

IN-DEPTH: SignalStar-GeoMx protocol

This protocol outlines the required reagents and step-by-step procedures for performing Orion imaging followed by GeoMx spatial transcriptomics on the same tissue section. This was originally described in the publication “*Same-Slide Spatial Multi-Omics Integration Reveals Tumor Virus-Linked Spatial Reorganization of the Tumor Microenvironment*”, with modifications adapted from protocols provided by SignalStar (Cell Signaling Technology) and Bruker Corporation (NanoString, GeoMx). Please cite when using this protocol.

Reagents checklist:

Reagent	Catalog #	Storage
Dako pH9.0 Antigen Retrieval Buffer	Agilent (S236784-2)	4°C
Nuclease-free water	Invitrogen (10977015)	RT
10X PBS (pH 7.4)	Gibco (70011069)	RT
Xylene	Sigma (534056)	RT
100% Ethanol	Fisher Scientific (07-678-007)	RT
SignalStar™ Midplex IHC Buffer Kit	Cell Signaling Technology (29414)	-20°C
SignalStar™ Oligo-Antibody (Antibody target of choice)	Cell Signaling Technology	-20°C
Tris Buffered Saline with Tween 20 (TBST-10X)	Cell Signaling Technology (9997)	RT
10% NBF Fixation Buffer	EMS Diasum (15740-04)	RT
DAPI	Cell Signaling Technology (4083)	4°C
20X SSC Buffer	Sigma (SRE0068)	RT
HybriSlip hybridization covers (22 mm x 40 mm x 0.25 mm)	Grace Bio-Labs (714022)	RT
100% formamide	ThermoFisher (AM9342)	4°C
GeoMx Instrument Buff Kit PCLN (Buffer S, Buffer R)	NanoString (100474)	RT for Buffer S, 4°C for Buffer R
GeoMx Whole Transcriptome Atlas - Human RNA for NGS	NanoString (121401102)	-20°C
GeoMx Nuclear Stain Morphology Kit (SYTO13)	NanoString (121300303)	-20°C
DSP collection plate	NanoString (100473)	RT
96-well PCR plate	Applied Biosystems (4483354)	RT
Plate seal	Applied Biosystems (4306311)	RT
GeoMx seq Code Pack - Primer plates: AB/CD/EF/GH - Seq Code Master Mix	NanoString (121400202/121400203/121400204)	-20°C
AMPure beads	Beckman Coulter (A63881)	4°C
DNA LoBind tubes (1.5 ml)	Eppendorf (022431021)	RT
Elution Buffer (0.05% Tween-20, 10mM Tris pH 8.0 in nuclease-free water)	/	RT
Hematoxylin	StatLab (HXMMHPT)	RT
Blueing Solution	StatLab (HXB00588E)	RT
Eosin Y Solution	StatLab (STE0243)	RT

DAY 1**Tissue preparation and antibody staining****1. Deparaffinize:**Time needed:
1 hour

- a. Bake FFPE tissue sections in an oven at 70°C for 1 hr.
2. While the tissue sections are baking, prepare the PT module for use later:
 - a. Prepare 1X Dako pH 9.0 Antigen Retrieval Buffer from the 10X stock using nuclease-free water in a slide holder.
 - b. Cover the slide holder with a lid or tin foil to prevent contamination of buffer with surrounding PBS.
 - c. Transfer the slide holder containing the buffer into the PT module.
 - d. Set the following retrieval program:

Prewarm	75°C
Heat	97°C, 20 mins
Cool	65°C

- e. Ensure that the PT module is filled with enough 1X PBS.
- f. Start prewarming the PT module.

