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Nova-ST Chip Preparation Protocol

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ABSTRACT

Nova-ST is a 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 (<https://www.biorxiv.org/content/10.1101/2024.02.22.581576v1>). 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 you 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.

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We use this protocol and it's working

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GUIDELINES

Section: HDMI sequencing

Oligonucleotide details:

HDMI32-Dral: CAAGCAGAAGACGGCATACGAGAT

TCTTCCCTACACGACGCTTCCGATCT

NNVNBNVNNVNNVNNVNNVNNVNNNNNN

TCTTGACTACAGCACCCCTCGACTCTCGC TTTTTTTTTTTTTTTTTTTTTTTTT TTTAAA

GACTTCACCAGTCCATGAT GTGTAGATCTCGTGGTCGCCGTATCATT

HDMI32-Dral was ordered using Ultramer service from IDT. Upon receipt, the HDMI-32 oligonucleotide were reconstituted in the Ultrapure water. The oligonucleotide was diluted to 10 nM before proceeding with the HDMI sequencing.

Read1-Dral: ATCATGGACTGGTGAAAGTC TTTAAA AAAAAAAAAAAAAAAAAAAAAAAA
GCGAGAGTCGAGGGTGCTGTAGTCACAAGA

Read1-Dral was also ordered using from IDT with PAGE purification. Upon receipt, the Read1-Dral oligonucleotide was reconstituted in the Ultrapure water. The oligonucleotide was diluted to 100 nM before proceeding with the HDMI sequencing.

The HDMI32-Dral was diluted to 10 nM and the library and qPCR is used to estimate the library concentration. The concentration estimated from qPCR is used to normalize the HDMIDral-32 library for the HDMI sequencing on Novaseq 6000 S4 flowcell.

Section: Preparation of Nova-ST chips

During the development of Nova-ST workflow we have developed two strategies for preparation of Nova-ST chips from the NovaSeq S4 flow cell and listed below are some consideration for choosing the appropriate method for cutting out the barcoded chips for Nova-ST workflow:

- **Manual cutting strategy:** As described in our pre-print, this method is based on semi-automated glass cutting method, where we have used the NOMAD 3 CNC milling machine from Carbide3D to score the glass surface into a 1cm x 1cm cutting grid. This CNC milling machine comes with a 130W spindle and we used a diamond drag bit from the CNC milling machine manufacturer for scoring the glass surface. Post scoring, we used traditional glass running pliers (Speedwox, Amazon) to cut the glasses into 1 cm x 1 cm chip tiles. We also tried the manual cutting strategy proposed in Open-ST,

another high resolution spatial transcriptomics workflow (<https://www.biorxiv.org/content/10.1101/2023.12.22.572554v1>). Both of these strategies, despite the numerous cutting optimizations and fine-tunings, we have found this procedure usually resulted in quite varied results, especially while cutting larger chip size of 1 cm² tiles. Cutting of the thicker glass section is much more challenging to get precise cuts. In addition, the physical manipulation of the chips during the cutting process also introduces artifacts on the functionalized surface of the chip, which would directly affect the performance of the assay. Also, the first step of separating the think and thick glass layers is also quite challenging with uneven cuts and breakages. All-in-all, we find the manual glass cutting strategy to produce sub-optimal glass cuts and therefore resulting in reduced recovery of ST chips. In order to improve the chip recovery and to optimize the chip cutting strategy we adopted and moved to a more automated workflow.

- **Automated glass dicing strategy:** In this new strategy, we use a wafer dicing instrument (DISCO DAD Automatic dicing saw; DAD 3220) to accurately cut the NovaSeq chips to varied dimensions. This instrument gives a great flexibility in cutting any desired dimensions. This instrument should commonly be available in electrical and electronics lab facilities. DAD 3220 employs a high-speed spindle (1.5 kW) fitted with an extremely thin diamond blade. The thickness of the cutting blades used varies with the material being cut, and in the case of glass cutting this is about  . The optimized glass dicing strategy provides close to 100% chip recovery. Manual intervention in the strategy is minimal and thus the artifact

The protocol describes the details on both the cutting strategies. If access to the wafer dicing instrument is a possibility, its highly recommended to use this for cutting of Nova-ST chips due to the high reliability and reproducibility in getting accurately cut Nova-ST chips.

MATERIALS

1. Reagents

- ☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**
- ☒ KAPA Library Quantification Kit for Illumina® Platforms **Kapa Biosystems Catalog #KK4835**
- ☒ Illumina NovaSeq 6000 S4 kit; 35 cycles **Illumina, Inc. Catalog #20044417**
- ☒ Sodium hydroxide, 10N aq. soln., Thermo Scientific Chemicals **Thermo Fisher Scientific Catalog #J63736.AE**
- ☒ Tris-HCl, 1M Solution, pH 8.0, Molecular Biology Grade, Ultrapure, Thermo Scientific Chemicals **Thermo Fisher Scientific Catalog #J22638.AE**
- ☒ DOW SYLGARD 184, 1.1KG Silicone Elastomer, Flowable, Sylgard® 184, RT Cure, Transparent, Container, **Dow Corning Catalog #101697**
- ☒ Drai - 10,000 units **New England Biolabs Catalog #R0129L**
- ☒ Parafilm **Contributed by users**
- ☒ TE pH 7.5 **IDT Technologies Catalog #11-01-02-02**
- ☒ 1 Liter IDTE pH 8.0 (1X TE Solution) **Integrated DNA Technologies, Inc. (IDT) Catalog #11-05-01-09**
- ☒ Exonuclease I (E.coli) - 15,000 units **New England Biolabs Catalog #M0293L**
- ☒ Quick CIP – 5,000 units **New England Biolabs Catalog #M0525L**
- ☒ 3M 201E 48MM Masking Tape, Crepe Paper, Cream, 48 mm x 50 m **3M corporation Catalog #201E 48MM**
- ☒ RNaseZap® **Thermo Scientific Catalog #AM9780**
- ☒ DNAZap™ PCR DNA Degradation Solutions **Thermo Fisher Catalog #AM9890**
- ☒ Tris (1 M), pH 7.0, RNase-free **Thermo Fisher Catalog #AM9851**
- ☒ 1M Tris-HCl pH 7.5 **Thermo Fisher Scientific Catalog #15567027**
- ☒ Tris (1 M), pH 8.0, RNase-free **Thermo Fisher Catalog #AM9856**

