IN-DEPTH: CODEX-GeoMx protocol

This protocol outlines the required reagents and step-by-step procedures for performing CODEX 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 Akoya Biosciences (PhenoCycler Fusion) 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)	4ºC
Nuclease-free water	Invitrogen (10977015)	RT
S2 Buffer (2.5 mM EDTA, 0.5× DPBS, 0.25% BSA, 0.02% NaN3, 250 mM NaCl, 61 mM Na2HPO4, 39 mM NaH2PO4)	/	4ºC
10X PBS (pH 7.4)	Gibco (70011069)	RT
Xylene	Sigma (534056)	RT
100% Ethanol	Fisher Scientific (07-678-007)	RT
BBDG Buffer (5% normal donkey serum, 0.05% NaN3 in 1× TBS-T)	/	-20ºC
Mouse IgG (B1) (50 µg/mL mouse IgG, diluted from 1 mg/ml stock in S2 Buffer)	Sigma (I5381-10mg)	-20ºC
Rat IgG (B2) (50 µg/mL rat IgG, diluted from 1 mg/ml stock in S2 Buffer)	Sigma (I4141-10mg)	-20ºC
Sheared salmon sperm DNA (500 µg/ml)	ThermoFisher (AM9680)	-20ºC
Oligo Block Buffer (50nM, diluted from stock with 500 nM of each oligo in 1× TE pH 8.0)	/	4ºC
Antibody Diluent (5% Donkey Serum, 0.05% NaN3 in 1× TBS-T)	/	-20ºC
FFPE Block Buffer (0.05 mg/ml mouse IgG, 0.05 mg/ml rat IgG, 0.5 mg/ml ssDNA, 100nM of each oligo in S2 Buffer)	/	4ºC
Hoechst 3342 Solution	Invitrogen (H3570)	4ºC
Plate Buffer (500 µg/mL sheared salmon sperm DNA in 1× CODEX buffer)	/	4ºC
16% PFA	EMS Diasum (15740-04)	RT (4 ºC when opened)
100% MeOH (ice cold aliquot in 50ml Falcone tubes)	Sigma (179337)	-20ºC
BS3 Final fixation	ThermoFisher (21580)	-20ºC
1X CODEX Buffer (10mM Tris pH 7.5, 0.02% NaN3, 0.1% Triton X-100, 10 mM MgCl2-6H2O, 150mM NaCl)	/	RT
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)	4ºC
GeoMx Instrument Buff Kit PCLN (Buffer S, Buffer R)	NanoString (100474)	RT for Buffer S, 4ºC for Buffer R
GeoMx Whole Transcriptome Atlas - Human RNA for NGS	NanoString (121401102)	-20ºC
GeoMx Nuclear Stain Morphology Kit (SYTO13)	NanoString (121300303)	-20ºC
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)	-20ºC
AMPure beads	Beckman Coulter (A63881)	4ºC
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.

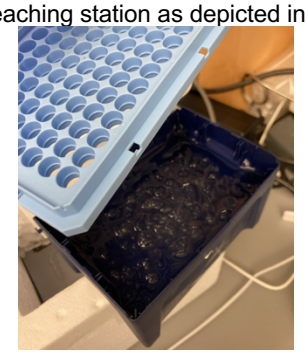
3. **Rehydration:** *Time needed: ~45 mins*
   a. Prepare 95%, 80% and 75% EtOH by diluting 100% EtOH with nuclease-free water.
   b. Examine the tissue sections to ensure all the FFPE wax has melted.
   c. Incubate the tissue sections twice with xylene for 5mins each.
   d. Dip the tissue sections for 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. ddH_2O (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: ~30 mins*
   a. Wash tissue sections with S2 Buffer in a coplin jar for 20 mins on a belly dancer.
   b. Proceed to blocking buffer preparation during S2 Buffer wash.
   c. Transfer tissue sections to another coplin jar filled with 1X TBST and wash for 5 mins on a belly dancer.

