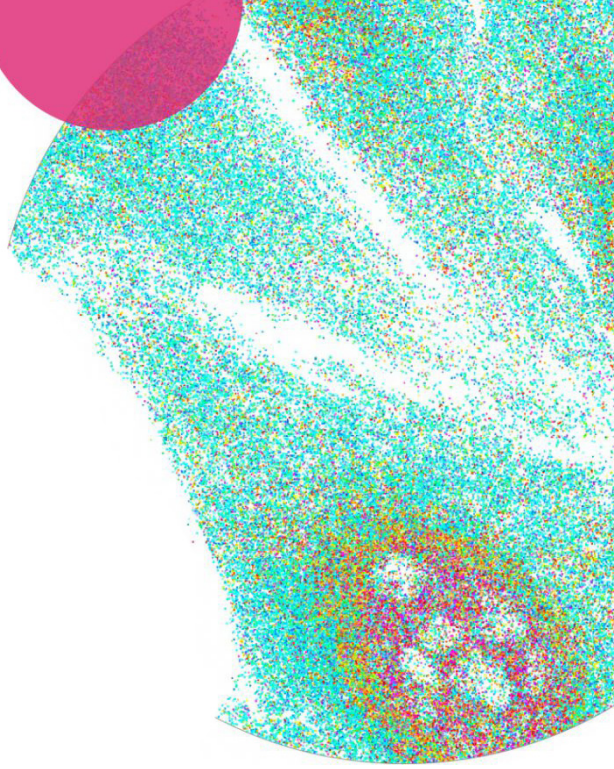
91600003 Rev F



MERSCOPE® User Guide

Cultured Cells Sample Preparation

Vizgen Materials

10500115	MERSCOPE Slide Box, 10 slides – Suspended cells MERSCOPE
10500001	MERSCOPE Slide Box, 20 slides – Suspended cells MERSCOPE
10500002	Slide (Non-beaded) Box, 20 slides – Adherent cells MERSCOPE
10400012	Sample Prep Kit, 20 samples
10400118	MERSCOPE Cell Boundary Stain Kit, 20 samples – Optional
10400009	MERSCOPE Cell Boundary Staining Kit, 20 samples – Optional
10400106 - 10400111	MERSCOPE Protein Stain Kits, 20 samples – Optional *To detect user-provided primary antibodies raised in mouse, rabbit, goat, rat, human, and chicken*
10400001	MERSCOPE 140 Gene Panel, 20 samples
10400002	MERSCOPE 300 Gene Panel, 20 samples
10400003	MERSCOPE 500 Gene Panel, 20 samples
10400125	MERSCOPE 1000 Gene Panel, 20 samples

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NOTICES

Document number	91600003
Document revision	Rev F
Revision date	June 2023

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VERSION CHANGE NOTES

Summary of substantive changes from Rev E to Rev F

Brief description of change	Page(s)
Compatibility note on potential nonspecific binding of MERSCOPE Protein Stains to same-species samples	15
Blue box reminder not to degas Gel Embedding Premix	38
Correction of Gel Coverslip part number	17, 38

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INTRODUCTION

MERFISH Technology

Multiplexed error-robust fluorescence in situ hybridization (MERFISH) is a spatially resolved single-cell transcriptome profiling technology. MERFISH combines the power of single-cell transcriptomics with spatial biology by directly visualizing and counting RNA transcripts from 100s to >10,000 genes in cells or tissue sections.

MERFISH advances the power of single-molecule fluorescence in situ hybridization (smFISH) – the gold standard of RNA quantification – with error robust barcoding, combinatorial labeling, and sequential imaging to greatly expand the multiplexing capacity. MERFISH enables researchers to map the molecular, cellular, and functional composition of biological systems with spatial context.

The Vizgen MERSCOPE Platform Solution

The Vizgen MERSCOPE Platform Solution is comprised of the MERSCOPE Gene Panel Design Software, MERSCOPE reagent kits, the MERSCOPE Instrument, the MERSCOPE Analysis Computer, and the MERSCOPE Vizualizer software to streamline the acquisition of high quality MERFISH data and data interpretation.

Reagent kits facilitate sample preparation. The automated MERSCOPE Instrument integrates high-resolution imaging and fluidics to automatically acquire a full MERFISH dataset. The MERSCOPE Vizualizer software automates image processing and offers interactive visualization tools to explore MERFISH data.

The MERSCOPE Workflow

Begin by either designing a MERSCOPE Custom Gene Panel or ordering a MERSCOPE Predesigned Gene Panel.

Design a custom gene panel. The intuitive MERSCOPE Gene Panel Design Software makes recommendations on your custom gene panel for optimal performance in a MERFISH measurement. For example, the software highlights genes that are too short or whose abundance may be too high and could cause optical crowding artifacts. Vizgen is currently offering custom gene panels for up to 140 genes, up to 300 genes, up to 500 genes, and up to 1000.

Order your MERSCOPE Gene Panel. MERSCOPE Custom Gene Panels and MERSCOPE Predesigned Gene Panels are delivered in a ready-to-use format. Once the gene panel is finalized, users will be able to download their panel-specific MERSCOPE Codebook to the MERSCOPE Instrument.

Prepare your sample. Vizgen's sample preparation user guides step you through sample preparation on MERSCOPE Slides.

Load and run the MERSCOPE Instrument. The MERSCOPE Slide is assembled into the MERSCOPE Flow Chamber and then loaded into the instrument along with a MERSCOPE Imaging Cartridge. Users define regions of interest on the MERSCOPE Slide and initiate the fully automated instrument run.

