APR 10, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.bp2l61ojkvqe/v1

Protocol Citation: Angela R.S. Kruse, Morad C Malek, Jamie Allen, Melissa Farrow, Jeff Spraggins 2023. GeoMx-NGS Manual RNA Slide Preparation Protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bp2l61ojkvqe/v1

Protocol status: Working
We use this protocol and it's working

Created: Aug 23, 2022

Last Modified: Apr 10, 2023

PROTOCOL integer ID: 69072

GeoMx-NGS Manual RNA Slide Preparation Protocol

Angela R.S. Kruse[1], Morad C Malek[1], Jamie Allen[1], Melissa Farrow[1], Jeff Spraggins[1]

[1]Vanderbilt University

Morad C Malek
Vanderbilt University

ABSTRACT

This protocol describes the preparation of tissue samples for spatial transcriptomics via the Nanostring GeoMx Digital Spatial Profiler.

Expected outcome: Tissue samples probed with RNA probes that are ready for subsequent spatial sampling.

GUIDELINES

Be sure to clean all surfaces with RNase-away to prevent cross-contamination with RNA probes or RNA degradation.

MATERIALS

EQUIPMENT

	A	B	C
	Equipment	Source	Part Numbers
	Baking oven	Quincy Lab, Inc. (or comparable) Boekel Scientific ACDBio	Various GC models
	Hybridization oven including hybridization chamber *: Boekel Scientific RapidFISH Slide Hybridizer or HybEZ II Hybridization System l HybEZ oven l Humidity control tray l EZ-Batch Wash Tray l EZ-Batch Slide Holder l HybEZ Humidifying Paper (2 sheets)	Boekel Scientific ACDBio	240200 for 120V or 240200-2 for 230V 321710/321720 310012 321717 321716 310025
	Water bath (up to at least 37°C)	Various	Various

	A	B	C
	UV Light Box (optional)	VWR	Various
	5-quart Steamer	Nesco or Hamilton Beach Note: Products from other vendors may require testing and optimization.	ST-25F 37530Z

Note

* These hybridization ovens are designed to keep the slides hydrated and maintain a precise
temperature overnight. NanoString does not recommend the use of any other hybridization
ovens for GeoMx RNA DSP. Please contact NanoString with any concerns.

MATERIALS

	A	B	C
	Materials	Source	Part Numbers
	Pipettes for 5–1,000 µL	Various	Various
	12-channel P20 multi-channel pipette	Various	Various
	Filter Tips (RNase/DNase free)	Various	Various
	Microtubes	Sarstedt (or comparable)	72.785.005
	Superfrost Plus microscope slides	Fisher Scientific (or comparable)	12-550-15
	Tissue Tek Slide Stain Dish (plastic Coplin Jars) or equivalent (recommend ~16)	Sakura (or comparable)	25608-904 or 25608-906
	Instant-read digital thermometer	Various	Various
	Humidity Chamber	Simport	M920-1

	A	B	C
	Benchtop protector sheet (fits inside the hybridization oven, optional)	Fisher Scientific (or comparable)	15235101
	HybriSlip hybridization covers (22 mm x 40 mm x 0.25 mm) Note: this has been validated by NanoString. Do not use other products	Grace Bio-Labs	714022
	RNase AWAY	ThermoFisher	7003PK
	Heat/cold protectant handling glove	Various	Various
	Forceps (for slide handling)	Various	Various
	Aluminum foil	Various	Various

REAGENTS

Reagent	Source/Part Number	Storage
DEPC-treated water	ThermoFisher, AM9922 (or comparable)	RT
10X phosphate buffered saline pH 7.4 (PBS)	Sigma-Aldrich, SKU P5368-10PAK, P5368-5X10PAK (or comparable)	RT
10% neutral buffered formalin (NBF) 	EMS Diasum, Cat # 15740-04 (or comparable)	RT
100% deionized formamide 	ThermoFisher , AM9342 **or** VWR, VWRV0606 (or comparable)	4°C (bring to RT before opening)
20X SSC (DNase, RNase free)	Sigma-Aldrich, S6639	RT
Proteinase K 	Proteinase K from Ambion, 2546, **or** Thermo Fisher, AM2548 Note: Use of Proteinase K from any other vendor will require optimization of incubation times and concentration.	-20°C
1X Tris-EDTA pH 9.0	eBioscience™ IHC Antigen Retrieval Solution - High pH, 00-4956-58 (10X - will need to be diluted with DEPC-treated water)	4°C
Tris base	Sigma-Aldrich, 10708976001 (or comparable)	RT
Glycine	Sigma-Aldrich, G7126 (or comparable)	RT
Citrisolv **or** Xylene 	Fisher Scientific, Cat # 04-355-121 Sigma Aldrich, SKU 183164-100ML or 183164-500ML (or comparable)	RT
100% ethanol (EtOH): ACS grade or better 	Various	RT
10% Tween 20	Teknova, T0710 (or comparable)	RT

