chip to dry. Then proceed for the cryo-sectioning of the tissue.

7 Tissue sectioning for Nova-ST chips:

- Follow the general guidelines and good practices for sectioning of a  tissue section. Follow the guidelines and recommendations in [10X Genomics Visium Tissue Preparation guide](#).

Additional useful resources in: [Cryosectioning of OCT Embedded Tissue Block](#).

7.1 Label a 3cm petridish with the chip number and couple of glass slides and place them into cryostat.
Allow them to cool to cryostat temperature for atleast 30 mins.

- 7.2 Once the glass slide has cooled to cryostat temperature, add 2 drops of OCT on the glass slide to hold the Nova-ST chip on the glass slide and to prevent the chip from sliding away from the slide.

Note

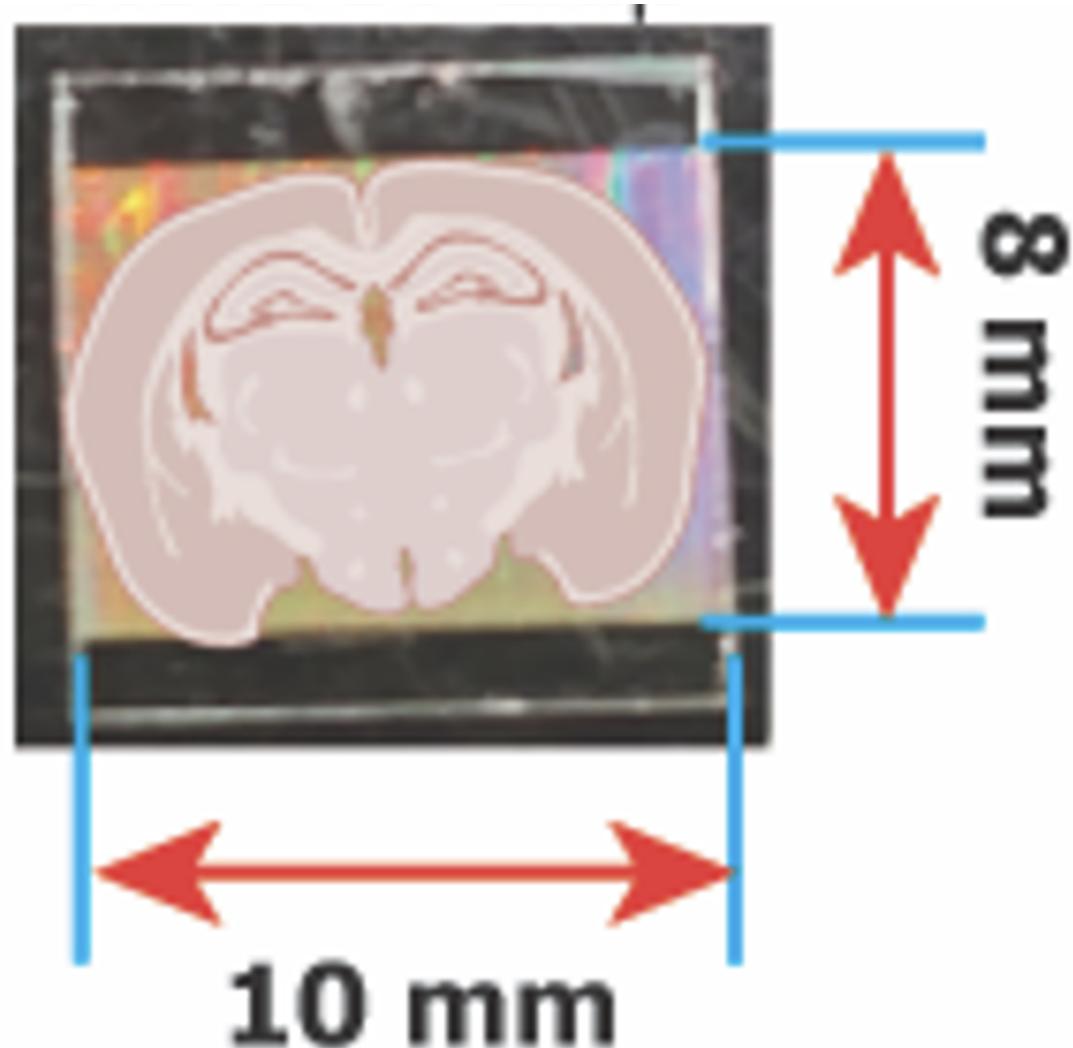
This is important for cryostats where the sample stage of the cryostat is inclined. Example: [Epredia CryoStar NX70](#). For these cryostats freeze the drop of OCT on the glass side with a distance of ~1 cm to hold the Nova-ST chips and preventing it from sliding (as shown below).