2. Equipments

Equipment**NovaSeq 6000**

NAME

DNA Sequencer

TYPE

Illumina

BRAND

-

SKU

<https://emea.illumina.com/systems/sequencing-platforms/novaseq.html>^{LINK}**Equipment****Bel-Art™ SP Scienceware™ Space Saver Vacuum Desiccators**^{NAME}

Vacuum Desiccators

TYPE

Bel-Art™ SP Scienceware™

BRAND

NA

SKU

Equipment**Diaphragm vacuum pump, VACUUBRAND®** NAME

Vacuum pump TYPE

VACUUBRAND BRAND

NA SKU

Equipment**Incubator** NAME

Memmert BRAND

Incubator IN55 SKU

HDMI Sequencing

1 Before Starting

- 1.1** The NovaSeq S4 reagents stored at $\text{−20 }^{\circ}\text{C}$ is taken out and thawed at $4\text{ }^{\circ}\text{C}$ for atleast 16:00:00 - 20:00:00 before sequencing. Prior to sequencing these reagents are removed and warmed to $25\text{ }^{\circ}\text{C}$ for atleast $00:30:00$.

- 1.2** The NovaSeq S4 flowcell stored at 4 °C is thawed to Room temperature at least 00:30:00 before sequencing.
- 1.3** Thaw HDMI32-Dral and Read1-Dral oligonucleotides to Room temperature, and prepare 20 µL of 100 micromolar (µM) of Read1-Dral read primer and prepare 200 µL of 1 micromolar (µM) of HDMI32-Dral oligonucleotide for HDMI sequencing. The 1 micromolar (µM) of HDMI32-Dral oligonucleotide is subject to qPCR quantification for library normalization

2 Sequencing

- 2.1** Prepare NaOH for denaturation (Mix the content well).

	A	B	C	D
	Component	Stock Concentration	Final Concentration	Volume
	NaOH	4 M	0.2 M	5 ul
	Nuclease Free Water (NFW)			95 ul

- 2.2** Prepare Tris 8 (Mix the content well)

	A	B	C	D
	Component	Stock Concentration	Final Concentration	Volume
	Tris 8.0	1 M	10 mM	2 ul
	NFW			198 ul

- 2.3** Prepare Tris 8 for neutralization (Mix the content well)

A	B	C	D
Component	Stock Concentration	Final Concentration	Volume
Tris 8.0	1 M	0.4 M	40 ul
NFW			60 ul

3 [M] 1.5 nanomolar (nM) of normalized HDMI library is prepared by diluting the qPCR quantified HDMI32-Dral oligonucleotide using 10 mM Tris pH 8.0 buffer.

4 Denaturing of the HDMI sequencing library is performed by adding [M] 0.2 Molarity (M) NaOH to a final concentration of [M] 0.04 Molarity (M).

5 Incubate for 00:08:00.

6 Neutralize the denatured HDMI library by adding [M] 0.4 Molarity (M) Tris pH 8 solution to a final concentration of [M] 67 millimolar (mM). The final concentration of the HDMI library prior to loading on sequencer is [M] 300 picomolar (pM).

7 Prepare the Read1-Dral primer concentration to [M] 0.3 micromolar (μ M) by adding $\text{10.5 } \mu\text{L}$ of [M] 100 micromolar (μ M) of Read1-Dral primer to $\text{3489.5 } \mu\text{L}$ of the HT1 buffer from the NovaSeq 6000 kit. Mix well and load the primer to the custom read primer 1 position in the NovaSeq reagent cartridge.

8 Proceed for HDMI sequencing. The sequencing configuration used for reading the HDMI barcodes is:

A	B
Read Configuration	Number of bases
Read 1	37
Index 1	0
Index 2	0

A	B
Read 2	0

- 9 At the **end of 34 cycles**, perform manual abortion of the run by pressing the option of "End run without Wash".



- 10 The S4 flow cell is retrieved either for immediate downstream post processing steps, or it can also be stored safely at 4 °C for at least 2 weeks (based on our experience). Users not having direct access to NovaSeq 6000 instrument can instruct sequencing facility to perform the HDMI sequencing and arrange for refrigerated transport of the flowcell in its **original shipping case**. In order to prevent the flow channels from evaporation, the inlet and outlet of the flow cell is plugged with 1.5 mm Polydimethylsiloxane (PDMS) punch.



Preparation of the 1.5 mm PDMS plugs:

- 10.1 The monomer (part A) and catalyst (part B) of SYLGARD 184 Silicone Elastomer Kit is prepared in a 10:1 weight ratio in a plastic container.

- 10.2 Using a spatula, the components were mixed thoroughly till the PDMS become milky white texture.

- 10.3 To remove the air bubble from the PDMS mix, it is degassed in a vaccum chamber for a total duration of 02:00:00 .

- 10.4 Depending of the length of the biopsy needle, pour the degassed PDMS to a petridish to the appropriate level and incubate in a 80 °C oven for a total duration of 02:00:00 to polymerize PDMS.



10.5 Using a fine spatula (Sigma Aldrich, Cat. No. Z193216-2EA), pry out the polymerized PDMS block out of the petridish and place on aluminum foil.

10.6 Using a disposable biopsy punch (World Precision Instrument; Cat. No. 504647), create 1.5-2 mm PDMS punches. With help of fine tweezers, use these punches to block the inlet and the outlet of the NovaSeq HDMI flow cell.

Flow cell cleanup & Enzymatic treatment

11 Before Starting

11.1 Thaw the following components:

- 10x rCutSmart buffer
- Switch on the incubator to 37 °C .

11.2 Prepare the **1X rCutSmart buffer**. Mix the content well.

A	B	C	D
Component	Stock Concentration	Final Concentration	Volume
10x rCutSmart buffer	10X	1X	60 ul
NFW			540 ul

11.3 Prepare the **DraI mix** (without enzyme). Mix the content well.

A	B	C	D
Component	Stock Concentration	Final Concentration	Volume
10x rCutSmart buffer	10X	1X	60 ul

A	B	C	D
** Drai enzyme	20 U/ul	2 U/ul	60 ul
NFW			480 ul

- 11.4 Incubate both of the above buffer (without the enzyme, marked with **) at 37 °C for at least

00:30:00

- 12 Remove the sequencing reagents from the flow channels of the NovaSeq flow cell using vacuum liquid aspirator. Wash the flow channels of the sequencing flow cell with 200 µL of NFW. Repeat this step for a total of 3 times.