Data Processing and Visualization. The MERSCOPE Instrument Software (in combination with the MERSCOPE Analysis Computer) automatically processes the raw images to output spatial genomics measurements in a format ready for immediate downstream analysis. The output includes the list of all detected transcripts and their spatial locations in three dimensions (CSV files), mosaic images (TIFF), experiment metadata (JSON), output from the cell segmentation analysis: transcripts per cell matrix (CSV), cell metadata (CSV), cell boundaries (HDF5 [MERSCOPE Instrument Software v231 or earlier] or PARQUET [v232 or later]), and a binary for use with the MERSCOPE Vizualizer software. The MERSCOPE Platform Solution includes the MERSCOPE Vizualizer software for visualizing and analyzing data. The output files are also compatible with open-source tools for single-cell and spatial analysis.

Broad Application

The MERSCOPE Platform Solution has broad application in both fundamental biology and medicine – from basic science, to drug discovery, to clinical pathology. Find out more at https://vizgen.com/applications/.


Oncology


Immunology

Neuroscience


Infectious Disease


Developmental Biology & Regenerative Medicine

SAMPLE PREPARATION OVERVIEW

This user guide **is applicable to cultured cells**. Only the first step is different for adherent cells and suspended cells. It is not applicable to tissue sections, including fresh frozen, fixed frozen, or formalin-fixed paraffin-embedded (FFPE) tissue. Refer to the applicable user guide for other sample types. Vizgen supports mouse and human cell samples only.

I. MERSCOPE Slide Preparation and Fixation

Adherent cells are adhered to a MERSCOPE Slide (Non-beaded) by overnight incubation in a cell culture incubator and then fixed with fixation buffer. For suspended cells, a MERSCOPE Slide is used, and suspended cells are spun down on the slide surface in a perfusion chamber.

II. Permeabilization

The cells now on the slide are made permeable to the hybridization probes by permeabilization buffer. Users may utilize their own fixation and permeabilization protocols in the first two steps and 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.

III. Cell Boundary Staining and/or Protein Staining (Optional)

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.

Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS for **IMPORTANT** information. 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*).

Cell boundary staining conveniently marks cell boundaries to enable individual cell analysis. If cells are too dense or prefer to grow and clump together, cell boundary staining may be beneficial. However, if cells are adequately dispersed in a sample, it is generally not necessary to do this step.

IV. Encoding Probe Hybridization and V. Post Encoding Probe Hybridization Wash

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

VI. Gel Embedding

Gel embedding immobilizes the RNA (with bound encoding probes) and creates a protective layer so the RNA species cannot escape in subsequent steps. For adherent cells, fluorescent fiducials are incorporated at this step.