PREPARE REAGENTS

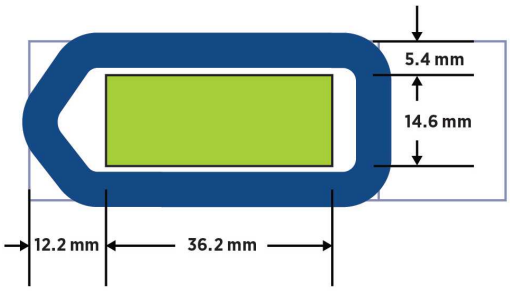
1

	A	B	C
	Reagent	Dilution	Storage

	A	B	C
	95% EtOH	Prepare 500 mL of 95% ethanol by adding 25 mL of DEPC- treated water to 475 mL of 100% ethanol. Change at least weekly.	RT
	1X PBS pH 7.4	Prepare 1 L of 1X PBS by combining 100 mL of 10X PBS and 900 mL of DEPC-treated water. Don't reuse.	4°C
	10% neutral buffered formalin (NBF)	Caution: NBF is hazardous, handle with care and minimize inhalation risks. Work with 10% NBF in the fume hood. OK to reuse.	RT
	Proteinase K	Prepare 1 µg/mL by adding 10 µL of 20 mg/mL Proteinase K to 200 mL of 1X PBS made with DEPC-treated water Note: Should be prepared fresh daily; don't reuse. Take care to pipette accurately. Inaccurate concentration of Proteinase K will affect assay performance. Serial dilutions are recommended.	RT
	NBF stop buffer	Prepare 24.5 g Tris base and 15 g Glycine to 2 L DEPC- treated water. Do not reuse. Solution will lose efficacy with repeated use.	RT
	2X SSC	Prepare 1 L of 2X SSC by combining 100 mL of 20X SSC and 900 mL of DEPC-treated water. Do not reuse.	RT
	2X SSC-T	Prepare 100 mL of 2X SSC by combining 10 mL of 20X SSC, 1 mL of 10% Tween-20, and 89 mL of DEPC-treated water. Do not reuse.	RT
	4X SSC	Prepare 1 L of 4X SSC by combining 200 mL of 20X SSC and 800 mL of DEPC-treated water. Do not reuse.	RT

Prepare Tissue Samples

2 Unstained tissue sections should be 5 µm thick on Superfrost Plus slides. Tissue sections must be placed in the Scan Area (the green area in the slide diagram) in the center of the slide and be no larger than 36.2 mm long by 14.6 mm wide. They should not overlap the slide gasket or the Tip Calibration area (this is the triangular region to the left of the green scan area in the slide diagram). If sections are larger than this size and/or placed off-center, the tissue located outside the Scan Area will not be scanned by the GeoMx DSP instrument.



3 Bake sections on slides in a 60°C drying oven for a minimum of 30 minutes prior to deparaffinization. Longer baking times may be necessary for some tissues to adhere to the slide; this should be empirically tested. If tissue falls off, then baking longer could help.

Deparaffinize and rehydrate FFPE tissue sections (31 minut..

31m

4 You will need the following items/reagents for this step: Steamer, Staining jars, 1X Tris EDTA (pH 9.0), DEPC-treated water, Citrisolv/Xylene, 100% EtOH, 95% EtOH, and 1x PBS. See the Equipment, Materials, and Reagents list for more details.

5 Fill the steamer reservoir up to the fill line with water. Place two staining jars inside, one containing 1X Tris EDTA (pH 9.0) and one containing DEPC-treated water (use sufficient volume to cover slides up to the label). Loosely cover each jar with aluminum foil instead of the jar lid; this allows for a thermometer reading in a later step. Preheat the steamer to 100°C.

The Nesco steamer takes 1 hour to heat the jars to a stable maximum temperature near 100°C. Final temperature can be checked by inserting a digital thermometer through the hole in the lid of the steamer .

6 Preheat the water bath to 37°C.

7 Deparaffinize and rehydrate FFPE tissue sections. Place the slides in a rack and gently perform the following washes using staining jars (see Figure 4).

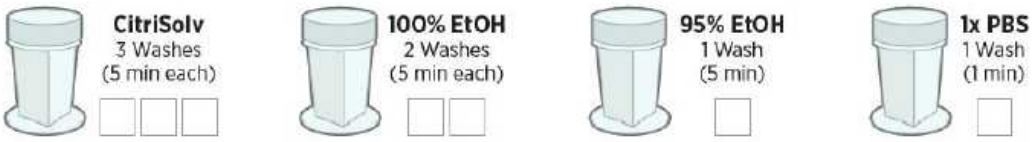


Figure 4: Wash steps

Ensure you have sufficient buffer to cover all slides in container for the washes below. After the last wash, slides can be stored in the 1x PBS for up to one hour.

