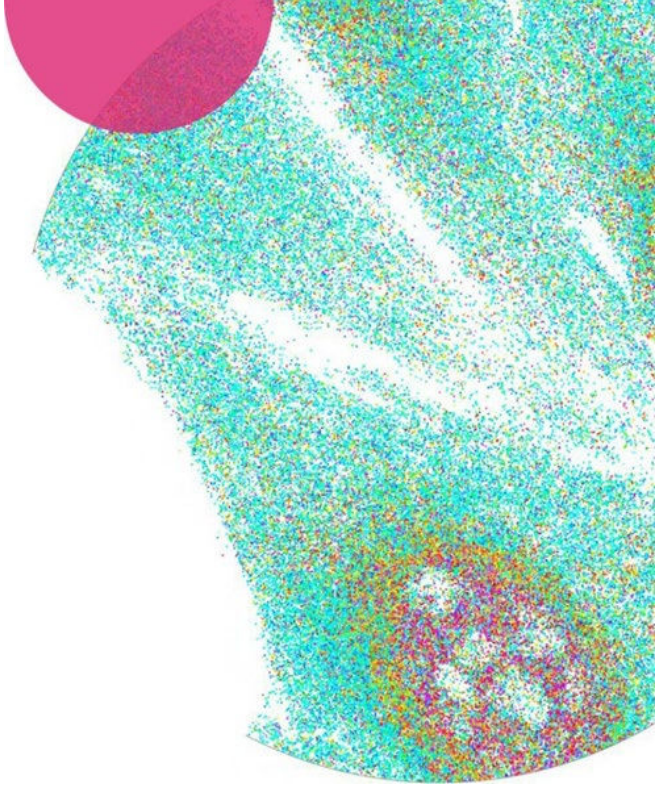

91600132 Rev A

MERFISH 2.0 Sample Preparation User Guide for Sectioned Tissue Samples

vizgen®

NOTICES

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SUPPORT

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TABLE OF CONTENTS

1 INTRODUCTION

1.1 The Vizgen MERSCOPE Platform Solution

The Vizgen MERSCOPE Platform provides an end-to-end solution for implementing the MERFISH technique for spatial transcriptomics, from sample preparation to data analysis and visualization (Figure 1). The first step in any project is to select your MERFISH gene panel using the Gene Panel Portal (Figure 1A). When you are ready to run your experiment, the tissue is first sectioned onto a MERSCOPE Slide (Figure 1B). Sample Preparation prepares the section for imaging on the MERSCOPE, which is covered in detail in this document (Figure 1C). Once the Slide is prepared, the MERSCOPE flow cell and Gene Imaging Cartridge are assembled and activated (Figure 1D), then analysis is performed on the MERSCOPE Instrument (Figure 1E). Analysis is performed on the MERSCOPE Analysis Computer and further exploration of the data is done through the MERSCOPE Vizualizer software (Figure 1F).

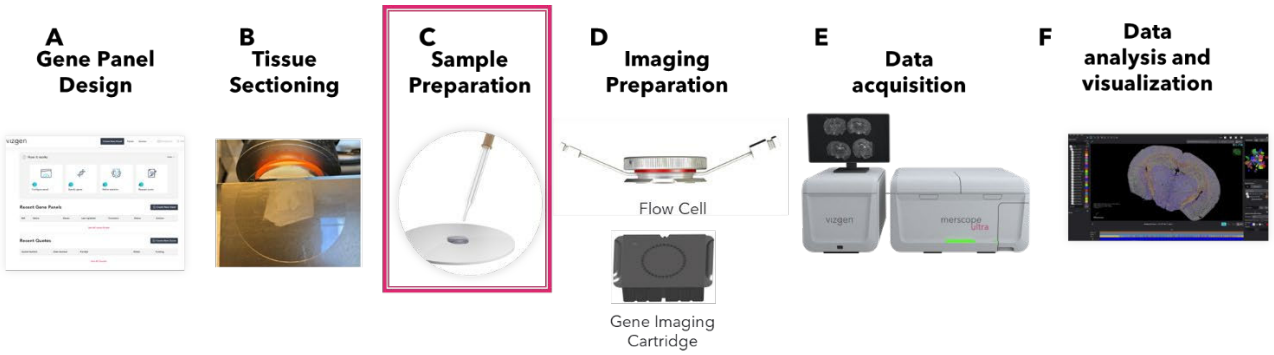

Figure 1. Overview of a MERFISH Platform project. This guide covers Sample Preparation (step C)

This user guide provides a detailed protocol to prepare tissue samples for MERFISH 2.0 chemistry and is applicable to **both formalin-fixed paraffin-embedded (FFPE) tissue** and **fresh frozen and paraformaldehyde (PFA)-fixed frozen tissue**. This guide is **not** applicable to cultured cells. The Sample Preparation protocol starts with sectioned slides and **does not** cover the histology workflow **prior** to MERFISH sample preparation or the instrument workflow **after** MERFISH sample preparation. Please refer to the appropriate documents for these steps.

For further guidance and detailed information on sectioning onto MERSCOPE Slides Vizgen has provided the following guides for reference:

- **FFPE tissues: MERSCOPE Tissue Preparation Guide:** Histology Guide for Preparing FFPE Tissue Samples for Experiments on the MERSCOPE Platform (PN 91600126)
- **Fresh and fixed frozen tissues:** MERSCOPE Tissue Preparation Guide: Histology guide for Preparing Fresh and Fixed Frozen Tissue Samples for Experiments on the MERSCOPE Platform (PN 91600129)

Step	User Guide	PN
Histological sectioning (FFPE)	MERSCOPE Tissue Preparation Guide: Histology Guide for Preparing FFPE Samples for Experiments on the MERSCOPE Platform	91600126
Histological sectioning (Fresh or Fixed Frozen)	MERSCOPE Tissue Preparation Guide: Histology Guide for Preparing Fresh and Fixed Frozen Tissue Samples for Experiments on the MERSCOPE Platform	91600129
Instrument Preparation and Operation (MERSCOPE)	MERSCOPE Instrument User Guide	91600001
Instrument Preparation and Operation (MERSCOPE Ultra)	MERSCOPE Ultra Instrument User Guide	91600131

The sample preparation workflow outlined in this Guide should serve as a general approach- most sample types require some optimization. For guidance on optimization, refer to the appendices in this User Guide, Vizgen technical notes, and/or contact your field application scientist or tech support for experimental planning.

2 MERFISH 2.0 CHEMISTRY OVERVIEW

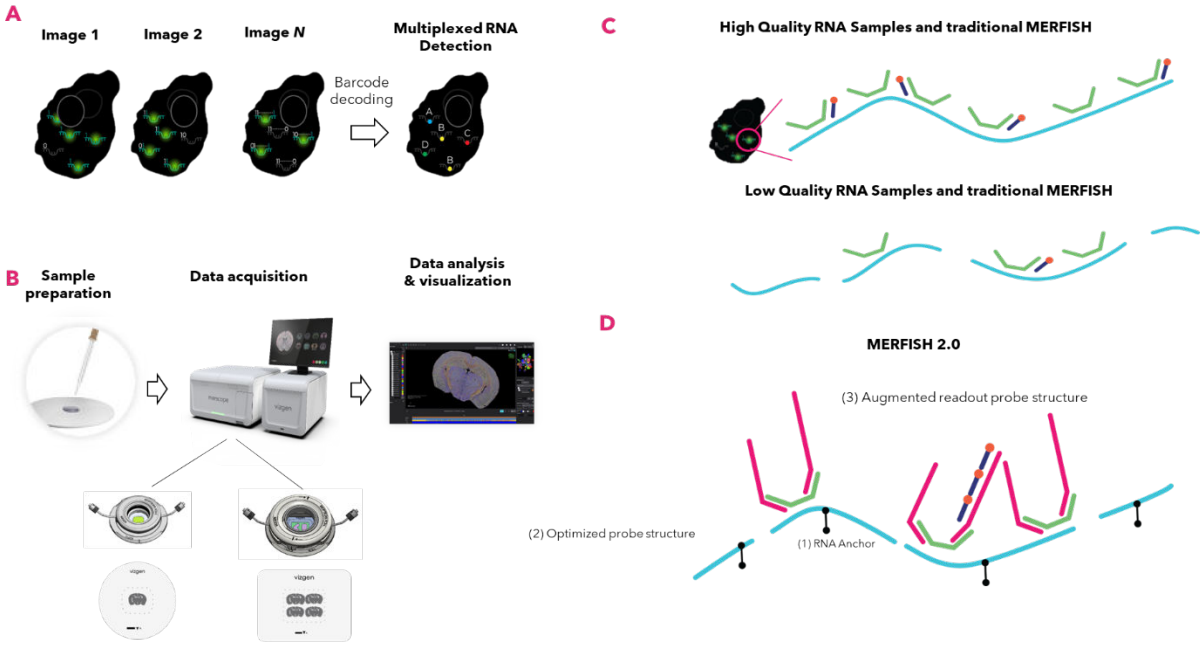

Figure 2. The MERFISH 2.0 chemistry overview.

MERFISH uses combinatorial labeling, binary barcoding and sequential imaging to image different mRNA species, which enables in situ profiling of hundreds to thousands of genes at single-molecular resolution (Figure 2A). In samples with high RNA quality, encoding probes

can tile along the target RNA transcripts, allowing the RNA transcripts to be detected faithfully in the original MERFISH 1.0 chemistry (Figure 2B). The MERFISH 2.0 chemistry and workflow improves upon MERFISH 1.0 by improving capture and detection of RNA, particularly in samples with lower RNA quality. Briefly, the MERFISH 2.0 chemistry introduces: 1) optimized anchoring to better capture RNA fragments; 2) optimized probe structure to enable efficient binding with the targets; and 3) enhanced readout probes that significantly increase the signal to noise ratio during imaging (Figure 2C). MERFISH 2.0 chemistry has been demonstrated to substantially improve the sensitivity of RNA detection as compared to the traditional MERFISH chemistry.

Samples processed with MERFISH 2.0 chemistry can be imaged either on the MERSCOPE Instrument or the MERSCOPE Ultra Instrument.

Instrument	Compatible Slides	Maximum Slide Tissue Size Compatibility
MERSCOPE Instrument	MERSCOPE Standard Slide V 2.0	1 cm^2
MERSCOPE Ultra Instrument	MERSCOPE Standard Slide V 2.0	1.25 cm^2
	MERSCOPE Large Slide V 2.0	3 cm^2

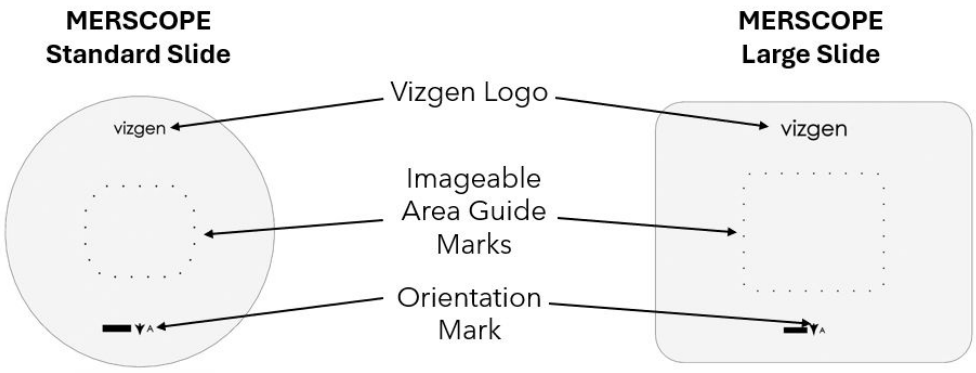

Figure 3. Diagram of both slides.

