IN-DEPTH: CODEX-VisiumHD protocol

This protocol outlines the required reagents and step-by-step procedures for performing CODEX imaging followed by VisiumHD 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 10x Genomics (VisiumHD). 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
Hematoxylin	StatLab (HXMMHPT)	RT
Blueing Solution	StatLab (HXB00588E)	RT
Eosin Y Solution	StatLab (STE0243)	RT
1N HCl	Sigma (1090571000)	RT
1X TE Buffer (pH 8.0)	Invitrogen (AM9849)	RT
Protease K Solution	Thermo Fisher Scientific (AM2546)	-20ºC
10% NBF Fixation Buffer	EMS Diasum (15740-04)	RT
NBF Stop Buffer (0.1M Tris and 0.1M Glycine in nuclease-free water)	/	RT
Dual Index Kit TS Set A, 96 rxn	10x Genomics (1000251)	-20ºC
Visium HD, Human Transcriptome, 6.5 mm	10x Genomics (1000675)	-20ºC (-80ºC for VisiumHD slides)
Visium 6.5mm Slide Cassette, 4 pk	10x Genomics (1000469)	RT
Visium CytAssist Tissue Slide Cassette, 4 pk, 6.5 mm	10x Genomics (1000471)	RT
10% Tween-20	Thermo Scientific (28320)	RT
1M Tris-HCl (pH 8.0)	Invitrogen (15568-025)	RT
0.8M KOH	Sigma (P4494-50ml)	RT
EB Buffer	Qiagen (19086)	RT
PCR 8-tube strip	USA Scientific (1402-4700)	RT
KAPA SYBR FAST qPCR Master Mix (2X)	Sigma (KK4600)	-20ºC
96-well PCR plate	Applied Biosystems (4483354)	RT
Plate seal	Applied Biosystems (4306311)	RT
SPRIselect Reagent	Beckman Coulter (B23317)	4ºC

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/).*

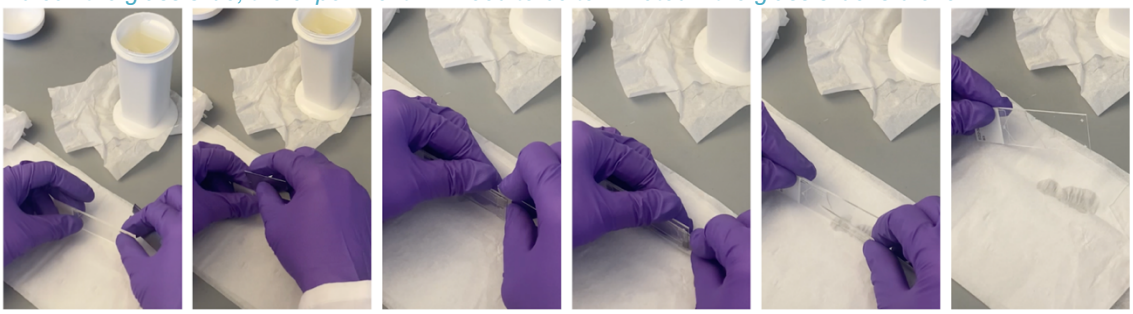
Day 4

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 2xSSC buffer.

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 VisiumHD processes.

It is strongly recommended to perform VisiumHD tissue preparation and probe hybridization immediately after CODEX imaging to preserve RNA quality.

TISSUE PREPARATION (VisiumHD)

Note: *There is no turning back once the tissue is processed for VisiumHD, when ready, proceed to Step.14.*

Time needed: ~30 mins

14. 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. Rehydrate tissue section by gently dipping it 12 times in 80% Ethanol followed by 70% Ethanol and finally with nuclease-free water.
    l. Mount the tissue section with a glass coverslip using nuclease-free water.
    m. Image the tissue section using a slide scanner.
    n. After the scan is completed, remove the coverslip gently by submerging the tissue section in nuclease-free water horizontally, the coverslip should slide off the tissue section by itself. ***Note:*** *Do not remove the coverslip by force as this will damage the tissue.*
    o. After the coverslip is removed, proceed to the next step.

15. Tissue destaining:

Time needed: ~20 mins

    a. Incubate tissue section with 0.1N HCl diluted from 1N HCl with nuclease-free water for 15 mins, at 42ºC, in a HybEz oven.
    b. After tissue destaining, rinse tissue section gently in a coplin jar filled with 1x TE buffer for 5 mins, twice.