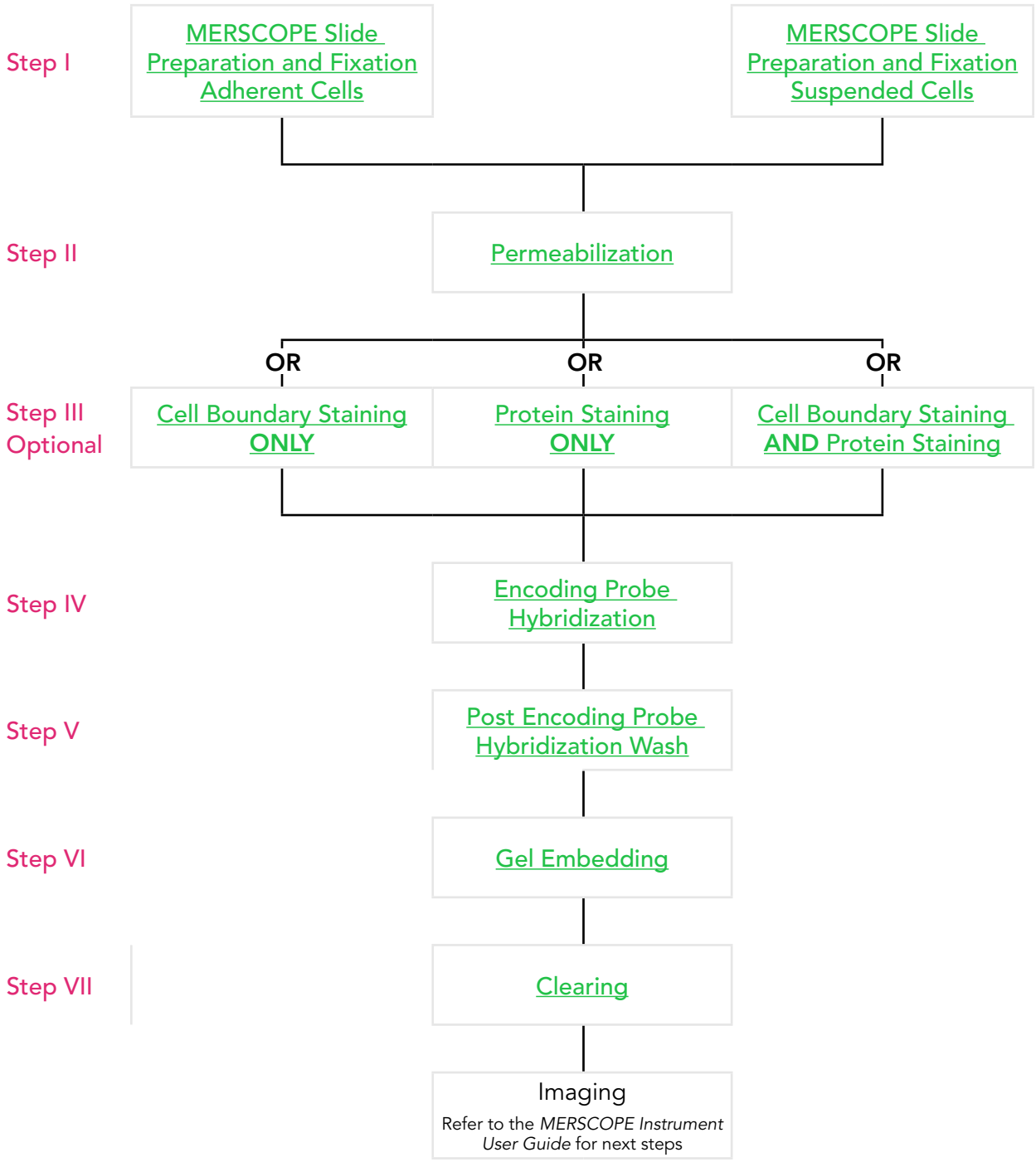
VII. Clearing

Clearing removes everything other than RNA and DNA and is critical to minimize the fluorescent background and thereby maximize signal. Users should use MERSCOPE Sample Verification Kits to optimize sample clearing protocols, especially for clearing resistant sample.

Next Steps

Refer to the *MERSCOPE Instrument User Guide* for next steps. In short, any final bench steps are performed and then MERSCOPE Slide with prepared sample is assembled into the MERSCOPE Flow Chamber and inserted in the instrument. The MERSCOPE Imaging Cartridge is loaded into the instrument and the fully automated instrument run is initiated.

SAMPLE PREPARATION WORKFLOW OPTIONS



TIMELINE

Day	Step	Bench time	Incubation time	Stopping points / storage
Day 0	I. MERSCOPE Slide Preparation – Adherent Cells	0.5 h	Overnight	-
Day 1	I. MERSCOPE Slide Preparation and Fixation	0.5 h	Up to 1 h	Fixed cells in 70% ethanol at 4°C up to a month
	II. Permeabilization	0.5 h	-	-
	III. Cell Boundary Staining and/or Protein Staining	1 h	3 x 1 h	-
	IV. Encoding Probe Hybridization	0.5 h	18 - 48 h	-
Day 2	V. Post Encoding Probe Hybridization Wash	0.25 h	2 x 0.5 h	-
	VI. Gel Embedding	0.5 h	1.5 h	
	VII. Clearing	0.25 h	24 h+*	Clearing Solution at 37°C Up to 7 days
Refer to the *MERSCOPE Instrument User Guide* for next steps				

A total of 3 days for sample preparation is based on the minimum times for Encoding Probe Hybridization and Clearing. If extra time needed for these steps, total days increase accordingly. The total of 3 days does not include overnight incubation of adherent cells and the MERSCOPE Slide (Non-beaded).

*Clearing incubation time depends on the resistance to clearing.

TECHNICAL TIPS

Experimental Planning

The MERSCOPE Instrument analyzes one sample at a time and imaging takes 0.5 – 1.5 days.

Sample preparation can be performed in batches and samples can be stored in Clearing Solution at 37°C for up to 7 days.

Minimize freeze-thaw cycles for reagents/kits stored at −20°C.

Refer to the TIMELINE for stopping points and sample storage conditions.

It is recommended to label samples on the bottom of the petri dish for future identification.

When approaching imaging, ensure the MERSCOPE Imaging Cartridge is thawed and the appropriate MERSCOPE Codebooks are available. Refer to the *MERSCOPE Instrument User Guide* for more information.

Cell Adhesion

Some cell types may not adhere strongly to the MERSCOPE Slide (Non-beaded). To facilitate adhesion, it is generally recommended to coat the MERSCOPE Slide (Non-beaded) with substrate before seeding the cells. Commonly used coating substrates include poly-lysine, collagen, fibronectin, laminin, or poly-arginine. Users may utilize their own coating substrate to facilitate cell adhesion.

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.

MERSCOPE Slide Handling

MERSCOPE Slides are fragile, 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.

Maintaining Humidity During Long Incubations

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

If a humidified incubator is not available, fill a small petri dish with nuclease-free water and place it together with the sample (covered in its 60-mm petri dish) within a 150-mm petri dish. Ensure the small petri dish has sufficient nuclease-free water daily.

Safety and Hazardous Steps

Safe laboratory practices should be followed at all times.

Formamide Wash Buffer is hazardous. Fixation buffers may contain hazardous materials. Perform steps using these materials 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

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, 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.

Step III Options

Identify the appropriate protocol for step III:

- Cell boundary staining **ONLY**
- Protein staining **ONLY**
- Cell boundary staining **AND** protein staining
- No staining (skip the step)

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.

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

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 cells of the same species (e.g., Anti-Mouse Aux 4 Protein Stain may show higher levels of nonspecific binding to mouse cells).
- Contact Vizgen Support (support@vizgen.com) for more information, if needed.

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.

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.