Note

While flowing the water through the channels, make sure to flow it slowly and evenly to ensure no air traps in the flow channel. Its important to make sure the channel surfaces are wet evenly. Also, ensure this for all the subsequent washes in this protocol. If you observe an air trap remove the liquid in the channels completely by using vacuum liquid aspirator and refill the channel again.

Between each wash, the liquid in the channels are completely aspirated using vacuum liquid aspirator. The channels should be dry after the aspiration.

- 13 Remove the NFW from the flow channels of the NovaSeq flow cell using vacuum liquid aspirator. Wash the flow channels of the sequencing flow cell with 140 µL of **1X rCutSmart buffer**.

- 14 Add 60 µL of Drai Enzyme to the **Drai mix** (enzyme marked in **) above to the buffer mix. Mix the content well using a P1000 pipette. Remove the fluid from the flow channels of the NovaSeq flow cell using vacuum liquid aspirator and load the flow cell channels with 140 µL **Drai mix** cocktail.

- 15 Absorb the excess fluids from the inlet & outlet of the sequencing flow cell and using a precision forcep (VWR Cat. No. BOCH1930), block the inlets and & outlets using the 1.5-2mm PDMS biopsy cores to prevent evaporation of the reagents. Apply scotch tape to ensure no evaporation losses.

16 Place the flow cell into a humidification chamber made with a large square petridish with some tissue towel paper soaked in NFW. Seal the petridish with parafilm tapes to completely seal the large petridish.

17 Incubate the flowcell in the sealed humidification chamber at 37 °C in an oven for a duration of 16:00:00 to 20:00:00 .



18 After 16:00:00 of incubation, check the flow channel and if air bubble are found in the flow channels, repeat go to step #25 with fresh **Drai mix** cocktail and incubate for an additional 04:00:00 .



19 Before Starting

19.1 Thaw the following components:

- 10x Exonuclease I buffer
- Switch on the incubator to 37 °C .

19.2 Prepare the **1X Exonuclease I buffer**. Mix the content well.

	A	B	C	D
	Component	Stock Concentration	Final Concentration	Volume
	10x Exonuclease I buffer	10X	1X	60 ul
	NFW			540 ul

19.3 Prepare the **Exonuclease I mix** (without enzyme). Mix the content well.

A	B	C	D
Component	Stock Concentration	Final Concentration	Volume
10x Exonuclease I buffer	10X	1X	60 μ L
NFW			493.25 μ L

19.4 Incubate both of the above buffer at 37 °C for at least 00:30:00.



20 Remove the **DraI mix** cocktail from the flow channels of the NovaSeq flow cell using vacuum liquid aspirator. Wash the flow channels of the sequencing flow cell with 200 μ L of NFW. Repeat this step for a total of 3 times.

Between each wash, the liquid in the channels are completely aspirated using vacuum liquid aspirator.

21 1. Remove the NFW from the flow channels of the NovaSeq flow cell using vacuum liquid aspirator. Wash the flow channels of the sequencing flow cell with 130 μ L of **1X Exonuclease I buffer**.

22 Add 30 μ L of to the Exonuclease I enzyme + 16.75 μ L Quick CIP enzyme to the **Exonuclease I mix** above. Mix the content well using a P200 pipette. Remove the fluid from from the flow channels of the NovaSeq flow cell using vacuum liquid aspirator and load the flow cell channels with 140 μ L **Exonuclease I mix** cocktail each.

23 Absorb the excess fluids from the inlet & outlet of the sequencing flow cell and using a precision forcep, block the inlets and & outlets using the 1.5-2mm PDMS biopsy cores to prevent evaporation of the reagents. Apply scotch tape to ensure no evaporation losses.

24 Place the flow cell into a humidification chamber made with a large square petridish with some tissue towel paper soaked in NFW.

25 Incubate the flow cell in the humidification chamber at 37 °C in an oven for a duration of



00:45:00

26 After the incubation, check the flow channel and if air bubble are found in the flow channels, repeat go to step #25 with fresh **Exonuclease I mix** cocktail and incubate for an additional 00:15:00.

27 Remove the **Exonuclease I mix** cocktail from the flow channels of the NovaSeq flow cell using vacuum liquid aspirator. Wash the flow channels of the sequencing flow cell with 200 µL of NFW. Repeat this step for a total of 3 times.

Between each wash, the liquid in the channels are completely aspirated using vacuum liquid aspirator.

28 The enzymatically treated S4 flow cell can then be processed directly for the Nova-ST chip preparation, or it can also be stored safely at 4 °C for at least 2 weeks (based on our experience).

If the flow cell is going to be stored, replace the NFW from the flow channels with 200 µL of IDT TE 8 buffer. Absorb the excess fluids from the inlet & outlet of the sequencing flow cell and using a precision forceps, block the inlets and & outlets using the 1.5-2mm PDMS biopsy cores to prevent evaporation of the reagents. Apply scotch tape to ensure no evaporation losses and proceed with the storage.

Please read the "**Guidelines & Warnings**" section, before the glass cutting step to prepare Nova-ST chips.

Manual cutting strategy for Nova-ST chip preparation

29 **Before Starting:**

29.1 Clean all the surfaces and tools being used for preparing the Nova-ST with 70% Ethanol followed by RNA zap and DNA zap.

29.2 Prepare 0.1N NaOH solution

	A	B	C	D
	Component	Stock Concentration	Final Concentration	Volume
	NaOH	10N	0.1N	5 ml
	NFW			495 ml

29.3 Prepare 0.1M Tris 7.5 solution

	A	B	C	D
	Component	Stock Concentration	Final Concentration	Volume
	Tris pH 7.5	1M	0.1M	50 ml
	NFW			450 ml

- 30 If the NovaSeq flow cell after the enzymatic treatment has been stored at 4 °C, remove the PDMS plugs and wash the flow channels of the sequencing flow cell with 200 µL of NFW. Repeat this step for a total of 3 times.