3. Rehydration:Time needed:
~45 mins

- a. Prepare 95%, 80% and 75% EtOH by diluting 100% EtOH with nuclease-free water.
- b. Examine the tissue sections to ensure all the FFPE wax has melted.
- c. Incubate the tissue sections twice with xylene for 5mins each.
- d. Dip the tissue sections for 10 times every 30 second for a total of 3 mins each following exactly the order listed below:
 - i. Xylene (x3 rounds)
 - ii. 100% EtOH (x2 rounds)
 - iii. 95% EtOH (x2 rounds)
 - iv. 80% EtOH
 - v. 70% EtOH
 - vi. ddH₂O (x3 rounds)

4. Heat-Induced Epitope Retrieval:Time needed:
~1 hour

- a. Transfer the tissue sections to the prewarmed slide holder containing 1X Dako pH 9.0 Antigen Retrieval Buffer in the PT module prepared in Step.2, and begin the retrieval program.
- b. After the PT module cools to 65°C, remove the slide holder together with the tissue sections from the PT module.
- c. Place the slide holder (with the tissue sections inside) onto a lab bench.
- d. Allow it to cool for 15 mins.

5. Wash:Time needed:
~5 mins

- a. Wash tissue sections with 1X PBS for 5 mins on a belly dancer.

6. Reagent preparation (Imaging Round 1):Time needed:
~ 30 mins

- a. Thaw SignalStar Kit components (including **all antibody targets** and **complementary oligos for Imaging Round 1**) and keep on ice. **Note: Antibody diluents and Amplification buffers are viscous, ensure the correct amount is pipetted when preparing the reaction solution.**

- b. Prepare SignalStar Antibody solution as follows:

Reagents	1 slide
SignalStar Antibody Diluent A	105 µl
SignalStar Antibody Diluent B	45 µl
SignalStar Oligo-Conjugated Antibody (x8)	1.5 µl
Each of SignalStar Complementary Oligo of Imaging Round 1 (x4)	1.5 µl
Total	168 µl

- c. Prepare SignalStar Amplification Solution 1 as follows:

Reagents	1 slide
SignalStar Amplification Buffer A	600 µl
SignalStar Amplification Buffer B	600 µl
SignalStar Amplification Oligo A-488	12 µl
SignalStar Amplification Oligo A-594	12 µl
SignalStar Amplification Oligo A-647	12 µl

SignalStar Amplification Oligo A-750	12 μ l
Total	1248 μ l

- a. Prepare SignalStar Amplification Solution 2 as follows:

Reagents	1 slide
SignalStar Amplification Buffer A	600 μ l
SignalStar Amplification Buffer B	600 μ l
SignalStar Amplification Oligo B-488	12 μ l
SignalStar Amplification Oligo B-594	12 μ l
SignalStar Amplification Oligo B-647	12 μ l
SignalStar Amplification Oligo B-750	12 μ l
Total	1248 μ l

- b. Rotate SignalStar Antibody solution, Amplification Solution 1 and 2 end-over-end for 20 min at RT and keep at RT.
- c. While rotating, prepare SignalStar Ligation Solution as follows:

Reagents	1 slide
SignalStar Ligation Buffer	75 μ l
T4 DNA Ligase (5 U/ μ L)	3 μ l
ATP (100 mM)	1.5 μ l
Nuclease-free water	70.5 μ l
Total	150 μ l

7. Antibody staining (Imaging Round 1):

- Remove tissue sections from 1X PBS. Carefully wipe away excess solution from the slide.
- Circle tissue area with Papien. **Note:** Do not draw too close to the tissue area as this might affect subsequent staining procedures.
- Briefly wash away excess Papien from the slide by incubating the tissue slide in a coplin jar filled with 1X PBS for 1 min.
- Remove the tissue section from 1X PBS. Carefully wipe away excess solution from the slide.
- Place the tissue slide flat with the tissue facing upwards a humid box.
- Carefully pipette 150 μ l of SignalStar Antibody solution onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papien barrier.
- Incubate at RT for 40 mins. **Note:** Incubation can also be done at 4°C overnight (16-24 hrs).
- Prepare 1X TBS-T by diluting the 10X stock in nuclease-free water.
- Prepare three coplin jars with each filled with 1X TBS-T, 10% NBF buffer and nuclease-free water, respectively.
- After incubation is complete, carefully flick the solution from slides and place the tissue slide in 1X TBST.
- Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.
- Transfer the slides in 10% NBF buffer and incubate for 5 mins at RT.
- Carefully flick the solution from slides and place the tissue slide in nuclease-free water.
- Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.