3 BEFORE YOU START: TIMING AND TIPS

3.1 MERFISH 2.0 Sample Preparation Workflow Timing

Day	Step	Hands-on time	Total time
1	7.1 Deparaffinization and Decrosslinking (FFPE only)	40 min	1.5 h
	7.2 Cell Staining* (optional)	1 h	4 h
	7.3 Anchoring Pretreatment	15 min	Overnight
2	7.4 Anchoring	30 min	2.5 h
	7.5 Gel Embedding⁺	30 min	2h
	7.6 Clearing‡	15 min	1+ days

3	7.7 Autofluorescence Quenching	15 min	3 h
	7.8 Encoding Probe Hybridization	15 min	Overnight
4	7.8 Post-Encoding Probe Wash	15 min	1 h
	7.9 Enhancer Probe Hybridization	15 min	Overnight
5	7.9 Post-Enhancer Probe Wash	15 min	20 min
5 or day of MERSCOPE run	DAPI staining and instrument operation§	1.5 h	3 h

A total of 5 days for sample preparation is based on the minimum times for Clearing. If extra time is needed for these steps, the total number of days increases accordingly.

*Users can perform cell boundary and/or protein staining. See Appendix I for additional protocols.

⁺See Appendix II for double gel embedding protocol

‡ Clearing incubation time depends on resistance of the tissue to clearing. Some resistant tissues may require additional digestion. See Appendix III for details on clearing and optimization. It is recommended to run the samples as soon as feasible, as extended storage in Clearing Solution may cause gel-related issues, which include but are not limited to gel bubbling.

§ See the relevant *MERSCOPE Instrument User Guide* for detailed instructions

3.2 Tissue Sectioning

The tissue of interest is sectioned and adhered to a MERSCOPE Slide. Tissue sectioning protocols are provided in the histology guides listed in Section 1.1.

3.3 Sample Verification

Users should use MERSCOPE Sample Verification Kits and MERSCOPE Protein Stain Verification Kits to verify that the sample preparation conditions are compatible with MERFISH imaging with the MERSCOPE Instrument.

4 MERFISH 2.0 SAMPLE PREPARATION WORKFLOW OVERVIEW

4.1 FFPE Tissue Only: Deparaffinization and Decrosslinking

Deparaffinization dissolves the paraffin and decrosslinking reverses the crosslinks originally introduced via formalin fixation.

4.2 All Tissues: Cell Boundary Staining and/or Protein Staining (Optional)

Tissue samples can be:

- Stained with cell boundary stain to mark cell boundaries and enable individual cell analysis, **AND/OR**
- Stained with protein stain for the co-detection of proteins and RNA in a single MERFISH experiment.

The standard MERFISH2.0 protocol provides details for staining with Vizgen's Cell Boundary Stains. For additional protocols and information on variations of this protocol, refer to Appendix I: Cell Boundary Staining and Protein Staining Tips. Users should use MERSCOPE

Protein Stain Verification Kits to evaluate whether their primary antibodies are suitable for use with a MERSCOPE Instrument, in addition to optimizing antibody concentrations for protein staining (refer to the *MERSCOPE Protein Stain Verification Kit User Guide, PN 91600103*).

4.3 Anchoring Pretreatment

Anchoring pretreatment primes the RNA for RNA anchoring.

4.4 RNA Anchoring

Formamide Wash Buffer denatures the RNA. Anchoring Buffer prepares RNA for gel embedding.

4.5 Gel Embedding

Gel embedding immobilizes the RNA and creates a protective layer so the RNA species cannot escape in subsequent steps.

4.6 Clearing

Clearing removes all tissue components other than RNA and DNA and is critical to minimize the autofluorescence background and thereby maximize signal. The optimal clearing protocol depends on the tissue sample type, and resistant samples may require an additional digestion step. More information on how to optimize clearing can be found in Appendix III: Clearing for Resistant Tissues and Clearing Optimization and in the tech note *Optimizing Tissue Digestion and Clearing for the MERSCOPE Platform* (PN 91700121).

We **strongly** recommended optimizing clearing conditions using MERSCOPE Sample Verification Kits prior to starting your MERFISH experiment. Some tissues may not reach complete transparency. If previous verification runs have shown positive results, we recommend proceeding with your experiment after 3 days of clearing even if the tissue is not fully transparent.

The standard MERFISH 2.0 protocol includes the clearing protocol for non-resistant tissue and optional digestion.

4.7 Autofluorescence Quenching

Autofluorescence background frequently occurs in a variety of tissues and is often caused by lipofuscin or lipofuscin-like pigments, which can interfere with MERFISH imaging. If autofluorescence is prevalent, the sample should be placed in the MERSCOPE Photobleacher to remove the background signal when the sample is in Clearing Solution. Human tissues are more likely to have autofluorescence background than mouse tissues. Users should use the MERSCOPE Sample Verification Kits to evaluate the tissue's autofluorescence.

4.8 Encoding Probe Hybridization

Formamide Wash Buffer denatures the RNA to allow encoding probe binding. The encoding probes hybridize to the RNA within the sample. Adequate washing minimizes background.

4.9 Enhancer Probe Hybridization

Enhancer Probe hybridization facilitates signal amplification, thereby increasing detection on the MERSCOPE platform. Adequate post enhancer probe hybridization washes minimize background.

4.10 Sample Storage (If Necessary)

Sample preparation can be performed in batches. Return batched samples to Clearing Solution supplemented with proteinase K for storage, if necessary. Samples can be stored in Clearing Solution at 37°C after Encoding Probe Hybridization Wash and after Enhancer Probe Hybridization Wash. **Total storage time in Clearing Solution should not exceed 4 days**, as extended storage in Clearing Solution may cause gel deformations.

4.11 Next Steps: Preparation and Running MERSCOPE (see MERSCOPE User Guide)

Refer to the *MERSCOPE Instrument User Guides* listed in Section 1.1 for next steps, briefly listed below:

1. Initialize and wash MERSCOPE (Instrument Guide Sections 7.1 -7.3)
2. Thaw cartridge and staining reagent* (Instrument Guide Section 7.4)
3. Stain sample with DAPI and polyT reagent* (Instrument Guide Section 7.6)
4. Configure MERSCOPE run* (Instrument Guide Section 7.7)
5. Assemble flow cell* (Instrument Guide Section 7.9)
6. Activate cartridge* (Instrument Guide Section 7.8)
7. Load flow cell and select ROI* (Instrument Guide Section 7.9)
8. Run MERSCOPE* (Instrument Guide Section 7.13)

*wetlab steps on day of MERSCOPE run

5 TECHNICAL TIPS

5.1 Experimental Planning

The MERSCOPE Instrument analyzes one sample at a time and imaging takes 0.5 – 2 days.

Sample preparation can be performed in batches and samples can be stored in Clearing Solution at 37°C after Encoding Probe Hybridization Wash and after Enhancer Probe Hybridization Wash. **Total storage time in Clearing Solution should not exceed 4 days, as extended storage in Clearing Solution may cause gel deformations.**

On the day of imaging, ensure 1) the MERFISH 2.0-compatible MERSCOPE Imaging Cartridge for the correct gene panel size is thawed, 2) The MERFISH 2.0 Staining Reagent is

thawed; and the MERFISH 2.0 MERSCOPE Codebooks are available. Refer to the *MERSCOPE Instrument User Guide* for more information.

The Clearing step depends on tissue type. Select step for Clearing based on the sample tissue type.

Refer to the table in Section 3.1 Workflow Timing for stopping points.

5.2 General Workflow Tips

- Approximate time and temperature conditions are provided when exact conditions have not been rigorously validated. Minimum or maximum incubation times are noted in steps where the timing is critical to the success of the protocol. "Overnight" incubations are approximately 16 hours. "Room temperature" refers to approximately 25°C.
- Minimize freeze-thaw cycles for reagents/kits stored at −20°C.
- Label samples on the bottom of the petri dish for future identification.
- Clean scissors, forceps, and any other equipment that will be in contact with the sample, buffers, or parafilm with RNaseZap and ethanol prior to use.

5.3 Sample Quality

It is recommended to evaluate RNA integrity/quality before doing experiments (e.g., using an Agilent TapeStation System or Agilent Bioanalyzer System). On average, higher RIN values in fixed frozen tissues and fresh frozen tissues or higher DV200 scores in FFPE tissues are associated with better MERFISH data generation. This recommendation is in addition to optimization using the Sample Verification kit prior to the first MERFISH experiment (Section 3.3).

5.4 RNase Decontamination

MERFISH measurements are sensitive to RNase activity. RNase contamination of any materials or reagents will degrade data quality.

Samples should be prepared in an area decontaminated with RNaseZap solution.

It is recommended to use RNase-free disposables, e.g., RNase-free media bottles (VWR PN 82051-594) for preparing buffers.

5.5 MERSCOPE Slide Handling

MERSCOPE Slides are fragile, be sure to handle with care. MERSCOPE Slides may be handled with tweezers or, if handling with gloved fingers, hold the edges to minimize the potential of touching the sample.

5.6 Histology and Sectioning

Careful histological technique is critical to obtaining high quality data. Histologists should be mindful of RNase contamination and maintaining an RNase-free environment.

5.7 Ensuring Full Coverage of MERSCOPE Slides During Washes

The MERSCOPE slide must be fully submerged in buffer at each wash step in the protocol. Visually confirm that the liquid covers the entire area of the slide when changing buffers. If parts of the slide float out of the buffer, swirl the buffer in the petri dish or manually submerge the slide with tweezers.

5.8 Maintaining Humidity During Long Incubations

Anchoring Pretreatment, RNA Anchoring, Clearing, Encoding Probe Hybridization, and Enhancer Probe Hybridization steps involve long incubation times. It is important that the samples do not dry out during incubation.

If a humidified incubator is not available, create a humidified environment with a large 150 mm petri dish. Place 1-2 Kimwipes in the dish with 5-10 mL of nuclease-free water. Place the sealed petri dish containing the sample within 150 mm humidified petri dish (Figure 4). Place in an oven at the appropriate temperature. Ensure the petri dish has sufficient nuclease-free water daily to prevent the sample drying out.

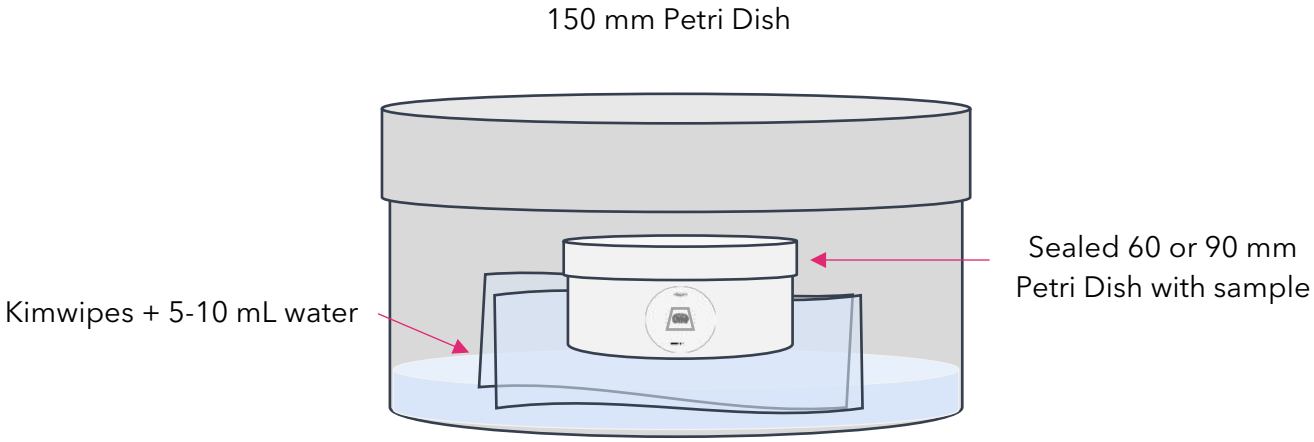

Figure 4. Creating a humidified chamber with a 150mm petri dish.