- 7.3 Place the Nova-ST chip on the glass slide cooled inside the cryostat for 00:03:00 prior to 3m sectioning.

- 7.4 Place the sectioned tissue and adjust the position of the tissue to ensure the desired region of interest falls within the functional area of the Nova-ST chip as shown below:





Once the tissue section is positioned correctly on the Nova-ST chip, lift the glass slide and place a finger on the slide underneath where the chip and tissue are located. The tissue melts over the chip surface. Once the chip is completely thawed on the surface of the Nova-ST chip, place the chip back into the cryostat to freeze the tissue. Using a sharp forcep place the chip into the pre-cooled 3 cms petridish inside the cryostat. Perform this gently and carefully to ensure not to disturb the functional surface of the Nova-ST while handling the chip.

7.5

If proceeding with spatial transcriptomics workflow proceed with step 9, else transfer the chip in the 3 cm petridish to dry ice and transfer to $\text{–}80\text{ }^{\circ}\text{C}$ for storage. This tissue can be stored at $\text{–}80\text{ }^{\circ}\text{C}$ for 3 weeks.

Tissue hematoxylin and eosin staining & permeabilization

1h

- 8** If the chip with the tissue is stored at -80 °C , remove the chip and place it on dry ice till the chip is ready for downstream processing.

9 Preparation for H&E staining:

Things to prepare:

- Put the PCR block at 37°C and place the 10X Genomics thermocycler adapter (PN:3000380) into the block and let the adapter equilibrate to 37°C.
- Thaw the 100mg/ml of pepsin stock on ice.
- Put two ovens at 37 °C (permeabilization) & 42 °C (first strand synthesis).
- Prewarm 0.1N HCl to 37 °C .
- Thaw dNTP Mix

A	B
Eosin Mix	Volume
Eosin Y Solution	100 ul
Tris-Acetic Acid Buffer (0.45 M, pH 6.0)	900 ul
Total	1000 ul

- Thaw 1x Maxima H- RT buffer.
- Transfer 12 ml Methanol into a 6 cm petridish (per sample) and place the petridish into -20 °C for atleast 15 mins.

- 10** ▪ Using a sharp forceps transfer the Nova-ST chip/s to the thermocycler adapter at 37 °C (without closing the lid of the PCR block). Ensure not to disturb the functional surface of the Nova-ST while handling the chip. Incubate for **2 mins** to thaw the tissue on the Nova-ST chip.

- 11** ▪ After the incubation, transfer the chip into the methanol at -20 °C . Perform the methanol fixation 30m for 00:30:00 .

- 12** During the methanol fixation, prepare the following for the H&E staining:

- Fill 3X 1 L beakers with Milli-Q water
- Prepare 1X 50 ml falcon with Milli-Q water (per sample)
- Prepare Eosin Mix.

A	B
Eosin Mix	Volume
Eosin Y Solution	100 ul
Tris-Acetic Acid Buffer (0.45 M, pH 6.0)	900 ul
Total	1000 ul

13 After 00:30:00 fixation in methanol, using a sharp forceps, transfer the Nova-ST chip from methanol paper towel to remove methanol from bottom of the chip.

