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

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NanoString GeoMx DSP TMA-TNP protein assay

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ABSTRACT

This protocol outlines the NanoString GeoMx DSP protein assay that was applied in the Human Tumor Atlas Network (HTAN) Tissue MicroArrary (TMA) -TransNetwork Project (TNP).

The TMA-TNP evaluates various characterization and analytics methodologies on a large array of breast tumor samples representing a broad spectrum of disease state and subtype. A commercially available anonymized breast tumor TMA was purchased and serial sections were distributed. Participating HTAN Centers characterized the FFPE specimens using various imaging platforms and generated a spatially resolved cell type/state census using each center's method of choice. Data was recorded in a common repository to enable joint analysis.

The protocol that immediately precedes this one for TMA-TNP Phase 3 (when this NanoString GeoMx DSP protein assay protocol was first tested) can be found at: dx.doi.org/10.17504/protocols.io.6qpvr6992vmk/v1. It describes FFPE block serial sectioning, slide processing and TMA sample distribution.

In this protocol, the DSP protein assay was performed with the following human marker panels: Immune cell profiling core, Immune activation status, Immune-Oncology (IO) Drug target, Cell death, Pan-tumor, MAPK and PI3K/AKT panels including OHSU custom panel (Cell cycle and DNA damage). A total of 87 protein targets were evaluated in the TMA samples. Three compartments (Immune, Tumor and Stroma) in each TMA core were analyzed to determine cell-to-cell interactions in the tissues.

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Keywords: NanoString, GeoMx, Digital Spatial Profiler, HTAN, TMA, DSP, cell profiling, cell type

MATERIALS

1. Materials
Slide Baking Oven
Antigen retrieval pressure cooker
Humidity chamber
Thermocycler
Centrifuge (up tp 2,000g)

2. Reagents
Xylene
Ethanol
TBS
TBS-T
Citrate buffer pH6.0
10% Neutral Buffered Formalin (NBF)
DEPC-treated water

3. Nanostring reagents
GeoMx Instrument Buffer kit / Collection plate (Item no. 100474)
FFPE slide prep kit (Buffer W / Buffer S) (Item no. 121300312)

Human Immune cell profiling core panel (Item no. 121300101)
Human Immune activation status panel (Item no. 121300103)
Human IO Drug target panel (Item no. 121300102)
Human Cell death panel (Item no. 121300112)
Human MAPK panel (Item no. 121300111)
Human PI3K/AKT panel (Item no. 121300113)
Human Pan-tumor panel (Item no. 121300105)
OHSU SQ-35430 custom protein panel

Solid tumor Morphology kit (Anti-PanCK / Anti-CD45 / SYTO-13) (Item no. 121300301)

Hybridization CodeSets (A-H) (Item no. 121300401)

Master kit / Hybridization buffer (Item no. 100052)

FFPE slide sample preparation

1h 59m

1 FFPE slide preparation

1.1 Bake FFPE slides at 60 °C for 01:00:00 . 1h

1.2 Deparaffinize by sequential incubation with Xylene (00:03:00 twice), 100% EtOH (00:01:0), 95% EtOH (00:01:00) and 70% EtOH (00:01:00). 9m

1.3 Briefly rinse the slides with diH_2O (distilled water) and incubate with 1x Citrate antigen retrieval bu (Epitope retrieval buffer, pH 6.0) for 00:15:00 at high pressure in a pressure cooker. 15m

1.4 Take out the slides from the pressure cooker and let them cool down to room temperature aroun 00:25:00 . 25m

1.5 Wash the slides 5 times with TBS-T for 00:02:00 each. 10m

Antibody panel incubation

1h 59m

2 DSP protein panel and visualization marker incubation

2.1 Mark off the entire tissue section with a hydrophobic pen to create a reagent boundary.

2.2 Block the slides for 01:00:00 at room temperature using the Nanostring blocking buffer (Buffe 1h
W)

2.3 Freshly prepare each commercial protein panel in 1:25 dilution and each visualization marker (PanCK and CD45) in 1:40 dilution with Buffer W to a volume of 220ul per slide.

