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GeoMX Digital Spatial Profiler Whole Transcriptome Assay for Human Pancreas

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DISCLAIMER

Refer to Nanostring online user manuals for additional information
(<https://nanostring.com/support/nanostring-university/>).

ABSTRACT

Purpose:

This protocol details processing fixed paraffin sections from human pancreas for the Nanostring GeoMX digital spatial profiler (DSP) whole transcriptome assay (WTA). This technology is based upon hybridization of samples with RNA reagents composed of >20,000 probes for full coverage of protein coding mRNA. The RNA probes are designed with complementary RNA sequences and UV cleavable linkers and DSP barcodes. Morphology markers are used to identify cell types of interest with nuclear counterstaining permitting tissue spatial information. Sample regions of interest (ROI) are defined based on the 4 morphology markers and each ROI is illuminated with UV light thereby cleaving hybridized RNA probes. Cleaved probes are aspirated via microcapillary and deposited into a unique well of a 96-well collection plate. The collection plate is used for next generation sequencing (NGS).

Scope:

This document was written following GeoMX- NGS guidelines (university.nanostring.com) with minor modifications for the University of Florida Molecular Pathology Core.

The entire workflow is described for manual slide staining and GeoMX DSP instrument use. Steps for library construction, sequencing, bioinformatics, and data analysis are in outline form only. Library preparation and sequencing are outsourced to the University of Florida ICBR facility.

Expected outcome:

RNA suitable for bulk RNA-sequencing will be obtained from fixed human pancreas sections within regions of interest and subareas defined by cell morphology markers.

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KEYWORDS

pancreas, FFPE, GeoMX, Nanostring, whole transcriptome assay, morphology, insulin, cytokeratin, CD31, fluorescence, hybridization, nCounter, HuBMAP, TMC-PNNL/UF, RNAsequencing, transcriptomics, DNA, UF ICBR, bioinformatics, Illumina, spatial transcriptomics, islet, exocrine pancreas, endothelium, duct

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IMAGE ATTRIBUTION

Human pancreas stained with panCytokeratin, UCHL-1 (PGP9.5), and CD31 with Syto13 nuclear marker.

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GUIDELINES

Maintain the DSP instrument according to vendor recommendations including weekly wash buffer changes.

Maintain a RNase-free work space.

Validate morphology markers using assay conditions with antigen retrieval and RNA probe hybridization steps but mock RNA probe solution.

Prepare all solutions using DEPC-water and RNase-free supplies.

Clean equipment with RNase AWAY, rinse with DEPC-treated water, and air-dry.

MATERIALS TEXT

Chemicals (store at RT except where noted)

Deparaffinization reagents (xylene or substitute, ethanols (100%, 95%))

10xPBS pH 7.4 (PBS, FisherScientific BP399-1 or comparable)

20x SSC (DNase RNase free, Sigma, S6639)

10x Tris-EDTA pH 9.0 Antigen Retrieval Solution (Invitrogen 00495658, FisherScientific 50-187-83)

Proteinase K (20 mg/ml, ThermoFisher, AM2548, **-20 °C**)

10% neutral buffered formalin (NBF, FisherScientific, 50-980-502 (EMS 1574004))

Formamide (deionized or molecular grade, FisherScientific, AM9342) store at **4 °C**

DEPC-treated water (DECP water, FisherScientific, BP561-1)
 RNase AWAY (FisherScientific, 7003PK)
 Tween 20 (FisherScientific, BP337-100)
 TRIS-base (Sigma, 10708976001 or equivalent)
 Glycine (Sigma G7126 or equivalent)

Solution preparations

1xPBS (PBS, 1 liter)- add 100ml 10xPBS to 900ml DEPC water, store **4 °C**
 1xAntigen Retrieval Solution (100ml)- add 10ml 10xTris-EDTA to 90ml DEPC water for final 10mM Tris, 1mM EDTA, pH 9.0)
 Proteinase K 1 µg/mL (200ml)- add 10 µL of 20 mg/mL Proteinase K stock to 200 mL of 1X PBS; prepare working solution fresh daily, take care to pipette carefully).
 NBF stop buffer (0.1M, 1 liter)- dissolve 12.25 g Tris base and 7.5 g Glycine to final 1 L DEPC water. Do not reuser. Store 1 month.
 10% Tween 20- prepare according to <https://dx.doi.org/10.17504/protocols.io.bfzmjp46>, store up to 12 weeks in dark container
 2xSSC (1 liter)- add 100ml 20x SSC and 900ml DEPC water.
 2xSSC-T (100ml)- add 10ml 2x SSC, 1ml 10% Tween-20, and 89ml DEPC water.
 4xSSC (1 liter)- add 200ml 20x SSC and 800 ml DEPC water.
 2x SSC/50% formamide- add 100ml each 4x SSC and 100% formamide

Morphology markers

Primary antibodies are conjugated to fluorophores for cell types of interest based on fluorophores compatible with the DSP instrument (AF488, AF532, AF594, AF647 or similar). Use up to 3 conjugated primaries with suitable nuclear marker. Nuclear markers are SYTO 13 (AF488, S7575) or SYTO 83 (AF532, S11364) (FisherScientific, 5mM in DMSO).