16. Protease K treatment: *Time needed: ~25 mins*

a. Prepare 2µg/ml protease K by diluting the stock with 1X PBS (pH 7.4).
b. Remove tissue section and gently absorb the excess TE buffer using a Kimwipe.
c. Incubate tissue section with the 2ug/ml protease K prepared in Step.16a for 20 mins at 40°C in a HybEz oven.
d. After tissue digestion, immediately rinse tissue section by dipping it gently in a jar filled with nuclease-free water for 3 times. Repeat this for two more rounds with a new jar of nuclease-free water each round.
e. Proceed immediately to NBF fixation.

17. NBF fixation: *Time needed: ~20 mins*

a. Remove excess nuclease-free water by wiping the tissue section gently with a Kimwipe.
b. Fix the tissue section by submerging it in a coplin jar filled with 10% NBF buffer. Incubate for 1 min at RT.
c. Stop the fixation by washing the tissue section with NBF Stop Buffer for 5 mins at RT on a belly dancer. Repeat this for 1 more time using a new jar of NBF Stop Buffer.
d. Wash the tissue section once with 1X PBS (pH 7.4) for 5 mins at RT on a belly dancer.

All steps beyond this point are based entirely on the VisiumHD protocol from 10x Genomics:

18. Probe hybridization: *Time needed: 16-24 hours*

a. Thaw the following reagents:
   i. FFPE Hyb Buffer (#2000423): Thaw and keep at RT, pipette 10 times to mix thoroughly *Note: Do not vortex.*
   ii. Human WT Probes v2-RHS (#2000657): Thaw and keep at RT, vortex to mix.
   iii. Human WT Probes v2-LHS (#2000658): Thaw and keep at RT, vortex to mix.
b. Remove tissue section from the coplin jar and wipe away excess PBS using a tint wipe.
c. Place the tissue section in the VisiumHD cassette and adjust the 6.5mmx6.5mm grid such that it is on top of the tissue region of interest.
d. Add 150 µl of 1X PBS (pH 7.4) to the grid well.
e. Prepare pre-hybridization mix as follows:

Pre-hybridization mix	**2 slides (+10%)**
Nuclease-free water	295.3 µl
10X PBS, pH 7.4	33 µl
10% Tween-20	1.7 µl
Total	**330 µl**

f. Carefully pipette away all 1X PBS (pH 7.4) from the grid well.
g. Add 150 µl pre-hybridization mix slowly along the side of the well without introducing bubbles to the tissue section.
h. Apply a pre-cut VisiumHD seal on the tissue cassette and let the tissue section incubate with the pre-hybridization mix for 15 mins at RT.
i. While incubating, place the low-profile thermocycler adaptor in the thermal cycler and prepare the following program:
   i. Set lid temperature to 50°C.
   ii. Program:

Step	**Temperature**	**Run time**
Pre-equilibrate	50°C	Hold
Hybridization	50°C	Overnight 16-24hrs
Post Hybridization wash	50°C	Hold

   iii. Start pre-equilibrate step.

j. Prepare probe hybridization mix as follows:

Probe Hybridization Mix	**2 slides (+10%)**
Nuclease-free water	22 µl
FFPE Hyb Buffer (#2000423)	154 µl
Human WT Probes v2-RHS (#2000657)	22 µl
Human WT Probes v2-LHS (#2000658)	22 µl
Total	**220 µl**

k. Pipette 10 times to mix thoroughly and centrifuge briefly to bring down all solution to the bottom.
l. Keep the Probe Hybridization Mix at RT until use.
m. After pre-hybridization incubation finishes, gently pipette away all pre-hybridization mix from the grid well.

n. Add 100ul Probe Hybridization Mix slowly along the side of the well without introducing bubbles to the tissue section.
o. Apply a pre-cut VisiumHD seal on the tissue cassette.
p. Place the tissue cassette on the low-profile thermocycler adaptor on the thermal cycler.
q. Close the thermal cycler lid and skip the pre-equilibrate step to initiate hybridization overnight (**16-24hrs**).