Perform target retrieval (15 - 25 minutes)

25m

8 You will need the following items/reagents for this step: Steamer, Staining jars, 1X Tris EDTA (pH 9.0), DEPC- treated water (all pre- heated in the previous step) and 1X PBS . See the

Equipment, Materials, and Reagents list for more details.

9 Without removing the lid, place an instant-read digital thermometer through the vents in the steamer lid and pierce the aluminum foil covering the Tris EDTA. Ensure the Tris EDTA has reached ~99°C. Reset the steamer's timer to ensure consistent heating during incubation.

10 Once the Tris EDTA has reached the appropriate temperature, carefully remove the steamer lid and jar covers. Dip the slides into the DEPC-treated water for 10 seconds; this step assists in maintaining the temperature of the retrieval buffer. Quickly transfer the slides to the Tris- EDTA.

11 Incubate the slides. Incubation times and temperatures may differ by tissue and may need to be empirically determined. The table below lists starting conditions (see Table 5). If the tissue type you wish to use is not listed, start with the default conditions: 15 min @ 100°C.

Table 5: Target retrieval times by tissue type

Tissue Type	Target Retrieval in Tris-EDTA
Breast	20 min
Cell pellets	5 min
Colorectal	20 min
Melanoma	20 min
NSCLC	20 min
Prostate tumor	20 min
Tonsil	15 min

12 When the timer is finished, move slides to room temperature PBS immediately.

13 Wash in room temperature PBS for 5 minutes Slides can be stored up to 1 hour in the PBS.

Expose RNA targets (10 - 30 minutes)

30m

14 You will need the following items/reagents for this step: Staining jars, water bath, Proteinase K, and 1X PBS. See the Equipment, Materials, and Reagents list for more details.

15 Dilute Proteinase K. Proteinase K concentration and incubation times differ by tissue and may need to be empirically determined. Modifications for certain tissues listed below (see Table 6). If the tissue type you wish to use is not listed, start with the default conditions: 1 µg/mL for 15 min. Note that Proteinase K solution must be diluted fresh immediately before use.

Table 6: Proteinase K digest concentrations and times by tissue type

Tissue Type	Proteinase K Digest
Breast	0.1 µg/mL for 15 min
Cell pellets	0.1 µg/mL for 5 min
Colorectal	1 µg/mL for 15 min
Melanoma	1 µg/mL for 15 min
NSCLC	1 µg/mL for 15 min
Prostate tumor	1 µg/mL for 15 min
Tonsil	1 µg/mL for 15 min

16 Place proteinase K in a Coplin jar and warm it to 37°C in water bath.

17 Incubate slides in proteinase K at 37°C using the time and concentration or tissue- dependent conditions listed in the table above (see Table 6).

18 Wash in 1X PBS for 5 minutes. Proceed to the next step immediately.

Postfix—Preserve tissue morphology for soft tissues (20 mi... 20m

19 You will need the following items/reagents for this step: Staining jars, 10% NBF, NBF Stop Buffer and 1X PBS. See the Equipment, Materials, and Reagents list for more details.

20 Post-fix the tissue by performing the washes listed in the figure below (see Figure 7).

Slides can be stored in the final PBS wash up to 1 hour at RT or up to 6 hours at 4°C.

In Situ Hybridization (overnight)

16h

21 You will need the following items/reagents for this step: Hybridization chamber, hybridization oven, Buffer R, RNA Probe Mix, and DEPC-treated water. See the Equipment, Materials, and Reagents list for more details.

Prepare buffers: Warm Buffer R to room temperature (RT) before opening. Thaw RNA detection probes on ice. Before use, mix thoroughly by pipetting. Once thawed, do not re-freeze probes. Refrigerate at 4°C for up to 3 months.

Prepare the hybridization chamber according to product instructions. The hybridization ovens listed in the Equipment section are not light-permeable. If your chamber is light-permeable, minimize light exposure (e.g., by wrapping the lid in aluminum foil).

22 Make hybridization solution (n= number of slides) (see Table 7).
If adding custom targets to the RNA assay, add the appropriate volume of custom RNA spike-in (up to two, see below). Add DEPC-treated water if needed to bring volume up to the final volume.

Table 7: Hybridization solution equation

	Buffer R	RNA Probe Mix	Custom spike-in (if applicable)	Custom spike-in (if applicable)	DEPC-treated H_2O (if applicable)	Final Volume
No custom spike-in	200 µL x *n*	25 µL x *n*	n/a	n/a	25 µL x *n*	250 µL x *n*
One custom spike-in	200 µL x *n*	25 µL x *n*	12.5 µL x *n*	n/a	12.5 µL x *n*	250 µL x *n*
Two custom spike-ins	200 µL x *n*	25 µL x *n*	12.5 µL x *n*	12.5 µL x *n*	n/a	250 µL x *n*

23 Clean all equipment with RNase AWAY and allow to dry or rinse with DEPC-treated water (see Important note, above). The hybridization chamber can be a key source of contamination by oligos. Arrange fresh Kimwipes on bottom of the chamber.