Description	Vendor	Catalog #
Insulin, Mouse anti-all, AF488 [IRDN/794]	Novus Biologicals	NBP2-47726AF488
Insulin, Mouse anti-all, AF488 [ICBTACLS]	FisherScientific	50-112-4641
Insulin, Mouse anti-all, AF594 [IRDN/794]	Novus Biologicals	NBP2-47726AF594
Cytokeratin (PanCK), Mouse anti-all, AF532 [AE-1/AE-3]	Novus Biologicals	NBP2-33200AF532
Cytokeratin (PanCK), Mouse anti-all, AF594 [AE-1/AE-3]	Novus Biologicals	NBP2-33200AF594
CD31, Mouse anti-hu, AF647 [JC/70A]	Abcam	ab215912
Chromogranin A (CGA), Mouse anti-all, AF594 [Chr-A(C-12)]	Santa Cruz	Santa Cruz
UCLH1 (PGP9.5), Mouse anti-all, AF594 [31A3]	Novus Biologicals	NBP2-33130AF594

Table Primary antibodies for GeoMX WTA assay in human pancreas. PanCK is also sold in morphology kits from Nanostring.

Lab Supplies

Superfrost Plus slides (FisherScientific, 22-035813)
 Glass desiccator for slide drying and storage

Kimwipes
RNAse-free beakers and cylinders to prepare buffers
Coplin jars or slide trays
Pipettes (5-1000, 12-channel P20) and filter tips (DNase/RNase free)
Reagent reservoir, 20ml, sterile (FisherScientific, 8094)
Microcentrifuge tubes (DNase/RNase free)
Microcentrifuge rack
Slide hybridization covers (22mm x 40mm x 0.25mm thick)(Hybrislip, Grace Bio-labs, 714022)
Adhesive PCR plate foils (ThermoFisher, AB0626)

Nanostring GeoMX DSP WTA reagents and kits

GeoMX collection plate (4pk, 100473)
GeoMX instrument buffer kit (100474)
GeoMX RNA slide prep kit FFPE (Buffers S, W, R; 121300313) ⚡ 4 °C
GeoMX Seq Code Pack ⚡ -20 °C
GeoMx NGS RNA Whole Transcriptome Atlas - Human (121401102) ⚡ -20 °C

Equipment

Oven for drying slides
Leica XL slide autostainer or alternative.
IHC retrieval steamer (FisherScientific, NC1314441 or alternative)
Waterbath (FisherScientific, FSGPD02)
Slide incubation chamber (black or opaque)
HybEZ II slide hybridization chamber (ACDBio, 310010)
Nanostring GeoMX DSP instrument
PCR thermocycler
Centrifuge with rotor for 96-well PCR plates (at least 2000 x g)
Minicentrifuge for quick spins

SAFETY WARNINGS



Follow Environmental Health and Safety protocols for all hazardous chemicals (xylene, formalin, formamide).

DISCLAIMER:

Refer to Nanostring online user manuals for additional information
(<https://nanostring.com/support/nanostring-university/>).

BEFORE STARTING

Preheat waterbath to ⚡ 37 °C .

Prepare 1x Antigen Retrieval Solution and preheat in steamer while slides are dewaxing.

- 1 This procedure details processing of paraffin slides, in set of 4, for GeoMX WTA by manual methods. Methods using the Leica BOND autostainer are detailed on the Nanostring website. Paraffin blocks containing 4% paraformaldehyde fixed human pancreas are screened by H&E staining and histopathology review <https://www.protocols.io/view/human-pancreas-histopathology-assessment-n92ld956xg5b/v1>.

Wash steps in this protocol may be abbreviated such as one wash= 1 x and three washes= 3 x. Room temperature (RT) is used except where different temperature is noted.