Time needed:
~ 1 hour
(or overnight,
16-24 hours)

8. Amplification (Imaging Round 1):

- Remove the tissue section from nuclease-free water. Carefully wipe away excess solution from the slide.
- Place the tissue slide flat with the tissue facing upwards a humid box.
- Carefully pipette 150 μ l of Amplification Solution 1 onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papien barrier.
- Incubate at RT for 8 mins.
- Prepare a coplin jar with filled with nuclease-free water.
- After incubation is complete, carefully flick the solution from slides and place the tissue slide in nuclease-free water.
- Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.
- Remove the tissue section from nuclease-free water. Carefully wipe away excess solution from the slide.
- Place the tissue slide flat with the tissue facing upwards a humid box.
- Carefully pipette 150 μ l of Amplification Solution 2 onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papien barrier.
- Incubate at RT for 8 mins.
- Prepare a coplin jar with filled with nuclease-free water.
- After incubation is complete, carefully flick the solution from slides and place the tissue slide in nuclease-free water.

Time needed:
~ 3 hours

- n. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.
- o. Repeat step a-n for a total of **8 rounds** of amplification.

9. Ligation (Imaging Round 1):

Time needed:
~ 25 mins

- a. Remove the tissue section from nuclease-free water. Carefully wipe away excess solution from the slide.
- b. Place the tissue slide flat with the tissue facing upwards a humid box.
- c. Carefully pipette 150 µl of SignalStar Ligation Buffer onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papen barrier.
- d. Incubate at RT for 20 mins.
- e. Prepare a coplin jar with filled with nuclease-free water.
- f. After incubation is complete, carefully flick the solution from slides and place the tissue slide in nuclease-free water.
- g. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.

10. SignalStar imaging (Imaging Round 1):

Time needed:
~ 2 hours

- a. Prepare DAPI solution by diluting the stock to 0.5µg/ml in 1X PBS.
- b. Transfer the tissue slide to a coplin jar filled with 1X TBS-T for 30 sec.
- c. Remove the tissue section from 1X TBS-T. Carefully wipe away excess solution from the slide.
- d. Place the tissue slide flat with the tissue facing upwards a humid box.
- e. Carefully pipette 150 µl of DAPI solution (working concentration) onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papen barrier.
- f. Incubate at RT for 5 mins **in dark**.
- g. After incubation is complete, carefully flick the solution from slides and place the tissue slide in 1X TBS-T.
- h. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.
- i. Remove the tissue section 1X TBS-T. Carefully wipe away excess solution from the slide using a KimWipe.
- j. Mount the tissue slide with a glass coverslip with **1X TBS-T**. Slightly seal the coverslip around with nail polish if necessary.
- k. Begin imaging immediately.

11. Coverslip removal after imaging:

Time needed:
~ 15 mins

- a. Dip a Q-tip with xylene and gently remove the nail polish around the coverslip.
- b. Transfer the tissue section in a coplin jar of 1X TBS-T.
- c. Incubate for 5 mins at RT on a belly dancer at low speed.
- d. Let the coverslip detach from the tissue slide by itself. Do not remove it by force as this may damage the tissue section.
- e. When the coverslip has detached, transfer the tissue slide to a new coplin jar containing 1X TBS-T.

