Quick Reference Guide

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ViewRNA™ ISH Tissue 2-Plex Assay

Fresh Frozen or OCT-Embedded Frozen Samples

IMPORTANT: If running ViewRNA ISH Tissue 2-Plex Assay for the first time, please refer to the *ViewRNA ISH Tissue 2-Plex Assay User Manual to* review assay guidelines and detailed procedures.

Part 1: Sample Preparation and Target Probe Hybridization

Step		Task
1	**Prepare and Chill 10% NBF**	▪ Prepare 200 mL of 10% NBF in 1X PBS and chill at 4 °C for 1 hr
2	**Fix Tissue Overnight 16-18 hr**	▪ Transfer frozen tissue slides into chilled 10% NBF and incubate at 4 °C for 16-18 hr
3	**Preparation for Part 1**	▪ Verify hybridization system is set to 40 °C and appropriately humidified ▪ Prepare: ▫ 2 L 1X PBS ▫ 200 mL 50% ethanol ▫ 200 mL 70% ethanol ▫ 4 L Wash Buffer ▫ 200 mL Storage Buffer ▪ Ensure availability of: ▫ 1 L ddH_2O ▫ 200 mL Gill's Hematoxylin I ▫ 200 mL of 3 µg/mL DAPI in 1X PBS (optional, for fluorescence detection) ▪ Thaw probe set(s) on ice ▪ Prewarm 40 mL 1X PBS and Probe Set Diluent QF to 40 °C ▪ Optional for 1-day assay: ▫ Prewarm PreAmplifier Mix QT, Amplifier Mix QT, and Label Probe Diluent QF to 40 °C ▫ Briefly spin down Label Probe 1-AP, Label probe 6-AP, and Blue Reagents, place on ice ▫ Bring Fast Red Tablets, Napthol Buffer, and AP Enhancer Solution to RT ▫ Prepare 1 L 0.01% ammonium hydroxide under a fume hood
4	**Wash Slides 5 min**	Wash slides 2 times in 1X PBS, 1 min each wash
5	**Tissue Dehydration 30 min**	▪ Sequentially soak the rack of slides in 50%, 70%, and then 100% ethanol at RT without agitation, 10 min each time ▪ Remove slides from 100% ethanol and drain on a paper towel. ▪ Bake entire slide rack at 60 °C for 60 min
6	**Draw Hydrophobic Barrier 40 min**	▪ Create hydrophobic barrier ▪ Allow slides to air dry at RT for 20-30 min
7	**Protease Digestion and Fixation 30-50 min, depending on optimized time**	▪ Prepare 1:100 working protease solution in prewarmed 1X PBS ▪ Add working protease solution to slides ▪ Incubate at 40 °C for optimal time determined in the pretreatment assay optimization procedure ▪ Wash slides 2 times in 1X PBS, 1 min each wash ▪ Fix slides in 10% NBF at RT for 5 min under a fume hood ▪ Wash slides 2 times in 1X PBS, 1 min each wash
8	**Target Probe Set Hybridization 2 hr 10 min**	▪ Prepare 1:40 working probe set solution in prewarmed Probe Set Diluent QT ▪ Add working probe set solution to slides ▪ Incubate at 40 °C for 2 hr
9	**Wash Slides 8 min**	Wash slides 3 times in Wash Buffer, 2 min each wash

Step		Task
10	**Optional Stop Point** **1 min**	▪ Store slides in Storage Buffer at RT for up to 24 hr. Cover dish with lid or sealing film to prevent evaporation. ▪ Store 1X PBS and Wash Buffer at RT for use in Part 2