For the custom antibody panel, the final concentration at 250ng/ml per antibody is calculated and adjusted to the final volume of 220ul.

2.4 Incubate slides with all antibody panels and visualization markers for Overnight at 4 °C 16h

Post fixation and DNA staining 1h 18m

3 Post fixation

3.1 After antibody incubation, wash the slides 3 times with TBS-T for 00:03:00 each. 9m

3.2 Fix the slides with 10% NBF (Neutral buffered Formalin) for 00:30:00 at RT. 30m

3.3 Wash 3 times with TBS-T for 00:03:00 each, then stain the samples with SYTO-13, prepared 18m
1:10 dilution in TBS for 00:15:00 at RT.

3.4 Slides are briefly washed 2 times with TBS-T for 00:03:00 each. 6m

Loading samples to DSP

4 DSP running preparation

4.1 Scrape the hydrophobic barrier off with a scalpel or a straight-edged razor.

4.2 Place slides on the GeoMx slide tray and clean the back of the slide with 70% EtOH.

4.3 Once the gasket is sealed, place 6 mL of Buffer S on slides.

4.4 Load the GeoMx slide tray onto DSP platform.

GeoMx operation

6h 30m

5 Slide scan, ROI (Region of Interest) selection and AOI (Area of Interest) segmentation.

5.1 Log onto GeoMx software and start with **“New / Continue Run”.**

5.2 After loading slides with the collection plate information, DSP is ready to scan slides. 30m

5.3 A slide scan name is created and panel/visualization marker information is selected as below: 10m

In the **Probe Reagent Kit** field, in any order select **Human Immune Cell Profiling Protein Core**, **Human Immune Activation Status Protein**, **Human IO Drug Target Protein**, **Human Cell Death Protein**, **Human MAPK Signaling Protein**, **Human Pan-Tumor Protein**, **Human PI3K/AKT Signaling Protein**, **and SQ-35430 OHSU Custom**

1. Select the FITC/525 nm, Cy5/568 nm and Texas Red/615 nm channels.
2. For **FITC/525 nm, select SYTO 13** as fluorophore, DNA as biological target and enter 50 as exposure time.
3. For **Cy5/568 nm, select Alexa 532 as fluorophore, PanCK** as biological target and enter 300 as exposure time.
4. For T**exas Red/615 nm, select Alexa 594 as fluorophore, CD45** as biological target and enter 300 as exposure time.
5. Select FITC/525 nm as focus channel

5.4 When the scan area for each slide has been adjusted with sensitivity setting, select **Scan**. 30m

6 ROI selection and AOI segmentation

6.1 After scanning is done, each color channel intensity is adjusted to show visualization markers alo 10m with tissue or cell line property.

6.2 Each ROI is determined and selected by pathologist's guide, and drawn with circle (maximum 660 30m radius), rectangle (maximum 660x785um) or polygonal shape (maximum 660x785um).

TNP TMA slide contains total 88 cores.

Due to the limitation of scan area in the slide loading slot, only 44 cores per slide can be scanned and collected.

Two TMA slides (88 cores were embedded and slightly shifted to either left or right side of slide to cover half of 88

cores in each slide) are required to collect all cores.

6.3 In segment menu, 3 segmentation classes (Immune, Tumor and Stroma) are added and paramet 10m are set in the following order:

For Immune segmentation, Alexa 594 (CD45) is set to positive **("+")** and others set to ignore **("0")** for the first collection
For Tumor segmentation, Alexa 532 (PanCK) is set to positive **("+")** and others set to ignore **("0")** for the second collection
For Stroma segmentation, FITC 525 (SYTO 13) is set to positive **("+")** and others are set to ignore **("0")** for the third collection

Then click **Generate Segments.**

6.4 Once all segments are automatically generated, each channel parameter needs to be manually adjusted with pathologist's input to confirm if the segmentation is correctly done. 1h 30m

Caution: less than 20 cells in each segment is removed from collection due to threshold for low signal.