Day 5

19. Post-hybridization wash: *Time needed: ~20 mins*

a. Thaw the following reagents (from -20C):
   i. FFPE post-hybr wash buffer (#2000424): Thaw and keep at RT, vortex to mix.
   ii. 2x Probe ligation buffer (#2000445): Thaw and keep at RT, vortex to mix.
   iii. Post-ligation wash buffer (#2000419): Thaw and keep at RT, vortex to mix.
   iv. Probe ligation enzyme (#2000425): Thaw and keep on ice**.**
b. Aliquot FFPE post-hybr wash buffer (495 µl per sample) into 0.2ml PCR tubes and pre-heat at 50ºC in the thermal cycler.
c. Prepare 2xSSC buffer (if not prepared) and keep at RT.
d. When probe is hybridized for 16-24hrs, perform the following steps:
   Note: *Continue the "hold" step from the ongoing hybridization protocol on the thermal cycler. Maintain the temperature at 50ºC throughout the following washes.*
   i. Remove the tissue cassette from the thermal cycler and peel back the slide seal.
   ii. Gently pipette away all probe hybridization mix from the grid well.
   iii. Immediately add 150 µl pre-heated FFPE post-hybr wash buffer slowly along the side of the well without introducing any bubbles to the tissue section.
   iv. Reapply the slide seal and place the tissue cassette back to the thermal cycler.
   v. Close the thermal cycler lid and incubate for 5 mins.
e. **Repeat Step.19d for a total of 3 times.**
f. After the washes were finished, remove tissue cassette from the thermal cycler on and peel back the slide seal.
g. Gently pipette away all FFPE post-hybr wash buffer from the grid well.
h. Add 150 µl 2x SSC buffer slowly along the side of the well without introducing any bubbles to the tissue section.
i. Reapply the slide seal and place the tissue cassette on the benchtop to let it cool to RT (~3 mins).

20. Probe ligation: *Time needed: ~75 mins*

a. Place the thermal cycler adaptor to the thermal cycler and initiate the following program:
   i. Set the lid temperatures to 37ºC or turn it off if there is no such option.
   ii. Program:

Step	Temperature	Run time
Pre-equilibrate	37ºC	Hold
Ligation	37ºC	1hr
Hold	4ºC	Hold

b. Prepare Probe Ligation Mix shortly before use and add the reagents in the order listed:

Probe Ligation Mix	2 slides (+10%)
Nuclease-free water	52.8 µl
2x Probe Ligation Buffer (#2000445)	66 µl
Probe Ligation Enzyme (#2000425)	13.2 µl
Total	**132 µl**

c. Peel off the slide seal from the tissue cassette and gently pipette away all 2x SSC buffer from the grid well.
d. Slowly add 60 µl Probe ligation mix to the well along the side without introducing bubbles.
e. Tap the tissue cassette gently to ensure all the tissue region of interest is uniformly covered with the Probe ligation mix.
f. Apply a new pre-cut slide seal to the tissue cassette.
g. Place the tissue cassette on the low-profile thermocycler adaptor on the thermal cycler.
h. Close the thermal cycler lid and skip the pre-equilibrate step to initiate ligation.

21. Preparation of VisiumHD slide (part 1):

a. During ligation step, remove the mylar bag containing the VisiumHD slide mailer from -80 ºC storage.
b. Remove the VisiumHD slide mailer from the mylar bag and place it upright on a benchtop to allow it to thaw at RT for 30-60 mins. ***Note:*** *the slide mailer should remain capped throughout this step.*

22. Post-Ligation Wash: *Time needed: ~20 mins*

a. After the ligation step has finish, remove the tissue cassette from the thermal cycler.
b. Prepare the thermal cycler with the following program:
    i. Set lid temperature to 57°C.
    ii. Program:

Step	Temperature	Run time
Incubate	57°C	Hold

c. Aliquot post-ligation wash buffer to a 0.2ml PCR tube (110 µl per sample) and pre-heat to 57°C in the thermal cycler. This pre-heated post-ligation wash buffer will be needed in Step.22j.
d. Peel off the VisiumHD seal and gently pipette away all probe ligation mix from the grid well.
e. Immediately add 100 µl RT post-ligation wash buffer along the side of the grid well without introducing bubbles to the tissue section. ***Note:*** *this is NOT the pre-heated post-ligation wash buffer.*
f. Apply a new pre-cut VisiumHD slide seal to the tissue cassette.
g. Place the tissue cassette on the low-profile thermocycler adaptor on the thermal cycler.
h. Close the thermal cycler lid and incubate at 57°C for 5 mins.
i. After the incubation has finish, remove the tissue cassette from the thermal cycler.
j. Peel back the VisiumHD slide seal and gently pipette away all post-ligation wash buffer from the grid well.
k. Slowly add 100 µl pre-heated post-ligation wash buffer (from Step.22c) along the side of the grid well without introducing bubbles to the tissue section.
l. Reapply the VisiumHD slide seal and place the tissue cassette on the low-profile thermocycler adaptor on the thermal cycler.
m. Close the thermal cycler lid and incubate at 57°C for 5 mins.
n. After the incubation has finish, remove the tissue cassette from the thermal cycler.
o. Peel back the VisiumHD slide seal and gently pipette away all post-ligation wash buffer from the grid well.
p. Slowly add 150 µl 2x SSC buffer along the side of the grid well without introducing bubbles to the tissue section.
q. Reapply the VisiumHD slide seal and place the tissue cassette at RT for 5 mins.
r. Store the tissue cassette at 4°C while preparing the VisiumHD slides.