14 Add 150 µL of 2-Propanol to the chip. Ensure the chip is completely covered in the liquid. Incubate for
 00:01:00

15 After the incubation, decant out 2-Propanol and by means of the sharp forceps, transfer the Nova-ST chip to fresh paper towel. Allow the 2-Propanol on the Nova-ST chip to dry-out for 00:03:00. Make sure the chip is completely dry. DONOT exceed 00:05:00.

16 Perform the rest of the H&E staining of the tissue sections on the Nova-ST using [Methanol Fixation, H&E Staining & Imaging for Visium Spatial Protocols](#).

Some changes to be noted:

- All the volumes of the reagents to be added to Nova-ST chip is 130 µL
- For wash of the chip with the Milli-Q water, make sure to hold the chip firmly in the region of the chip which doesn't have tissue on it and submerge into water. If the chip is not held firmly, the chip can slip away and get lost.
- For imaging of Nova-ST chip, after drying the stained Nova-ST chip, place the chip on microscopic glass slide, and proceed with imaging.
- During imaging make sure to identify and note down the Nova-ST chip label.
- Note the time for imaging, prior to permeabilization step. DONOT to exceed 01:00:00.

Tissue permeabilization and First Strand Synthesis

17 Preparations:

Prepare the following mixes (excluding the items marked with **, these items are added just before addition to Nova-ST chip) and incubate for at least 00:15:00 in a 37 °C oven.

- Prepare RT wash buffer below (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
Maxima RT buffer	5	X	1	80
**RNase Inhibitor (Lucigen)	40	U.ul	0.5	5
NFW				315
				400

- Prepare 0.1X SSC below (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
SSC	20	X	0.1	2
**RNase Inhibitor (Lucigen)	40	U.ul	0.5	5
NFW				393
				400

- Prepare RT First Strand Mix (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
Maxima RT buffer	5	X	1.05	84
**RNase Inhibitor (Lucigen)	40	U.ul	1.05	10.5
Ficoll PM-400	20	%	4.5	90
**dNTPs (Thermo)	25	mM	1.05	16.8

	A	B	C	D	E
**Maxima H-Rtase	200		10	20	
NFW				178.7	
				400	

Prepare 0.65U/ul working stock of Pepsin enzyme by diluting the 100 mg/ml enzyme stock with 0.1N HCl (

(2) warmed to (37 °C). Check the pH of the diluted enzyme mix and ensure (2).

18

With the help of a sharp forceps transfer the Nova-ST chip after imaging to a 3 cm petridish and add the (120 µL pre-warmed 0.65U/ul Pepsin mix onto the chip (add drop-by-drop outside the tissue area). Ensure to spread the liquid evenly on the surface of the Nova-ST chip. Incubate the chip with the enzyme for the optimized permeabilization time in the (37 °C) oven. During the incubation time, add the remaining reagents in the table (**).

19

After the incubation time, immediately remove the enzyme from the surface of the Nova-ST chip with repeated aspiration using a P200 pipette. Ensure not to disturb or scratch the functional surface of the chip.

20

With the help of sharp forceps, transfer the Nova-ST chip to a clean paper towel, to remove the liquid at the bottom of the chip and then transfer the chip to a fresh 24 well plate with the tissue facing up.

21

Add 0.1X SSC buffer, drop wise, to the corners of the chip to wash the chip. Remove and discard 0.1X SSC buffer. Repeat this step with the 1X RT wash buffer.

22

Add 1X RT first strand mix, drop wise, to the corners of the chip in the 24 well plate. Ensure that the chip completely submerged in the liquid. Add 1 ml of NFW to the neighboring wells. Cut 4 square patches of parafilm M. Place the patch on the wells to seal the well with Nova-ST chip. Seal the 24 well plate with parafilm tape and proceed with the incubation to complete the first strand synthesis:

(42 °C for (16:00:00 - 20:00:00).

Note

After about 01:00:00 of incubation, check for air bubble on the surface of the Nova-ST chip. If there is air bubble over the tissue surface, gently hold the chip on a side and tilt the chip of the liquid to remove the air bubble. Place the chip back into the liquid, seal the 24 well plate with parafilm tape and proceed with the rest of the incubation.