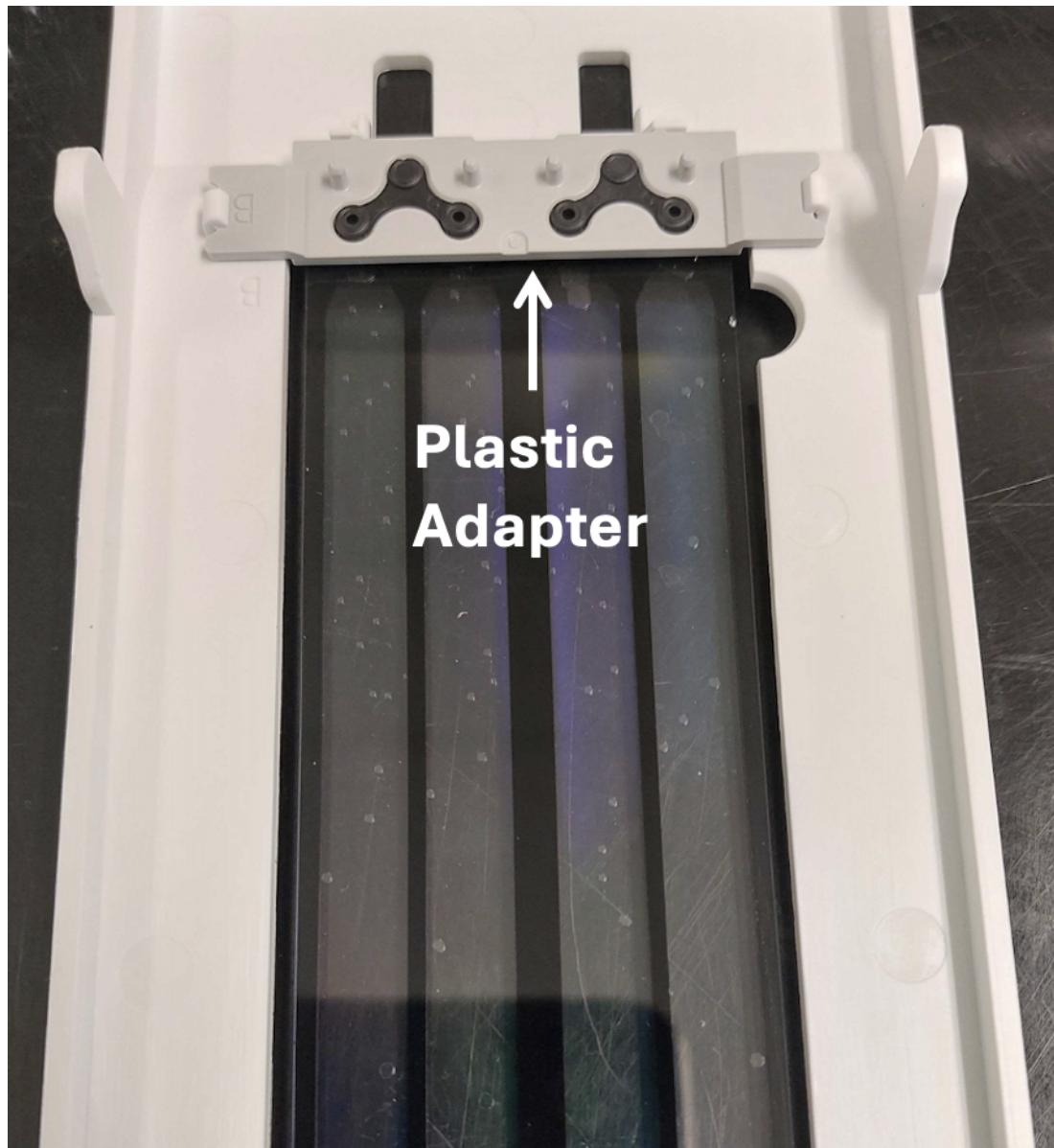
Between each wash, the liquid in the channels are completely aspirated using vacuum liquid aspirator.

- 31 The orientation of the chip is marked with the QR code present in the corner of the NovaSeq flow cell.





- 32 The flow cell is removed from its plastic adapter by releasing the plastic clip holding the chip. The flow cell is then incubated at  50 °C for  00:20:00 to dry the flow channels.



- 33 Using a scalpel, gently pry between the thin & thick glass layers which is separated by a black gasket. This has to be done on all sides of the flow cell. By gentle prying, the layers would slowly separate on all the

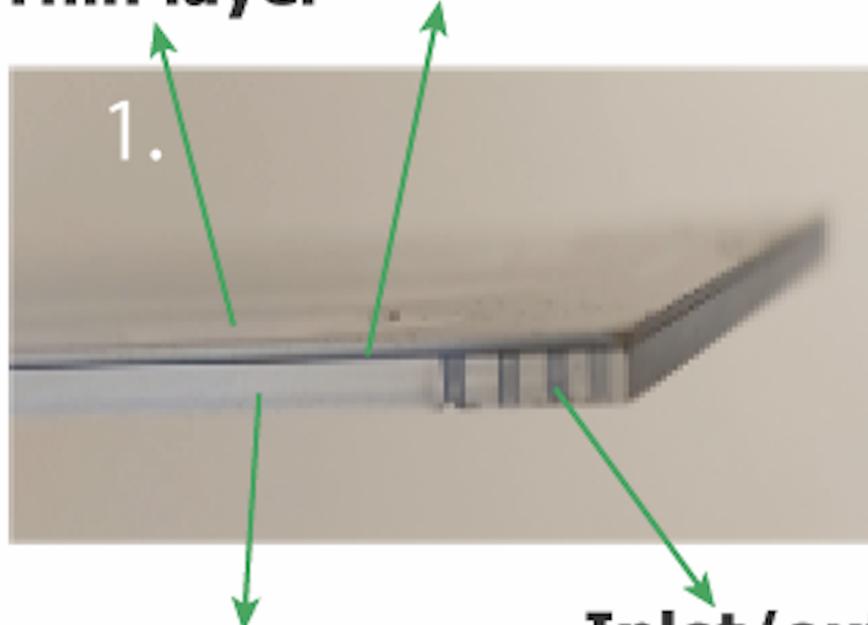


sides. Once the layers have separated significantly, hold them and gradually separate the layers by hand till the thin and thick layers have separated completely.

Note

It's crucial to perform this step with at most care & diligence. The thin glass layer is very fragile and this section can encounter uneven stress if not handled carefully and could result in breakage or shattering.