MATERIALS

Vizgen Materials

MERSCOPE Slide Box, 10 slides	10500115	Storage
MERSCOPE Slide, 10 x 1 slides	20400001	–20°C, horizontally

MERSCOPE Slide Box, 20 slides	10500001	Storage
MERSCOPE Slide, 20 x 1 slides	20400001	–20°C, horizontally

MERSCOPE Slide (Non-beaded) Box, 20 samples	10500002	Storage
MERSCOPE Slide (Non-beaded), 20 x 1 sample	20400002	–20°C, horizontally
Fluorescent Fiducial Premix	20300025	4°C, protected from light

MERSCOPE Sample Prep Kit, 20 samples	10400012	Storage
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	20300003	4°C
Gel Coverslip	30200004	Room temperature
Digestion Premix, 5 x 4 samples*	20300005	–20°C upon receipt

*This reagent is not used in cultured cell sample preparation.

Sample Prep Wash Buffer and Formamide Wash Buffer are used in the imaging and verification workflows.

MERSCOPE 140 Gene Panel, 20 samples	10400001	Storage
MERSCOPE 140 Gene Panel Mix, 5 x 4 samples	20300006	–20°C

MERSCOPE 300 Gene Panel, 20 samples	10400002	Storage
MERSCOPE 300 Gene Panel Mix, 5 x 4 samples	20300007	–20°C

MERSCOPE 500 Gene Panel, 20 samples	10400003	Storage
MERSCOPE 500 Gene Panel Mix, 5 x 4 samples	20300008	–20°C

MERSCOPE 1000 Gene Panel, 20 samples	10400125	Storage
MERSCOPE 1000 Gene Panel Mix, 5 x 4 samples	20300141	–20°C

MERSCOPE Cell Boundary Stain Kit, 20 samples	10400118	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	−20°C
Cell Boundary Primary Stain Mix	20300010	−20°C
Cell Boundary Secondary Stain Mix	20300011	−20°C

*Minimize freeze-thaw cycles

MERSCOPE Anti-Mouse Protein Stain Kit, 20 samples	10400106	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Mouse Aux 4 Protein Stain	20300101	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Anti-Rabbit Protein Stain Kit, 20 samples	10400107	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Rabbit Aux 5 Protein Stain[a]	20300102	–20°C
*Minimize freeze-thaw cycles		
[a]Not compatible with the MERSCOPE Cell Boundary Stain Kit		

MERSCOPE Anti-Goat Protein Stain Kit, 20 samples	10400108	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Goat Aux 6 Protein Stain	20300103	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Anti-Rat Protein Stain Kit, 20 samples	10400109	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Rat Aux 7 Protein Stain	20300104	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Anti-Human Protein Stain Kit, 20 samples	10400110	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Human Aux 8 Protein Stain	20300105	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Anti-Chicken Protein Stain Kit, 20 samples	10400111	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Chicken Aux 9 Protein Stain	20300106	–20°C
*Minimize freeze-thaw cycles		

Safety Data Sheets are available online at https://vizgen.com/

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
Triton X-100, Molecular Biology Grade	VWR	PAH5141
Solutions, consumables & small laboratory equipment		
Grace Bio-Labs CoverWell perfusion chamber *For suspended cells*	Millipore-Sigma	GBL622501-40EA
RNaseZap RNase Decontamination Solution	Thermo Fisher	AM9782
Petri Dish, 60 x 15 mm, Sterile	VWR	25382-687
Petri Dish, 150 x 15 mm	VWR	76081-554
Gel Slick Solution	VWR	12001-812
Parafilm M	VWR	102091-164
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.

General Laboratory Equipment

General laboratory equipment should be used per manufacturer's instructions.

- Fume hood
- 37°C cell culture incubator (humidified)[a,b]
- 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] (e.g., VWR 10055-006)
- Vacuum trap system (e.g., VWR 76207-602)
- Vacuum pumps (e.g., Thomas Scientific 1162B24)
- Benchtop centrifuge
- Centrifuge for harvesting suspended cells and compatible with a centrifuge plate adapter
- 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)
- Cryotome

a. Multiple temperatures are needed throughout the protocol. Therefore, it is recommended to have equipment dedicated to each temperature.

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, fill a small petri dish with nuclease-free water and place it together with the sample (covered in its 60-mm petri dish) within a 150-mm petri dish. Ensure the small petri dish has sufficient nuclease-free water daily.

c. If using an alternate make/model, it should be large enough to accommodate the MERSCOPE Imaging Cartridge: 8 × 11 in (20 × 28 cm).

Additional Buffer Recipes

These buffers are not provided in Vizgen kits.

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

Used in Step I and Step III (when included) – 5 mL per sample needed each time			
Fixation Buffer*	**1 sample**	**5 samples**	**10 samples**
10X PBS	4 mL	4 mL	8 mL
32% paraformaldehyde (formaldehyde) solution	5 mL	5 mL	10 mL
Nuclease-free water	31 mL	31 mL	62 mL

*Make fresh every time used

Used in Permeabilization Buffer (below) for Step II – 100 µL per sample needed			
25% Triton X-100*	**1 sample**	**5 samples**	**10 samples**
Triton X-100, molecular biology grade	10 mL	10 mL	10 mL
Nuclease-free water	30 mL	30 mL	30 mL

*Can be stored at room temperature for up to a year

Used in Step II – 5 mL per sample needed			
Permeabilization Buffer	**1 sample**	**5 samples**	**10 samples**
10X PBS	0.5 mL	2.5 mL	5 mL
25% Triton X-100	0.1 mL	0.5 mL	1 mL
Nuclease-free water	4.4 mL	22 mL	44 mL

Used in Step VI – 25 µL per sample needed	Prepare fresh aliquots every time*		
10% w/v Ammonium Persulfate Solution	**1 sample**	**5 samples**	**10 samples**
Ammonium persulfate‡	100 mg	100 mg	100 mg
Nuclease-free water	1 mL	1 mL	1 mL

*Only 25 µL per sample is needed. 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.