Note: *The tissue section at this step can be stored at 4°C for up to 24 hrs. However, it is highly recommended to proceed to the next steps immediately.*

Once you start the following steps, CytAssist will need to be started within 2hrs

23. VisiumHD slide preparation (part 2): *Time needed: ~10 mins*

a. Note the following before starting:
    i. The VisiumHD slide is covered with hydrogel, take extra caution not to scratch it.
    ii. Without opening the mailer, visually inspect if there is any crack on the slide.
    iii. The slide must be used after it has been washed. The unwashed slides are still useable after three freeze-thaw cycles.
    iv. DO NOT recap the mailer once it is opened.
b. Prepare 0.1X SSC buffer and 1X PBS (pH 7.4) and keep at RT.
c. Gently open the slider mailer and perform the following washes:
    i. Add 7ml 0.1X SSC buffer slowly and gently along the side of the mailer on one side.
    ii. Repeat the previous step for the other side such that both sides of the VisiumHD slides are submerged in 0.1X SSC buffer.
    iii. Incubate at RT for 1 min without capping the mailer.
    iv. Remove the 0.1X SSC buffer by securing the slide in place with one finger and pour the buffer out.
d. **Repeat Step.23c for a total of 3 times.**
e. Gently remove the VisiumHD slides from the mailer and dry the back of the slides with lint-free wipes.
f. Place the VisiumHD slide into the blue VisiumHD cassette.
g. Slowly add 100 µl 0.1X SSC buffer along the side of the grid well without scratching the slide.
h. Apply a new pre-cut VisiumHD seal to the cassette and incubate at RT until ready for equilibration.

24. Probe release and extension: *Time needed: ~1 hour*

a. Thaw the following reagents:
    i. 2X RNase Buffer (#2000411): Thaw and keep at RT, pipette to mix, DO NOT vortex.
    ii. Extension Buffer (#2000409): Thaw and keep at RT, vortex to mix.
    iii. Perm Enzyme B (#3000553): Thaw and keep at RT, pipette to mix, DO NOT vortex.
    iv. RNase Enzyme (#3000605): Thaw and keep on ice, pipette to mix. DO NOT vortex.
    v. Extension Enzyme (#2000389): Thaw and keep on ice, pipette to mix. DO NOT vortex.
b. Turn on the CystAssist and open the lid.

c. Clean the CystAssist briefly with lint-free wipes.
d. Press **New Run** and enter the assay info. (30 mins at 37°C is recommended for most application)
e. Prepare 10% Eosin as follows. Vortex and centrifuge briefly. Eosin should be made fresh each run.

10% Eosin	**2 slides (+10%)**
Alcoholic Eosin	33 µl
1X PBS	297 µl
Total	**330 µl**

f. Prepare slide equilibration mix as follows (Maintain the final mix on ice):

Slide Equilibration Mix	**2 slides (+10%)**
Nuclease-free water	96.8 µl
2X RNase buffer (#2000411)	110 µl
RNase Enzyme (#3000605)	13.2 µl
Total	**220 µl**

g. Peel off the slide seal from the blue VisiumHD cassette and gently pipette away all of the 0.1X SSC buffer from each well.
h. Slowly add 100 µl Slide Equilibration Mix along the slide of each well.
i. Gently tap the cassette to ensure uniform coverage.
j. Apply a new slide seal to the cassette and incubate at RT for 10 mins.
k. Prepare Probe Release Mix during incubation as follows (Maintain the final mix on ice):