Thin layer Flow channel Gasket



Thick layer

Inlet/outlet Bores

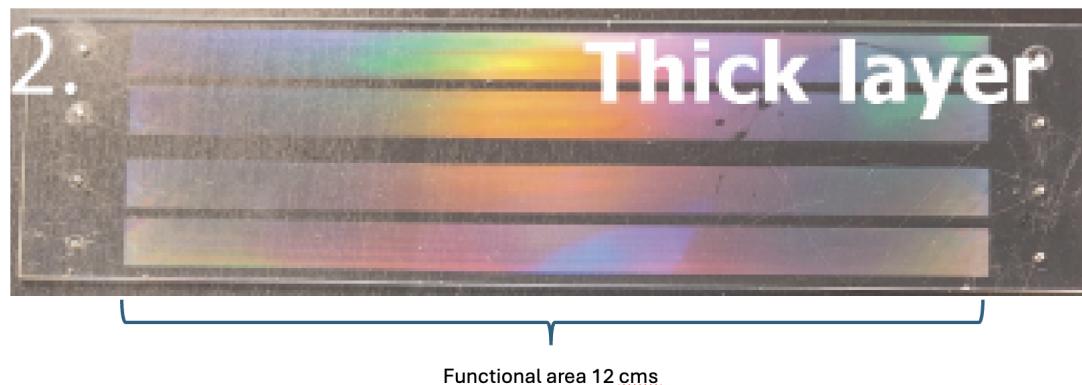
34

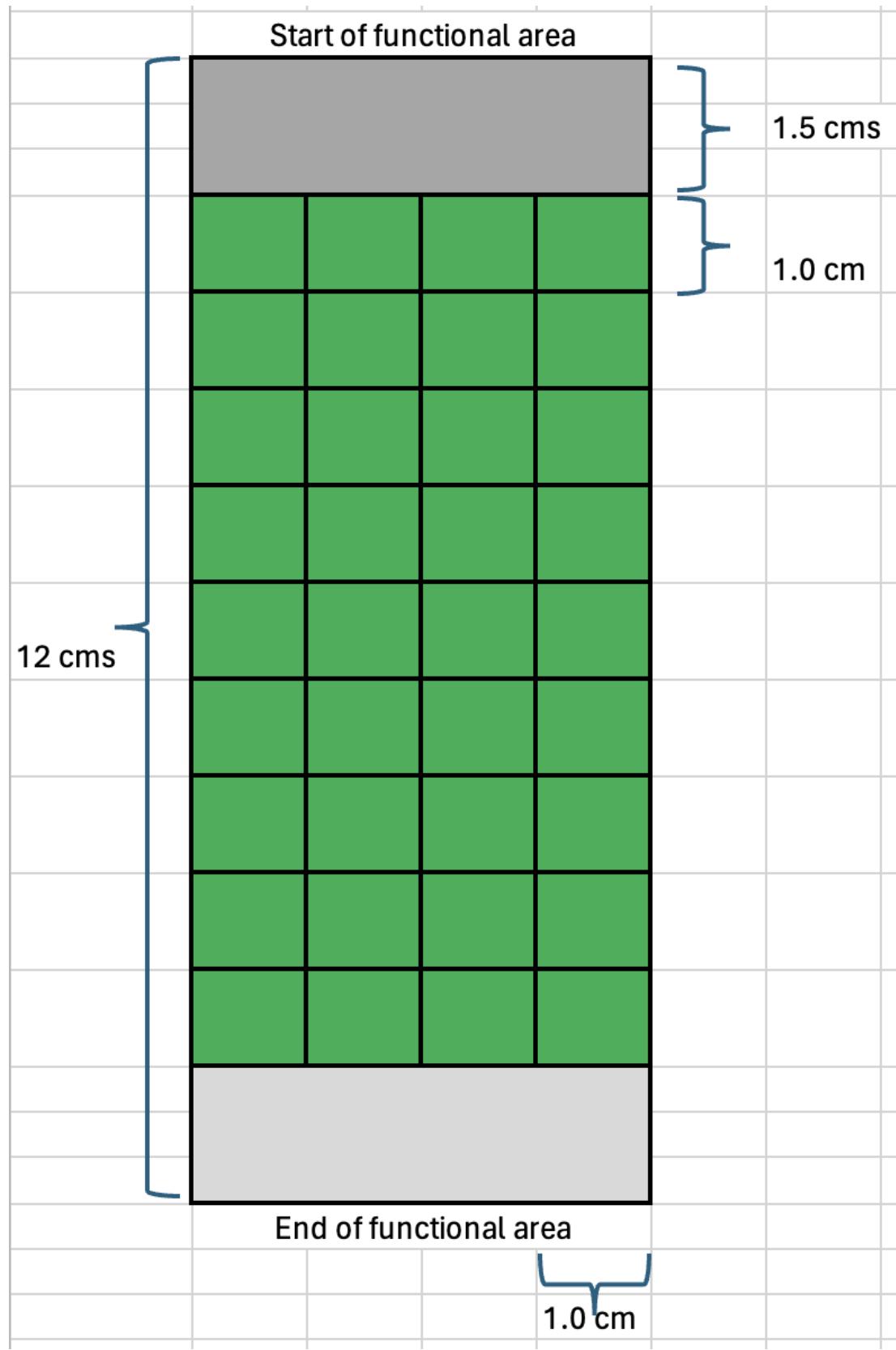
After separating the layers, the glass slides are glued to paper masking tapes. The excess paper tapes around the glass slides are trimmed away.

**Note**

The masking tapes are used to keep the chip together during the cutting process. It's important to do this to ensure the chips are not lost or flipped during the cutting process. It's extremely difficult to identify the correct functionalized surface of the chip if it's flipped during the manipulation.

- 35 The scoring pattern was created using the machine suppliers dedicated software, Carbide Create. The glass plates dimensions were defined in the software as well as the desired scoring pattern. The pattern used for scoring both the glass slides:





The green colored area is cut for the preparing Nova-ST chips. The area shaded with grey corresponds to non-functional area and is excluded from cutting.

36

The glass slides were aligned and fixed onto the bread-board stage of the instrument. As per manufacturers protocol, the x, y and z coordinates of the spindle is set. The scoring depths was then adapted according to the glass thickness and the direction. The thicker glass plate (1.2mm thick) was scored with a 0.6mm depth in the width direction (shortest side) and 0.2mm depth in the length direction. For the thin section (thickness 0.3mm), depths of 0.4mm and 0.1mm were used, respectively. Each score was performed with a single pass of the tool.