SAMPLE PREPARATION PROTOCOL

The protocol considers each MERSCOPE Slide a sample.

Each sample is prepared in a separate petri dish.

Incubations are stationary and at room temperature unless stated otherwise. 5-mL buffer and reagent volumes are sufficient to cover a MERSCOPE Slide in a 60-mm petri dish.

CHOOSE STEP I BASED ON CELL TYPE – ADHERENT OR SUSPENDED

For adherent cells, use a MERSCOPE Slide (Non-beaded).

For suspended cells, use a MERSCOPE Slide.

I. MERSCOPE Slide Preparation and Fixation – Adherent Cells

MERSCOPE Slides (Non-beaded) (PN 20400002) are stored at −20°C. Warm up at room temperature for 15 min before use. Unused MERSCOPE Slides (Non-beaded) should be stored at −20°C. ***DO NOT*** *use a MERSCOPE Slide (PN 20400001) for adherent cells.*

Refer to Additional Buffer Recipes for fixation buffer.

1. Place a MERSCOPE Slide (Non-beaded) into a 60-mm petri dish. Place in a cell culture hood under UV to sterilize for 1 h.
2. Seed cells at 40% confluency (~200,000 cells in 5 mL media). Allow cells to adhere by incubating at 37°C overnight in a cell culture incubator and grow to ~70% confluency.
3. Aspirate the culture media. Add **5 mL** fixation buffer.
4. Fix the cells at room temperature for 15 min.
5. Wash **2x** with **5 mL** 1X PBS, incubate 2 min each wash.

Fixed cells can be stored in 70% ethanol at 4°C up to a month.

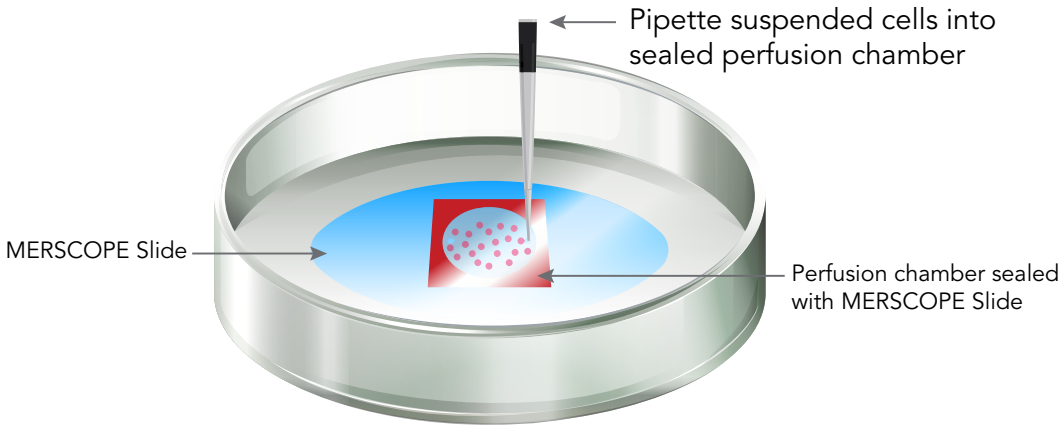
To prepare samples for storage, aspirate the 1X PBS after the second wash and add 5 mL 70% ethanol.

I. MERSCOPE Slide Preparation and Fixation – Suspended Cells

1. Harvest the suspended cells by centrifugation (e.g., 250 x g for 5 min or based on the optimal force for a specific cell type). Discard the culture media and resuspend the cells in 1X PBS to make a 2 million cells/mL suspension.

> *MERSCOPE Slides (PN 20400001) are stored at −20°C. Warm up at room temperature for 15 min before use. Unused MERSCOPE Slides should be stored at −20°C.* **DO NOT** *use a MERSCOPE Slide (Non-beaded) for suspended cells.*
>
> *Refer to Additional Buffer Recipes for fixation buffer.*

2. Peel the backing off a perfusion chamber and place the perfusion chamber at the center of the MERSCOPE Slide. Press the perfusion chamber to create a seal with the MERSCOPE Slide.
3. Mix the cell suspension and fixation buffer in 1:1 v/v ratio to make a 1 million cells/mL suspension. Pipette **200 µL** cell suspension into the perfusion chamber.



4. Place the petri dish in a centrifuge plate adapter, secure the petri dish with tape, and centrifuge to spin the cells down (e.g., 250 x g for 5 min or based on the optimal force for a specific cell type).
5. Carefully peel the perfusion chamber off the MERSCOPE Slide. Aspirate the solution. Add **5 mL** fixation buffer.
6. Fix the cells at room temperature for an additional 15 min.
7. Wash **2x** with **5 mL** 1X PBS, incubate 2 min each wash.

> *Fixed cells can be stored in 70% ethanol at 4°C up to a month.*
>
> *To prepare samples for storage, aspirate the 1X PBS after the second wash and add 5 mL 70% ethanol.*

II. Permeabilization

1. **IF** continuing directly from fixation, aspirate the 1X PBS and go to step 3.
2. **IF** continuing from storage in 70% ethanol, aspirate the ethanol and wash **2x** with **5 mL** 1X PBS, incubate 2 min each wash. Aspirate the 1X PBS and go to step 3.
3. Add **5 mL** permeabilization buffer (refer to Additional Buffer Recipes).
4. Incubate at room temperature for 10 min.
5. Wash **2x** with **5 mL** 1X PBS, incubate 2 min each wash.
6. Proceed immediately to the next step.