Probe Release Mix	**2 slides (+10%)**
2X RNase buffer (#2000411)	20 µl
RNase Enzyme (#3000605)	17.5 µl
Total	**37.5 µl**

l. Once the incubation has finished, peel off the slide seal and gently pipette away all Slide Equilibration Mix from each well.
m. Remove the VisiumHD slide from the blue cassette and gently wipe the back of the slide with lint-free wipes.
n. Immediately load the VisiumHD slide against the groove of the stage inside the CystAssist with the slide facing upwards. The label should be on the right.
o. Allow the VisiumHD slide to dry on the stage for 10 mins.
p. After that, inspect each well to ensure there is no liquid remaining within the well. Continue to dry if any liquid is presence.
q. Remove the tissue cassette from Step.22r from 4°C.
r. Gently pipette away all of the 2XSSC buffer from the grid well.
s. Slowly add 100 µl 10% Eosin along the slide of each well.
t. Gently tap the cassette to ensure the well is uniformly covered with the 10% Eosin. Allow it to incubate for 1 min at RT.
u. After incubation, gently pipette away all of the 10% Eosin from the grid well.
v. Remove the tissue section from the cassette and rinse it with 1ml 1X PBS (pH7.4) using a P1000 pipette. ***Note:*** *Do not pipette directly onto the tissue.*
w. Repeat Step.24v for a total of 3 times.
x. Carefully wipe away any excess PBS on the slide with lint-free wipes.
y. Ensure the tissue section is completely dry prior to the next step.
z. Load the tissue section into the CystAssist and perform slide alignment. ***Note:*** *This should be completed before the VisiumHD slide is completely dried.*
aa. After that, ensure the VisiumHD slide is now completely dry.
bb. Perform the following within 5 mins:
    i. Add 2.5 µl Perm Enzyme B to 37.5 µl of Probe Release Mix prepared in Step.24k. Mix thoroughly without introducing bubbles. Centrifuge for a full 5 seconds.
    ii. Slowly add 17 µl of Probe Release Mix into the center of the spacer on the VisiumHD slide without introducing any bubbles.
    iii. Close the CystAssist lid and press **Next.**
    iv. Press the **Play button** on the screen to start the run.

cc. During the CystAssist run, prepare the following:
    i. Place the low-profile thermal cycler adaptor in the thermal cycler.
    ii. Set the lid temperature to 53°C.
    iii. Program:

Step	**Temperature**	**Run time**
Pre-equilibrate	53°C	Hold
Probe Extension 1	53°C	30 mins
Cool Down	4°C	3 mins

Hold	4°C	Hold
Probe Extension 2	53°C	30 mins
Cool Down	4°C	3 mins
Hold	4°C	Hold

dd. Prepare Probe Extension Mix as follows (Maintain the final mix on ice):

Probe Extension Mix	**2 slides (+10%)**
Extension Buffer (#2000409)	323.4 µl
Extension Enzyme ($2000389)	6.6 µl
Total	**330 µl**

ee. Open the CysAssist lid and remove the VisiumHD slide from the stage.
ff. Rinse the capture area with 1ml EB buffer. **DO NOT** pipette directly on the capture area.
gg. **Repeat Step.24ff for a total of 3 times**.
hh. Place the VisiumHD into the blue cassette.

25. Probe Extension: *Time needed: ~75 mins*

a. Slowly add 75 µl Probe Extension Mix along the side of each well. Gently tap the blue cassette to achieve uniform coverage.
b. Apply a new slide seal to the blue cassette.
c. Place the tissue cassette on the low-profile thermocycler adaptor on the thermal cycler.
d. Close the thermal cycler lid and initiate Probe Extension 1.
e. After the first Cool Down step, remove the blue cassette from the thermal cycler.
f. Peel back the slide seal and gently pipette away all of the Probe extension mix from the well.
g. Slowly add 75 µl Probe Extension Mix along the side of each well. Gently tap the blue cassette to achieve uniform coverage.
h. Reapply the slide seal.
i. Place the tissue cassette on the low-profile thermocycler adaptor on the thermal cycler.
j. Close the thermal cycler lid and initiate Probe Extension 2.

