FFPE Tissue Staining and Reporter Plate preparation

1. Prepare Pre-Staining Reagents

a. Prepare **Humidity Chamber** by using an empty pipette tip box. Add a paper towel to the bottom, fill with water to cover paper towel. Rinse and dry tray. Cover with lid.
b. For every 1 sample coverslip, fill **2 wells** with **5mL** of **Hydration Buffer.**
c. For every 1 sample coverslip, fill **1 well** with **5mL** of **Staining Buffer.**
d. Prepare 2 containers containing the required volume of Histo-Choice Clearing 1x.
e. Prepare 2 containers containing the required volume of 100% Ethanol/Reagent Alcohol.
f. Prepare one container **each** containing the required volume of **90%**, **70%**, **50%**, and **30%** Ethanol/Reagent Alcohol diluted in ddH20.
g. Prepare 2 containers containing the required volume of ddH2O.
h. Prepare **1x Citrate Buffer** in ddH20.

Sample(s)

- Tissue adhered on poly-L-lysine coated coverslip. Referred to as Sample Coverslip

Akoya Materials

- CODEX® Staining Kit
  - Hydration Buffer
  - Staining Buffer
  - N, G, J, & S Blockers
- CODEX® Antibodies and Custom-Conjugated Antibodies

Materials NOT Included in Kits

Consumables/tools:
- Bent tip tweezers
- 6-well plates
- 1.5 mL Eppendorf tubes
- 50ml Glass Beaker
- Ice bucket
- Humidity Chamber
- Aluminum foil

Instrumentation:
- Pressure Cooker
- Heating Plate
- Fume Hood

Solvents:
- Ethanol
- Histo-Choice Clearing 1x reagent.

Chemicals/Buffers:
- 1x Citrate Buffer
- Tris EDTA *(optional)*
- Coverglass staining rack
- 10 Solvent-resistant containers with lids

2. Prepare Sample Coverslip – Deparaffinization and Hydration

a. Place sample coverslip(s) on **55°C** hot plate with tissue facing up for **20-25 mins**.
b. Place sample coverslip(s) on the coverglass staining rack and wait for **5 mins** to allow the tissue(s) to cool down.
c. Place the sample coverslip(s) in each of the 10 solvents for **5 mins** each in the following order:

1	2	3	4	5	6	7	8	9	10
Histo-clear or Xylene	Histo-clear or Xylene	100% Ethanol	100% Ethanol	90% Ethanol	70% Ethanol	50% Ethanol	30% Ethanol	ddH20	ddH20

d. Fully immerse the staining rack in 50 mL beaker of **1x Citrate Buffer** and tightly cover with foil.
e. Place beaker in a Pressure Cooker. Fill pressure cooker with water halfway up the height of the beaker.
f. Set the pressure cooker to the high-pressure protocol and let the tissue cook for **20 mins**. Let it cool to RT (~10 mins).
h. Incubate the sample coverslip in ddH20 for two minutes.
i. Incubate the sample coverslip in another container of ddH20 for 2 minutes.
j. Incubate the sample coverslip in the first well of **Hydration Buffer** for 2 minutes.
k. Incubate the sample coverslip in the second well of **Hydration Buffer** for 2 minutes.
l. Place sample coverslip in **Staining Buffer** for **20-30 mins**. Prepare solutions in **Step 3** during this incubation.

3. Stain Tissue

a. Prepare a stock solution of **CODEX® Blocking Buffer** for the Antibody Cocktail Solution.

CODEX® Blocking Buffer	2 Samples	4 Samples	6 Samples	8 Samples	10 Samples
Staining Buffer [µL]	362	724	1086	1448	1810
N Blocker [µL]	9.5	19	28.5	38	47.5
G Blocker [µL]	9.5	19	28.5	38	47.5
J Blocker [µL]	9.5	19	28.5	38	47.5
S Blocker [µL]	9.5	19	28.5	38	47.5
Total [µL]	400	800	1200	1600	2000

b. Calculate the **volume** of Antibody per sample coverslip.
c. Subtract that Antibody **volume** from 200 µL. This is the CODEX® Blocking Buffer needed per sample.
d. The final volume of the Antibody Cocktail Staining Solution is a total of 200 µL per tissue.
e. Pipette the **CODEX® Blocking Buffer** volume into 1.5mL tube.
f. Pipette each **Antibody** into the CODEX® Blocking Buffer to create the **Antibody Cocktail Solution**. Vortex gently.
g. Place the sample coverslip tissue side up on the **Humidity Chamber.**
h. Pipette **190 µL** of the **Antibody Cocktail Solution** to the top corner of the sample coverslip. The liquid will cover the entire tissue. Be careful not to pipette the solution directly on the tissue, and do not create bubbles.
i. Cover the **Humidity Chamber** and **incubate for 3 hours at RT**.