Exonuclease treatment

1h

23 Preparations:

Things to prepare:

- Put an oven at 37 °C .
- Thaw 10X Exonuclease buffer
- Thaw NEB buffer 2
- Thaw 25 mM dNTP mix.
- RPE Randomer 100 micromolar (µM) (Refer Table S1)
- Thaw KAPA HiFi HotStart on ice
- Thaw the RPEPCR*Forward primer 100 micromolar (µM) (Refer Table S1)
- Thaw the RPEPCR*Reverse primer 100 micromolar (µM) (Refer Table S1)

Prepare the following mixes (excluding the items marked with **, these items are added just before addition to Nova-ST chip) and incubate for at least 00:15:00 in a 37 °C oven.

- Prepare 0.1X SSC below (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
SSC	20	X	0.1	5
NFW				995

- Prepare 1X Exo-I buffer (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
Exo I buffer	10	X	1	40
NFW				360

- Prepare Exo-I reaction Mix (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
** Exo I	20		1	20
Exo I buffer	10	X	1	40
NFW				340
				400

Prior to adding the mixes to the 24 well plate, add the remaining reagents in (**) to the respective tubes.

24 After the completion of the FSS step, remove the FSS reagents from the 24 well plate.

25 Add 0.1X SSC buffer, drop wise, to the corners of the chip to wash the chip. Remove and discard 0.1X SSC buffer. Repeat this step with the 1X Exo-I buffer.

26 Add Exo-I reaction Mix, drop wise, to the corners of the chip in the 24 well plate. Ensure that the chip is completely submerged in the liquid. Seal the 24 well plate with parafilm tape and proceed with the incubation to complete the exonuclease reaction:

37 °C for 00:45:00 .

1h

During the exonuclease incubation, prepare the following tissue removal mix (excluding the items marked with **, these items are added just before addition to Nova-ST chip) and incubate for at least 00:15:00 in a 37 °C oven.

- Prepare 1X Tissue removal mix (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
Tris pH 8.0	1000	mM	100	40
NaCl	5000	uM	200	16
SDS	10	%	2	80
EDTA	500	mM	5	4
**Proteinase K	800	mU.ul	16	8

A	B	C	D	E
NFW				252
				400

Tissue removal step

2h

- 27 After the completion of exonuclease reaction step, remove the Exo-I reagents from the 24 well plate. Remove as much liquid as possible.

- 28 Add pre-warmed 1X Tissue removal mix, drop wise, to the corners of the chip in the 24 well plate. Ensure that the chip is completely submerged in the liquid. Seal the 24 well plate with parafilm tape and proceed with the incubation to complete the tissue removal reaction:

1h

⌚ 37 °C for ⏳ 01:00:00 .

Note

After about ⏳ 00:15:00 of incubation, check for air bubble on the surface of the Nova-ST chip. If there are air bubbles over the tissue surface, gently hold the chip on a side and tilt the chip out of the liquid to remove the air bubble. Place the chip back into the liquid, seal the 24 well plate with parafilm tape and proceed with the rest of the incubation.

During the tissue removal incubation, prepare the following denaturation and neutralization mixes:

- Prepare 0.1N NaOH below (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
NaOH	10	N	0.1	100
NFW				9900
				10000

- Prepare 100 mM Tris pH 7.5 (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
Tris pH 7.5	1000	mM	100	1000

A	B	C	D	E
NFW				9000
				10000

Note

After the complete incubation for tissue removal, before proceeding with the next step, check the functional surface of Nova-ST chip. If there is remnants of tissue on the chip, continue with the tissue removal reaction for an additional 00:30:00 or longer till the tissue is removed (removal time needs to optimized).

First strand denaturation & Second strand synthesis

5m

- 29 During the tissue removal incubation step prepare the following second strand synthesis mix (excluding the items marked with **, these items are added just before addition to Nova-ST chip) and incubate for at least 00:15:00 in a 37 °C oven.

