Quick Reference Guide

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QuantiGene ViewRNA miRNA ISH Cell Assay

24-Well Format for Adherent Cells

IMPORTANT: If running QuantiGene ViewRNA miRNA ISH Cell Assay for the first time, please refer to the appropriate *QuantiGene ViewRNA miRNA ISH Cell Assay User Manual*, based on your sample type and processing format, to review assay guidelines and detailed procedures.

Part 1: Sample Preparation

Step	Task
Step 1. Coating Glass Coverslips with Poly-L-Lysine **1 hr**	▪ Prepare 25 mL of 0.01% poly-L-lysine solution ▪ Ensure availability of 100% ethanol, sterile 1X PBS, 24-well tissue culture plate, 12-mm glass coverslips, fine-tipped forceps ▪ Sterilize coverslips in 24-well plate with 100% ethanol for 15 min at RT ▪ Remove ethanol and air dry coverslips completely for 15 min at RT ▪ Coat coverslips with diluted poly-L-lysine for 15 min at RT ▪ Wash coverslips 3 times in 1X PBS ▪ Air dry coated coverslips O/N at RT in laminar flow hood
Step 2. Culturing Adherent Cells on Poly-L-Lysine Coated Coverslips **20 min**	▪ Seed cells on coated coverslips ▪ Culture cells O/N to 70-90% density

Part 2: Pretreatment and Target Probe(s) Hybridization

Step	Task
Step 3. Fix Samples **1 hr 15 min**	▪ Prepare 1L of 1X PBS, 11 mL of 4% formaldehyde in 1X PBS ▪ Wash cells 2 times with 1X PBS ▪ Fix cells in 4% formaldehyde for 1 hr at RT (in a fume hood) ▪ Remove formaldehyde and wash 3 times with 1X PBS
Step 4. Prepare Buffers and Reagents during Sample Fixation **10 min**	▪ Set a dry incubator to 40 ± 1 °C ▪ Equilibrate EDC to RT ▪ Pre-warm Probe Set Diluent QF to 40 °C ▪ Thaw Probe Set(s) and place on ice ▪ Place Protease QS on ice ▪ Prepare 800 mL of Wash Buffer, 30 mL Storage Buffer ▪ Ensure availability of 50%, 70%, and 100% ethanol if dehydrating cells for storage or rehydrating cells for the assay ▪ Ensure availability of Cross-Linking Buffer QM, Detergent Solution QC
Step 5. Cross-Link with EDC **1 hr 30 min**	▪ Equilibrate cells with Cross-Linking Buffer QM 2 times, 10 min each at RT with rocking ▪ Prepare 0.16M EDC just before use ▪ Cross-link target with 0.16 M EDC for 1 hr at RT, rocking ▪ Wash cells 3 times with 1X PBS
Step 6. *Optional.* Dehydrate/ Rehydrate **10-20 min**	▪ Dehydrate cells if storing sample: 50% EtOH 2 min -> 70% EtOH, 2 min -> 100% EtOH, 2 min -> Fresh 100% EtOH and store at -20 °C ▪ Rehydrate cells for assay if starting with dehydrated sample: 100% EtOH, 2 min -> 70% EtOH, 2 min -> 50% EtOH, 2 min -> 1X PBS, 10 min

Step	Task
Step 7. Permeabilize with Detergent and Digest with Protease **40 min**	▪ Permeabilize cells with Detergent Solution QC for 10 min at RT ▪ Wash cells 2 times with 1X PBS ▪ Prepare Working Protease Solution ▪ Treat cells with Working Protease Solution for 10 min at RT ▪ Wash cells 3X with 1X PBS
Step 8. Hybridize Target Probe(s) **3 hrs**	▪ Prepare Working Probe Set(s) Solutions ▪ Hybridize cells with Working Probe Solutions for 3 hrs at 40 °C
Step 9. Wash Samples **10 min**	▪ Wash cells 3 times in Wash Buffer, soaking 3 min each
Step 10. Stop Point **1 min**	▪ Store samples in Storage Buffer, covered with lid and seal with parafilm at 4 °C. Do not exceed 24 hrs

Part 3. Amplification and Detection

Step	Task
Step 11. Prepare Additional Buffers and Reagents **10 min**	▪ Prewarm PreAmplifier Mix QM, Amplifier Mix QM, and Label Probe Diluent QF to 40 °C ▪ Equilibrate the following reagents to RT: ▫ AP Enhancer, Fast Red Tablets, Naphthol Buffer ▫ Cell samples ▪ Thaw, mix and place on ice 100X DAPI and Label Probe 4-Alexa 488 ▪ Prepare 11 mL 1X DAPI and store on ice protected from light ▪ Place Label Probe 1-AP on ice ▪ Ensure availability of Wash Buffer and 1X PBS ▪ Ensure availability of Prolong® Gold Antifade Reagent
Step 12. Wash Storage Buffer off Samples **5 min**	▪ Wash cells 2X with Wash Buffer
Step 13. Hybridize with PreAmplifier **1 hr**	▪ Prepare Working PreAmplifier Mix Solution ▪ Hybridize cells with Working PreAmplifier Mix Solution for 1 hr at 40 °C
Step 14. Wash Samples **10 min**	▪ Wash cells 3X with Wash Buffer, soaking 3 min each
Step 15. Hybridize with Amplifier **1 hr**	▪ Prepare Working Amplifier Mix Solution ▪ Hybridize cells with Working Amplifier Mix Solution for 1 hr at 40 °C
Step 16. Wash Samples **10 min**	▪ Wash cells 3X with Wash Buffer, soaking 3 min each
Step 17. Hybridize with Label Probe(s) **1 hr**	▪ Prepare Label Probe(s) Working Solution ▪ Hybridize cells with Label Probe(s) Working Solution for 1 hr at 40 °C
Step 18. Wash Samples **20 min**	▪ Wash cells 3 times in Wash Buffer, soaking 3 min each for the first 2 washes and 10 min for the final wash
Step 19. Fast Red Development **1 hr 20 min**	▪ Incubate cells with AP-Enhancer for 5 min at RT; prepare Fast Red Substrate ▪ Remove AP-Enhancer, add Fast Red Substrate and incubate at 40 °C for 45 min ▪ Wash cells 2 times with 1X PBS ▪ Fix cells with 4% formaldehyde for 10 min at RT (in a fume hood) ▪ Wash cells 3 times with 1X PBS

Step	Task
Step 20. DAPI Counter Stain **5 min**	▪ Stain cells with 1X DAPI for 5 min at RT ▪ Wash cells once with 1X PBS and replace with fresh 1X PBS
Step 21. Mount Samples on Glass Slides **15 min**	▪ Mount glass coverslips to microscope glass slide ▪ Cure samples overnight at RT (protected from light) ▪ View under microscope ▫ DAPI for nuclei: EX 387/11 nm, EM 447/60 nm (recommended Semrock P/N DAPI-11060B) ▫ ALEXA 488 for mRNA: EX 485/20 nm, EM 524/24 nm (recommended Semrock P/N FITC-2024B) ▫ Fast Red for miRNA: EX 530/40 nm, EM 590/40 nm (recommended Semrock P/N TRITC-B) ▫ ALEXA 750 for mRNA: EX 708/75 nm, EM 809/81 nm (recommended Semrock P/N Cy7-B)

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