26. Probe Elution: *Time needed: ~15 mins*

a. Prepare 0.08M KOH as follows (Maintain the final mix at RT):

KOH Mix	**2 slides (+10%)**
Nuclease-free water	108.9 µl
0.8M KOH	1.1 µl
Total	**110 µl**

b. After Probe Extension 2 is completed, remove the blue cassette from the thermal cycler.
c. Peel off the slide seal and gently pipette away all of the Probe Extension Mix from the well.
d. Slowly add 150 µl EB buffer along the side of the well and gently pipette away all of the EB buffer from the well right after.
e. Slowly add 50 µl 0.08M KOH prepared in Step.26a along the side of the well. Gently tap the blue cassette to ensure uniform coverage.
f. Incubate at RT for 10 mins.
g. During the incubation, transfer 3 µl 1M Tris-HCl (pH 8.0) to one PCR-strip tube (one for each sample). Label accordingly.
h. After incubation has completed, transfer all solution from each well of the blue cassette to the designated PCR tube prepared in the step above. ***Note:*** *Each PCR tube containing Tris-HCl should be designated for a single sample only.*
i. Pipette gently to mix and place on ice until ready for amplification.

27. Pre-amplification: *Time needed: ~20 mins*

a. Thaw the following reagents:
    i. TS Primer Mix B (#2000537): Thaw and keep at RT, vortex to mix.
    ii. Amp Mix B (#2000567): Thaw and keep on ice, vortex to mix.
    iii. Beckman Coulter SPRIselect Reagent: Keep at RT.
b. Prepare Pre-Amplification Mix as follows. Add reagents in the order listed and maintain the final mix on ice:

Pre-amplification Mix	**2 slides (+10%)**
Nuclease-free water	42.9 µl
Amp Mix B (#2000567)	55 µl
TS Primer Mix B (#2000537)	5.5 µl
Total	**103.4 µl**

c. Add 47 µl Pre-Amplification Mix to each sample-containing PCR tube from Step.26i. Pipette to mix thoroughly.
d. Incubate in a thermal cycler with the following program:
    i. Set the lid temperature to 105°C:
    ii. Program:

Step	Temperature	Run time
1	98°C	3 mins
2	98°C	15 sec
3	63°C	20 sec
4	72°C	30 sec
5	Repeat step 2-4 for 9 times	10 cycles
6	72°C	1 min
7	4°C	Hold

    iii. Begin amplification.

28. SPRIselect cleanup: *Time needed: ~20 mins*

a. Prepare 80% Ethanol. ***Note:*** *This should be prepared fresh every time.*
b. Vortex thoroughly to resuspend the SPRIselect reagent.
c. Add 120 µl SPRIselect reagent to each pre-amplified sample from Step.27 (each tube should have 100 µl).
d. Pipette to mix well and allow it to incubate at RT for 5 mins.
e. Place the tube on the magnetic stand-HIGH side for 3 mins or until solution clears.
f. The beads now contain the sample.
g. Carefully remove the solution without disturbing the beads.
h. Gently add 300 µl 80% Ethanol to the PCR tube without disturbing the beads. Incubate for 30 sec.
i. Carefully remove the solution without disturbing the beads.
j. Gently add 200 µl 80% Ethanol to the PCR tube without disturbing the beads. Incubate for 30 sec.
k. Carefully remove the solution without disturbing the beads.
l. Centrifuge briefly and place the PCR tube on the magnetic stand-LOW side for 1 min.
m. Remove any remaining ethanol without disturbing the beads.
n. Open the PCR tube lid and let the beads air dry for 2 mins. ***Note:*** *It is highly recommended not to exceed 2 mins as this will affect beads recovery.*
o. Remove the PCR tubes from the magnetic stand.
p. Gently add 105 µl EB buffer to each PCR tube. Pipette thoroughly to resuspend the beads.
q. Incubate at RT for 2 mins.
r. Place the PCR tube on the magnetic stand-HIGH side for 2 mins or until solution clears.
s. The solution now contains the cDNA sample.
t. Carefully transfer 100 µl sample to a new PCR tube without disturbing the beads.
u. Store at -20°C until amplification.

Note: *These are double-stranded cDNA which are stable for a long time.*

This is the only safe stop point.