- Prepare 1X Second Strand Synthesis mix (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
NEB Buffer 2	10	X	1.1	44
RPE Randomer	100	uM	11	44
** dNTPs (Thermo)	25	mM	1.1	17.6
**Klenow exo (-) Fragment	5	U/ul	0.55	44
NFW				250.4
				400

- 30 After the completion of the tissue removal reaction step, remove the tissue removal reagents from the 24 well plate. Remove as much liquid as possible.

- 31 Add 3 ml of NFW drop-by-drop onto the surface of the Nova-ST chip. Remove and discard the wash. Repeat this step for a total of 3X times

- 32 Add 3 ml of 0.1N NaOH drop-by-drop to the Nova-ST chip. For completion of the denaturation incubate the chip for  00:05:00 . Remove and discard the wash. Repeat this step for a total of 3X times 5m

Note

After the addition of 0.1N NaOH to the well, using a P1000, pipette 5X times on to the surface of the chip and leave the chip for incubation for  00:05:00 .

- 33 Add 3 ml of 100 mM Tris pH 7.5 drop-by-drop onto the surface of the Nova-ST chip. Remove and discard the wash. Repeat this step for a total of 3X times.

- 34 Finally, add 3 ml of NFW drop-by-drop onto the surface of the Nova-ST chip. Remove and discard the wash. Repeat this step for a total of 3X times. Proceed **immediately** with the second strand synthesis.

- 35 After the completion of last wash of the Nova-ST chip with NFW, with the help of sharp forcep, transfer the Nova-ST chip to a clean paper towel, to remove the liquid at the bottom of the chip and then transfer the chip to a fresh 24 well plate with the tissue facing up.

- 36 Add pre-warmed second strand synthesis mix, drop wise, to the corners of the chip in the 24 well plate.  37 °C for  02:00:00 . Ensure that the chip is completely submerged in the liquid. Cut 4 square patches of parafilm M. Place the patch on the wells to seal the well with Nova-ST chip. Seal the 24 well plate with parafilm tape and proceed with the incubation to complete the second strand synthesis:

Note

After about  00:30:00 of incubation, check for air bubble on the surface of the Nova-ST chip. If there are air bubbles over the tissue surface, gently hold the chip on a side and tilt the chip out of the liquid to remove the air bubble. Place the chip back into the liquid, seal the 24 well plate with parafilm tape and proceed with the rest of the incubation.

37 During the second strand synthesis incubation prepare the following denaturation mixes:

- Prepare 0.1N NaOH below (per reaction):

A	B	C	D	E
	Stock	Unit	Final	Volume
NaOH	10	N	0.1	50
NFW				4950
				5000

38 After the completion of the second strand synthesis reaction step, remove the SSS reagents from the 24 well plate. Remove as much liquid as possible.

39 After the completion of SSS reaction step, with the help of a sharp forcep, transfer the Nova-ST chip to a clean paper towel, to remove the liquid at the bottom of the chip and then transfer the chip to a 3 cm petridish and proceed immediately with the random primer extension product from the the second strand synthesis. Ensure not the disturb the functional surface of the Nova-ST chip, while handling with the forceps

40 Add  90 µL of 0.1N NaOH, drop-by-drop to corners of the Nova-ST chip. **Ensure not to spill out the 0.1N NaOH outside the Nova-ST chip.** With a low bind pipette set to  70 µL, pipette mix the denaturant on the chip 5X times and incubate for  00:05:00. After incubation, by repeated aspiration with a low bind pipette, remove as much liquid as possible from the surface of the Nova-ST chip and transfer the denatured RPE extract to a low bind 1.5 ml eppendorf tube. (The petridish can be tilted to one of the corners of the chip to facilitate the withdrawal of the RPE product) Ensure not the disturb the functional surface of the Nova-ST chip while with-drawing the RPE extract from the surface of the Nova-ST chip.

41 Repeat the above **step 41**, two additional time for a total of 3X times. Collect all the RPE extract to the same 1.5 ml eppendorf lo-bind tube. To maximize the RPE collect, after the final wash, transfer the chip into a 50 ml falcon and spin the tube at for  500 x g, 00:01:00. Extract the RPE extract and collect it into the same low bind 1.5 ml eppendorf tube.