24 Wet the Kimwipes with 2X SSC or DEPC-treated water. Take care that the Kimwipes and 2X SSC do not contact the slides. Hybridization solution can wick off of the slides if in contact with Kimwipes or liquid. Kimwipes should be thoroughly damp, but standing buffer should not be present.

25 One at a time, remove slides from PBS, wipe away excess liquid, and set in hybridization chamber. Take care not to let the slides dry out.

26 Add 200 µL hybridization solution to each slide. Take care not to introduce any bubbles.

27 Gently apply a Grace Bio-Labs HybriSlip. Start by setting one edge of the coverslip down in solution on the slide, then gradually laying down the rest of the coverslip to avoid the formation of air bubbles.

28 Repeat steps 4–6 for each slide.

29 Close hybridization chamber, insert into oven, and clamp into place. Incubate at 37°C overnight (16–24 hours).

Perform stringent washes to remove off-target probes (90 ...

1h 30m

30 You will need the following items/reagents for this step: Water bath, 4X SSC, and formamide. See the Equipment, Materials, and Reagents list for more details.

31 Before you begin, ensure water bath is set to 37°C for later use. Warm 100% formamide to room temperature before opening. Once formamide is at room temperature, make Stringent Wash by mixing equal parts 4X SSC and 100% formamide. Fill two staining jars with Stringent Wash and preheat them in 37°C water bath.

32 Dip the slides in 2X SSC-T and allow the coverslips to slide off by themselves. Minimize the time in SSC-T; continue to the wash steps within 5 minutes. Slides with coverslips that have not come off after 5 minutes can be moved to stringent wash. Coverslips should come off in the first stringent wash.

33 Perform the washes listed in the figure below (see Figure 11). After the last wash, slides can be stored in 2X SSC for up to one hour.

Figure 11: Stringent washes to remove off-target probes

Add morphology markers (100 minutes)

1h 40m

34 You will need the following items/reagents for this step: Humidity chamber, Buffer W, nuclear stain (SYTO 13), morphology markers, and 2x SSC . See the Equipment, Materials, and Reagents list for more details.
Before you begin: Remove nuclear stain (SYTO 13) from the freezer (stored at -20°C) and allow to warm to room temperature on bench.

IMPORTANT: Before using the humidity chamber in the following steps, clean it with RNase Away. Prep the humidity chamber by lining with Kimwipes wetted with 2x SSC.

35 Block with Buffer W. Move one slide at a time to the tray. Transfer tray to the humidity chamber for antibody staining. Cover tissue with up to 200 µL Buffer W and leave at RT for 30 minutes. Cover to prevent light exposure.

Ensure adequate Buffer W surrounds the edges of the tissues so they don't dry out. Use a pipette

tip to gently move the solution so there is a 2-3 mm border around the tissue. A hydrophobic barrier can be used at this step, but must be carefully removed with a razor blade after antibody incubation.

36 Picofuge thawed nuclear stain (SYTO 13) for at least 1 minute to bring the solution to the bottom of the vial and precipitate insoluble particles. Do not pipette from the bottom of the vial. Close all vials tightly and store the remaining nuclear stain (SYTO 13) at -80°C.

37 Prepare morphology marker solution (n = number of slides)(see Table 8). Prepare up to 220 µL per slide.

Table 8: Morphology marker solution equation

Nuclear stain (SYTO 13)	Morphology Marker 1	Morphology Marker 2	Other Marker (optional)	Buffer W	Total Volume
22 µL x *n*	5.5 µL x *n*	5.5 µL x *n*	...	187 µL x *n* *	220 µL x *n*

*If a different number of detection Ab or morphology marker tubes are used, Buffer W amount needs to
be adjusted to bring total volume up to 220 µL per slide.

38 Spin morphology marker solution and antibodies by flicking and spinning down.

39 Remove Buffer W by tapping slide onto a Kimwipe.

40 Cover tissue with antibodies (up to 200 µL). Stain for 1 hour in the humidity chamber at RT. Cover to prevent light exposure.

41 Wash 2 times for 5 min in 2x SSC.

When the slides are prepared, load them immediately on the GeoMx DSP (see the GeoMx DSP

Instrument User Manual).

If needed, slides can be stored at 4°C in 2xSSCT for 6 hours or 2xSSC for one week.

42 Once GeoMx DSP aspirates are collected, the collection plate may be stored at -20°C until ready for library preparation.