12. SignalStar Fluorescent Removal:

Time needed:
~ 2.5 hours

- a. Turn on and pre-warm the HybEZ Oven to 37°C.
- b. Arrange fresh Kimwipes on the bottom of the HybEZ Tray, then wet with nuclease-free water. **Note:** *Ensure no excess water is floating on the Kimwipes to avoid mixing with probes on the tissue section.*
- c. Thaw 10X dsDNase Buffer and keep on ice. Bring dsDNase from -20°C to ice.
- d. Prepare SignalStar Fluorescent Removal Solution as follows:

Reagents	1 slide
10X dsDNase Buffer	15 µl
dsDNase	1.5 µl
Nuclease-free water	133.5 µl
Total	150 µl

- e. Remove the tissue section from 1X TBS-T. Carefully wipe away excess solution from the slide.
- f. Place the tissue slide flat with the tissue facing upwards the HybEZ tray.
- g. Carefully pipette 150 µl of SignalStar Fluorescent Removal Solution onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papen barrier.
- h. Close the hybridization chamber and incubate at 37°C for 2 hrs.
- i. While incubating, proceed to the next part to prepare reagents for imaging round 2.
- j. After incubation is complete, carefully flick the solution from slides and place the tissue slide in nuclease-free water.
- k. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.

13. Reagent preparation (Imaging Round 2):Time needed:
~ 30 mins

- a. Thaw SignalStar Kit components (including **complementary oligos for Imaging Round 2, no antibodies are needed**) and keep on ice. *Note: Antibody diluents and Amplification buffers are viscous, ensure the correct amount is pipetted when preparing the reaction solution.*
- b. Prepare SignalStar Oligo solution as follows:

Reagents	1 slide
SignalStar Antibody Diluent A	105 μ l
SignalStar Antibody Diluent B	45 μ l
Each of SignalStar Complementary Oligo of Imaging Round 2 (x4)	1.5 μ l
Total	156 μl

- c. Prepare SignalStar Amplification Solution 1 as follows:

Reagents	1 slide
SignalStar Amplification Buffer A	600 μ l
SignalStar Amplification Buffer B	600 μ l
SignalStar Amplification Oligo A-488	12 μ l
SignalStar Amplification Oligo A-594	12 μ l
SignalStar Amplification Oligo A-647	12 μ l
SignalStar Amplification Oligo A-750	12 μ l
Total	1248 μl

- d. Prepare SignalStar Amplification Solution 2 as follows:

Reagents	1 slide
SignalStar Amplification Buffer A	600 μ l
SignalStar Amplification Buffer B	600 μ l
SignalStar Amplification Oligo B-488	12 μ l
SignalStar Amplification Oligo B-594	12 μ l
SignalStar Amplification Oligo B-647	12 μ l
SignalStar Amplification Oligo B-750	12 μ l
Total	1248 μl

- e. Rotate SignalStar Oligo solution, Amplification Solution 1 and 2 end-over-end for 20 min at RT and keep at RT.
- f. While rotating, prepare SignalStar Ligation Solution as follows:

Reagents	1 slide
SignalStar Ligation Buffer	75 μ l
T4 DNA Ligase (5 U/ μ L)	3 μ l
ATP (100 mM)	1.5 μ l
Nuclease-free water	70.5 μ l
Total	150 μl

14. Antibody staining (Imaging Round 2):Time needed:
~ 45 mins

- o. Remove tissue sections from 1X nuclease-free water. Carefully wipe away excess solution from the slide.
- p. Place the tissue slide flat with the tissue facing upwards a humid box.
- q. Carefully pipette 150 μ l of SignalStar Oligo solution onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papan barrier.
- r. Incubate at RT for 40 mins.
- s. Prepare a coplin jar filled with nuclease-free water.
- t. After incubation is complete, carefully flick the solution from slides and place the tissue slide in nuclease-free water.
- u. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.

15. Amplification (Imaging Round 2):Time needed:
~ 3 hours

- a. Remove the tissue section from nuclease-free water. Carefully wipe away excess solution from the slide.
- b. Place the tissue slide flat with the tissue facing upwards a humid box.
- c. Carefully pipette 150 μ l of Amplification Solution 1 onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papan barrier.
- d. Incubate at RT for 8 mins.
- e. Prepare a coplin jar with filled with nuclease-free water.
- f. After incubation is complete, carefully flick the solution from slides and place the tissue slide in nuclease-free water.
- g. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.