42 Estimate the volume of the RPE extract collected in the denaturation steps above. This volume usually amounts to  250 µL. Neutralize the RPE extract by adding  70 µL of 1M Tris 7.0. Vortex the

content in the 1.5 ml eppendorf tube to mix the content. Incubate for 00:01:00 . (Scale the volumes accordingly)

43 Perform Ampure XP purification as below:

28m 30s

Important: Ensure to vortex and mix the Ampure XP beads well before adding for purification.

1. To purify cDNA add 1.8:1 ratio of Ampure XP beads to sample (576 µL of Ampure XP), and mix by vortexing.

2. Incubate at Room temperature for 00:10:00 .

3. Spin down and place on magnet and allow beads to be capture on the magnet. Place on magnet to separate the beads for 00:08:00

4. Discard supernatant, and wash once with 1500 µL of **freshly prepared 80% ethanol**. After 00:00:30 remove and discard the 80% ethanol.

5. Repeat **step 4**, 1X additional time.

6. Allow the bead pellet to dry for 00:10:00 . Intermittently remove the 80% ethanol flowing down from the bead pellet to facilitate the bead pellet drying.

7. Elute cDNA in 44.5 µL of elution buffer. Elute out 42 µL of cDNA library for RPE cDNA product to a fresh PCR tube.

Random Primer Extension product amplification

11m 30s

44

Prepare PCR mix for RPE PCR:

Note: The KAPA HiFi DNA polymerase with hot start has very low 3-5' exonuclease activity. Still it is recommended to prepare the PCR mix on ice.

A	B	C	D	E
	Stock	Unit	Final	Volume
KAPA HiFi Hotstart ready mix	2	X	1	50
RPEPCR*F primer	100	uM	1	1
RPEPCR*R primer	100	mM	1	1
Purified RPE product				42
dH2O				6
				100

Prepare the PCR mix above and transfer $\text{PCR } 58 \mu\text{L}$ of PCR master mix to the PCR tube having the eluted RPE product.

Quick centrifugation to collect reaction to the bottom of the strip tube, before running the following PCR program in a thermocycler for RPE PCR amplification.

A	B	C	D
Step	Temperature	Time	Cycles
Initial Denaturation	95°C	3 minutes	1x
Denaturation	95°C	30 seconds	
Annealing	60°C	1 minute	14 cycles
Elongation	72°C	1 minute	
Final Elongation	72°C	5 minutes	1x
Hold	12°C	Hold	1X

45 Perform Ampure XP purification as below:

Important: Ensure to vortex and mix the Ampure XP beads well before adding for purification.

11m 30s



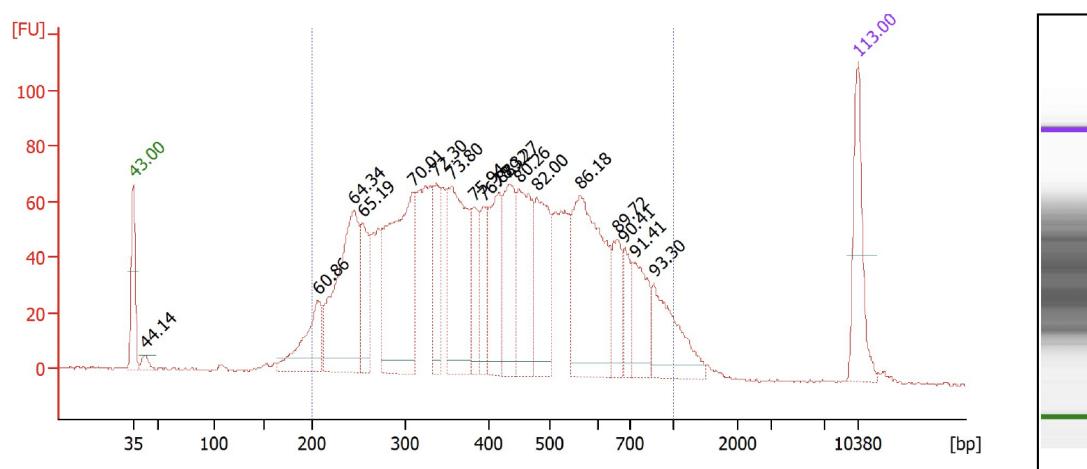
- To purify cDNA add 0.8:1 ratio of Ampure XP beads to sample ($\text{Ampure XP } 80 \mu\text{L}$), and mix by gently pipetting using P200.
- Incubate at Room temperature for $00:05:00$.
- Spin down and place on magnet and allow beads to be capture on the magnet. Place on magnet to separate the beads for $00:04:00$.
- Discard supernatant, and wash once with $200 \mu\text{L}$ of **freshly prepared 80% ethanol**. After $00:00:30$ remove and discard the 80% ethanol.
- Repeat **step 4**, 1X additional time.
- Spin down the tube to bring down the beads to the bottom of the tube. Place on the magnet to remove the residual 80% ethanol, without disturbing the bead pellet.
- Allow the bead pellet to dry for $00:02:00$.
- Elute cDNA in $25.5 \mu\text{L}$ of elution buffer. Elute out $25 \mu\text{L}$ of RPE PCR to a fresh lo-bind 1.5 ml eppendorf tube.