Optional: Circle tissue area with Pappen.
***Note:** Do not draw too close to the tissue area as this might affect subsequent staining procedures.*

6. **Blocking and Photobleaching:** *Time needed: ~1 hour*
   a. Remove BBDG, salmon DNA, B1 and B2 buffers from -20°C, thaw on ice. Vortex to mix.
   b. Remove Oligo block from 4 °C, keep on ice until use.
   c. Set up the blocking and photobleaching station as depicted in the image:


   d. Depending on the size of the tissue, 200µl of blocking buffer is normally needed for each tissue section.

e. Prepare blocking buffer as follows:

Reagent	Stock conc.	Working conc.	Volume (1 tissue section)
BBDG	/	/	150 µl
Oligo block	500nM	50nM	20 µl
ssDNA	20X	1X	10 µl
Mouse IgG / B1	20X	1X	10 µl
Rat IgG / B2	20X	1X	10 µl

c. Add 100µl of blocking buffer to cover each tissue section.
d. Place tissue section in a humid box (with ice slurry) and photobleach it under happy light for 1 h.
e. Replenish blocking buffer to the tissue section after 30 mins or whenever necessary to prevent tissue section from drying.
f. Proceed to the next step while waiting for blocking and photobleaching to finish.

Note: *Ensure temperature is below 37°C throughout the entire process. Stop the procedure and let the tissue to cool for 10 mins if the temperature goes beyond 37°C.*

7. **Primary antibody preparation:** *Time needed: ~40 mins*
   a. Remove antibody diluent buffer from -20°C, thaw on ice. Vortex to mix.
   b. Remove all conjugated antibodies from 4°C and centrifuge at 12,500 x *g* for 8 mins.
   c. In an empty 1.5ml epitube, pipette and transfer 300µl of antibody diluent.
   d. Add the desired concentration of each conjugated antibody into the epitube. Mix well by pipetting gently.
   e. Pre-wet a 50-kDa centrifugal filter column with 400 µl S2 buffer and centrifuge at 12,500 x *g* for 2 mins.
   f. Carefully pipette the remaining liquid inside the filter, discard the column flow-through.
   g. Transfer the antibody mix to the pre-wetted 50-kDa centrifugal filter column and centrifuge at 12,500 x *g* for 8 mins.
   h. To collect the antibodies, invert the 50-kDa filter into a new collection tube and centrifuge at 12,500 x *g* for 1 min. ***Note:*** *Do not discard the filter at this stage.*
   i. Measure the volume of antibodies and calculate the amount of antibody diluent needed with the following formula:

      Total staining volume - volume of antibodies - ¼ total staining volume FFPE Block

   j. Add the amount of antibody diluent needed to the 50-kDa filter from Step.7h and pipette gently to collect any residual antibodies.
   k. Invert the 50-kDa filter into the collection tube containing the collected antibodies and centrifuge at 12,500 x *g* for 1 min.
   l. Add FFPE block to the collected antibodies. Mix well by pipetting gently.
   m. Pre-wet a 0.1 µm centrifugal filter column with 400 µl S2 buffer and centrifuge at 12,500 x *g* for 2 mins.
   n. Transfer the centrifugal filter into a new 1.5ml epitube. Discard the collection tube.
   o. Transfer the collected antibodies into the pre-wetted 0.1 µm centrifugal filter column.
   p. Centrifuge the 0.1 µm centrifugal filter column at 12,500 x *g* for 2 mins to remove antibody aggregates.
   q. The column flow-through now contains the antibody mix.

Note: *Antibodies should be treated with extra care. Avoid any bubbles during pipetting.*

8. **Primary antibody staining:** *Time needed: 16-24 hours*
   a. After blocking and photobleaching, remove excess blocking buffer by soaking gently with a Kimwipe.
   b. Place the tissue section in a humid box.
   c. Add the antibody mix prepared in Step.7 onto tissue.
   d. Seal the humid box with parafilm and incubate at 4°C for 16-24 h.