Choose *step III based on the extent of additional staining. Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS for* ***IMPORTANT*** *information, including antibody requirements and compatibility.*

If the sample does not require cell boundary staining and/or protein staining, proceed directly to step IV. Encoding Probe Hybridization.

III. Cell Boundary Staining ONLY

When preparing blocking solutions and primary/secondary staining solutions, use the included Block Buffer C Premix (PN 20300100) that is included with Cell Boundary Stain Kit (PN 10400118).

Maintain Cell Boundary Primary Stain Mix, Cell Boundary Secondary Stain Mix, 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.

Refer to Additional Buffer Recipes for fixation buffer.

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

1. Aspirate the 70% ethanol. Add **5 mL** 1X PBS.
2. Prepare Blocking Solution:

Blocking Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL

3. Aspirate the 1X PBS to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
4. Add **100 µL** Blocking Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 Blocking Solution is not spread across the cells, lift and then lower the parafilm with tweezers until the Blocking Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Blocking Solution may wick away into the petri dish.

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

Primary Staining Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL
Cell Boundary Primary Stain Mix (PN 20300010)	1 µL	5 µL	10 µL

7. Use tweezers to remove the parafilm.
8. Aspirate the solution to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
9. Add **100 µL** Primary Staining Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 cells, lift and then lower the parafilm with tweezers until the Primary Staining Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Primary Staining Solution may wick away into the petri dish.*

10. Incubate at room temperature for 1 h.
11. Use tweezers to remove the parafilm.
12. Wash **3x** with **5 mL** 1X PBS, incubate 5 min on a rocker each wash.
13. Prepare Secondary Staining Solution:

Secondary Staining Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL
Cell Boundary Secondary Stain Mix (PN 20300011)	3 µL	15 µL	30 µL

14. Aspirate the 1X PBS to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.

15. Add **100 µL** Secondary Staining Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 cells, lift and then lower the parafilm with tweezers until the Secondary Staining Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Secondary Staining Solution may wick away into the petri dish.

16. Incubate at room temperature for 1 h.
17. Use tweezers to remove the parafilm.
18. Wash **3x** with **5 mL** 1X PBS, incubate 5 min on a rocker each wash.
19. Aspirate the 1X PBS. In a fume hood, add **5 mL** fixation buffer to fix the stained cells at room temperature for 15 min.
20. Wash **2x** with **5 mL** 1X PBS, incubate 5 min each wash.
21. Proceed immediately to the next step (IV. Encoding Probe Hybridization).

Choose *step III based on the extent of additional staining. Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS for* ***IMPORTANT*** *information, including antibody requirements and compatibility.*

If the sample does not require cell boundary staining and/or protein staining, proceed directly to step IV. Encoding Probe Hybridization.

III. 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.

Refer to Additional Buffer Recipes for fixation buffer.

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

1. Aspirate the 70% ethanol. Add **5 mL** 1X PBS.
2. Prepare Blocking Solution:

Blocking Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL

3. Aspirate the 1X PBS to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
4. Add **100 µL** Blocking Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 Blocking Solution is not spread across the cells, lift and then lower the parafilm with tweezers until the Blocking Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Blocking Solution may wick away into the petri dish.

5. Incubate at room temperature for 1 h.

6. Prepare Primary Staining Solution:

Primary Staining Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 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	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 CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS and the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.

7. Use tweezers to remove the parafilm.
8. Aspirate the solution to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
9. Add **100 µL** Primary Staining Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 cells, lift and then lower the parafilm with tweezers until the Primary Staining Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Primary Staining Solution may wick away into the petri dish.

10. Incubate at room temperature for 1 h.
11. Use tweezers to remove the parafilm.
12. Wash **3x** with **5 mL** 1X PBS, incubate 5 min on a rocker each wash.

13. Prepare Secondary Staining Solution:

Secondary Staining Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL
Protein Stain(s) Select among[a]: Anti-Mouse Aux 4 (PN 20300101) Anti-Rabbit Aux 5 (PN 20300102) Anti-Goat Aux 6 (PN 20300103) Anti-Rat Aux 7 (PN 20300104) Anti-Human Aux 8 (PN 20300105) Anti-Chicken Aux 9 (PN 20300106)	1 µL of each	5 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.

14. Aspirate the 1X PBS to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
15. Add **100 µL** Secondary Staining Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 cells, lift and then lower the parafilm with tweezers until the Secondary Staining Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Secondary Staining Solution may wick away into the petri dish.

16. Incubate at room temperature for 1 h.
17. Use tweezers to remove the parafilm.
18. Wash **3x** with **5 mL** 1X PBS, incubate 5 min on a rocker each wash.
19. Aspirate the 1X PBS. In a fume hood, add **5 mL** fixation buffer to fix the stained cells at room temperature for 15 min.
20. Wash **2x** with **5 mL** 1X PBS, incubate 5 min each wash.
21. Proceed immediately to the next step (IV. Encoding Probe Hybridization).

Choose *step III based on the extent of additional staining. Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS for* ***IMPORTANT*** *information, including antibody requirements and compatibility.*

If the sample does not require cell boundary staining and/or protein staining, proceed directly to step IV. Encoding Probe Hybridization.

III. 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.*

For cell boundary staining ***AND*** *protein staining, use Blocking Buffer C Premix (PN 20300100) included in MERSCOPE Protein Stain Kits.*

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.