5.9 Safety and Hazardous Steps

Safe laboratory practices should be followed at all times. Follow your institution's guidelines for working with all materials used in the MERFISH 2.0 workflow.

Formamide Wash Buffer is hazardous. Fixation buffers, Pre-Anchoring Activator, and Enhancer Wash Buffer contain hazardous materials and steps using these materials should be performed in a fume hood. The reagents used in gel embedding also contain hazardous materials.

Safety Data Sheets for Vizgen Materials are available online at https://portal.vizgen.com.

6 MATERIALS

6.1 Vizgen Materials

Kits contain sufficient reagent for 10 samples for MERSCOPE Standard Slide V 2.0, 5 samples for MERSCOPE Large Slide V 2.0 unless otherwise noted.

MERSCOPE Standard Slide Box V 2.0, 10 Slides	**10500132**	**Storage**
MERSCOPE Standard Slide V 2.0, 10 x 1 slides	20400117	−20°C, horizontally
MERSCOPE Large Slide Box V 2.0, 5 Slides	**10500131**	**Storage**
MERSCOPE Large Slide V 2.0, 5 x 1 slides	20400118	−20°C, horizontally
MERSCOPE Tissue Sample Prep Kit	**10400194**	**Storage**
Deparaffinization Buffer	20300112	4°C
Decrosslinking Buffer	20300115	4°C
Conditioning Buffer	20300116	4°C
Sample Prep Wash Buffer	20300001	4°C
Formamide Wash Buffer	20300002	4°C, protected from light
Gel Embedding Premix	20300004	4°C, protected from light
Clearing Premix	20300114	4°C
Pre-Anchoring Activator*	20300113	−20°C upon receipt, protected from light
Anchoring Buffer	20300117	−20°C upon receipt
Digestion Premix *	20300005	−20°C upon receipt
*Minimize freeze-thaw cycles. Sample Prep Wash Buffer and Formamide Wash Buffer are used in the imaging and verification workflows.		
MERSCOPE Gel Coverslip Kit	**10500137**	**Storage**
25x25mm^2 Large Gel Coverslip	10500130	Room Temperature
20mm Diameter Gel Coverslip	30200004	Room Temperature
MERSCOPE 140 Gene Panel V 2.0, 10 samples	**10400178**	**Storage**
MERSCOPE 140 Gene Panel, V 2.0	20300195	−20°C
Enhancer Probe Mix	20300194	−20°C
Probe Dilution Buffer	20300193	−20°C
Enhancer Wash Buffer	20300192	4°C (shipped at -20°C; store at 4°C after opening)

MERSCOPE 300 Gene Panel V 2.0, 10 samples	**10400179**	**Storage**
MERSCOPE 300 Gene Panel, V 2.0	20300196	−20°C
Enhancer Probe Mix	20300194	−20°C
Probe Dilution Buffer	20300193	−20°C
Enhancer Wash Buffer	20300192	4°C (shipped at -20°C; store at 4°C after opening)
MERSCOPE 500 Gene Panel V 2.0, 10 samples	**10400180**	**Storage**
MERSCOPE 500 Gene Panel, V 2.0	20300197	−20°C
Enhancer Probe Mix	20300194	−20°C
Probe Dilution Buffer	20300193	−20°C
Enhancer Wash Buffer	20300192	4°C (shipped at -20°C; store at 4°C after opening)
MERSCOPE 1000 Gene Panel V 2.0, 10 samples	**10400181**	**Storage**
MERSCOPE 1000 Gene Panel, V 2.0	20300198	−20°C
Enhancer Probe Mix	20300194	−20°C
Probe Dilution Buffer	20300193	−20°C
Enhancer Wash Buffer	20300192	4°C (shipped at -20°C; store at 4°C upon receipt)
MERSCOPE Photobleacher	**10100003**	
MERSCOPE Photobleacher Universal Tray	10700287	

MERSCOPE Cell Boundary Stain Kit	**10400118**	**Storage**
Blocking Buffer C Premix	20300100	−20°C
Cell Boundary Primary Stain Mix	20300010	−20°C
Cell Boundary Secondary Stain Mix	20300011	−20°C

- 20 samples for MERSCOPE Standard Slide V 2.0, 10 samples for MERSCOPE Large Slide V 2.0
- Minimize freeze-thaw cycles

MERSCOPE Anti-Mouse Protein Stain Kit	**10400106**	**Storage**
Blocking Buffer C Premix	20300100	–20°C
Anti-Mouse Aux 4 Protein Stain	20300101	–20°C

- 20 samples for MERSCOPE Standard Slide V 2.0, 10 samples for MERSCOPE Large Slide V 2.0
- Minimize freeze-thaw cycles

MERSCOPE Anti-Rabbit Protein Stain Kit	**10400107**	**Storage**
Blocking Buffer C Premix	20300100	–20°C
Anti-Rabbit Aux 5 Protein Stain[a]	20300102	–20°C

- 20 samples for MERSCOPE Standard Slide V 2.0, 10 samples for MERSCOPE Large Slide V 2.0
- Minimize freeze-thaw cycles

[a]Not compatible with the MERSCOPE Cell Boundary Stain Kit

MERSCOPE Anti-Goat Protein Stain Kit	**10400108**	**Storage**
Blocking Buffer C Premix	20300100	–20°C
Anti-Goat Aux 6 Protein Stain	20300103	–20°C

- 20 samples for MERSCOPE Standard Slide V 2.0, 10 samples for MERSCOPE Large Slide V 2.0
- Minimize freeze-thaw cycles

MERSCOPE Anti-Rat Protein Stain Kit	**10400109**	**Storage**
Blocking Buffer C Premix	20300100	–20°C
Anti-Rat Aux 7 Protein Stain	20300104	–20°C

- 20 samples for MERSCOPE Standard Slide V 2.0, 10 samples for MERSCOPE Large Slide V 2.0
- Minimize freeze-thaw cycles

MERSCOPE Anti-Human Protein Stain Kit	**10400110**	**Storage**
Blocking Buffer C Premix	20300100	–20°C
Anti-Human Aux 8 Protein Stain	20300105	–20°C

- 20 samples for MERSCOPE Standard Slide V 2.0, 10 samples for MERSCOPE Large Slide V 2.0
- Minimize freeze-thaw cycles

MERSCOPE Anti-Chicken Protein Stain Kit	**10400111**	**Storage**
Blocking Buffer C Premix	20300100	–20°C
Anti-Chicken Aux 9 Protein Stain	20300106	–20°C

- 20 samples for MERSCOPE Standard Slide V 2.0, 10 samples for MERSCOPE Large Slide V 2.0
- Minimize freeze-thaw cycles

Safety Data Sheets are available online at portal.vizgen.com/

6.2 Required User Supplied Materials and Recommended Suppliers

Item	Vendor	Part number
Buffers and additives		
32% Paraformaldehyde (Formaldehyde) Solution	EMS	15714
Ammonium Persulfate *Refresh stock every 6 months*	Millipore-Sigma	09913-100G
Ethyl Alcohol, Pure (200 proof)	Millipore-Sigma	E7023-6X500ML
N,N,N′,N′-Tetramethylethylenediamine (TEMED) *Refresh stock every 6 months*	Millipore-Sigma	T7024-25ML
RNase Inhibitor, Murine	NEB	M0314L
Proteinase K, Molecular Biology Grade	NEB	P8107S
Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher	AM9625
UltraPure DNase/RNase-free Distilled Water	Thermo Fisher	10977015
Solutions, consumables & small laboratory equipment		
RNaseZap RNase Decontamination Solution	Thermo Fisher	AM9782
Petri Dish, 60 x 15 mm, Sterile	VWR	25382-687
Petri Dish, 90 x 15 mm, Sterile	VWR	10062-878
Petri Dish, 150 x 15 mm	VWR	76081-554
Gel Slick Solution*	VWR	12001-812
Parafilm M	VWR	102091-164
Gel coverslip, 20mm diameter, round	Fisher	NC0308916
Gel coverslip, 22mm diameter, round	VWR	10200-036
Gel coverslip, 25X25 mm, square	Corning	CLS285525-100EA
*If Gel Slick cannot be obtained, Sigmacote (Sigma-Aldrich, SL2-25ML) is a useful alternative. If using Sigmacote, use the manufacturer's protocol in Section 7.5.1 **Glass coverslips of the stated dimensions can be obtained from any manufacturer provided they are thin glass (spec. No. 1, 1.5, or 2).		
Hobby Blades *2x are provided with the MERSCOPE Instrument*	VWR	80094-372 and 103301-802
Cleaning tissue (Kimwipe or similar)	VWR*	21913-214*
High Precision Tweezers *1x are provided with the MERSCOPE Instrument*	Techni-Tool	758TW462
Serrated Tweezers *1x are provided with the MERSCOPE Instrument*	Techni-Tool	758TW450

EMS, Electron Microscopy Sciences. NEB, New England BioLabs. *Alternative to Kimwipe.		

6.3 General Laboratory Equipment

General laboratory equipment should be used according to the manufacturer's instructions.

- Fume hood
- 37°C cell culture incubator (humidified)[a]
- 47°C cell culture incubator (humidified)[a,b]
- 37°C incubator in a fume hood[a,b] (e.g., VWR 10055-006)
- 47°C incubator in a fume hood[a,b]
- 55°C dry oven[a] (e.g., VWR 10055-006)
- 90°C dry oven[a] (e.g., Fisher Scientific S13043)
- Vacuum trap system (e.g., VWR 76207-602)
- Vacuum pumps (e.g., Thomas Scientific 1162B24)
- Benchtop centrifuge
- Rocker
- Vortexer
- Analytical balance
- Water bath[c] (e.g., VWR 76308-896)
- Tube/bottle weight (e.g., VWR 47748-174)
- Benchtop cooler (e.g., VWR 414004-286)
- Cold plate (e.g., VWR 25608-942)
- Hot plate (e.g., VWR 89090-188)
- Drying rack (e.g., Millipore-Sigma Z743685)
- Tweezers, scissors, dissecting needles, and sectioning tools
- Microtome

a. Various incubation temperatures are needed throughout the protocol. Therefore, it is recommended to have equipment dedicated to each temperature incubation.
b. The 'cell culture incubator' and the 'incubator in a fume hood' may be the same piece of equipment. If a humidified incubator is not available, create a humidified environment as shown in Section 5.8.
c. If using an alternate make/model water bath, it should be large enough to accommodate the MERSCOPE Imaging Cartridge: 8 × 11 in (20 × 28 cm).

6.4 Additional Buffer Recipes

These buffers are not provided in Vizgen kits.

Commonly used buffers – make with nuclease-free water
70% Ethanol
90% Ethanol
1X PBS

7 MERFISH 2.0 SAMPLE PREPARATION PROTOCOL

The protocol considers each MERSCOPE Slide a sample. **Standard** refers to MERSCOPE Standard Slide V 2.0, and **Large** refers to MERSCOPE Large Slide V 2.0 in this Guide.

Each sample is prepared in a separate petri dish. A 60 mm petri dish is used for Standard Slide V 2.0 with 5 mL volumes to cover the slide. A 90 mm petri dish is used for MERSCOPE Large Slides V 2.0 with 10 mL volumes to cover the slide.

Incubations are stationary and at room temperature unless stated otherwise.

Symbol	Description
	Multiple options to proceed; depends upon experimental setup
	Note timing
	Overnight incubation or stopping point
	Visually inspect samples before proceeding
	Tip
	Critical step – follow instructions carefully

DAY 1

7.1 Deparaffinization and Decrosslinking for FFPE samples

If using fixed frozen and fresh frozen tissues, skip this step and proceed directly to Cell Boundary Staining

1. Add (**Standard**: **500 µL**; **Large: 1 mL**) of Deparaffinization Buffer (PN 20300112) onto the center of the FFPE tissue section. Ensure the Deparaffinization Buffer covers the whole tissue section and try to prevent the Deparaffinization Buffer from flowing beneath the MERSCOPE Slide V 2.0. Incubate at **55°C** (oven) for **5 min**.
2. Aspirate the Deparaffinization Buffer and repeat step 1.