Note

It is important to note that the depth of cut defined in the software is not the actual depth. The actual depth differs due to the spring that retracts at the glass contact.

37

Retrieve the glass slides from the CNC machine. Using the glass running pliers (SPEEDEOX), place the glass slides such that the score lines align with the reference line on the pliers. Adjust the set screws of the pliers to control the width of the jaws such that the glass layer sits flushed against the jaws when held between it. Apply gentle pressure, to break the glass along the score lines. Firstly, break the glass slide along the width (shorter length). Then, break along the long score lines to produce 1cm² Nova-ST chip tiles.



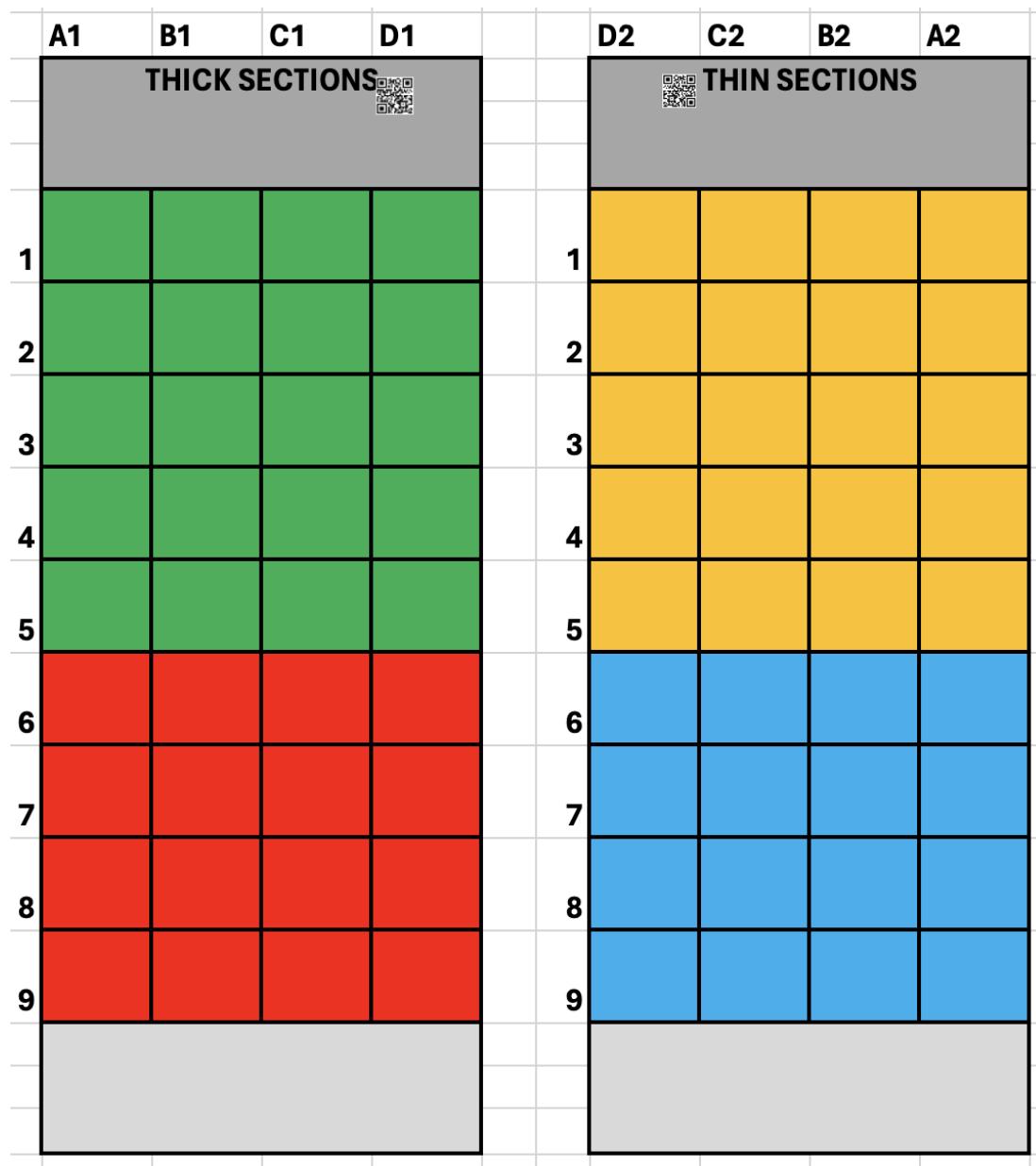
Note

It's important to use the replacement rubber tips to ensure that the functional surface of the Nova Seq chip are not damaged or chipped during the processing steps. Also be sure to apply gently pressure to break the glass. Higher pressure can result in the undesired cuts in the thick glass layer.

38

Prepare **4x 24-well plate** for transferring the chips. After cutting the chips, with help of sharp forceps gently remove the Nova-ST chips from the adhesive tape, without damaging the functional surface of the Nova-ST chip. Transfer the Nova-ST chips to the wells of 24 well plate according to an example color scheme described below:





Nova-ST chips derived from each of the colored regions go to respective 24 well plate.

The wells where the Nova-ST chips are stored are labeled according to their location in the S4 flowcell. This is needed for the identification of the location of the chips in the original NovaSeq flow cell. Example, the **Nova-ST chip 1A1** is derived from the thick glass slide and is closest to the outlet port.

Note

It's important to handle the transfer of the thin Nova-ST chips to the 24 well plate, as they are very delicate and can break easily

- 39 After the chip transfer to the corresponding 24 well plates are completed, proceed with the wash steps below:

- 39.1 Using a multi-channel pipette, add 1 ml of NFW to the wells of the 24 well plate. After wash, discard NFW to a collection reservoir.



Note

Ensure all the chips are completely submerged in the liquid. The thin chips are light and buoyant and sometimes tends to float. If a Nova-ST chip floats, submerge the chip using a pipette tip and be sure to use a non-functional area to submerge the chip. Adding liquid directly to the chip surface helps with submerging the chips during the washes.

repeat the step for a total of 3X times.

- 39.2 Using a multi-channel pipette, add 1 ml of **0.1N NaOH solution** to the wells of the 24 well plate. 5m



Ensure all the chips are completely submerged in the liquid. Incubate the chips for **⌚ 00:05:00** at **🌡 Room temperature**. After incubation discard the **0.1N NaOH solution** from the wells to a collection reservoir. After each wash remove as much as liquid possible from the wells.

repeat the step for a total of 3X times.