Part 2: Signal Amplification and Detection

Step		Task
11	**Preparation for Part 2** **10 min**	▪ Pour Gill's Hematoxylin I into a clear staining dish, store at RT protected from light. ▪ Optional – Prepare 200 mL 3 µg/mL DAPI, store at 4 °C until use or place on ice ▪ Prewarm PreAmplifier Mix QT, Amplifier Mix QT and Label Probe Diluent QF to 40 °C ▪ Place Label Probe 1-AP, Label Probe 6-AP, and Blue reagents on ice ▪ Bring Fast Red Tablets, Naphthol Buffer, AP Enhancer, and Blue Buffer to RT ▪ Prepare 1 L 0.01% ammonium hydroxide in ddH_20
12	**Wash Slides** **8 min**	Remove slides from Storage Buffer and wash 3 times with Wash Buffer with constant and vigorous agitation, 2 min each wash
13	**PreAmplifier Hybridization** **35 min**	Add PreAmplifier Mix QT directly to slides, incubate at 40 °C for 25 min
14	**Wash Slides** **8 min**	Wash slides 3 times in Wash Buffer with constant and vigorous agitation, 2 min each wash
15	**Amplifier Hybridization** **20 min**	Add Amplifier Mix QT directly to slides, incubate at 40 °C for 15 min
16	**Wash Slides** **8 min**	Wash slides 3 times in Wash Buffer with constant and vigorous agitation, 2 min each wash
17	**Label Probe 6-AP Hybridization** **20 min**	▪ Prepare 1:1000 working Label Probe 6-AP solution ▪ Add working Label Probe 6-AP solution to slides, incubate at 40 °C for 15 min
18	**Wash Slides** **12 min**	Wash slides 3 times in Wash Buffer with constant and vigorous agitation, 3 min each wash
19	**Apply Fast Blue Substrate** **35 min**	▪ Prepare Fast Blue Substrate ▪ Add Fast Blue Substrate to slides, incubate at RT in the dark for 30 min
21	**Wash Slides** **12 min**	Wash slides 3 times in Wash Buffer with constant and vigorous agitation, 3 min each wash
21	**Quench Label Probe 6-AP** **35 min**	▪ Add AP Stop QT to slides, incubate at RT in the dark for 30 min ▪ Wash slides 2 times in 1X PBS, 1 min each wash ▪ Rinse slides in Wash Buffer for 1 min
22	**Label Probe 1-AP Hybridization** **20 min**	▪ Prepare 1:1000 working Label Probe 1-AP solution in prewarmed Label Probe Diluent QF ▪ Add working Label Probe 1-AP solution to slides, incubate at 40 °C for 15 min
23	**Wash Slides** **12 min**	Wash slides 3 times in Wash Buffer, 3 min each wash
24	**Apply Fast Red Substrate** **45 min**	▪ Add AP-Enhancer to slides and incubate at RT for 5 min ▪ Prepare Fast Red Substrate (1 Fast Red Tablet/5 mL Napthol Buffer) ▪ Decant AP-Enhancer and add Fast Red Substrate to slides, incubate at 40 °C for 30 min ▪ Wash slides in 1X PBS for 1 min

Step		Task	
25	**Counterstain** **25 min**	▪ Incubate slides in Gill's Hematoxylin I stain at RT for 5-10 sec ▪ Wash slides 3 times in ddH_2O, 1 min each wash ▪ Incubate in 0.01% ammonium hydroxide at RT for 10 sec ▪ Wash slides in ddH_2O for 1 min ▪ Optional – Incubate slides in DAPI at RT for 1 min, wash slides in ddH_2O for 1 min ▪ Let slides completely air dry at RT (~20 min)	
26	**Mount and Image** **40 min**	**DAKO Ultramount:** **For no coverslipping** ▪ Add Ultramount to tissue sections ▪ Place slides in a 70 °C oven/incubator for 10-30 min ▪ Observe under brightfield or fluorescence microscope ▪ Store slides at RT **For post mounting with coverslip** ▪ Follow the no coverslip procedure ▪ Allow the slides to come to RT ▪ Apply Histomount directly on top of the dried Ultramount ▪ Place coverslip ▪ Air dry at RT for 15 min ▪ Observe under brightfield or fluorescence microscope ▪ Store slides at RT	**Innovex Advantage Mounting Media:** ▪ Add Advantage Mounting Media to cover glass and invert tissue slide to cover ▪ Flip over, allow slides to dry ▪ Seal all four edges with nail polish ▪ Observe under brightfield or fluorescence microscope ▪ Store slides at RT

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