Quality control check

- 46** Check the RPE PCR cDNA library content and quality on a Agilent Bioanalyzer, using High Sensitivity DNA Analysis chips and concentration of the library using [Qubit Fluorometric Quantification](#).

Expected result

Usually, the Qubit value of the spatial RPE cDNA library is **>5ng/ul** and a typical bioanalyzer trace of the RPE cDNA library:



Indexed library preparation

47 Preparations:



Things to prepare:

- Thaw KAPA HiFi HotStart on ice
- Thaw the OAK-MO-primer-X.X primer [M] 10 micromolar (μM) (Refer Table S1)
- Thaw the WTA1*R primer [M] 10 micromolar (μM) (Refer Table S1)

Estimate the molarity of the RPE PCR cDNA (from **step 45**):

1. Average fragment size (estimated with BioAnalyzer).
2. Library concentration (Qubit measurement)

$$\text{Library concentration (nM)}: \frac{\text{Library concentration (ng/uL)}}{660 \times \text{Average fragment size (bps)}} \times 10^6$$

Prepare 10nM normalized RPE cDNA library for index PCR

Prepare Index PCR mix:

Note: The KAPA HiFi DNA polymerase with hot start has very low 3-5' exonuclease activity. Still it is recommended to prepare the mix on ice.

A	B	C	D	E
	Stock	Unit	Final	Volume
KAPA HiFi Hotstart ready mix	2	X	1	25
OAK-MO-primer-X.X	10	uM	1	5
WTA1*R	10	uM	1	5
Purified RPE product	10	nM	2	10
dH2O				5
				50

Prepare the PCR mix above and transfer  40 μ L of PCR master mix to the PCR tube having the normalized [M] 10 nanomolar (nM) RPE product.

Quick centrifugation to collect reaction to the bottom of the strip tube, pipette mix the reaction before running the following PCR program in a thermocycler for Index PCR amplification.

A	B	C	D
Step	Temperature	Time	Cycles
Initial Denaturation	95*C	3 minutes	1x
Denaturation	95*C	30 seconds	
Annealing	60*C	30 seconds	10 cycles
Elongation	72*C	30 seconds	
Final Elongation	72*C	5 minutes	1x
Hold	12*C	Hold	1X

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Perform Ampure XP purification as below:

10m 30s



Important: Ensure to vortex and mix the Ampure XP beads well before adding for purification.