Slide preparation

- 2 Cut paraffin block to fresh sample.
Section at $\rightarrow 5 \mu\text{m}$ and place sections centrally on Superfrost Plus slides as shown in Fig. 1. Tissue samples should have maximum width and length of ~ 14 and 36 mm, respectively, for inclusion within the GeoMX instrument slide holder gasket. Tissue outside the gasket region should be carefully removed without creating folds or leaks to gasket seal. Air dry mounted slides overnight before user or storage.
Recommended storage is for < 2 weeks in a desiccator at $\rightarrow 4^\circ\text{C}$.

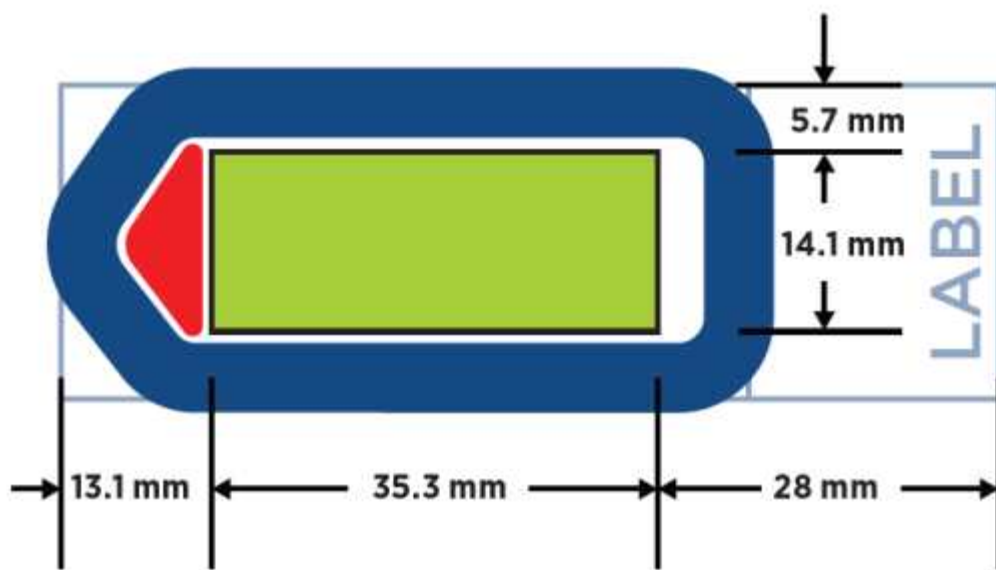


Fig. 1. Tissue section placement on glass slide (GeoMX DSP Manual Slide Preparation User Manual).



- 3 Bake slides at $\rightarrow 60^\circ\text{C}$ for $\rightarrow 01:00:00$ immediately before use.

1h

Slide deparaffinization




- 4 Dewax and rehydrate sections using slide tray stations or a Leica XL slide autostainer.

Do not allow tissue sections to dry once rehydrated and avoid touching tissue when drying around edges.

- 4.1 Xylene 3 x  00:05:00 . CitriSolv or another xylene substitutes can be used. 5m
- 4.2 100% Ethanol 2 x  00:05:00 5m
- 4.3 95% Ethanol 2 x  00:05:00 5m
- 4.4 PBS 1 x  00:01:00 1m

Antigen retrieval

- 5 Prepare Antigen Retrieval Solution (1x Tris EDTA, pH 9) in Coplin jar.
Pre-heat in steamer.

- 5.1 Transfer slides to Antigen Retrieval Solution jar and steam for  00:20:00 . 20m
- 5.2 Immediately transfer slides to PBS.
- 5.3 Wash slides in PBS 1x  00:05:00 . 5m
- 5.4  1h

Slides can be stored in PBS up to  01:00:00 .

RNA retrieval

- 6 RNA retrieval solutions needed: 10m
Proteinase K working solution: Place in Coplin jar and prewarm to δ 37 °C for \odot 00:15:00 in water bath.
NBF stop solution in two Coplin jars
PBS wash

6.1 Incubate slides in Proteinase K jar at δ 37 °C for \odot 00:15:00 in water bath. 15m


6.2 Wash PBS 1 x \odot 00:05:00 . Proceed to next step immediately. 5m

7 Post-retrieval fixation for pancreas

7.1 Incubate in 10% NBF for \odot 00:05:00 . 5m

7.2 Incubate in NBF stop buffer 2 x \odot 00:05:00 . 5m

7.3 Wash PBS 1 x \odot 00:05:00 . 5m

7.4  1h

Slides can be stored in PBS wash solution up to \odot 01:00:00 at RT or up to 6 hrs at δ 4 °C .

RNA probe hybridization and stringency washes 1d 8h 10m

- 8 Prepare RNA probe mixture in dedicated RNase-free area away from nCounter work or other NGS workflows.