- h. Remove the tissue section from nuclease-free water. Carefully wipe away excess solution from the slide.
- i. Place the tissue slide flat with the tissue facing upwards a humid box.
- j. Carefully pipette 150 µl of Amplification Solution 2 onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papan barrier.
- k. Incubate at RT for 8 mins.
- l. Prepare a coplin jar with filled with nuclease-free water.
- m. After incubation is complete, carefully flick the solution from slides and place the tissue slide in nuclease-free water.
- n. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.
- o. Repeat step a-n for a total of **8 rounds** of amplification.

16. Ligation (Imaging Round 1):

*Time needed:
~ 25 mins*

- a. Remove the tissue section from nuclease-free water. Carefully wipe away excess solution from the slide.
- b. Place the tissue slide flat with the tissue facing upwards a humid box.
- c. Carefully pipette 150 µl of SignalStar Ligation Buffer onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papan barrier.
- d. Incubate at RT for 20 mins.
- e. Prepare a coplin jar with filled with nuclease-free water.
- f. After incubation is complete, carefully flick the solution from slides and place the tissue slide in nuclease-free water.
- g. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.

17. SignalStar imaging (Imaging Round 2):

*Time needed:
~ 2 hours*

- a. Prepare DAPI solution by diluting the stock to 0.5µg/ml in 1X PBS.
- b. Transfer the tissue slide to a coplin jar filled with 1X TBS-T for 30 sec.
- c. Remove the tissue section from 1X TBS-T. Carefully wipe away excess solution from the slide.
- d. Place the tissue slide flat with the tissue facing upwards a humid box.
- e. Carefully pipette 150 µl of DAPI solution (working concentration) onto the tissue section **without introducing any bubbles**. Ensure the solution entirely covers the tissue and contained within the Papan barrier.
- f. Incubate at RT for 5 mins **in dark**.
- g. After incubation is complete, carefully flick the solution from slides and place the tissue slide in 1X TBS-T.
- h. Wash the tissue slide at RT for 30 sec on a belly dancer at low speed.
- i. Remove the tissue section 1X TBS-T. Carefully wipe away excess solution from the slide using a KimWipe.
- j. Mount the tissue slide with a glass coverslip with **1X TBS-T**. Slightly seal the coverslip around with nail polish if necessary.
- k. Begin imaging immediately.

18. Coverslip removal after imaging:

*Time needed:
~ 15 mins*

- f. Dip a Q-tip with xylene and gently remove the nail polish around the coverslip.
- g. Transfer the tissue section in a coplin jar of 1X TBS-T.
- h. Incubate for 5 mins at RT on a belly dancer at low speed.
- i. Let the coverslip detach from the tissue slide by itself. Do not remove it by force as this may damage the tissue section.
- j. When the coverslip has detached, transfer the tissue slide to a new coplin jar containing 1X PBS.

DAY 2/3

After SignalStar imaging, image quality assessment will be performed. Re-imaging might be needed for markers not passing the quality check. Proceed to the next step ONLY when all markers have passed quality check.

Note: It is strongly recommended to perform GeoMx tissue preparation and probe hybridization immediately after SignalStar imaging to preserve RNA quality.

TISSUE PREPARATION (GeoMx)

Note: There is no turning back once the tissue is processed for GeoMx, when ready, proceed to Step.19. All GeoMx steps are based entirely on the GeoMx protocol from Bruker Cooperation.

*Time needed:
16-24 hours*

19. Probe hybridization:

- a. Turn on and pre-warm the HybEZ Oven to 37°C.
- b. Prepare and thaw the following reagents:
 - i. GeoMx RNA detection probes: Thaw and keep on ice. **Do not vortex and re-freeze probes.** Once thawed, they can be stored at 4°C for up to 3 months.
 - ii. Record probe's lot number. It is needed for setting up GeoMx RNA capture in the latter steps.
 - iii. Buffer R: Warm to RT. **Do not vortex.**
- c. Prepare Hybridization solution as follows:

Hybridization solution	2 slides + 10%
Buffer R	60 µl
RNA detection probes	7.5 µl
Custom probes or nuclease-free water	7.5 µl <i>(Note: For 1 custom probe set, add 3.75 µl probes with 3.75 µl nuclease-free water; For 2 custom probe sets, add 3.75 µl each of the probe set.)</i>
Total	75 µl

- d. Gently mix by pipetting **without introducing any bubbles.**
- e. Arrange fresh Kimwipes on the bottom of the HybEZ Tray, then wet with nuclease-free water. **Note: Ensure no excess water is floating on the Kimwipes to avoid mixing with probes on the tissue section.**
- f. Remove tissue section from 1X PBS, gently wipe away excess 1X PBS and place in the HybEZ Tray.
- g. Slowly pipette 70 µl Hybridization solution to the tissue section **without introducing any bubbles.**
- h. Peel off the plastic film from the Grace Bio-Labs HybriSlip and gently place the side covered by the plastic film over the tissue section **without introducing any bubbles.**
- i. Close the hybridization chamber and incubate at 37°C overnight (16-24 hrs).

DAY 4

Note: From henceforth, minimize exposure of the tissue sections to light.

Time needed:
~40 mins

All wash steps on a belly dancer below should be set at low speed.

Ensure that cell type annotation and the phenotype maps are ready at this point, and have a clear sense of which ROIs to select based on study design and tissue morphology.

20. Post-hybridization wash and nuclear staining:

- a. Prepare the following:
 - i. 2x and 4x SSC Buffers by diluting the 20x stock in nuclease-free water.
 - ii. Two coplin jars of Stringent Wash Buffer (40 ml each) by mixing 1:1 of 100% formamide with 4x SSC buffer.
 - iii. Prewarm Stringent Wash Buffer in a 37°C water bath.
 - iv. Remove SYTO13 from the -20°C freezer and thaw at RT. Make 100nM SYTO13 by diluting the stock in 2x SSC buffer.
 - v. One coplin jar of 2x SSC buffer.
- b. Remove the tissue sections from the HybEZ Oven.
- c. Gently dip the tissue section in the coplin jar containing 2x SSC buffer. Allow the coverslip to fall off by itself. **Proceed to the next step immediately once the coverslip has fallen off.**
- d. Transfer the tissue section to the pre-warmed Stringent Wash Buffer at 37°C.
- e. Incubate for 5 mins.
- f. Repeat incubation using the 2nd coplin jar containing the pre-warmed Stringent Wash Buffer.
- g. Transfer the tissue sections to the coplin jar containing 2x SSC buffer, wash tissue sections for 5 mins on a belly dancer.
- h. Stain tissue sections with the 100nM SYTO13 prepared in the previous step for 5 mins at RT in the dark.
- i. Wash tissue sections twice in 2x SSC buffer for 5 mins each on a belly dancer.

21. GeoMx transcript collection:

Time needed:
~3 hours per
plate

- a. Load and secure the tissue section into the slide holder accordingly. Slowly add 3ml Buffer S along the side of the holder to the tissue section. **Note:** *Be careful not to add directly on top of the tissue as this may cause damage.*
- b. Gently clean the bottom of each tissue section with 70% EtOH using a KimWipe, then wipe off with another dry KimWipe.
- c. Follow the instructions from the GeoMx DSP for scanning, image capture and drawing ROI. **Note:** *Exposure of 10ms for SYTO13 was used in our study. Adjust the exposure accordingly.*
- d. After drawing the ROIs, export the omi.tiff files and record the x- and y-coordinates of each of the ROI drawn.
- e. Generate the phenotype maps based on the ROI info and save each ROI per folder in a thumb drive. **Note:** *Refer to <https://sizunjianglab.github.io/IN-DEPTH/> for more details.*
- f. Import the phenotype maps of each of the ROI to the GeoMx DSP one by one accordingly. **Note:** *This is extremely important, importing all phenotype maps at once will freeze the GeoMx DSP.*
- g. After importing the phenotype maps of each ROI, carefully inspect whether the alignment is correct.
- h. Begin transcript collection as per GeoMx DSP instructions.
- i. Replace the collection plate as soon as possible after a plate has been filled up. One full plate will take around 3 hours to complete collection.
- j. Dry the plate by leaving on a clean bench top overnight or incubating in a thermocycler at 65°C for 1 hr.
- k. Check that the DSP collection plate wells are **completely dry** and seal it with a plate seal.
- l. Store the dried aspirates at -20°C until ready for library construction.