Refer to Additional Buffer Recipes for fixation buffer.

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

1. Aspirate the 70% ethanol. Add **5 mL** 1X PBS.
2. Prepare Blocking Solution:

Blocking Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL

3. Aspirate the 1X PBS to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
4. Add **100 µL** Blocking Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 Blocking Solution is not spread across the cells, lift and then lower the parafilm with tweezers until the Blocking Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Blocking Solution may wick away into the petri dish.

5. Incubate at room temperature for 1 h.

6. Prepare Primary Staining Solution:

Primary Staining Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL
Cell Boundary Primary Stain Mix (PN 20300010)	1 µL	5 µL	10 µL
User-provided primary antibody raised in[a-d]: • Mouse • Goat • Rat • Human • Chicken	1 µL of each	5 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 CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS and the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.

7. Use tweezers to remove the parafilm.
8. Aspirate the solution to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
9. Add **100 µL** Primary Staining Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 cells, lift and then lower the parafilm with tweezers until the Primary Staining Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Primary Staining Solution may wick away into the petri dish.

10. Incubate at room temperature for 1 h.
11. Use tweezers to remove the parafilm.
12. Wash **3x** with **5 mL** 1X PBS, incubate 5 min on a rocker each wash.
13. Prepare Secondary Staining Solution:

Secondary Staining Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL
Cell Boundary Secondary Stain Mix (PN 20300011)	3 µL	15 µL	30 µL
Protein Stain(s) Select among[a-b]: Anti-Mouse Aux 4 (PN 20300101) Anti-Goat Aux 6 (PN 20300103) Anti-Rat Aux 7 (PN 20300104) Anti-Human Aux 8 (PN 20300105) Anti-Chicken Aux 9 (PN 20300106)	1 µL of each	5 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. 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.

14. Aspirate the 1X PBS to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
15. Add **100 µL** Secondary Staining Solution onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 cells, lift and then lower the parafilm with tweezers until the Secondary Staining Solution is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the Secondary Staining Solution may wick away into the petri dish.

16. Incubate at room temperature for 1 h.
17. Use tweezers to remove the parafilm.
18. Wash **3x** with **5 mL** 1X PBS, incubate 5 min on a rocker each wash.

19. Aspirate the 1X PBS. In a fume hood, add **5 mL** fixation buffer to fix the stained cells at room temperature for 15 min.
20. Wash **2x** with **5 mL** 1X PBS, incubate 5 min each wash.
21. Proceed immediately to the next step (IV. Encoding Probe Hybridization).

IV. Encoding Probe Hybridization

Maintain the applicable MERSCOPE Gene Panel Mix 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. Aspirate the 1X PBS and wash **1x** with **5 mL** Sample Prep Wash Buffer (PN 20300001).
2. Aspirate Sample Prep Wash Buffer and add **5 mL** Formamide Wash Buffer (PN 20300002), incubate at 37°C for 30 min in an incubator in a fume hood.
3. Aspirate the Formamide Wash Buffer to dry the MERSCOPE Slide without scraping the cells. The cells should not be completely dry for more than 1 min.

Aspirate all the residual solution without disrupting the cells to avoid diluting the MERSCOPE Gene Panel Mix.

4. Add **50 µL** MERSCOPE Gene Panel Mix onto the center of the cells. Use scissors to cut a piece of parafilm 2×2 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 MERSCOPE Gene Panel Mix is not spread across the cells, lift and then lower the parafilm with tweezers until the MERSCOPE Gene Panel Mix is spread across the cells. The parafilm should fit within the MERSCOPE Slide, otherwise the MERSCOPE Gene Panel Mix may wick away into the petri dish.

5. Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.
6. Seal the petri dish with parafilm and place in a humidified 37°C cell culture incubator for at least 18 h and a maximum of 48 h. **DO NOT** let the sample dry out.

If a humidified incubator is not available, fill a small petri dish with nuclease-free water and place it together with the sample (covered in its 60-mm petri dish) within a 150-mm petri dish. Ensure the small petri dish has sufficient nuclease-free water daily.

V. Post Encoding Probe Hybridization Wash

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

1. Remove the parafilm and add **5 mL** Formamide Wash Buffer (PN 20300002).
2. Incubate at 47°C for 30 min in an incubator in a fume hood.
3. Aspirate the Formamide Wash Buffer. Add **5 mL** Formamide Wash Buffer.
4. Incubate at 47°C for 30 min in an incubator in a fume hood.
5. Wash **1x** with **5 mL** Sample Prep Wash Buffer (PN 20300001), incubate 2 min.
6. Proceed immediately to the next step.

VI. Gel Embedding

Gel embedding reagents contain hazardous materials.

***DO NOT** degas Gel Embedding Premix.*

Ensure Fluorescent Fiducial Premix is well mixed using a benchtop vortexer to re-suspend particles before aliquoting.

1. **IF** suspended cells, start at step 2. **IF** adherent cells, prepare Fluorescent Fiducial Solution. Aspirate the Sample Prep Wash Buffer. Add **5 mL** Fluorescent Fiducial Solution to the petri dish, and allow the fiducial beads to settle onto the cells for 10 min.