DAY 2-3

CODEX imaging

9. **CODEX reporter plate preparation:** *Time needed: ~30 mins*
   a. Calculate the amount of CODEX plate buffer that is needed (250 µl * total # cycles).
   b. Prepare CODEX Plate Buffer by diluting Hoechst 3342 in Plate Buffer at 1:300.
   c. Remove the reporter oligos needed from 4°C and centrifuge at 12,500 x g for 2 mins.
   d. Dilute reporter oligos at 1:10 for each cycle accordingly based on the following calculations:
      i. 250µl of Plate Buffer for blank cycles.
      ii. 247.5µl Plate Buffer for cycles with one reporter.
      iii. 245µl Plate Buffer for cycles with two reporters.
   e. Add reporter mix for each cycle accordingly to a black 96-well plate.
   f. Seal the reporter plate with an Aluminum microplate seal.

g. Keep at 4°C until ready for CODEX imaging.

10. Post-stain washes and fixation: *Time needed: ~1 hour*

a. After antibody incubation has finished, remove the tissue section from the humid box.
b. Gently wash the tissue section with S2 buffer in a coplin jar twice for 2 mins each on a belly dancer.
c. Prepare 200µl of 1.6% PFA in 1X PBS from 16% PFA stock and 10X PBS.
d. Remove the tissue section from the coplin jar, gently wipe off excess buffer with a Kimwipe.
e. Place the tissue section in a humid box and add 1.6% PFA, PBS onto the tissue.
f. Cover the humid box with tin foil and incubate for 10 mins at room temperature.
g. After incubation has finished, remove the tissue section from the humid box.
h. Gently remove the excess 1.6% PFA, PBS by soaking with a Kimwipe.
i. Gently rinse the tissue section with 1X PBS in a coplin jar twice and wash for 2 mins on a belly dancer.
j. Remove 100% MeOH aliquot from -20°C, fill a slider mailer and place it in ice.
k. Transfer the tissue section from the coplin jar after wash to the slide mailer containing the ice-cold MeOH and incubate for 5 mins in ice.
l. After incubation has finished, remove the tissue section from the MeOH and gently remove the excess MeOH by soaking with a Kimwipe.
m. Gently rinse the tissue section with 1X PBS in a coplin jar twice and wash for 2 mins on a belly dancer.
n. Prepare 150µl fresh final fixative per tissue section by diluting CODEX BS3 final fixative with 1X PBS at 1:50.
o. Remove the tissue section from the coplin jar, gently wipe off excess buffer with a Kimwipe.
p. Place the tissue section on a humid box and add 75µl fresh final fixative to each tissue section.
q. Cover the humid box with tin foil and incubate at room temperature for 20 mins.
r. At the 10-min mark, add the remaining 75µl fresh final fixative to each tissue section, and continue the incubation.
s. After incubation has finished, remove the tissue section from the humid box and gently remove the excess fixative by soaking with a Kimwipe.
t. Gently wash the tissue section with 1X CODEX buffer in a coplin jar twice for 5 mins each.
u. Store the tissue section in 1X CODEX buffer at 4°C until ready for imaging.

Note: *1.6% PFA in 1X PBS must be prepared by diluting from 10X PBS.*

Final fixative BS3 must be prepared immediately before fixation.

11. Flow cell assembly: *Time needed: ~20 mins*

a. If Pappen was used to encircle the tissue area, gently scrape away the Pappen marks with the wooden end of a Q-tip. Be extremely careful not to disrupt or touch the tissue.
b. Remove any sticker label from the tissue section. ***Note:*** *This is very important, slight elevation from the sticker will result in flow cell or sample slide breakage.*
c. Remove flow cell from the case and dispose the plastic cover slide.
d. Visually inspect to ensure there is no cracks on the flow cell. Use a dust blower to remove any dust on the flow cell.
e. Peel off the plastic frame that covers the adhesive on the flow cell.
f. Place the flow cell on the stage of the Assembly Device with the adhesive side facing up.
g. Remove the tissue section from the coplin jar and gently wipe away any excessive buffer from the slide. Be extremely careful not to disturb the tissue. ***Note:*** *Be careful not to dehydrate the tissue.*
h. Align the tissue section with the flow cell with the sample facing down on top of the flow cell.
i. Slide the stage all the way into the Assembly Device and lower the lever in one single action all the way down.
j. Wait for 30 secs to ensure proper seal.
k. Raise the lever and transfer the flow cell-assembled tissue section to a coplin jar containing 1X CODEX buffer.
l. Incubate for at least 10 mins to allow the flow cell to completely adhere to the tissue section.
m. The tissue section is now ready for CODEX imaging.