8.1 Warm buffer R to RT before opening vial.

8.2 Thaw RNA Probe Mix on ice. Before use, mix thoroughly by pipetting. Store unused RNA detection probes at **4 °C** up to 6 months or re-freeze.

8.3 Make RNA probe hybridization solution as per Table 1. Before use, briefly vortex and quick spin down.

8.4

A	B	C	D
Buffer R (ul)	RNA Probe Mix (ul)	DEPC Water (ul)	Final Volume (ul)
200ul x N	25ul x N	25ul x N	250ul x n
800	150	50	1000

Table 1. RNA probe hybridization solution preparation for NGS readout. Amounts per slide are shown. Volumes needed for 4 slides are provided.

8.5 Prepare slide hybridization incubator with RNase AWAY and dry or rinse with DEPC water. Place Kimwipes on bottom and wet with 2 x SCC or DEPC water to create humid environment during overnight hybridization.

8.6 

The hybridization chamber can be a key point of contamination by oligo probes.

RNA probe hybridization and stringency washes 1d 8h 10m

8.7 Remove each slide from PBS, tilt and wipe off excess PBS, then place in slide holder of hybridization chamber. Work quickly to avoid tissue drying.

8.8 Immediately add 200 ul RNA probe solution to each slide. Do not introduce air bubbles during pipetting.

8.9 Apply a Hybrislip over each section by gently laying down over tissue, starting from one edge, and avoiding introduction of air bubbles.

8.10 Repeat steps 8.5-7 for each slide.
Close slide tray, insert into hybridization oven, and clamp in place.

8.11 Incubate for 🕒 **16:00:00** at 🌡 **37 °C** . Slides must remain under humid 16h
conditions to prevent drying out of hybridization solution. The chamber can be
placed in a ziplock bag for additional security.

9 Post-hybridization stringency washes.

Solutions needed:

2xSSC

2xSSC-T

2x SSC/50% formamide

9.1 Warm 100% formamide to RT before opening.
Prepare 2x SSC/50% formamide by mixing 100ml each of 4x SSC and 100%
formamide.
Add solution to 2 Coplin jars and preheat to 🌡 **37 °C** in water bath .

9.2 Dip slides in 2x SSC to remove coverslips. 5m
If they do not come off immediately, move to 2x SSC-T for maximum of
🕒 **00:05:00** .
If coverslip have not come off in 2x SSC-T, move slides to stringent washes
where coverslips should come off.

9.3 ⚠
Do not force off coverslips to avoid tissue damage.

9.4 Wash in 2xSSC/50% formamide 2 x 🕒 **00:25:00** at 🌡 **37 °C** in water bath. 25m

9.5 Wash in 2xSSC 2 x 🕒 **00:05:00** . 5m

9.6 ⏸ 1h

After last wash, slides can be stored in 2xSSC for up to 🕒 **01:00:00** .

- 9.7 Carefully wash all containers coming into contact with the hybridization solution (Proteinase K, formamide mixture 2XSSC) with RNase AWAY to prevent probe contamination. Use separate Coplin jars for different probe mixtures.

Morphology marker incubation

1d 8h 10m

- 10 Incubate sections with up to 3 morphology markers (MM) and one DNA counterstain to define cell types of interest. Morphology markers are validated by in-house testing using the RNA assay conditions.
Clean humidity chamber with RNase AWAY.

10.1

Thaw nuclear marker, vortex then spin down for at least 1 minute to pellet any precipitates. Pipette from top of vial only. Close tightly after use and refreeze at **-20 °C**.

10.2

Remove one slide from 2xSSC and tap clean on stacked clean towels to ^{30m} remove excess buffer.

Cover section with 200ul Buffer W for **00:30:00** in dark slide chamber while preparing morphology marker solution. Make sure Buffer W extends 2-3 mm past section borders.

10.3

Prepare MM solution. Mix solution by flicking followed by quick spin down.

A	B	C	D	E	F	G
	Nuclear Marker	MM1	MM2	MM3*	Buffer W**	Total Volume
Slide n	22 ul x n	5.5 ul x n	5.5 ul x n	5.5 ul x n	187ul x n	220 ul x n
1	22	5.5	5.5	0	187	220
2	44	11	11		374	440
3	66	16.5	16.5		561	660
4	88	22	22		748	880

Table 2. Morphology marker solution preparation. Volumes are in ul for up to 4 slides.

*Optional third MM. Adjust Buffer W amount when using 3 MM. MM: morphology marker.

10.4

Working with one slide at a time, remove Buffer W by tapping slide edge on stacked towels then add 200ul morphology marker solution/slide in humidity chamber. Repeat for each slide.