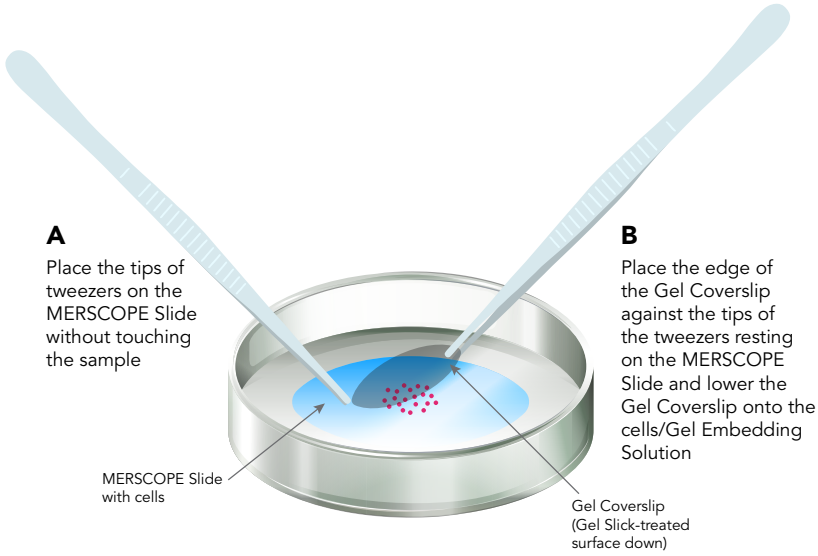
Fluorescent Fiducial Solution	1 sample	5 samples	10 samples
Fluorescent Fiducial Premix (PN 20300025)	1 µL	5 µL	10 µL
Sample Prep Wash Buffer (PN 20300001)	5 mL	25 mL	50 mL

2. Clean a Gel Coverslip (PN 30200004) by spraying with RNaseZap solution and wiping with a Kimwipe, followed by spraying 70% ethanol and wiping with a Kimwipe.
3. Add **100 µL** Gel Slick Solution 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.
4. Prepare Gel Embedding Solution:

Gel Embedding Solution	1 sample	5 samples	10 samples
Gel Embedding Premix (PN 20300004)	5 mL	25 mL	50 mL
10% w/v ammonium persulfate solution	25 µL	125 µL	250 µL
N,N,N',N'-tetramethylethylenediamine	2.5 µL	12.5 µL	25 µL
Refer to Additional Buffer Recipes for 10% w/v ammonium persulfate solution			

5. Aspirate the Fluorescent Fiducial Solution for adherent cells or the Sample Prep Wash Buffer for suspended cells. **Retain 100 µL** Gel Embedding Solution in a small tube. Add **the remainder of the 5 mL** Gel Embedding Solution to the sample, ensure the sample is fully covered, and incubate at room temperature for 1 min.
6. Using a pipette, transfer the majority of the Gel Embedding Solution to a waste tube (to monitor the gel formation).
7. Aspirate to dry the MERSCOPE Slide, leaving just enough liquid to cover the cells.
8. Add **50 µL** of the retained Gel Embedding Solution on the cells.
9. Place the tips of one pair of tweezers on an area of the MERSCOPE Slide without touching the cells. Use tweezers to pick up the 20-mm Gel Slick-treated Gel Coverslip. With the Gel Slick-treated side **facing down** toward the cells, 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 cells to spread the Gel Embedding Solution. If needed, adjust the Gel Coverslip so it is positioned in the center of the MERSCOPE Slide. Gently press the Gel Coverslip to squeeze out excess Gel Embedding Solution, and remove the extra Gel Embedding Solution by aspiration.



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. Avoid squeezing the Gel Coverslip too hard as it may damage the sample and result in gel that is too thin/no gel.

10. Incubate at room temperature for 1.5 h.

Monitor the Gel Embedding process in the waste tube. Gel starts to form within 1 h.

Repeat the Gel Embedding process from step 2 if:

- *no gel forms.*
- *it is not possible to remove the air bubbles. Air bubbles result in an empty area in the gel.*
- *too much Gel Embedding Solution is squeezed out and the gel is too thin and not visible.*

It is not necessary to remove the gel prior to repeating the Gel Embedding process.

11. Ensure **eye protection** is worn during this step. Gently brace the Gel Coverslip with tweezers in one hand and lift the 20-mm Gel Slick-treated Gel Coverslip with the sharp tip of a Hobby Blade and discard the Gel Coverslip appropriately.
12. Proceed immediately to the next step.

VII. Clearing

1. Warm Clearing Premix (PN 20300003) at 37°C for 30 min before use. The Clearing Premix should be a clear solution before use. If the solution is cloudy, warm and mix until the solution becomes clear. Prepare Clearing Solution:

Clearing Solution	1 sample	5 samples	10 samples
Clearing Premix (PN 20300003)	5 mL	25 mL	50 mL
Proteinase K	50 µL	250 µL	500 µL

2. Add **5 mL** Clearing Solution.
3. Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.
4. Seal the petri dish with parafilm and place in a humidified 37°C cell culture incubator for 24 h.

If autofluorescence is observed during imaging, consider transferring the petri dish to 47°C and incubate for 24 h.

DO NOT *incubate at 47°C >24 h otherwise the RNA will begin to degrade – this is important to remember if clearing over the weekend.*

If the cells are still not transparent, transfer the petri dish back to 37°C until the cells have cleared.

SAMPLES CAN BE STORED OR CLEARED IN CLEARING SOLUTION

*AT **37°C** FOR UP TO **7 DAYS***

REPLENISH** THE CLEARING SOLUTION **AFTER 4 DAYS

REFER TO THE MERSCOPE INSTRUMENT USER GUIDE FOR NEXT STEPS