Day 6

29. Cycle number determination – qPCR: *Time needed: ~30 mins*

a. Thaw KAPA SYBR FAST qPCR Master Mix, maintain on ice.
b. Dilute TS Primer Mix B 1:10 in nuclease-free water. 1 µl is needed for one reaction.
c. Prepare qPCR mix as follows:

qPCR Mix	1 reaction	3 reactions + 10%
KAPA SYBR FAST qPCR Master Mix	5 µl	16.5 µl
Diluted TS Primer Mix B	1 µl	3.3 µl
Nuclease-free water	3 µl	9.9 µl
Total	9 µl	29.7 µl

Note: *ROX reference dye may be required for certain qPCR system.*

d. Add 9 µl qPCR Mix to a well of a qPCR plate (one tube per sample, a well for negative control may be included)
e. Dilute 1:5 pre-amplified sample from Step.28 in nuclease-free water. Pipette to mix thoroughly and centrifuge briefly.
f. Transfer 1 µl diluted sample (nuclease-free water for negative control) to the qPCR plate containing qPCR Mix prepared in Step.29d. Pipette to mix thoroughly.
g. Apply qPCR seal and centrifuge briefly.
h. Prepare a qPCR system with the following protocol:

i. Set lid temperature to 105ºC.
ii. Program:

Step	Temperature	Run Time
1	98ºC	3 mins
2	98ºC	5 sec
3	63ºC Read signal	30 sec
4	Go to step 2 for a total of 25 cycles	

iii. Place the qPCR plate in the thermal cycler and begin the program.

i. To determine the Cq value, set the y-axis to linear scale and plot RFU on the y-axis (if a dye was not used; Rn if dye was used).
j. The Cq value should be set along the exponential phase of the amplification plot, at ~25% of the peak fluorescence value.
k. Round Cq value up to the nearest whole number and add one extra cycle as the optimal amplification cycle number to be used in the next step.

30. Sample index PCR: *Time needed: ~45 mins*

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Note: Refer to 10x Genomics for combination of indices for low variation i.e., 1-4 samples per sequencing run.
b. Prepare Amplification Master Mix as follows:

Amplification Master Mix	2 reactions + 10%
Nuclease-free water	99 µl
Amp Mix B	55 µl
Total	154 µl

c. Pipette 10 times to mix thoroughly. Keep the master mix on ice until use.
d. Add 70 µl Amplification Master Mix to a tube of a PCR strip-tube per sample.
e. Add 25 µl of each pre-amplified sample from Step.28 to the PCR tube containing the Amplification Master Mix (one for each sample).
f. Add 5 µl of an individual Dual Index TS Set A to each tube.
g. Pipette mix and centrifuge briefly.
h. Set up the thermal cycler with the following program:
i. Set the lid temperature to 105.
ii. Program:

Step	Temperature	Run Time
1	98ºC	3 mins
2	98ºC	15 secs
3	63ºC	20 secs
4	72ºC	30 secs
5	Go to Step 2, use the Cq value determined in Step.29k	
6	72ºC	1 min
7	4ºC	Hold

iii. Close the thermal cycler lid and begin the program.

Note: *Only samples within +/- 1 cycle numbers can be combined in a single index-PCR run using the higher cycle number.*

31. Post-Sample Index cleanup – SPRIselect: *Time needed: ~20 mins*

a. Prepare 80% Ethanol. ***Note:*** *This should be prepared fresh every time.*
b. Vortex thoroughly to resuspend the SPRIselect reagent.
c. Add 85 µl SPRIselect reagent to each indexed sample from Step.30 (each tube should have 100 µl).
d. Pipette to mix well and allow it to incubate at RT for 5 mins.
e. Place the tube on the magnetic stand-HIGH side for 3 mins or until solution clears.
f. The beads now contain the sample.
g. Carefully remove the solution without disturbing the beads.
h. Gently add 200 µl 80% Ethanol to the PCR tube without disturbing the beads. Incubate for 30 sec.
i. Carefully remove the solution without disturbing the beads.
j. Gently add 200 µl 80% Ethanol to the PCR tube without disturbing the beads. Incubate for 30 sec.
k. Carefully remove the solution without disturbing the beads.
l. Centrifuge briefly and place the PCR tube on the magnetic stand-LOW side for 1 min.
m. Remove any remaining ethanol without disturbing the beads.
n. Open the PCR tube lid and let the beads air dry for 2 mins. ***Note:*** *It is highly recommended not to exceed 2 mins as this will affect beads recovery.*

o. Remove the PCR tubes from the magnetic stand.
p. Gently add 27 µl EB buffer to each PCR tube. Pipette thoroughly to resuspend the beads.
q. Incubate at RT for 2 mins.
r. Place the PCR tube on the magnetic stand-LOW side for 2 mins or until solution clears.
s. The solution now contains the indexed sample.
t. Carefully transfer 25 µl sample to a new PCR tube without disturbing the beads.
u. Store at -20°C for long-term storage.
v. Samples are now ready for QC and sequencing.