DAY 5**22. Library construction:**

Time needed: ~2
hours per plate

- a. After GeoMx has finished collecting aspirates and all plates were dried, centrifuge the plates at 1000xg for 30 sec.
- b. Carefully remove the plate seal and rehydrate each well with 10 µl nuclease-free water. **Note:** *Use different pipette tips for individual wells to avoid contamination. Well A01 of each plate is automatically assigned as NTC and it should be empty.*
- c. Reseal the plate and centrifuge at 1000xg for 30 sec. Allow each aspirate to solubilized by incubating at RT for 10 mins. Centrifuge at 1000xg for 30 sec once incubation is done.
- d. While incubating, thaw Primer Plates and 5X PCR Master Mix at RT. **Note:** *Each DSP collection plate must be assigned to a unique Primer Plate (A-H).*
- e. Centrifuge Primer Plate and 5X PCR Master Mix at 1000xg for 30 sec.
- f. Using a new 96-well PCR plate per collection plate, set up the PCR reaction for each well as follows:

Reaction	Volume
Primer	4 µl
Rehydrated aspirate	4 µl
5X PCR Master Mix	2 µl
Total	10 µl

- g. Pipette 10 times to mix thoroughly and seal the PCR plates with a new plate seal.
- h. Centrifuge PCR plates at 1000 xg for 30 sec.
- i. Store PCR plates at 4°C until ready for reaction.
- j. Begin PCR reaction in a thermal cycler with the following program:
 - i. Set the lid temperature to 100 °C.
 - ii. Program:

Step		Temperature	Run time
1	UDG incubation	37°C	30 mins
2	UDG deactivation	50°C	10 mins
3	Initial denaturation	95°C	3 mins
4	Denaturation	95°C	15 sec
5	Anneal	65°C	60 sec
6	Extend	68°C	30 sec
7	Return to Step 4 for a total of 18 times		
8	Final extension	68°C	5 mins
9	Hold	12°C	∞

- iii. Initiate the program.