10.5 Incubate slides in dark slide chamber for 🕒01:00:00

1h

11 Tap slide edge on towels to remove MM solution then wash slides in 2x SSC 2 x 🕒00:05:00^{5m}.

11.1 Submerge slides in 2x SSC for 2 x 🕒00:05:00. Load slides in DSP within^{6h 5m} 🕒06:00:00 of last wash for optimal results.

11.2 Alternatively, store slides in 2xSSC for up to 2 days at 🌡4 °C in dark.

11.3 

Slides must be stored in dark to avoid cleavage of DSP tags by UV light.

GeoMX DSP run 1d 8h 10m

12 Start run by selecting New/Continue Run under Data Collection in the GeoMx DSP Control Center.


12.1 Clean the bottom of slide with 70% ethanol and place slide in slide holder with slide label towards front of instrument.

12.2 Complete loading slides then lower slide tray clamp. Cover sections with 6 ml Buffer S. Record slots for each slide.

12.3 Load a 96-well aspiration plate in DSP. Check solution containers are filled and waste container is empty.

13 Follow prompts in the GeoMx DSP Control Center to identify slides.

- 13.1 Name slides according to protocol
<https://dx.doi.org/10.17504/protocols.io.n2bvj6dnblk5/v1>
Include UF HuBMAP sampleID and morphology marker abbreviations in order of channels followed by date (MMDDYY). Example: P1-21_DNA_PanCK_INS_CD31_121021.
- 13.2 Upload unzipped configuration files to DSP kits used for Morphology and WTA kits used in slide preparations (nanosttring/dsconfigfiles). Config files must match names of assays used on slides.
- 13.3 Define scan area for each sample using x- and y-sliders and scan. The minimum tissue slide filters particles and small tissue areas from scan. The sensitivity slider adjusts intensity of tissue identification. 2h
- 13.4 Draw regions of interest (ROI) using geometric, segmented or other tools. Maximum ROI size is ~ **650 μ m** diameter circle.
- 13.5 Segment ROIs for AOI (area of illumination) for cell types of interest based on positive or negative selection by morphology markers. Minimum of ~100 nuclei/AOI is recommended for sufficient RNA.

Insulin is used to designate beta-cells in the endocrine compartment, CD31 is used to define endothelium, and panCK is used to define ductal epithelium in the exocrine compartment. Acinar cells are defined by the lack of staining for CD31 and PanCK in the exocrine ROI.
- 13.6 Exit scan workspace and approve ROI to start ROI collection.  **02:00:00** 2h
- 13.7 After ROI/AOI collections are completed, follow prompts to remove slide holder and collection plate.
- 13.8 Remove buffer from each slide with pipette and dispose. Open clamps and unload slides. Store slides in 2x SSC at **4 °C** up to 7 days.
- 13.9 Clean slide holder as directed and replace in instrument. Follow prompts to close instrument door.

- 13.10 Fill in the GeoMX Seq Code plate to be used with the plate barcode in the Readout Group Information grid.
Click update button and finalize and download readout package buttons to USB drive.

Slide reuse

1d 8h 10m

14



RNA assay slides can be restained following UV exposure for 3 minutes to strip tags from bound probes followed by washes using with 2xSSC-T. Proceed with additional staining.

NGS library and sequencing

- 15 Transfer collection plate to the University of Florida ICBR Gene Expression and Genotyping Core or other NGS core facility for library preparation and Illumina sequencing.
Refer to Nanostring user manual for additional information.

- 16 Library will be prepared after indexing PCR with pooling and purification.

- 16.1 Library will be evaluated for quality and fragment size distribution using a BioAnalyzer 2100 (Agilent).


- 17 Core facility will perform Illumina sequencing.

- 18 Core facility will process Illumina FASTQ sequencing files and transfer back to laboratory.

Data analysis

- 19 Share FASTQ files and annotation file with data analyst who will analyze RNAseq data using R scripts for GeoMx DSP workflow ([Bioconductor - GeoMxWorkflows](https://dx.doi.org/10.17504/protocols.io.8epv59bong1b/v1)). Data analysis may also be performed with GeoMX DSP software on auxiliary server.

- 19.1 Manage annotations (tags). Add columns to further define regions and cell types from morphology markers.

- 
- 19.2 Run various testing including QC, data scaling, normalization, background, statistical tests, and ratios.
 - 19.3 Analyze in spatial context using spatial deconvolution software and scRNAseq data.
 - 19.4 Complete analysis and upload data to HuBMAP HIVE.