- 39.3 Using a multi-channel pipette, add **0.1M Tris 7.5 solution** to the wells of the 24 well plate. Ensure all the chips are completely submerged in the liquid. After incubation discard the **0.1M Tris 7.5 solution** from the wells to a collection reservoir. After each wash remove as much as liquid possible from the wells.

repeat the step for a total of 3X times.

- 39.4 Using a multi-channel pipette, add 1 ml of 1x IDT TE **⌚ pH 8** to the wells of the 24 well plate. Ensure all the chips are completely submerged in the liquid. After wash, discard the TE buffer to a collection reservoir.

- 39.5 Using a multi-channel pipette, add 1 ml of 1x IDT TE **⌚ pH 8** to the wells of the 24 well plate. Ensure all the chips are completely submerged in the liquid. Seal the 24 well plate with parafilm M tapes and store the chips at **🌡 4 °C**.



Automatic dicing strategy for Nova-ST chip preparation

5m

40 Before Starting:

- 40.1** Clean all the surfaces and tools being used for preparing the Nova-ST with 70% Ethanol followed by RNA zap and DNA zap.

40.2 Prepare 0.1N NaOH solution

A	B	C	D
Component	Stock Concentration	Final Concentration	Volume
NaOH	10N	0.1N	5 ml
NFW			495 ml

40.3 Prepare 0.1M Tris 7.5 solution

A	B	C	D
Component	Stock Concentration	Final Concentration	Volume
Tris pH 7.5	1M	0.1M	50 ml
NFW			450 ml

- 41** If the NovaSeq flow cell after the enzymatic treatment has been stored at 4 °C , remove the PDMS plugs and wash the flow channels of the sequencing flow cell with 200 µL of NFW. Repeat this step for a total of 3 times.

Between each wash, the liquid in the channels are completely aspirated using vacuum liquid aspirator.

- 42** The orientation of the chip is marked with the QR code present in the corner of the NovaSeq flow cell.

- 43 The NovaSeq flow is mounted on dicing tape which has a sticky backing that holds the flow cell on a thin sheet metal frame, to prepare for dicing process. The thick glass side of the flow cell is glued on to the adhesive film. In order to reduce the adjustment for alignment in the dicing machine, the flow cell is glued on to the dicing tape, pre-aligned.
- 44 The metal frame with flow cell is fixed to the dicing stage. The x-y- and θ alignment is performed. The origin for the start of the dicing is initiated. Cutting speed is set to 1 mm/s and dicing process is initiated.

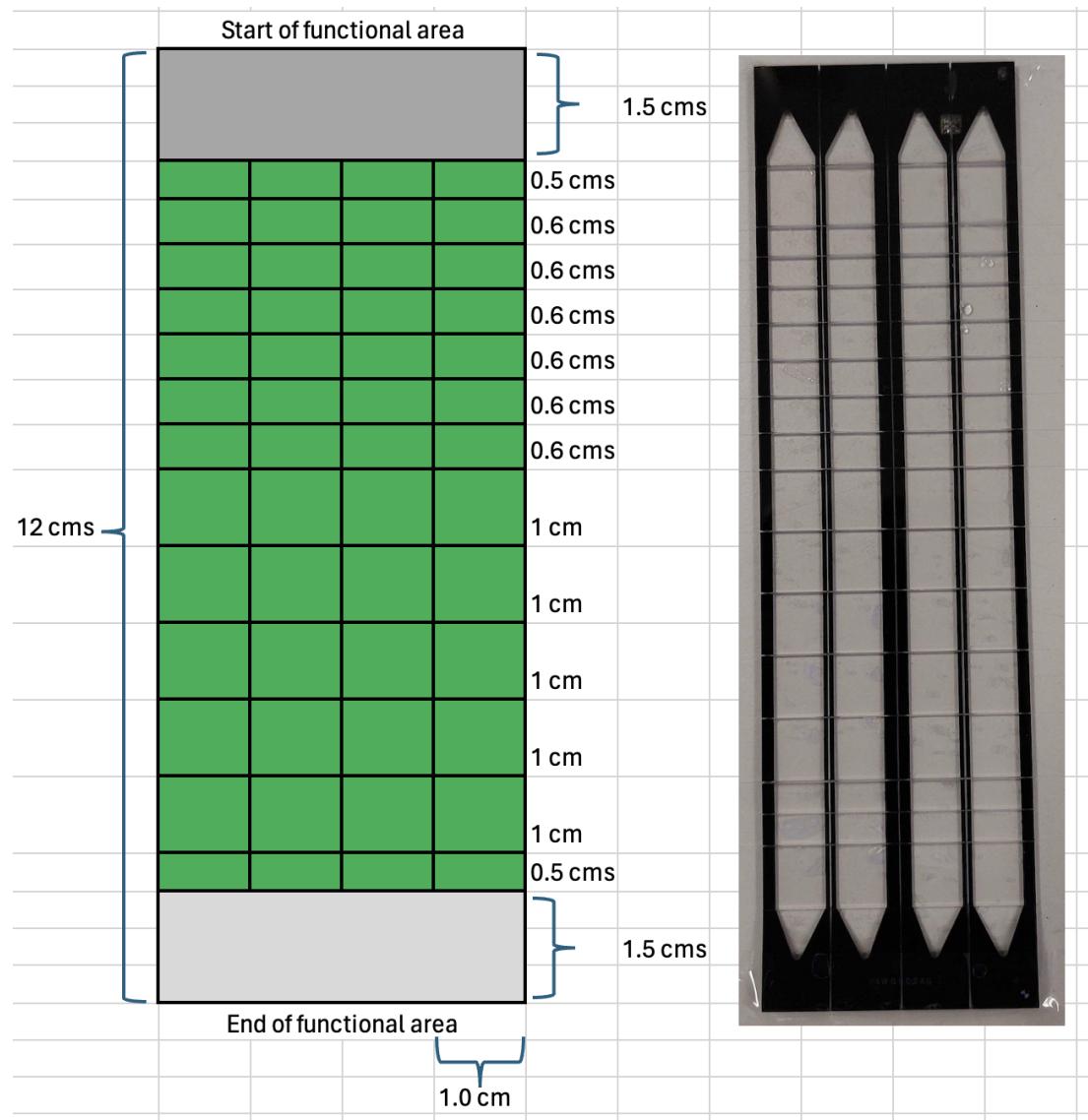


45

Firstly, the dicing is performed along the length of the NovaSeq flow cell and its cut into slabs of 1 cm thick.



