RNAScope HiPlex Experiment Checklist for FRESH FROZEN Samples					
Method: RNAscope HiPlex					
Experiment Name			Sample Info		
DATE:					
Sample Preparation Steps					
1	Remove the slides from-80ºC, ***immediately*** Post-fixation	4% PFA/PBS	60 min	**RT**	Coplin Jars
2	Rinse **X2**	PBS	5 min	RT	
3	Dehydration				
		a 50% EtOH (50 ml)	5min	RT	
		b 70% EtOH (50 ml)	5min	RT	
		c 100% EtOH (50 ml)	5min	RT	
		d 100% EtOH (50 ml)	5min	RT	
		e 100% EtOH (50 ml)	5min	RT	
	Slides may be stored in 100% Ethanol at -20ºC for 1 week				
4	Air Dry on absorbent paper with section face-up	*NA*	5min	RT	
5	Create hydrophobic barrier, let barrier dry completely	*NA*	5min	RT	
	Place a humidifying paper in humidity control tray and wet completely with distilled water				
6	Load slides into ACD EZ-Batch Slide Holder	*NA*	Briefly	RT	
7	Protease Digestion	Protease IV (kit)	30min	RT+hum	
8	Rinse **X2**	PBS	2 min	RT	
HiPlex Protocol					
Round 1	**Prepare 3 L 1 X Wash Buffer**				
	Warm up RNAscope HiPlex probe stocks and diluent at 40 ºC for about 10 min				
	Prepare target probe with warmed HiPlex probe stocks and diluent, mix well and cool to RT before use				
	Set HybEZOven and prepared humidity control tray at 40ºC				
	Set RNAscpe HiPlex Amp 1-3 and RNAscope HiPlex Fluoro T1-T4 V2 reagents at RT				
	Bring RNAscope HiPlex FFPE Reagent to RT, Prepare 2.5-5% FFPE reagent by using 1:40-1:20 ratio of FFPE reagent to 4X SSC				
9	**Probe Incubation**	Diluted Probes	2 hrs	40°C	
10	Wash **X2**	Wash Buffer	2 min	RT	
11	Amplification	(kit reagents)			
		a) AMP1	30 min	40°C	
		b) *WASH Buffer*	*2x2 min*	*RT*	
		c) AMP2	30 min	40°C	
		d) *WASH Buffer*	*2x2 min*	*RT*	
		e) AMP3	30 min	40°C	
		f) *WASH Buffer*	*2x2 min*	*RT*	
12	FFPE Reagent (optional)	diluted 2.5-5% in 4 X SSC	30 min	RT	
13	Wash **X2** (optional, only FFPE Reagent)	Wash Buffer	2 min	RT	
14	RNAscope HiPlex Fluoro T1-T4 V2	Kit Reagent	15 min	40°C	
15	Wash **X2**	Wash Buffer	2 min	RT	
16	Nuclear Staining	DAPI (Kit Reagent)	**30 SEC**	**RT**	
15	Cover Glass	Prolong Gold			
	Store slides in the dark at 2-8 ºC				
16	Image slides for round 1	Slide Scanner	6 hrs		
Round 2	**Prepare 500 ml 4 X SSC, 1 L 0.5% PBST**				
	Set RNAscpe HiPlex Amp 1-3 and RNAscope HiPlex Fluoro T5-T8 V2 reagents and FFPE reagent (Optional) at RT				
	Prepare 10% cleaving solution v1 by diluting with 4X SSC				
17	Remove Coverslips: Soak slides until the cover slips fall off the slides	4 X SSC	1-2 hrs	RT	
18	Rinse	4 X SSC	briefly	RT	
19	Cleave the T1-T4 V2 fluorophores	10% diluted in 4X SSC	15 min	RT	
20	Wash with PBST **X2**	0.5% PBST	2 min	RT	
19	Cleave the T1-T4 V2 fluorophores	10% diluted in 4X SSC	15 min	RT	
20	Wash with PBST **X2**	0.5% PBST	2 min	RT	
21	FFPE Reagent (optional)	diluted 2.5-5% in 4 X SSC	30 min	RT	
22	Wash **X2** (optional, only for FFPE Reagent)	Wash Buffer	2 min	RT	
23	RNAscope HiPlex Fluoro T5-T8 V2	Kit Reagent	15 min	40°C	
24	Wash **X2**	Wash Buffer	2 min	RT	
25	Nuclear Staining	DAPI (Kit Reagent)	**30 SEC**	**RT**	
26	Cover Glass	Prolong Gold			
	Store slides in the dark at 2-8 ºC				
27	Image slides for round 2	Slide Scanner	6 hrs		
Round 3	**Prepare 1 L 0.5% PBST**				
	Set RNAscpe HiPlex Amp 1-3 and RNAscope HiPlex Fluoro T9-T12 reagents and FFPE reagent (Optional) at RT				
	Prepare 10% cleaving solution v1 by diluting with 4X SSC				
28	Remove Coverslips: Soak slides until the cover slips fall off the slides	4 X SSC	1-2 hrs	RT	
29	Rinse	4 X SSC	briefly	RT	
30	Cleave the T5-T8 V2 fluorophores	10% diluted in 4X SSC	15 min	RT	
31	Wash with PBST **X2**	0.5% PBST	2 min	RT	
32	Cleave the T5-T8 V2 fluorophores	10% diluted in 4X SSC	15 min	RT	
33	Wash with PBST **X2**	0.5% PBST	2 min	RT	
34	FFPE Reagent (optional)	diluted 2.5-5% in 4 X SSC	30 min	RT	
35	Wash **X2** (optional, only for FFPE Reagent)	Wash Buffer	2 min	RT	
36	RNAscope HiPlex Fluoro T9-T12 V2	Kit Reagent	15 min	40°C	
37	Wash **X2**	Wash Buffer	2 min	RT	
38	Nuclear Staining	DAPI (Kit Reagent)	**30 SEC**	**RT**	
39	Cover Glass	Prolong Gold			
	Store slides in the dark at 2-8 ºC				
40	Image slide for round 3	Slide Scanner	6 hrs		

Prepare fresh-frozen tissue sections

1. Remove tissue and cut to fit into cryomolds.
2. Freeze the specimen on dry ice or in liquid nitrogen, isopentane, or 2-methyl butane within 5 MIN of tissue harvest.
3. Embed the frozen tissue in cryo-embedding medium (OCT) or Tissue Freezing Medium (TFM):
   a. Add two drops of OCT into a cryomold.
   b. Place the frozen tissue on the OCT in the correct orientation for cutting.
   c. Add more OCT to fill the cryomold. Do not allow any air bubbles to form.
   d. Hold the block with forceps on the surface of the liquid nitrogen or isopentane cooled by dry ice or liquid nitrogen, or place the cryomold on dry ice.
4. Store the frozen block in an air-tight container at –80°C prior to sectioning. Embedded tissue may be stored for up to three months.
5. Section the block:
   e. Equilibrate block to –20°C in a cryostat ~1 HR.
   f. Cut 10–20 µm thick sections and mount onto SUPERFROST PLUS SLIDES.
   g. Dry the sections at 60 –120 MIN at –20°C to retain tissue adherence.
6. Store the sections in slide boxes wrapped air-tight with aluminum foil or zip-lock bags at –80°C until use.