12. CODEX imaging *Time needed: 1-3 days*

a. Refer to PhenoCycler Fusion protocol from Akoya Biosciences for detailed procedure on setting up the software.

Note: *Exposure times for each antibody markers should be titrated prior to the actual experiment. For reference, please refer to the Supplementary Table 6_Antibody Panel from the original publication "Same-Slide Spatial Multi-Omics Integration Reveals Tumor Virus-Linked Spatial Reorganization of the Tumor Microenvironment" and previous studies from our lab (https://sizunjianglab.com/publications/).*

Day 4

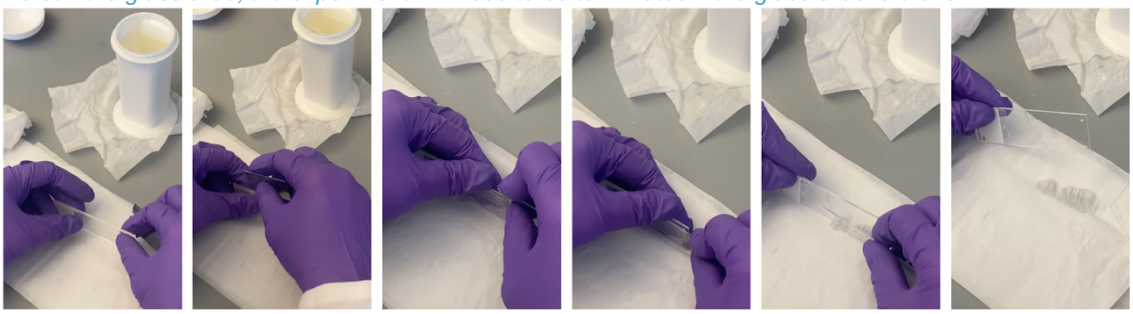
After CODEX imaging, image quality assessment will be performed. Re-imaging might be needed for markers not passing the quality check. Proceed to the next step when all markers have passed quality check.

Time needed: ~30 mins

13. Flow cell removal after fusion:

a. Prepare 2xSSC buffer by diluting 20X SSC buffer stock with nuclease-free water.
b. Remove the tissue section from the PhenoCycler Fusion slide carrier and transfer it into a coplin jar containing 2xSSC buffer.
c. Use a razor to remove the flow cell. Dip the tissue section in 2xSSC buffer as needed to ensure that the tissue section does not dry up. *Note: the flow cell will break during the process, it is completely normal.*
d. After the flow is removed from the tissue section, use a Q-tip soaked with methanol to scrap of the residual adhesive from the slide.
e. After the adhesive is removed, transfer the tissue sections into a new coplin jar containing 1X PBS.

Note: *Tutorial video on flow cell removal can be found in https://sizunjianglab.github.io/IN-DEPTH/ . Be careful not to break the glass slide, the experiment will need to be terminated if the glass slide is broken.*



It is important to remove as much residual adhesive from the tissue section as possible, remaining adhesive might affect subsequent GeoMx processes.

It is strongly recommended to perform GeoMx tissue preparation and probe hybridization immediately after CODEX 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.14. All GeoMx steps are based entirely on the GeoMx protocol from Bruker Cooperation.*

Time needed: 16-24 hours

14. 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 *(**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.)*
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 5

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.

15. 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.

16. <u>GeoMx transcript collection:</u>

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 6

17. <u>Library construction:</u>

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 4ºC until ready for reaction.
j. Begin PCR reaction in a thermal cycler with the following program:
   i. Set the lid temperature to 100 ºC.
   ii. Program:

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

   iii. Initiate the program.

18. 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.*
a. 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

aa. Pipette 20 times to mix thoroughly.
bb. Pulse centrifuge the tube for 1 sec and incubate at RT for 5 mins.
cc. 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.
dd. Transfer as much solution from each tube to a new LoBind tube without disturbing the beads.
ee. The solution now contains the library.
ff. 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

19. 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 filed 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.