Without detaching the separated 1 cm slab from the dicing tape, the tape assembly is turned by 90^0 angle and the dicing is repeated along the width of the NovaSeq flow cell and it cut into desired dimensions. An example of a recent dicing pattern used by our lab:



Note

To ensure the glass layers are diced through the flow cell completely, observe for the score pattern on the dicing tape. If there is no score pattern on the dicing tape the NovaSeq chip has not been diced properly.

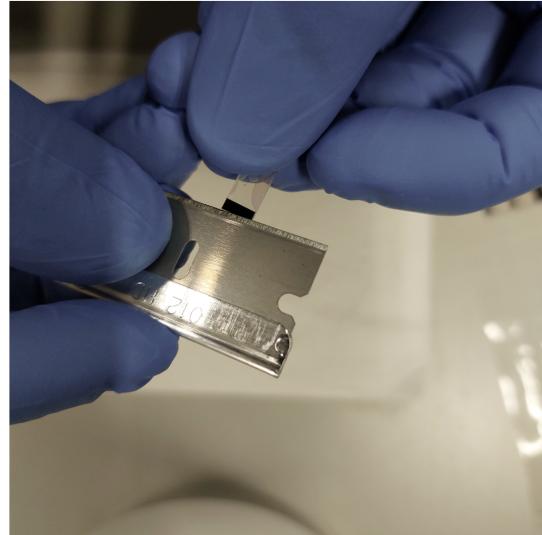
- 46 After the dicing steps are completed, the diced NovaSeq flow cell is removed from the instrument and the dicing tape is cut out to retrieve the diced NovaSeq flow cell.
- 47 Prepare **6x24-well plate** for transferring the chips. For the dicing schema described in step 46, the following plate configuration is used:

Outlet (QR code)				Outlet (QR code)			
Thick sections				Thin sections			
A1	B1	C1	D1	A2	B2	C2	D2
1				10 mm	1		
2				5 mm	2		
3				5 mm	3		
4				6 mm	4		
5				6 mm	5		
6				6 mm	6		
7				6 mm	7		
8				6 mm	8		
Plate 1							
Plate 2							
Plate 3							
Plate 4							
Plate 5							
Plate 6							
9				10 mm	9		
10				10 mm	10		
11				10 mm	11		
12				10 mm	12		
13				10 mm	13		
14				5 mm	14		
15				5 mm	15		
16				10 mm	16		
Inlet				Inlet			
Thick sections				Thin sections			

The wells where the Nova-ST chips are stored are labeled according to their location in the S4 flowcell. This is needed for the identification of the location of the chips in the original NovaSeq flow cell. Example, the **Nova-ST chip 3A1** is derived from the thick glass slide and is closest to the outlet port.

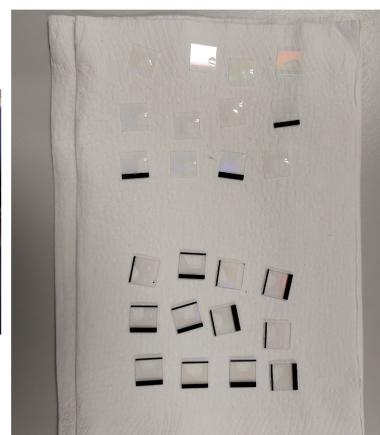
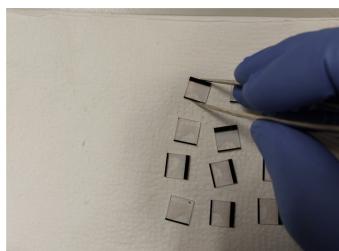
48

With help of sharp forceps gently remove the Nova-ST chips from the adhesive dicing tape. The Nova-ST chips at this stage is still bonded with the thin and thick sections still bonded. Using a fresh razor, pry gently at the side between the thin and thick section (as shown below). A gentle push is sufficient to separate the layers. **Ensure not to disturb/damage the function surface of the Nova-ST chips.**



49

Once the layers have been separated, place the separated chip on a paper towel with the functional surface facing up. After separating a batch of chips, using a sharp forceps, transfer the chips to the respective 24 well plate, with the functional surface of the chips facing upwards.



50 After all the chip transfer has been performed to the corresponding 24 well plates, proceed with the wash steps below:

50.1 Using a multi-channel pipette, add 1 ml of NFW to the wells of the 24 well plate. After wash, discard NFW to a collection reservoir.

Note

Ensure all the chips are completely submerged in the liquid. The thin chips are light and buoyant and sometimes tends to float. If a Nova-ST chip floats, submerge the chip using a pipette tip and be sure to use a non-functional area to submerge the chip. Adding liquid directly to the chip surface helps with submerging the chips during the washes.

repeat the step for a total of 3X times.

50.2 Using a multi-channel pipette, add **0.1N NaOH solution** to the wells of the 24 well plate. Ensure all the chips are completely submerged in the liquid. Incubate the chips for  00:05:00 at  Room temperature . After incubation discard the **0.1N NaOH solution** from the wells to a collection reservoir. After each wash remove as much as liquid possible from the wells.

repeat the step for a total of 3X times.

50.3 Using a multi-channel pipette, add **0.1M Tris 7.5 solution** to the wells of the 24 well plate. Ensure all the chips are completely submerged in the liquid. After incubation discard the **0.1M Tris 7.5 solution** from the wells to a collection reservoir. After each wash remove as much as liquid possible from the wells.

repeat the step for a total of 3X times.

50.4 Using a multi-channel pipette, add 1 ml of 1x IDT TE  to the wells of the 24 well plate. Ensure all the chips are completely submerged in the liquid. After the wash, discard the TE buffer to a collection reservoir.

50.5 Using a multi-channel pipette, add 1 ml of 1x IDT TE  to the wells of the 24 well plate.  Ensure all the chips are completely submerged in the liquid. Seal the 24 well plate with parafilm M tapes and store the chips at  4 °C .

Data processing

51 Details on data processing, post HDMI sequencing can be found here <https://github.com/aertslab/Nova-ST>