

Fresh Frozen Tissue Staining and Reporter Plate preparation

1. Prepare Pre-Staining Reagents

- 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.
- For every 1 sample coverslip have **1 x 50ml** glass beaker for Acetone.
- For every 1 sample coverslip, fill **2 wells** with **5ml** of **Hydration Buffer**.
- For every 1 sample coverslip, fill **1 well** with **5ml** of **Staining Buffer**.
- For every 1 sample coverslip, have **1 well** for **5ml** of **Pre-Staining Fixing Solution** for step 2g.
- Place **antibodies** and **Blockers** on **Ice**.

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

Additional Materials

- Acetone
- 16% PFA
- Bent tip tweezers
- 6-well plates
- Drierite beads
- 1.5 ml tubes
- Glass Beaker
- Ice bucket
- Humidity Chamber

2. Prepare Sample Coverslip

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

- Place sample coverslip in **Pre-Staining Fixing Solution** for **10 mins**.
- Dunk sample coverslip in first **Hydration Buffer 3 times** to remove fixative.
- Dunk sample coverslip in second **Hydration Buffer 3 times** to remove fixative.
- Place sample coverslip in **Staining Buffer** for **20 mins**. Prepare solutions in **Step 3** during this incubation.

3. Stain Tissue

- Prepare a stock solution **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

- Calculate the **volume** of Antibody per sample coverslip.
- Subtract that Antibody **volume** from 210μl. This is the CODEX® Blocking Buffer per sample.
- The final volume of the Antibody Cocktail Staining Solution is a total of 210 μl per tissue.
- Pipette the **CODEX® Blocking Buffer** volume into a low bind tube.
- Pipette each **Antibody** into the CODEX® Blocking Buffer to create the **Antibody Cocktail Solution**. Vortex gently.
- Place the sample coverslip tissue side up on the **Humidity Chamber**.
- Pipette **200μl** 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.
- Cover the **Humidity Chamber** and **Incubate for 3 hours at RT**.

Akoya Materials

CODEX® Staining Kit

- Staining Buffer
- Storage Buffer

Additional Materials

- 4°C Methanol
- 1X PBS
- 16% PFA
- 6-well plates
- Ice bucket

4. Prepare Post-Staining Reagents

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

5. Wash and Fix Antibodies

- Place sample coverslip in first **Staining Buffer** for **2 mins** to rinse unbound antibodies.
- Place sample coverslip in second **Staining Buffer** for **2 mins** to rinse unbound antibodies.
- Prepare **Post-Staining Fixing Solution** and place **5ml** 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

- Place sample coverslip in **Post-Staining Fixing Solution** for **10 mins**.
- Dunk sample coverslip in each **1 x PBS** 3 times for a total of 9 dunks to remove fixative.
- Add **5 ml** of cold (**~4°C**) **methanol** to one well per sample keeping the 6-well TC plate on ice.
- Place sample coverslip in **Methanol** for **5 mins**
- Dunk sample coverslip in each **1 x PBS** 3 times for a total of 9 dunks to remove fixative.
- Prepare the **Final Fixative Solution** by diluting all the 20µl of the **CODEX® Fixative Reagent** in 1 ml of 1x PBS.
- Place sample coverslip in **Humidity Chamber**.
- Pipette **190µl** of the **Final Fixative 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 please be careful to not create bubbles.
- Cover the **Humidity Chamber** and **Incubate for 20 mins**.
- Dunk sample coverslip in each **1 x PBS** 3 times for a total of 9 dunks to remove fixative.
- Store tissue in **5ml** of **Storage Buffer**. Place at 4°C up to 2 weeks.

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

- Determine antibody distribution across cycles. Maintain one dye type per cycle.
- 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

- For each individual cycle, label an amber tube with the associated well number (for example, "A1").
- Add the **Reporter Stock Solution** to each amber tube.

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

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

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