3. Aspirate the Deparaffinization Buffer and add Deparaffinization Buffer (**Standard**: **500 µL**; **Large: 1 mL**) onto the center of the tissue section Incubate at **room temperature** for **5 min**.
4. Aspirate the Deparaffinization Buffer.
5. Wash **3x** with 100% ethanol (**Standard**: **5 mL**; **Large: 10 mL**), incubate **2 min** each wash.

If Deparaffinization Buffer is underneath the MERSCOPE Slide V 2.0, gently lift the slide to ensure oil droplets from underneath are also removed. Oil droplet removal can be facilitated by transferring to a new labeled petri dish.

6. **IF** oil droplets are observed after 3x washes, repeat 100% ethanol washes until no oil droplets are observed.
7. Wash **1x** with 90% ethanol (**Standard**: **5 mL**; **Large: 10 mL**), incubate 2 min.
8. Wash **1x** with 70% ethanol (**Standard**: **5 mL**; **Large: 10 mL**), incubate 2 min.

NOTE: *If tissue is prone to detachment, dry the slide at room temperature for 30 minutes to 1 hour after the 70% ethanol wash step. Inspect tissue carefully for detachment before proceeding*

Samples can be stored in 70% ethanol at 4°C for up to 1 day.

9. Aspirate the 70% ethanol and add Decrosslinking Buffer (PN 20300115) (**Standard**: **5 mL**; **Large: 10 mL**).
10. Aspirate and add Decrosslinking Buffer (**Standard**: **5 mL**; **Large: 10 mL**). Place the petri dish in an incubator at **90°C** for **15 min**.

DO NOT *heat above 95°C. Monitor temperature carefully before and during incubation – multiple thermometers can help monitor temperature.*

11. The petri dish is hot and should be handled accordingly: Remove the petri dish from the oven and cool on the bench for **5 min**.
12. Proceed to Cell Staining. If no staining is needed for your experiment, proceed to 7.3: Anchoring Pre-treatment.

If proceeding directly to Step III, thaw Pre-Anchoring Activator at room temperature for 30 min

7.2 Cell Boundary Staining

Cell Boundary Staining outlines the cell membrane and facilitates cell segmentation and **is recommended for most tissues except adult mouse brain or adult human brain**. Other protein stains can be used alone or in combination with Cell Boundary Staining.

The protocol for Cell Boundary Staining is provided below.

Refer to Appendix I: Cell Boundary Staining and Protein Staining Tips for additional protocols and other **IMPORTANT** information, including antibody requirements and compatibility.

If the sample does not require cell boundary staining and/or protein staining, proceed directly to step 7.3: Anchoring Pretreatment.

1. Prepare Blocking Solution:

	Standard		Large	
Sample number	1	5	1	5
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	200 µL	1000 µL
RNase inhibitor	5 µL	25 µL	10 µL	50 µL

2. Aspirate the buffer from the previous step (decrosslinking buffer for FFPE tissues, 70% ethanol for fixed frozen and fresh frozen tissues).
3. Add (**Standard: 5 mL**; **Large: 10 mL**) 1X PBS. Aspirate the 1X PBS to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
4. Add (**Standard**: **100 µL**; **Large: 200 µL**) Blocking Solution onto the center of the tissue section.
5. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**) (Figure 5). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

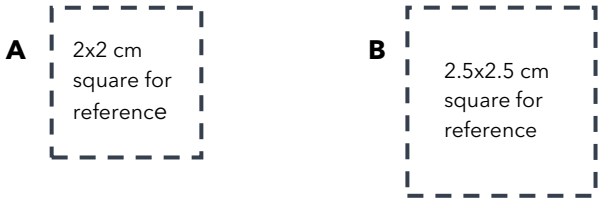

Figure 5. Reference size for **A)** Standard and **B)** Large

6. Incubate at **room temperature** for **1 h**.
7. Prepare Primary Staining Solution:

If the Blocking Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Blocking Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Blocking Solution may wick away into the petri dish.

	Standard		Large	
Sample number	1	5	1	5
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	200 µL	1000 µL
RNase inhibitor	5 µL	25 µL	10 µL	50 µL
Cell Boundary Primary Stain Mix (PN 20300010)	1 µL	5 µL	2 µL	10 µL

8. Use tweezers to remove the Parafilm.

9. Aspirate the solution to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
10. Add (**Standard**: **100 µL**; **Large: 200 µL**) Primary Staining Solution onto the center of the tissue section.
11. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

If the Primary Staining Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Primary Staining Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Primary Staining Solution may wick away into the petri dish.

12. Incubate at **room temperature** for **1 h**.
13. Use tweezers to remove the Parafilm.
14. Wash **3x** with 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**), incubate **5 min** on a rocker each wash.
15. Prepare Secondary Staining Solution:

	Standard		Large	
Sample number	1	5	1	5
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	200 µL	1000 µL
RNase inhibitor	5 µL	25 µL	10 µL	50 µL
Cell Boundary Secondary Stain Mix (PN 20300011)	3 µL	15 µL	6 µL	30 µL

16. Aspirate the 1X PBS to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
17. Add (**Standard**: **100 µL**; **Large: 200 µL**) Secondary Staining Solution onto the center of the tissue section.
18. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

If the Secondary Staining Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Secondary Staining Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Secondary Staining Solution may wick away into the petri dish

19. Incubate at **room temperature** for **1 h**.

20. Prepare Fixation Buffer:

Fixation Buffer* (**Standard**: **5mL; Large: 10 mL**)				
	Standard		**Large**	
Sample number	1	5	1	5
10X PBS	1 mL	3 mL	2 mL	6 mL
32% paraformaldehyde (formaldehyde) solution	1.25 mL	3.75 mL	2.5 mL	7.5 mL
Nuclease-free water	7.75 mL	23.25 mL	15.5 mL	46.5 mL
*Make fresh every time used				

21. Use tweezers to remove the Parafilm.
22. Wash **3x** with 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**), incubate **5 min** on a rocker each wash.
23. Aspirate the 1X PBS. In a fume hood, add Fixation Buffer (**Standard**: **5 mL**; **Large: 10 mL**) to fix the stained tissue section at **room temperature** for **15 min**.

*Thaw Pre-Anchoring Activator for Step 7.3 at **room temperature** for **30 min** before use.*

24. Wash **2x** with 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**), incubate **5 min** each wash.
25. Proceed to Anchoring Pretreatment.

7.3 Anchoring Pretreatment

Pre-Anchoring Activator contains an alkylating agent and dimethyl sulfoxide. Ensure Pre-Anchoring Activator is fully thawed, mixed, and spun down using a benchtop centrifuge before use.
Return unused reagents to the appropriate storage conditions but minimize freeze-thaw cycles.

1. Aspirate the buffer from the previous step (previous step varies based on experimental conditions).
2. Wash **2x** with Conditioning Buffer (PN 20300116) (**Standard**: **5 mL**; **Large: 10 mL**) to remove previous buffer.
3. Combine Conditioning Buffer and RNase inhibitor:

	Standard		**Large**	
Sample number	1	5	1	5
Conditioning Buffer	5 mL	25 mL	10 mL	50 mL
RNase inhibitor	5 µL	25 µL	10 µL	50 µL

4. Add Conditioning Buffer with RNase inhibitor to sample and incubate at **room temperature** for **15 min**.

5. Prepare Pre-Anchoring Reaction Buffer and mix well:

	Standard		Large	
Sample number	1	5	1	5
Conditioning Buffer (PN 20300116)	100 µL	500 µL	200 µL	1000 µL
Pre-Anchoring Activator (PN 20300113)	5 µL	25 µL	10 µL	50 µL
RNase inhibitor	5 µL	25 µL	10 µL	50 µL

6. First aspirate the majority of the Conditioning Buffer from the dish. Then carefully aspirate around the tissue section to remove excess liquid without touching the tissue section. The tissue section should not be completely dry for more than 1 min.
7. Add (**Standard**: **100 µL**; **Large: 200 µL**) Pre-Anchoring Reaction Buffer onto the tissue to cover the whole tissue.
8. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**) (Figure 6). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles. Seal the petri dish with Parafilm.

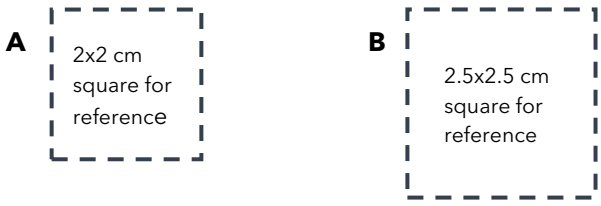

Figure 6. Reference size for **A)** Standard and **B)** Large

Even distribution of the Pre-Anchoring Reaction Buffer over the sample is critical for success. If it is not spread evenly across the tissue section, lift and then lower the Parafilm with tweezers until the Pre-Anchoring Reaction Buffer is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Pre-Anchoring Reaction Buffer may wick away into the petri dish.

Return unused reagents to −20°C storage but minimize freeze-thaw cycles.

9. In a 150-mm large petri dish, place 1-2 Kimwipes in the dish with 5-10 mL of nuclease-free water. This creates a humidified chamber to ensure sample does not dry out (Figure 7)
10. Place the sealed petri dish (**Standard**: **60 mm**; **Large: 90 mm**) containing the sample inside the 150mm petri dish. Seal the outer petri dish.

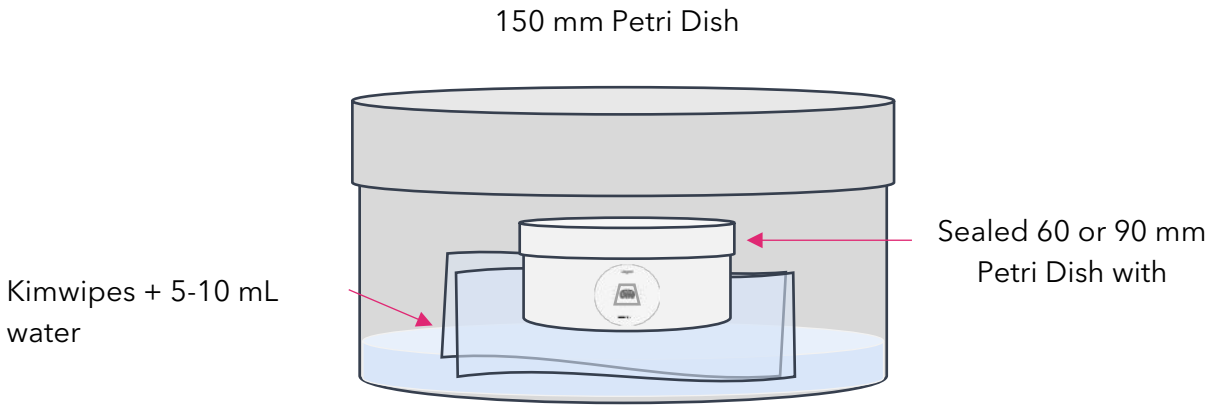

Figure 7. Humidified chamber inside a 150mm petri dish.

11. Cover the samples to protect from light.
12. Incubate on the bench at **room temperature overnight** (minimum of 16 h).

DAY 2

7.4 RNA Anchoring

Maintain Anchoring Buffer in a benchtop cooler until use. Spin down using a benchtop centrifuge before use.

Formamide Wash Buffer is hazardous. Perform these steps in a fume hood. Return unused reagents to −20°C storage but minimize freeze-thaw cycles.

