Quick Reference Guide

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ViewRNA™ ISH Tissue 2-Plex Assay

FFPE 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	**Bake Slides** **65 min**	▪ Label slides with pencil ▪ Bake slides at 60 °C for 60 min	
2	**Preparation for Part 1**	▪ Verify hybridization system is set to 40 °C and appropriately humidified ▪ Prepare: □ 3 L 1X PBS □ 200 mL 10% NBF in 1X PBS □ 4 L Wash Buffer □ 500 mL 1X Pretreatment Solution □ 200 mL Storage Buffer ▪ Ensure availability of: □ 600 mL 100% ethanol □ 1.4 L ddH_2O □ 600 mL xylene or 400 mL Histo-Clear □ 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, Blue Buffer, and AP Enhancer Solution to RT □ Prepare 1 L 0.01% ammonium hydroxide under a fume hood
3	**Deparaffinization** **30 min**	If using xylene (work in a fume hood): ▪ Incubate slides 3 times each for 5 min in xylene ▪ Wash slides 2 times in 100% ethanol, 5 min each wash ▪ Decant 100% ethanol from slides and let air dry	If using Histo-Clear: ▪ Bake slides at 80 ± 1 °C for 3 min ▪ Incubate slides 2 times, 5 min each in HistoClear ▪ Wash slides 2 times in 100% ethanol, 5 min each wash ▪ Decant 100% ethanol from slides and let air dry
4	**Draw Hydrophobic Barrier** **40 min**	▪ Create hydrophobic barrier ▪ Allow slides to air dry at RT for 20-30 min ▪ Heat 1X Pretreatment Solution to 90-95 °C while slides are drying	
5	**Heat Pretreatment** **10-25 min, depending on optimized time**	▪ Heat slides in 1X Pretreatment Solution at 90-95 °C for the optimal time determined in the pretreatment assay optimization procedure ▪ Wash slides 2 times in ddH_2O, 1 min each wash ▪ Transfer slides to 1X PBS **IMPORTANT:** Do not let the slides dry out from this point forward.	
6	**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	

Step		Task
7	**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
8	**Wash Slides** **8 min**	Wash slides 3 times in Wash Buffer, 2 min each wash
9	**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
10	**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
11	**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
12	**PreAmplifier Hybridization** **35 min**	Add PreAmplifier Mix QT directly to slides, incubate at 40 °C for 25 min
13	**Wash Slides** **8 min**	Wash slides 3 times in Wash Buffer with constant and vigorous agitation, 2 min each wash
14	**Amplifier Hybridization** **20 min**	Add Amplifier Mix QT directly to slides, incubate at 40 °C for 15 min
15	**Wash Slides** **8 min**	Wash slides 3 times in Wash Buffer with constant and vigorous agitation, 2 min each wash
16	**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
17	**Wash Slides** **12 min**	Wash slides 3 times in Wash Buffer with constant and vigorous agitation, 3 min each wash
18	**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
19	**Wash Slides** **12 min**	Wash slides 3 times in Wash Buffer with constant and vigorous agitation, 3 min each wash
20	**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
21	**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
22	**Wash Slides** **12 min**	Wash slides 3 times in Wash Buffer, 3 min each wash

<table>
<tr><th colspan="2">Step</th><th colspan="2">Task</th></tr>
<tr><td>23</td><td>Apply Fast Red Substrate
45 min</td><td colspan="2">▪ 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</td></tr>
<tr><td>24</td><td>Counterstain
25 min</td><td colspan="2">▪ 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)</td></tr>
<tr><td>25</td><td>Mount and Image
40 min</td><td>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
▪ Work under a fume hood and 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</td><td>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</td></tr>
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