AKOYA BIOSCIENCES® THE SPATIAL BIOLOGY COMPANY®

*We've rebranded some of our products. **CODEX**® is now **PhenoCycler**™.*

FFPE Tissue Staining and Reporter Plate preparation

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

Akoya Materials
- PhenoCycler Staining Kit
  - Hydration Buffer
  - Staining Buffer
  - N, G, J, & S Blockers
- PhenoCycler Antibodies and Custom-Conjugated Antibodies

Materials NOT Included in Kit
- Consumables/tools:
  - Bent tip tweezers
  - 6-well plates
  - 1.5 mL Eppendorf tubes
  - 50mL Pyrex Beaker
  - Ice bucket
  - Humidity Chamber
  - Aluminum foil
- Instrumentation:
  - Pressure Cooker
  - Heating Plate
  - Fume Hood
- Solvents:
  - Ethanol
  - Histo-Choice Clearing agent.
- Chemicals/Buffers:
  - 1X AR9 Buffer
  - Tris EDTA (optional)
  - Coverslip staining rack
  - 10 Solvent-resistant containers with lids

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** in the 6-well plate with **5mL** of **Hydration Buffer**.

c. For every 1 sample coverslip, fill **1 well** in the 6-well plate with **5mL** of **Staining Buffer**.

d. Prepare 2 containers of the solvent resistant containing the required volume of 1x Histo-Choice Clearing Agent.

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 ddH_20.

g. Prepare 2 containers containing the required volume of ddH_2O.

h. Prepare **1x AR9 buffer** in Pyrex beaker in ddH_20.

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 coverslip staining rack and wait 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
Histoclear	Histoclear	100% Ethanol	100% Ethanol	90% Ethanol	70% Ethanol	50% Ethanol	30% Ethanol	ddH_20	ddH_20

d. Fully immerse the staining rack in the 50 mL beaker of **1x AR9 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 incubate for 20 mins. Let it cool to RT (~10 mins).

g. Incubate the sample staining rack with coverslip in ddH_20 for 2 minutes.

h. Incubate the sample coverslip in another container of ddH_20 for 2 minutes.

i. Incubate the sample coverslip in the first well of **Hydration Buffer** for 2 minutes.

j. Incubate the sample coverslip in the second well of **Hydration Buffer** for 2 minutes.

k. 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 **PhenoCycler Blocking Buffer** for the **Antibody Cocktail Solution**.

PhenoCycler 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 **PhenoCycler 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 **PhenoCycler Blocking Buffer** volume into **1.5mL** tube.

f. Pipette each Antibody into the **PhenoCycler Blocking Buffer** to create the **Antibody Cocktail Solution**. Vortex gently.

Akoya Materials
- Staining Kit for PhenoCycler
  - Staining Buffer
  - Storage Buffer
  - Fixative Reagent

Materials NOT Included in Kit
- Solvents and Chemicals:
  - 4°C Methanol
  - 1X PBS
  - 16% PFA
- Plastic
  - Consumables/Tools:
  - 6-well plates
  - Ice bucket

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.

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 **5 mL** of **Storage Buffer** per sample coverslip.

5. Wash and Fix Antibodies

a. Immerse 2-3 times and then 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 **PhenoCycler 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.

Reporter Stock Solution	5	10	15	20
Nuclease free water [µL]	1220	2440	3660	4880
10X PhenoCycler 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

b. Prepare **Reporter Stock Solution** for the total number of cycles in the experiment.

c. For each individual cycle, label an amber tube with the associated well number (for example, "A1").

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

d. Add the appropriate volume of **Reporter Stock Solution** to each amber tube according to the table above.

e. Pipette **5 µL** of each Reporter to each corresponding tube to create a **Reporter Master Mix** per cycle.

f. Mix each tube by gentle pipetting.

g. Pipette **245 µL** of **Reporter Master Mix** from each tube into its corresponding well on the 96-well plate.

h. Cover the plate with the **foil seal**.

i. Store Reporter Plate at 4°C for up to two weeks.

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