6.5 Once all AOI segmentation is complete, Exit Scan Workspace button icon is clicked to approve ROI selection and samples are collected in 96-well plate. 3h

Hybridization

18h 10m

7 Hybridization

7.1 After completion of sample collection, the 96-well plate is finalized, removed from DSP and transferred to the PCR thermocycler. 1h
DNA oligo samples in 96-well plate are completely dehydrated at 60 °C for 01:00:00 in PCR thermocycler.

Caution: the plate lid of PCR thermocycler should be opened completely to avoid the contamination of DNA oligo from evaporation during this dry step.

7.2 Add 10 µL of DEPC-treated water to each well and resuspend the DNA oligo samples for 00:30:00 at RT. 30m

7.3 (1) Add and mix well 12 µL of each Probe A per panel to a 1.5ml Eppendorf tube (labeled Pr A tube) 10m

Since 7 commercial panels and 1 custom panel (consists of 2 Probe A) are used, the, total volume is 108 µL (= 12ul x 9 Probe A) in the Probe A tube.
(2) Add and mix well 12 µL of Probe B (Universal probe) with 87 µL of DEPC water in a fresh 1.5ml Eppendorf tube (labeled Probe B tube, total volume is 99 µL).
(3) Calculate and add 80 µL of hybridization buffer per row into a fresh 1.5ml Eppendorf tube (labeled Master mix tube). For 44 core samples, we collected a total 8 rows in the collection plate so total (= 80 x 8 rows) 640 µL of hybridization buffer was added.

The Probe/Master Mix is made by adding 64 µL from the Probe A tube and 64 µL from the Probe B into the Master mix tube (64ul + 64ul + 640ul).

7.4 The Hybridization CodeSet for each row (A-H) is taken out from -80 °C and thawed at RT 10m
84 µL of Probe / Master mix is added to each hybridization Codeset and mixed well.

7.5 Prepare the hybridization plate separately (96-well plate) 20m
8 µL of hybridization CodeSet mix was added to each well per each row in the hybridization plate.
3 µL of DNA oligo samples from the rehydrated plate (out of 10ul) was mixed into each hybridization Codeset (8ul + 3ul = total 11ul / well).

7.6 Seal the hybridization plate with aluminum foil using the microplate heat sealer and briefly spin it down in a centrifuge at 2000g.

7.7 Samples are hybridized at 67 °C for 16:00:00 in PCR Thermocycler and then kept at 16h
4 °C until loading in **nCounter MAX system.**

nCounter reading 5h 45m

8 nCounter preparation and reading

8.1 In the DSP server, the collection plate is finalized and the library preparation file for nCounter load 15m
is downloaded.
The sample loading volume is determined (it will vary) according to the area size calculation of total ROI/AOI in each column.
The Samples from each row (A-H) are collected and transferred into a 12-well strip PCR tube using a 12-channel multi-pipette.

8.2 Briefly, the samples are spun down in the 12-well PCR strip and then transferred to Nanostring's MA 3h
Prep-station to load samples onto the cartridge with the standard sensitivity setting.

8.3 The cartridge is transferred to nCounter to read the counts with defined **CDF (CodeSet Design I** 2h 30m
setting downloaded from the DSP plate information.

8.4 The **RCC (Reporter Code Count) file** from nCounter reading is downloaded to your PC and imported into the DSP server.

DSP data analysis

9 QC DSP data and analysis

9.1 Select and queue the slides to analyze using **"New Analysis"** in the DSP server.

9.2 Determine the New Analysis file name and save it in the designated folder.

9.3 Open an analysis file and perform the **QC** with preset parameters.

9.4 QC passed samples are processed and the QC file (CSV) is downloaded to a PC for further analysis.

Comment: All documents related with GeoMx DSP run can be found at https://university.nanostring.com/page/document-library