1. Disassemble the humidified chamber. Use tweezers to remove the Parafilm.
2. Wash **1x** with Sample Prep Wash Buffer (PN 20300001) (**Standard**: **5 mL**; **Large: 10 mL**).
3. Add Formamide Wash Buffer (PN 20300002) (**Standard**: **5 mL**; **Large: 10 mL**), incubate at **37°C** for **15 min** in an incubator in a fume hood.
4. Aspirate the majority of the Formamide Wash Buffer. Then carefully aspirate around the tissue section to remove excess liquid without touching the tissue section. The tissue section should not be completely dry for more than 1 min.
5. Add (**Standard**: **100 µL**; **Large: 200 µL**) Anchoring Buffer (PN 20300117) onto the center of the tissue section.
6. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

If the Anchoring Buffer is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Anchoring Buffer is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Anchoring Buffer may wick away into the petri dish.

7. Place the petri dish in a humidified **37°C** cell culture incubator for **2 hours**.
8. Use tweezers to remove the Parafilm.

9. Add Formamide Wash Buffer (**Standard**: **5mL**; **Large: 10 mL**).
10. Incubate at **47°C** for **10 min** in an incubator in a fume hood.
11. Wash **1x** with Sample Prep Wash Buffer (**Standard**: **5mL**; **Large: 10 mL**), incubate **2 min**.
12. Proceed to Gel Embedding.

7.5 Gel Embedding

Tips for successful Gel Embedding:

*We recommend using **20 mm** diameter gel coverslip for **MERSCOPE Standard Slide V 2.0**, and **25x25 mm²** gel coverslip for **MERSCOPE Large Slides V 2.0.***

Smaller Gel Coverslips are generally recommended to avoid diluting the Gene Panel Encoding Probes.

Make sure the Gel Coverslip can cover the tissue area while staying within the gasket of the flow cell.

Gel Coverslip size should not exceed 25mm in diameter.

*If Gel Slick cannot be obtained, Vizgen recommends **Sigmacote** from Sigma-Aldrich. If using Sigmacote, replace step 2 with the manufacture's recommended protocol.*

⚠ *Gel embedding reagents contain hazardous materials.*

1. Clean both sides of a Gel Coverslip by spraying with RNaseZap solution and wiping with a Kimwipe, followed by spraying 70% ethanol and wiping with a Kimwipe.
2. Add (**Standard**: **100 µL**; **Large: 200 µL**) Gel Slick Solution onto the Gel Coverslip.
3. Allow the Gel Slick Solution to evaporate for 10 min at room temperature. Wipe gently with a Kimwipe to remove any remaining film, liquid, or deposition from the glass. Use immediately after preparation.
4. Prepare an ammonium persulfate solution:

10% w/v Ammonium Persulfate Solution – (Standard: 25 µL; Large: 50 µL)*
Prepare fresh every time – do not reuse dissolved solution

	Standard		Large	
Sample number	1	5	1	5
Ammonium persulfate‡	100 mg	100 mg	100 mg	100 mg
Nuclease-free water*	1 mL	1 mL	1 mL	1 mL

*Adjust volume of nuclease-free water to mass of ammonium persulfate weighed out in each case to obtain 10% w/v solution.

‡Discard any ammonium persulfate that is more than 6 months old.

5. Prepare Gel Embedding Solution and mix thoroughly:

	Small		Large	
Sample number	1	5	1	5
Gel Embedding Premix (PN 20300004)	5 mL	25 mL	10 mL	50 mL
10% w/v ammonium persulfate solution	25 µL	125 µL	50 µL	250 µL
N,N,N′,N'-tetramethylethylenediamine	2.5 µL	12.5 µL	5 µL	25 µL

6. Aspirate the Sample Prep Wash Buffer.
7. Transfer **1 mL** Gel Embedding Solution to an Eppendorf tube.
8. Wash the sample with remainder of the Gel Embedding Solution, ensuring the sample is fully covered, and incubate at **room temperature** for **1 min**.
9. Using a pipette, transfer the majority of the Gel Embedding Solution wash to a new tube to monitor the gel formation.
10. Aspirate to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
11. Add (**Standard**: **100 µL**; **Large: 200 µL**) from the 1 mL of retained Gel Embedding Solution on the tissue section.
12. Place the tips of one pair of tweezers on an area of the MERSCOPE Slide V 2.0 without touching the tissue section. Use tweezers to pick up the Gel Slick-treated Gel Coverslip. With the Gel Slick-treated side **facing down** toward the tissue, place the edge of the Gel Coverslip against the tweezer tips resting on the MERSCOPE Slide, creating stability, and slowly lower the Gel Coverslip onto the tissue section to spread the Gel Embedding Solution. If needed, adjust the Gel Coverslip so it is positioned in the center of the slide (Figure 8).
13. Gently press the Gel Coverslip to squeeze out excess Gel Embedding Solution and remove the extra Gel Embedding Solution by aspiration.

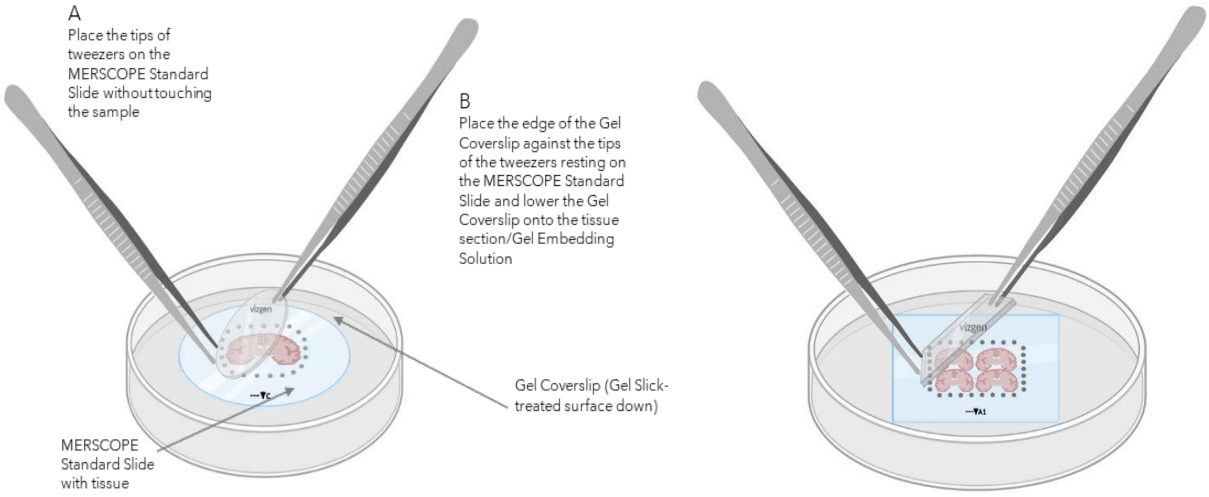

Figure 8. Steps to place Gel-coverslip onto tissue section on Standard and Large slides

Air bubbles in the gel solution inhibit gel polymerization. If a bubble forms, lift and then lower the Gel Coverslip to help the air bubbles escape.

***Gently** press the Gel Coverslip with tweezers or the back of a pipet tip to achieve a thin layer of gel between the Gel Coverslip and the MERSCOPE Slide V 2.0. Avoid squeezing the Gel Coverslip too hard as it may damage the sample and result in gel that is too thin/no gel. When properly aspirated, the coverslip will not glide over the sample when the petri dish is tilted.*

14. Incubate at **room temperature** for **1.5 h**. Monitor the Gel Embedding process in the waste tube. Gel starts to form within 1 h.
15. While the gel is forming, warm the Clearing Premix at **37°C**.
16. Ensure **eye protection** is worn during this step. Gently brace the Gel Coverslip with tweezers in one hand and lift the Gel Slick-treated Gel Coverslip with the sharp tip of a Hobby Blade and discard the Gel Coverslip appropriately.

Carefully inspect the gel before proceeding. Repeat the Gel Embedding process from step 1 if:

–no gel forms.

–bubbles are visible in the gel.

–too much Gel Embedding Solution was squeezed out and the gel is not visible.

Do not attempt to remove the gel prior to repeating the Gel Embedding process.

17. Proceed to Clearing.

7.6 Clearing

Choose Clearing protocol based on tissue type. Refer to Clearing in the Sample Preparation Overview for more information

Exact clearing conditions will need to be determined experimentally for each sample type. The clearing protocol below is a good starting point for many tissues. See Appendix III and the tech note *Optimizing Tissue Digestion and Clearing for the MERSCOPE Platform* (PN 91700121) for additional protocols and recommendations for optimizing clearing.

Particularly fibrotic or otherwise collagen-rich tissues can be difficult to clear, and we recommend **adding a digestion step prior to clearing.**

- **Mouse** tissues are **generally not resistant** to clearing. Known **exceptions** include mouse skin and bone tissues, which are generally resistant to clearing.
- **Human** tissues are **generally resistant** to clearing. Known **exceptions** include human liver, heart, spinal cord, and brain tissues, which are generally not resistant to clearing.

Optional Protocol for Digestion for Resistant Tissues

1. Thaw Digestion Premix. Ensure fully thawed and mixed, and spin down using a benchtop centrifuge before use.
2. Prepare Digestion Mix:

	Standard		Large	
Sample number	1	5	1	5
Digestion Premix (PN 20300005)	100 µL	500 µL	200 µL	1 mL
RNase inhibitor	2.5 µL	12.5 µL	5 µL	25 µL

3. Aspirate to dry the MERSCOPE Slide V 2.0 without touching the gel. Add Digestion Mix (**Standard**: **100 µL**; **Large: 200 µL**) onto the gel.
4. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.
    a. *If the Digestion Mix is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Digestion Mix is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide, otherwise the Digestion Mix may wick away into the petri dish.*
5. Incubate at 37°C for 2 h to 6h.
    a. *2 h digestion is suitable for most clearing-resistant tissue. However, if a tissue does not become transparent with 2 h Digestion Mix treatment and 24 h tissue clearing at 47°C, consider extending the Digestion Mix incubation time up to 6 hours facilitate tissue clearing.*
6. Use tweezers to remove the Parafilm. Aspirate the Digestion Mix. Proceed to Step 1 of **Clearing.**

1. Ensure the Clearing Premix (PN 20300114) has been pre-warmed at **37°C** for **30 min** before use (Step 7.5.13). If the solution is cloudy or is not homogeneous, warm and mix until the solution becomes clear.
2. Prepare Clearing Solution:

	Standard		Large	
Sample number	1	5	1	5
Clearing Premix (PN 20300114)	5 mL	25 mL	10 mL	50 mL
Proteinase K	50 µL	250 µL	100 µL	500 µL

3. Add Clearing Solution (**Standard**: **5 mL**; **Large: 10 mL**).
4. Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.

5. Seal the petri dish with Parafilm and place in a humidified **47°C** cell culture incubator for **18-24 h**.

DO NOT *incubate at 47°C for more than 24 hours, otherwise the RNA will begin to degrade - this is important to remember if clearing over the weekend.*

DAY 3

6. Inspect the sample. **IF** the tissue is transparent, directly proceed to the next step. **IF** the tissue is not transparent after 24 h, transfer the petri dish to a humidified **37°C** cell culture incubator and incubate until the tissue section becomes transparent (up to 3 days). Refresh the Clearing Solution each day to ensure optimal Proteinase K activity. See Appendix III for more information on clearing conditions.

*SAMPLES CAN BE STORED OR CLEARED IN CLEARING SOLUTION AT **37°C** FOR UP TO **4 DAYS.***

7.7 Autofluorescence Quenching (If Necessary)

1. Aspirate bubbles or condensation from the lid of the petri dish to minimize light scattering from above. Seal the petri dish with Parafilm.

ENSURE *the petri dish is sealed with Parafilm prior to autofluorescence quenching, otherwise the Clearing Solution may evaporate in the MERSCOPE Photobleacher.*

The sample remains in Clearing Solution for autofluorescence quenching.

2. Place the Parafilm-sealed petri dish in the MERSCOPE Photobleacher (PN 10100003). **ENSURE** there are no labels/writing/other items on the lid that may block the light.
3. Turn on the MERSCOPE Photobleacher and leave at room temperature for at least 3 hours. Highly autofluorescent tissue can be photobleached for up to 11.5 hours and left in the MERSCOPE Photobleacher overnight. Exact Photobleaching time may need to be experimentally determined for each sample type using the Sample Verification Kit.