23. Pooling and AMPure cleanup:

Time needed:
~ 90 mins

- a. Once PCR is completed, centrifuge each plate at 1000xg for 30 sec.
- b. For each PCR plate, pool the PCR products into two separate DNA LoBind tubes:
 - i. Tube 1 (Sample): Transfer 4 µl from each sample and NTC well (A01)
 - ii. Tube 2 (NTC): Transfer 6 µl from each NTC well (A01)
- c. Prepare the following **fresh** every time:
 - i. 5 ml 80% EtOH (v/v)
 - ii. 1 ml Elution Buffer
- d. Measure the exact volume in each LoBind tube using a pipette and record the volume. **Note:** *This step is critical, as it determines the volume of AMPure XP beads to be used, which directly influences the size selection of the DNA fragments during cleanup.*
- e. Resuspend the AMPure XP beads by vortexing until being completely resuspended.
- f. Add 1.2 times volume of AMPure XP beads into each LoBind tube containing the pooled samples accordingly.
- g. Carefully pipette 10 times to mix thoroughly.
- h. Pulse centrifuge the tube for 1 sec and incubate at RT for 5 mins.
- i. Pellet the beads by placing the tube into a magnetic stand for 5 mins. Ensure that the solution is clear before proceeding to the next step.
- j. Carefully open the tube and pipette away the solution from the tube **without disturbing the beads**.
- k. **The beads now contain the library.**
- l. Gently add 1 ml of 80% EtOH to each tube **without disturbing the beads**. Incubate for 30 sec and carefully pipette away the solution.
- m. Repeat Step.18l for a total of two times. **Note:** *Remove any residual EtOH with a P20 pipette if needed.*
- n. Air dry the beads for 5 mins in the magnetic stand. **Note:** *Do not dry it for over 5 mins as this will affect library recovery.*
- o. Remove the tube from the magnetic stand and resuspend the beads with 54 µl of Elution Buffer. Pipette 20 times to mix thoroughly.
- p. Pellet the beads by placing the tube into a magnetic stand for 5 mins. Ensure that the solution is clear before proceeding to the next step.
- q. Transfer 50 µl of solution from each tube to a **new** LoBind tube **without disturbing the beads**.
- r. **The solution now contains the library.**
- s. Add 60 µl of AMPure XP beads to each tube. Carefully pipette 10 times to mix thoroughly.
- t. Pulse centrifuge the tube for 1 sec and incubate at RT for 5 mins.
- u. Pellet the beads by placing the tube into a magnetic stand for 5 mins. Ensure that the solution is clear before proceeding to the next step.
- v. Carefully open the tube and pipette away the solution from the tube **without disturbing the beads**.
- w. **The beads now contain the library.**
- x. Gently add 1 ml of 80% EtOH to each tube **without disturbing the beads**. Incubate for 30 sec and carefully pipette away the solution.
- y. Repeat Step.18l for a total of two times. **Note:** *Remove any residual EtOH with a P20 pipette if needed.*
- z. Air dry the beads for 5 mins in the magnetic stand. **Note:** *Do not dry it for over 5 mins as this will affect library recovery.*
- aa. Remove the tube from the magnetic stand and resuspend the according to the number of wells pooled in that tube:

Number of wells	Volume of Elution Buffer to add
96	48 µl
48	24 µl
24	16 µl
12	12 µl
NTC	5 µl

- bb. Pipette 20 times to mix thoroughly.
- cc. Pulse centrifuge the tube for 1 sec and incubate at RT for 5 mins.
- dd. Pellet the beads by placing the tube into a magnetic stand for 5 mins. Ensure that the solution is clear before proceeding to the next step.
- ee. Transfer as much solution from each tube to a **new** LoBind tube **without disturbing the beads**.
- ff. **The solution now contains the library.**
- gg. Analyze sample purity and concentration using Tapestation or Bioanalyzer. The sequencing library can be stored at -20C until it is ready for sequencing.

After sequencing sample QC

24. Perform H&E staining and imaging:

- a. Incubate tissue section with Hematoxylin solution for 5 mins at RT.
- b. Wash tissue section by gently dipping it in a jar filled with nuclease-free water for 15 times. Repeat this step with new jar of nuclease-free water until the solution is clear of Hematoxylin.
- c. Remove tissue section from the jar and gently wipe away the excess water using a Kimwipe.
- d. Incubate tissue section with Blueing solution for 1 min, RT.
- e. Wash tissue section by gently dipping it in a jar filled with nuclease-free water for 15 times. Repeat this step for 2 more times using a new jar of nuclease-free water each time.
- f. Remove tissue section from the jar and gently wipe away the excess water using a Kimwipe.
- g. Submerge tissue section in a jar filled with 95% Ethanol and incubate for 1 min at RT.
- h. Remove tissue section from the jar and gently wipe away the excess ethanol using a Kimwipe.
- i. Incubate tissue section with Eosin solution for 1 min at RT.
- j. Wash tissue section by gently dipping it in a jar filled with 95% Ethanol for 12 times. Repeat this step for 2 times using a new jar of 95% Ethanol each time.
- k. Dehydrate tissue section by gently dipping it 12 times in 80% Ethanol followed by 95% Ethanol, 100% EtOH and finally with xylene.
- l. Mount the tissue section with a glass coverslip using xylene-based mounting solution.
- m. Allow the mount to adhere by laying the slide flat for at least 1 hr.
- n. Image the tissue section using a slide scanner.