1. To purify cDNA add 0.8:1 ratio of Ampure XP beads to sample (80 µL of Ampure XP), and and mix by gently pipetting up and down.
2. Incubate at Room temperature for 00:05:00 .
3. Spin down and place on magnet and allow beads to be capture on the magnet. Place on magnet to separate the beads for 00:03:00
4. Discard supernatant, and wash once with 200 µL of of **freshly prepared 80% ethanol**. After 00:00:30 remove and discard the 80% ethanol.
5. Repeat **step 4**, 1X additional time.
6. Spin down the tube to bring down the beads to the bottom of the tube. Place on the magnet to remove the residual 80% ethanol.
6. Allow the bead pellet to dry for 00:02:00 .
7. Elute cDNA in 100.5 µL of Elution Buffer. Elute out 100 µL of final index PCR library to a fresh lo-bind 300 µL pcr strip tubes.

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Perform another round of Ampure XP purification as below:

10m 30s



Important: Ensure to vortex and mix the Ampure XP beads well before adding for purification.

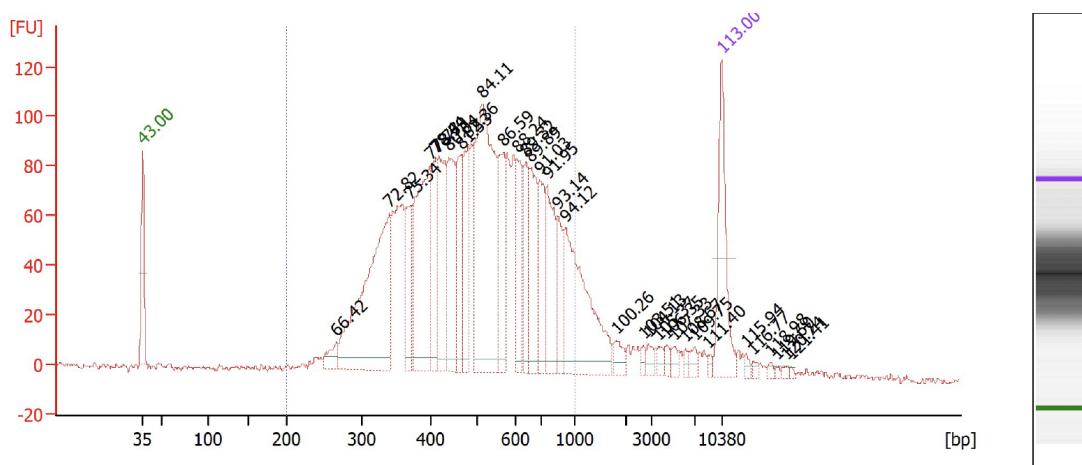
1. To purify cDNA add 1:1 ratio of Ampure XP beads to sample (100 µL of Ampure XP), and and mix by gently pipetting up and down.
2. Incubate at Room temperature for 00:05:00 .
3. Spin down and place on magnet and allow beads to be capture on the magnet. Place on magnet to separate the beads for 00:03:00
4. Discard supernatant, and wash once with 200 µL of of **freshly prepared 80% ethanol**. After 00:00:30 remove and discard the 80% ethanol.
5. Repeat **step 4**, 1X additional time.
6. Spin down the tube to bring down the beads to the bottom of the tube. Place on the magnet to remove the residual 80% ethanol.
6. Allow the bead pellet to dry for 00:02:00 .
7. Elute cDNA in 60.5 µL of Elution Buffer. Elute out 60 µL of final Index PCR product to a fresh lo-bind 1.5 ml eppendorf tube.

Quality control check

- 50 Check the sample index library content and quality on a Agilent Bioanalzyer, using High Sensitivity DNA Analysis chips and concentration of the library using **Qubit Fluorometric Quantification**.

Expected result

Usually, the Qubit value of the final Nova-ST sequencing library is **>20ng/ul** and a typical bioanalyzer trace of the final indexed Nova-ST library:



Nova-ST library sequencing

- 51 The sequencing ready library can be sequenced on any Illumina compatible sequencers. The sequencing is performed as paired end sequencing. Sequencing parameter used for sequencing of the Nova-ST library is:

A	B
Read Configuration	Number of bases
Read 1	33
Index 1	8
Index 2	8
Read 2	80

Data processing

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Details on data processing related to Nova-ST spatial processing can be found here:

<https://github.com/aertslab/Nova-ST>