7.8 Encoding Probe Hybridization

Probe Dilution Buffer is highly viscous and must be pipetted slowly.

Warm Probe Dilution Buffer at 37 ℃ for 5 minutes prior to use to increase the ease of pipetting.

Spin down Probe Dilution Buffer and MERSCOPE Gene Panel V 2.0 using a benchtop centrifuge before use.

Formamide Wash Buffer is hazardous. Perform these steps in a fume hood.

Return unused reagents to −20°C storage but minimize freeze-thaw cycles.

1. Ensure Probe Dilution Buffer has been pre-warmed and centrifuged, avoid introducing bubbles.
2. Aspirate the Clearing Solution and wash **3x** with Sample Prep Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**), incubating at **room temperature** for **5 min** each wash.
3. Aspirate Sample Prep Wash Buffer and add Formamide Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**), incubate at **37°C** for **30 min**.
4. Slowly combine the Gene Panel V 2.0 with the Probe Dilution Buffer (PN 20300193) to make the **Encoding Probe Mix.** Avoid introducing bubbles.

	Standard		Large	
Sample number	1	5	1	5
MERSCOPE Gene Panel, V 2.0	5 µL	25 µL	10 µL	50 µL
Probe Dilution Buffer (PN 20300193)	100 µL	500 µL	200 µL	1000 µL

5. Carefully mix the solution well by pipetting up and down 10 times. Pipet slowly to avoid introducing bubbles. Spin down the probe mixture with a centrifuge at room temperature for 1 minute at ≥10,000g.
6. Aspirate the majority of the Formamide Wash Buffer from the dish. Then carefully aspirate around the gel to remove excess liquid without touching the gel.
7. Slowly and carefully add Encoding Probe Mix (**Standard**: **100 µL**; **Large: 200 µL**) onto the center of the gel.

Aspirate all the residual solution in the dish and on the MERSCOPE Slide V 2.0 without disrupting the gel to avoid diluting the Encoding Probe Mix.

8. Use clean scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.
9. Place the lid on the petri dish (**Standard**: **60 mm**; **Large: 90 mm**). Seal the petri dish tightly with Parafilm.
10. Place 1-2 Kimwipes in the dish with 5-10 mL of nuclease-free water into a 150 mm dish.

11. Place the sealed petri dish (**Standard**: **60 mm**; **Large: 90 mm**) into the 150-mm dish. (See Figure 6).

Even distribution of the Probe Mix across the gel is critical to success. If the Probe Mix is not spread across the gel, lift and then lower the Parafilm with tweezers until the Probe Mix is spread across the gel. The Parafilm should fit within the MERSCOPE Slide V 2.0.

On Large slides, the full 200 µL of Gene Panel Mix is needed for optimal MERFISH results. Aim to contain the full 200 µL within the area of the gel.

12. Seal the 150 mm dish well with a long piece of Parafilm.
13. Place the 150 mm dish in a humidified **47°C** incubator **overnight** (minimum of 18 hours).

DAY 4

14. Disassemble the humidified petri dish setup and wipe off water outside the petri dish. Use tweezers to remove the Parafilm.
15. Add Formamide Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**).
16. Incubate at **47°**C for **30 min** in an incubator in a fume hood.
17. Aspirate the Formamide Wash Buffer. Add Formamide Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**).
18. Incubate at **47°C** for **30 min** in an incubator in a fume hood.
19. Proceed to Enhancer Probe Hybridization or Sample Storage (If Necessary).

7.9 Enhancer Probe Hybridization

Enhancer Probe Mix is highly viscous and must be pipetted slowly. Warm Enhancer Probe Mix at 37°C for 5 minutes prior to use to increase the ease of pipetting. Spin down Enhancer Probe Mix using a benchtop centrifuge before use.

Enhancer Wash Buffer and Formamide Wash Buffer are hazardous. Perform these steps in a fume hood.

Return unused reagents to −20°C storage but minimize freeze-thaw cycles.

1. Ensure Enhancer Probe Mix has been pre-warmed and centrifuge, avoid introducing bubbles
2. If proceeding directly from Encoding Probe Hybridization, aspirate the Formamide Wash Buffer and proceed to **Step 5**.
3. If samples were stored in Clearing Solution, aspirate the Clearing Solution and wash **3x** with Sample Prep Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**), at **room temperature** for **5 min** each wash.
4. Aspirate Sample Prep Wash Buffer and add Formamide Wash Buffer, incubate at **37°C** for **15 min**.
5. Aspirate the majority of the Formamide Wash Buffer from the dish. Then carefully aspirate around the gel to remove extra liquid without touching the gel.

Aspirate all the residual solution in the dish and on the MERSCOPE Slide V 2.0 without disrupting the gel to avoid diluting the Enhancer Probe Mix.

6. Add **(Standard: 100 µL; Large: 200 µL)** Enhancer Probe Mix onto the center of the gel.
7. Use scissors (clean with RNaseZap solution) to cut a piece of Parafilm (**Standard: 2×2 cm; Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

If the Enhancer Probe Mix is not spread across the gel, lift and then lower the Parafilm with tweezers until the Probe Mix is spread across the gel. The Parafilm should fit within the MERSCOPE Slide V 2.0.

8. Place the lid on the petri dish (**Standard**: **60 mm**; **Large: 90 mm**). Seal the petri dish tightly with Parafilm.
9. Place 1-2 Kimwipes in the dish with 5-10 mL of nuclease-free water into the 150 mm dish. Place the sealed petri dish (**Standard**: **60 mm**; **Large: 90 mm**) into a 150 mm dish (See Figure 6).
10. Seal the 150 mm petri dish with Parafilm and place in a humidified **37°C** cell culture incubator **overnight** (minimum of 16 h). **DO NOT** let the sample dry out.

DAY 5

11. Warm Enhancer Wash Buffer (PN 20300192) at **room temperature** for **15 min**.
12. Disassemble the humidified petri dish setup and wipe off water outside of the petri dish. Use tweezers to remove the Parafilm.
13. Add Enhancer Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**) to the petri dish.
14. Incubate at **47°C** for **20 min** in an incubator in a fume hood.
15. Wash **1x** with Enhancer Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**) and incubate at **47°C** for **20 min** in an incubator in a fume hood.

Store Enhancer Wash Buffer at 4°C after opening the Gene Panel V 2.0 Kit.

16. Proceed with MERSCOPE imaging (refer to the MERSCOPE Instrument User Guide) or Sample Storage (If Necessary).

7.10 Sample Storage (If Necessary)

1. Warm Clearing Premix at **37°C** for **30 min** before use. The Clearing Premix should be a clear solution before use. If the solution is cloudy, warm until the solution becomes clear.
2. Wash **1x** with Sample Prep Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**), incubate **2 min**.
3. Aspirate the Sample Prep Wash Buffer and add Clearing Premix (**Standard**: **5 mL**; **Large: 10 mL**) supplemented with Proteinase K (**Standard**: **50 µL**; **Large: 100 µL**).

1. *After Encoding Probe Hybridization Wash, samples can be stored in Clearing Premix in a 37°C humidified incubator for up to **3 DAYS***
2. *After Enhancer Probe Hybridization Wash, samples can be stored in Clearing Premix in a 37°C humidified incubator for up to **2 DAYS***
3. *Do not exceed a **TOTAL OF 4 DAYS** of storage in Clearing Solution.*

8 APPENDIX I: CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS

Cell boundary staining conveniently marks cell boundaries to enable individual cell analysis. If cells are adequately dispersed in a sample (e.g., in central nervous system tissue) it may not be necessary to do cell boundary staining.

The MERSCOPE Protein Stain Kits use oligonucleotide-conjugated secondary antibodies to detect user-provided primary antibodies and thereby enable the co-detection of proteins and RNA in a single MERFISH experiment.

8.1 Cell Boundary and Protein Staining Options

Identify the appropriate protocol for staining:

- Cell boundary staining **ONLY (Step 7.2)**
- Protein staining **ONLY (Step 8.2)**
- Cell boundary staining **AND** protein staining **(Step 8.3)**
- No staining (skip the step)

8.1.1 User-provided Primary Antibody Requirements and Verification

User-provided primary antibodies **MUST** be:

- Bovine serum albumin (BSA)-free, **AND**
- Compatible with immunohistochemistry (IHC).

Users should use MERSCOPE Protein Stain Verification Kits to evaluate whether their primary antibodies are suitable for use with a MERSCOPE Instrument, in addition to optimizing antibody concentrations. Refer to the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.

8.1.2 MERSCOPE Protein Stains

Vizgen supplies protein stains compatible with user-provided primary antibodies raised in a variety of host species.

Primary Antibody Raised In	Corresponding Protein Stain	Auxiliary Bit	Kit PN
Mouse	Anti-Mouse Aux 4 Protein Stain	Aux 4	10400106
Rabbit	Anti-Rabbit Aux 5 Protein Stain	Aux 5	10400107
Goat	Anti-Goat Aux 6 Protein Stain	Aux 6	10400108
Rat	Anti-Rat Aux 7 Protein Stain	Aux 7	10400109
Human	Anti-Human Aux 8 Protein Stain	Aux 8	10400110
Chicken	Anti-Chicken Aux 9 Protein Stain	Aux 9	10400111

8.1.3 Compatibility

- Rabbit primary antibodies (and therefore the MERSCOPE Anti-Rabbit Protein Stain Kit) are currently **NOT** compatible with concurrent cell boundary staining.

- Gene panels containing sequential gene encoding **MAY NOT** be compatible with certain MERSCOPE Protein Stain Kits. To identify potential conflicts:
  - *Locate the auxiliary bit assigned to sequential genes in your gene panel. Navigate to the panel summary page for a constructed gene panel in the MERSCOPE Gene Panel Design Software. Sequential genes are listed along with the assigned auxiliary bits.*
  - *Compare the auxiliary bits used by the sequential genes in the gene panel to the auxiliary bits of the protein stains (table above).* ***If the auxiliary bits overlap, the protein stain is not compatible with the gene panel.*** *E.g., if a sequential gene in the gene panel is assigned to Aux 4, the MERSCOPE Anti-Mouse Protein Stain is not compatible with the gene panel.*
- MERSCOPE Protein Stains may display higher levels of non-specific binding to tissue of the same species (e.g., Anti-Mouse Aux 4 Protein Stain may show higher levels of nonspecific binding to mouse tissue).
- Contact Vizgen Support (support@vizgen.com) for more information, if needed.

8.1.4 Primary Staining Solution for Protein Staining – Key Details

- Add the primary antibodies for **EACH** protein to be detected.
  - *E.g., in a single sample, if a protein is detected using a mouse primary antibody and a second protein is detected using a goat primary antibody, add the mouse primary antibody* ***AND*** *the goat primary antibody to the Primary Staining Solution.*
- Only 1 primary antibody **PER SPECIES** can be detected in a single MERFISH experiment. **DO NOT** add 2 primary antibodies raised in the same species.
- Optimal primary antibody concentration(s) should be determined during verification.
- Refer to the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.
- Cell boundary staining **AND** protein staining **ONLY**: Rabbit primary antibodies are currently **NOT** compatible with concurrent cell boundary staining and therefore should not be included in the Primary Staining Solution.

8.1.5 Secondary Staining Solution for Protein Staining – Key Details

- **ONLY** add the protein stain(s) against **EACH** primary antibody host species used in the Primary Staining Solution.
  - *E.g., if mouse, goat, and rat primary antibodies are used in the Primary Staining Solution, add the required volume of anti-mouse, anti-goat, and anti-rat protein stain to the Secondary Staining Solution.*
- Cell boundary staining **AND** protein staining **ONLY**: Rabbit primary antibodies are currently **NOT** compatible with concurrent cell boundary staining, and therefore Anti-Rabbit Aux 5 should not be included in the Secondary Staining Solution.

