AKOYA BIOSCIENCES®
THE SPATIAL BIOLOGY COMPANY®

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

FF 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
- Custom-Conjugated Antibodies

Materials NOT Included in Kit
- Solvents and Chemicals:
- Acetone
- 16% PFA
- Plastic Consumables/Tools:
- Bent tip tweezers
- 6-well plates
- Drierite absorbent beads
- 1.5 mL Eppendorf tubes
- 50 mL Glass Beaker Ice bucket
- Humidity Chamber

1. Prepare Pre-Staining Reagents

a. Prepare **Humidity Chamber** by using an emptypipette tip box. Add a paper towel to the bottom,fill with water to cover paper towel. Rinse and drytray. Cover with lid.
b. For every 1 sample coverslip have **1 x 50 mL** glass beaker for Acetone.
c. For every 1 sample coverslip, fill **2 wells** with **5 mL** of **Hydration Buffer**.
d. For every 1 sample coverslip, fill 1 well with 5 mL **Staining Buffer**.
e. For every 1 sample coverslip, have **1 well** for **5 mL** of **Pre-Staining Fixing Solution** for step 2g.
f. Place **antibodies** and **Blockers** on Ice.

2. Prepare Sample Coverslip

a. Obtain sample from freezer and place in prepared box of 1-2 cm Drierite beads for **2 mins**.
b. Dispense **10 mL of acetone** in a 50 mL beaker per each sample coverslip.
c. Incubate the sample coverslip in the corresponding beaker containing **Acetone** for **10 mins**.
d. Place sample coverslip faceup in **Humidity Chamber** for **2 mins** .
e. Place sample coverslip in first **Hydration Buffer** for **2 mins**.
f. Place sample coverslip in second **Hydration Buffer** for **2 mins.**
g. Prepare **Pre-Staining Fixing Solution** and place **5 mL** in each well

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

h. Place sample coverslip in **Pre-Staining Fixing Solution** for **10 mins**.
i. Submerge sample coverslip in first **Hydration Buffer 3 times** to remove fixative.
j. Submerge sample coverslip in second **Hydration Buffer 3** times to remove fixative.
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 **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 200µL. This is the **PhenoCycler** Blocking Buffer per sample.
d. The final volume of the Antibody Cocktail Staining Solution is a total of 200µL per tissue.
e. Pipette the **PhenoCycler Blocking Buffer** volume into a 1.5 mL tube.
f. Pipette each **Antibody** into the PhenoCycler Blocking Buffer to create the Antibody Cocktail Solution. Vortex gently.
g. Place the sample coverslip tissue side up on the Humidity Chamber.
a. Pipette **190µL** 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.
h. Cover the **Humidity Chamber** and **Incubate for 3 hours at RT**.

Akoya Materials
- PhenoCycler Staining Kit
- · 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

4. Prepare Post-Staining Reagents

a. For every 1 sample coverslip, fill **2 wells** with **5 mL** of S**taining 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. 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** .
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**
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.l of the **PhenoCycler Fixative Reagent** in 1 mL of 1x PBS.
j. Place sample coverslip in **Humidity Chamber**.
k. Pipette **200µL** 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	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

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

e. 235 µL 240 µL 245 µL 250 µL
f. Pipette **5µL** of each Reporter to each corresponding tube to create a Reporter Master Mix per cycle.
g. Mix each tube by gentle pipetting.
h. Pipette **245µL** of Reporter Master Mix from each tube into its corresponding well on the 96-well plate.
i. Cover the plate with the **foil seal**.
j. Store Reporter Plate at 4°C for up to two weeks.