Akoya Materials

CODEX® Staining Kit
- Staining Buffer
- Storage Buffer
- Fixative Reagent

Material NOT Included in Kits

Solvents and Chemicals:
- 4°C Methanol
- 1X PBS
- 16% PFA

Plastic Consumables/Tools:
- 6-well plates
- Ice bucket

4. Prepare Post-Staining Reagents

a. For every 1 sample coverslip, fill **2 wells** with **5 mL** of **Staining Buffer.**
b. For every 1 sample coverslip, fill **3 wells** with **5 mL** of **1x PBS.**
c. For every 1 sample coverslip, have **1 well** for **5 mL** of **Post-Staining Fixing Solution** for step 5c.
d. For every 1 sample coverslip, have **1 well** for **5 mL** of **Methanol** for step 5g.
e. Prepare sample storage container by filling **1 well** with **5ml** of **Storage Buffer** per sample coverslip.

5. Wash and Fix Antibodies

a. Place sample coverslip in first **Staining Buffer** for **2 mins** to rinse unbound antibodies**.**
b. Place sample coverslip in second **Staining Buffer** for **2 mins** to rinse unbound antibodies**.**
c. Prepare **Post-Staining Fixing Solution** and place **5 mL** in each well.

Post-Staining Fixing Solution	2 Samples	4 Samples	6 Samples	8 Samples	10 Samples
16% PFA [mL]	1	2	3	4	5
Storage Buffer [mL]	9	18	27	36	45
Total Volume [mL]	10	20	30	40	50

d. Place sample coverslip in **Post-Staining Fixing Solution** for **10 mins** at RT**.**
e. Submerge sample coverslip in each **1x PBS** 3 times for a total of 9 washes to remove fixative.
f. Add **5 mL** of cold **(~4°C) Methanol** to one well per sample keeping the 6-well TC plate on ice.
g. Place sample coverslip in **Methanol** for **5 mins** on ice.
h. Submerge sample coverslip in each **1x PBS** 3 times for a total of 9 washes to remove methanol.
i. Prepare the **Final Fixative Solution** by diluting all the 20 µL of the **CODEX® Fixative Reagent** in 1 mL of 1x PBS.
j. Place sample coverslip in **Humidity Chamber**.
k. Pipette **200 µL** of the **Final Fixative Solution** to the top corner of the sample coverslip. The liquid should cover the entire tissue. Be careful not to pipette the solution directly on the tissue, and be careful to not create bubbles.
l. Cover the **Humidity Chamber** and **Incubate for 20 mins**.
m. Submerge sample coverslip in each **1x PBS** 3 times for a total of 9 washes to remove fixative.
n. Store tissue in **5 mL** of **Storage Buffer.** Place at 4°C up to 5 days.

6. Reporters Plate for Corresponding Antibodies – One well per cycle

a. Determine antibody distribution across cycles. Maintain one dye type per cycle.
b. Prepare **Reporter Stock Solution** for the total number of cycles in the experiment.

Reporter Stock Solution	Cycles/Wells			
	5	10	15	20
Nuclease free water [µL]	1220	2440	3660	4880
10X CODEX® Buffer [µL]	150	300	450	600
Assay Reagent [µL]	125	250	375	500
Nuclear Stain [µL]	5	10	15	20
Total [µL]	1500	3000	4500	6000

Akoya Materials
- 96-well plate
- Foil Seals
- 10x CODEX® Buffer
- Assay Reagent
- Nuclear Stain
- Corresponding Reporters

Additional Materials
- Nuclease Free Water
- Amber 1.5ml tubes
- Ice bucket

b. For each individual cycle, label an amber tube with the associated well number (for example, “A1”).
c. Add the appropriate volume of **Reporter Stock Solution** to each amber tube according to the table below.

3 Reporters	2 Reporters	1 Reporter	Blanks
235 µL	240 µL	245 µL	250 µL

d. Pipette **5 µL** of each Reporter to each corresponding tube to create a Reporter Master Mix per cycle.
e. Mix each tube by gentle pipetting.
f. Pipette **245 µL** of Reporter Master Mix from each tube into its corresponding well on the 96-well plate.
g. Cover the plate with the **foil seal**.
h. Store Reporter Plate at 4°C for up to two weeks.