8.2 Protein Staining ONLY

Maintain user-provided primary antibodies per manufacturer's instructions. Maintain Protein Stains and RNase inhibitor in a benchtop cooler until use. Spin down using a benchtop centrifuge before use.

Thaw Blocking Buffer C Premix. Ensure fully thawed and mixed, and spin down using a benchtop centrifuge before use.

Return unused reagents to −20°C storage but minimize freeze-thaw cycles.

1. If working with FFPE tissues, aspirate the decrosslinking buffer. Add 1XPBS (**Standard**: **5 mL**; **Large: 10 mL**).
2. If working with fixed frozen and fresh frozen tissues, aspirate the 70% ethanol. Add 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**).
3. Prepare Blocking Solution:

	Standard		Large	
Blocking Solution	1	5	1	5
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	200 µL	1000 µL
RNase inhibitor	10 µL	50 µL	20 µL	100 µL

4. Aspirate the 1X PBS to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
5. Add Blocking Solution (**Standard**: **100 µL**; **Large: 200 µL**) onto the center of the tissue section.
6. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles (Figure 9).

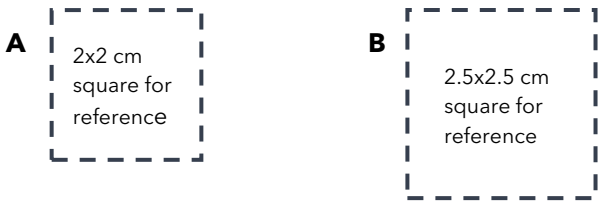

Figure 9. Reference size for **A)** Standard and **B)** Large.

7. Incubate at **room temperature** for **1 h**.

If the Blocking Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Blocking Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Blocking Solution may wick away into the petri dish.

8. Prepare Primary Staining Solution:

	Standard		Large	
Sample number	1	5	1	5
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	200 µL	1000 µL
RNase inhibitor	10 µL	50 µL	20 µL	100 µL
User-provided primary antibody raised in[a-c]: • Mouse • Rabbit • Goat • Rat • Human • Chicken	1 µL of each	5 µL of each	2 µL of each	10 µL of each

a. Add the primary antibodies for **EACH** protein to be detected. E.g., in a single sample, if a protein is detected using a mouse primary antibody and a second protein is detected using a goat primary antibody, add the mouse primary antibody **AND** the goat primary antibody to this Primary Staining Solution.

b. Only 1 primary antibody **PER SPECIES** can be detected in a single MERFISH experiment. **DO NOT** add 2 primary antibodies raised in the same species.

c. Optimal primary antibody concentration(s) should be determined during verification. Refer to the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.

9. Use tweezers to remove the Parafilm.
10. Aspirate the solution to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
11. Add Primary Staining Solution (**Standard**: **100 µL**; **Large: 200 µL**) onto the center of the tissue section.
12. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

If the Primary Staining Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Primary Staining Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Primary Staining Solution may wick away into the petri dish.

13. Incubate at **room temperature** for **1 h.**
14. Use tweezers to remove the Parafilm.
15. Wash **3x** with 1X PBS (**Standard: 5 mL**; **Large: 10 mL**), incubate **5 min** on a rocker each wash.

16. Prepare Secondary Staining Solution:

		Standard		Large	
Sample number		1	5	1	5
Blocking Buffer C Premix (PN 20300100)		100 µL	500 µL	200 µL	1000 µL
RNase inhibitor		10 µL	50 µL	20 µL	100 µL
Protein Stain(s) Select among[a]: Anti-Mouse Aux 4 Anti-Rabbit Aux 5 Anti-Goat Aux 6 Anti-Rat Aux 7 Anti-Human Aux 8 Anti-Chicken Aux 9	 (PN 20300101) (PN 20300102) (PN 20300103) (PN 20300104) (PN 20300105) (PN 20300106)	1 µL of each	5 µL of each	2 µL of each	10 µL of each

a. **ONLY** add the protein stain(s) against **EACH** primary antibody host species used in the Primary Staining Solution. E.g., if mouse, goat, and rat primary antibodies were used in the Primary Staining Solution, add the required volume of anti-mouse, anti-goat, and anti-rat protein stain to this Secondary Staining Solution.

17. Aspirate the 1X PBS to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
18. Add Secondary Staining Solution (**Standard: 100 µL**; **Large: 200 µL**) onto the center of the tissue section.
19. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

If the Secondary Staining Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Secondary Staining Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Secondary Staining Solution may wick away into the petri dish. petri dish.

20. Incubate at **room temperature** for **1 h**.
21. Use tweezers to remove the Parafilm.

22. Prepare Fixation Buffer:

Fixation Buffer* (**Standard**: **5 mL; Large: 10 mL**)				
	Standard		**Large**	
Sample number	1	5	1	5
10X PBS	1 mL	3 mL	2 mL	6 mL
32% paraformaldehyde (formaldehyde) solution	1.25 mL	3.75 mL	2.5 mL	7.5 mL
Nuclease-free water	7.75 mL	23.25 mL	15.5 mL	46.5 mL
*Make fresh every time used.				

23. Wash **3x** with 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**), incubate **5 min** on a rocker each wash.
24. Aspirate the 1X PBS. In a fume hood, add Fixation Buffer (**Standard**: **5 mL**; **Large: 10 mL**) to fix the stained tissue section at **room temperature** for **15 min**.

Thaw Pre-Anchoring Activator for Anchoring Pretreatment before use.

25. Wash **2x** with 1X PBS (**Standard**: **5mL**; **Large: 10 mL**), incubate **5 min** each wash.
26. Continue to Anchoring Pretreatment.

8.3 Cell Boundary Staining AND Protein Staining

Primary antibodies raised in rabbits (and therefore Anti-Rabbit Aux 5) CANNOT be used when protein staining is combined with cell boundary staining.

Maintain user-provided primary antibodies per manufacturer's instructions. Maintain Cell Boundary Primary Stain Mix, Cell Boundary Secondary Stain Mix, Protein Stains, and RNase inhibitor in a benchtop cooler until use. Spin down using a benchtop centrifuge before use.

Thaw Blocking Buffer C Premix. Ensure fully thawed and mixed, and spin down using a benchtop centrifuge before use.

Return unused reagents to −20°C storage but minimize freeze-thaw cycles.

1. If working with FFPE tissues, aspirate the decrosslinking buffer. Add 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**).
2. If working with fixed frozen tissues and fresh frozen tissues, aspirate the 70% ethanol. Add 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**).

3. Prepare Blocking Solution:

MERSCOPE Slide V 2.0	**Standard**		**Large**	
Blocking Solution	1	5	1	5
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	200 µL	1000 µL
RNase inhibitor	10 µL	50 µL	20 µL	100 µL

4. Aspirate the 1X PBS to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
5. Add Blocking Solution (**Standard**: **100 µL**; **Large: 200 µL**) onto the center of the tissue section.
6. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles (Figure 9)

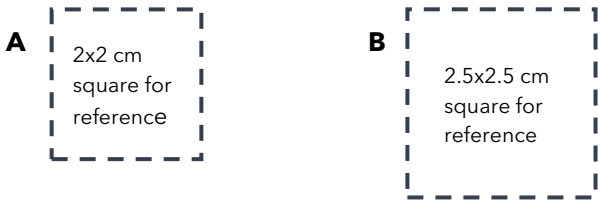

Figure 10: Reference size for **A)** Standard and **B)** Large.

7. Incubate at **room temperature** for **1 h.**

If the Blocking Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Blocking Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Blocking Solution may wick away into the petri dish.

8. Prepare Primary Staining Solution:

	Standard		**Large**	
Sample number	1	5	1	5
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	200 µL	1000 µL
RNase inhibitor	10 µL	50 µL	20 µL	100 µL
Cell Boundary Primary Stain Mix (PN 20300010)	1 µL	5 µL	2 µL	10 µL

User-provided primary antibody raised in[a-d]: • Mouse • Goat • Rat • Human • Chicken	1 µL of each	5 µL of each	2 µL of each	10 µL of each

a. Add the primary antibodies for **EACH** protein to be detected. For example, in a single sample, if a protein is detected using a mouse primary antibody and a second protein is detected using a goat primary antibody, add the mouse primary antibody **AND** the goat primary antibody to this Primary Staining Solution.
b. Only 1 primary antibody **PER SPECIES** can be detected in a single MERFISH experiment. **DO NOT** add 2 primary antibodies raised in the same species.
c. Rabbit primary antibodies are currently **NOT** compatible with concurrent cell boundary staining, and therefore not listed here.
d. Optimal primary antibody concentration(s) should be determined during verification. Refer to the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.

9. Use tweezers to remove the Parafilm.
10. Aspirate the solution to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
11. Add Primary Staining Solution (**Standard**: **100 µL**; **Large: 200 µL**) onto the center of the tissue section.

If the Primary Staining Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Primary Staining Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Primary Staining Solution may wick away into the petri dish.

12. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.
13. Incubate at **room temperature** for **1 h**.
14. Use tweezers to remove the Parafilm.
15. Wash **3x** with 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**), incubate **5 min** on a rocker each wash.
16. Prepare Secondary Staining Solution:

	Standard		**Large**	
Sample number	1	5	1	5
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	200 µL	1000 µL

RNase inhibitor		10 µL	50 µL	20 µL	100 µL
Cell Boundary Secondary Stain Mix (PN 20300011)		3 µL	15 µL	6 µL	30 µL
Protein Stain(s) Select among[a]:					
Anti-Mouse Aux 4	(PN 20300101)	1 µL of each	5 µL of each	2 µL of each	10 µL of each
Anti-Goat Aux 6	(PN 20300103)				
Anti-Rat Aux 7	(PN 20300104)				
Anti-Human Aux 8	(PN 20300105)				
Anti-Chicken Aux 9	(PN 20300106)				

a. **ONLY** add the protein stain(s) against **EACH** primary antibody host species used in the Primary Staining Solution. For example, if mouse, goat, and rat primary antibodies were used in the Primary Staining Solution, add the required volume of anti-mouse, anti-goat, and anti-rat protein stain to this Secondary Staining Solution.

b. Rabbit primary antibodies are currently **NOT** compatible with concurrent cell boundary staining, and therefore Anti-Rabbit Aux 5 is not listed here.

17. Aspirate the 1X PBS to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
18. Add Secondary Staining Solution (**Standard**: **100 µL**; **Large: 200 µL**) onto the center of the tissue section.
19. Use scissors to cut a piece of Parafilm (**Standard: 2×2 cm**; **Large: 2.5×2.5 cm**). Use tweezers to peel off the Parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

If the Secondary Staining Solution is not spread across the tissue section, lift and then lower the Parafilm with tweezers until the Secondary Staining Solution is spread across the tissue section. The Parafilm should fit within the MERSCOPE Slide V 2.0, otherwise the Secondary Staining Solution may wick away into the petri dish.

20. Incubate at **room temperature** for **1 h**.
21. Use tweezers to remove the Parafilm.
22. Wash **3x** with 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**), incubate **5 min** on a rocker each wash.
23. Prepare Fixation Buffer:

Fixation Buffer* (**Standard**: **5 mL; Large: 10 mL**)				
	Standard		**Large**	
Sample number	1	5	1	5
10X PBS	1 mL	3 mL	2 mL	6 mL
32% paraformaldehyde (formaldehyde) solution	1.25 mL	3.75 mL	2.5 mL	7.5 mL

Nuclease-free water	7.75 mL	23.25 mL	15.5 mL	46.5 mL
*Make fresh every time used				

24. Aspirate the 1X PBS. In a fume hood, add Fixation Buffer (**Standard: 5mL; Large: 10 mL**) to fix the stained tissue section at **room temperature** for **15 min**.
25. Wash **2x** with 1X PBS (**Standard**: **5 mL**; **Large: 10 mL**), incubate **5 min** each wash.

*Thaw Pre-Anchoring Activator for Step III at **room temperature** for **30 min** before use.*

26. Continue to Anchoring Pretreatment).

9 APPENDIX II: DOUBLE GEL EMBEDDING FOR RESCUING GEL DEFORMATION

9.1 Overview

Gel deformations can occur before or after tissue clearing in the form of bubbles, ripples, or blebbing over regions of the tissue and can be reversed using a second gel embedding step immediately prior to loading the sample on the MERSCOPE Platform. These deformations are typically visible by eye, appear out of focus over the tissue regions in the DAPI/Poly-T image, and are missing transcripts in areas where gel deformation is observed.

Generally, gel deformations are not readily observed on the gel unless samples have spent multiple days in clearing

The deformation can be tissue specific, with fatty or collagen-rich tissue more likely to exhibit this phenotype after tissue clearing. These deformations can be suppressed by forming another gel under light pressure over the top of the old, deformed gel.

We strongly recommend visually inspecting the gel before loading to MERSCOPE flow cell and performing double gel embedding step if gel deformation is observed prior to MERSCOPE imaging (Figure 11).

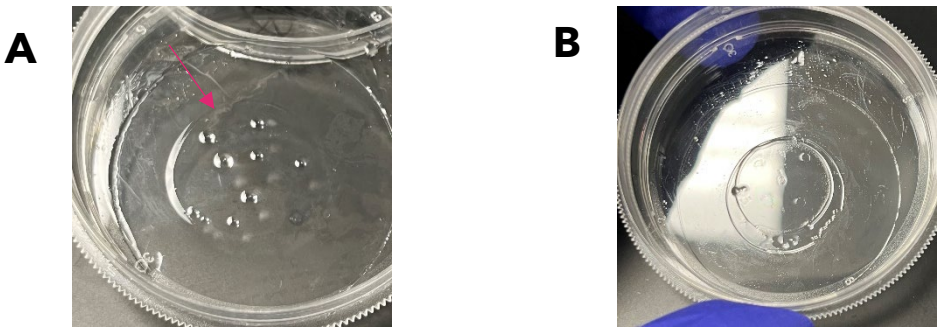

Figure 11. Gel embedding step visualization. **A)** Gel deformation after clearing (bubbles) **B)** After double gel embedding

9.2 Double Gel Embedding Protocol

The following steps outline the double gel embedding workflow. This workflow should only be applied to mitigate gel deformation occurring during or after clearing and visualized as bubbles or blebbing over the tissue.

Gel embedding reagents contain hazardous materials.

At any point after gel embedding, particularly before imaging, if gel deformations such as bubbles or blebbing are observed:

1. Clean a Gel Coverslip (**Standard: 22-mm round Coverslips, Large: 25×25 mm Coverslips**) by spraying with RNaseZap solution and wiping with a Kimwipe, followed by spraying 70% ethanol and wiping with a Kimwipe.

2. Add Gel Slick Solution (**Standard**: **100 µL**; **Large: 200 µL**) onto the Gel Coverslip. Allow the Gel Slick Solution to evaporate for 10 min at room temperature. Wipe gently with a Kimwipe to remove any remaining film, liquid, or deposition from the glass. Use immediately after preparation.
   NOTE: If Gel Slick cannot be obtained, Vizgen recommends **Sigmacote** from Sigma-Aldrich. If using Sigmacote, replace step 2 with the manufacture's recommended protocol.
3. Prepare ammonium persulfate solution:

10% w/v Ammonium Persulfate Solution – (Standard: 25 µL; Large: 50 µL)* Prepare fresh every time				
	Standard		**Large**	
Sample number	1	5	1	5
Ammonium persulfate‡	100 mg	100 mg	100 mg	100 mg
Nuclease-free water*	1 mL	1 mL	1 mL	1 mL

*Adjust volume of nuclease-free water to mass of ammonium persulfate weighed out in each case to obtain 10% w/v solution.

‡Discard any ammonium persulfate that is more than 6 months old.

4. Prepare Gel Embedding Solution and mix thoroughly:

	Standard		**Large**	
Sample number	1	5	1	5
Gel Embedding Premix (PN 20300004)	5 mL	25 mL	10 mL	50 mL
10% w/v ammonium persulfate solution	25 µL	125 µL	50 µL	250 µL
N,N,N′,N'-tetramethylethylenediamine	2.5 µL	12.5 µL	5 µL	25 µL

5. Aspirate the Clearing Solution/Clearing Premix/Formamide Buffer (from sample preparation) and ensure all Clearing Solution/Clearing Premix/Formamide Buffer is aspirated from the petri dish.
6. Wash **2x** with Sample Prep Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**).
7. Aspirate the Sample Prep Wash Buffer.
8. Transfer **1 mL** Gel Embedding Solution in am Eppendorf tube.
9. Wash the sample with the remainder of the Gel Embedding, ensuring the sample is fully covered, and incubate at **room temperature** for **1 min**.
10. Using a pipette, transfer the majority of the Gel Embedding Solution wash to a waste tube (to monitor the gel formation).
11. Aspirate to dry the MERSCOPE Slide V 2.0, leaving just enough liquid to cover the tissue section.
12. Add (**Standard**: **100 µL**; **Large: 200 µL**) of the 1 mL of retained Gel Embedding Solution on the tissue section **directly on top of the existing gel.**
13. Place the tips of one pair of tweezers on an area of the MERSCOPE Slide V 2.0 without touching the tissue section. Use tweezers to pick up the Gel Slick-treated Gel Coverslip (**Standard**: **22 mm round**; **Large: 25×25 mm square**). With the Gel Slick-treated side

facing down toward the gel, place the edge of the Gel Coverslip against the tweezer tips resting on the gel, creating stability, and slowly lower the Gel Coverslip onto the gel to spread the Gel Embedding Solution. If needed, adjust the Gel Coverslip so it is positioned in the center of the MERSCOPE Slide V 2.0. Gently press the Gel Coverslip to squeeze out excess Gel Embedding Solution and remove the extra Gel Embedding Solution by aspiration (Figure 12).

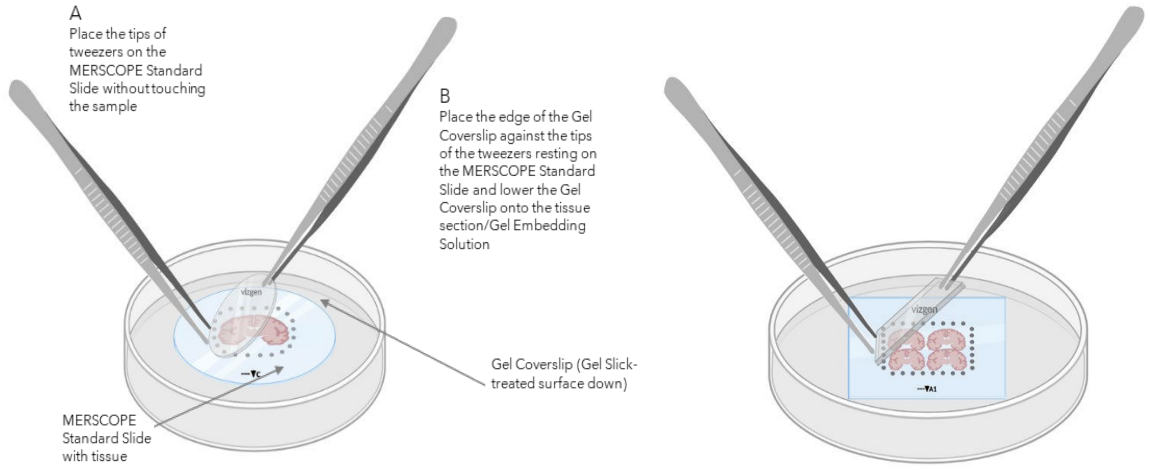

Figure 12. Step to place Gel-coverslip onto tissue section on Standard and Large slides.

Air bubbles in the gel solution inhibit gel polymerization. If a bubble forms, lift, and then lower the Gel Coverslip to help the air bubbles escape.

Squeeze the Gel Coverslip gently to remove the Gel Embedding Solution that seeps out and leaves only a thin layer of gel between the Gel Coverslip and the MERSCOPE Slide V 2.0. Avoid squeezing the Gel Coverslip too hard as it may damage the sample and result in gel that is too thin/no gel.

14. Once the Gel Coverslip is placed on the polymerizing gel mix and the excess is aspirated, fill a 15mL conical tube **cap** (1.2 g) with 500 µL RNAse-free water.
15. Carefully place the cap **directly on top of the gel coverslip.**
16. Place the top of the petri dish back over the sample and incubate for **1.5 h** at **room temperature**. Monitor the Gel Embedding process in the waste tube. Gel starts to form within 1 h.
17. Ensure **eye protection** is worn during this step. Gently brace the Gel Coverslip with tweezers in one hand and lift the Gel Slick-treated Gel Coverslip with the sharp tip of a Hobby Blade and discard the Gel Coverslip appropriately.
18. Remove coverslip and wash **1x** with Sample Prep Wash Buffer (**Standard**: **5 mL**; **Large: 10 mL**).
19. Proceed to the next step in the sample prep. All regions under the newly polymerized gel should be eligible for imaging.

10 APPENDIX III: CLEARING FOR RESISTANT TISSUES AND CLEARING OPTIMIZATION

Tissue clearing is a critical step for achieving high quality, high resolution MERFISH data. Proteinase K facilitates tissue clearing, with higher concentrations clearing more effectively. The exact conditions for clearing may need to be optimized for each tissue type. For non-resistant tissues, the default clearing conditions are a 1:100 dilution of Proteinase K incubated at 47°C overnight.

To optimize clearing, the Proteinase K concentration, incubation temperature, and incubation time may be adjusted. The total clearing time can be accelerated by increasing the effective concentration of Proteinase K. We recommend testing a range of dilution factors from 1:50, 1:20 or 1:4 in a smaller volume of Clearing Premix, as long as the total volume is >250 µL.

More detailed information can be found in *Optimizing Tissue Digestion and Clearing for the MERSCOPE Platform* (PN 91700121)

An example for an **accelerated clearing protocol** is provided on the next page.

This protocol can be used in conjunction with digestion and/or standard overnight clearing conditions:

1. Warm Clearing Premix (PN 20300114) at **37°C** for **30 min** before use. The Clearing Premix should be a clear solution before use. If the solution is cloudy or not homogeneous, warm and mix until the solution becomes clear. Prepare Clearing Solution:

	Standard		Large	
Sample number	1	5	1	5
Clearing Premix (PN 20300114)	200 µL	1 mL	400 µL	2 mL
Proteinase K	50 µL	250 µL	100 µL	500 µL

2. Add Clearing Solution onto the gel (**Standard: 250 µL; Large: 500 µL**).
3. Cover the sample with a square of Parafilm (**Standard: 2x2 cm; Large 2.5x2.5 cm**)
4. Place the lid on the petri dish. Seal the petri dish tightly with Parafilm. Spray the outside with 70% ethanol to sterilize.
5. Place the petri dish in a humidified 47°C cell culture incubator for **1-4 hours**. Check samples every hour to assess clearing progression.
6. Add 5 mL of Clearing Solution and proceed to Autofluorescence Quenching (if necessary) or Encoding Probe Hybridization.
7. **IF** the tissue is not transparent, transfer the petri dish to a humidified 37°C cell culture incubator and incubate until the tissue is cleared or until the tissue section becomes transparent

Some tissues may not be completely transparent after 3 days. We recommend optimizing clearing conditions using Sample Verification Kits prior to your MERFISH experiment. If previous Verification results were positive after 3 days of clearing, we recommend proceeding after 3 days in clearing even if the tissue is not completely transparent, as gel deformations are more likely to occur over time.

*SAMPLES CAN BE STORED OR CLEARED IN CLEARING SOLUTION AT **37°C** FOR UP TO **4 DAYS TOTAL.***