TECH NOTE

Combined Workflow of GeoMx® Cancer Transcriptome Atlas and RNAscope™ Assays

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Introduction

Tumor biology is complicated by interactions between the host, the tumor, and the tumor microenvironment (TME). Investigating these interactions and the role different cell populations play is key to understanding tumor pathology and identifying potential therapeutics. Spatial technologies, such as immunohistochemistry (IHC) and in situ hybridization (ISH), are key tools in translational oncology but have historically been limited by the number of targets analyzed at one time. RNA-Seq and other Next Generation Sequencing (NGS) technologies generate high-plex data but lack spatial context. The GeoMx® Cancer Transcriptome Atlas (CTA) for use with the GeoMx Digital Spatial Profiler (DSP) enables high-plex data combined with spatial information using an NGS readout. CTA detection probes consist of a targeting oligo for in situ RNA analysis and an NGS-compatible GeoMx DSP barcode separated by a UV-photocleavable linker for ex situ RNA quantification (**FIGURE 1**). After hybridization of CTA probes and morphology markers to a slide-mounted tissue section, the user selects region of interest (ROI) based on the immunofluorescence and the experimental question at hand. After ROI selection, the DSP barcodes are released from each ROI upon UV exposure. Released tags are quantitated with NGS and counts are mapped back to tissue location, yielding a spatially-resolved digital profile of analyte abundance. The CTA covers 1,834 RNA targets with critical relevance across tumor biology, the immune response, and the tumor microenvironment.

GeoMx RNA assays, such as CTA, are compatible with antibody staining for tissue visualization and ROI selection[1], however the mild proteinase K digestion during slide preparation does digest some antigens. Furthermore, many biological targets such as chemokines and cytokines are difficult to raise antibodies against. Here, we describe how to circumvent these issues by using the RNAscope™ ISH assay (Advanced Cell Diagnostics; ACD) as a morphology marker to guide ROI selection in the GeoMx CTA assay. The RNAscope assay employs a unique signal amplification strategy that allows for the visualization of single molecules of RNA with high sensitivity. The single molecules are visualized as punctate dots and provide morphological context at the single cell level. High specificity is achieved through stringent probe design that requires sets of probes to bind adjacently on a transcript. Each RNAscope probe consists of an average of 20 of these pairs. Furthermore, the RNAscope assay is widely used in formalin-fixed paraffin-embedded (FFPE) and fresh frozen tissues and is compatible with tyramide signal amplification (TSA). TSA increases the sensitivity of the assay, particularly in FFPE tissues, and results in fluorophores that are covalently bound to the molecular scaffold, thus enabling the labeling of slides with GeoMx DSP CTA detection probes while maintaining RNAscope signal. More information can be found in the GeoMx and ACD Manuals. Using ACD's catalog of >21,000 ISH probes, users are able to select ROIs guided by nearly any RNA target[3].

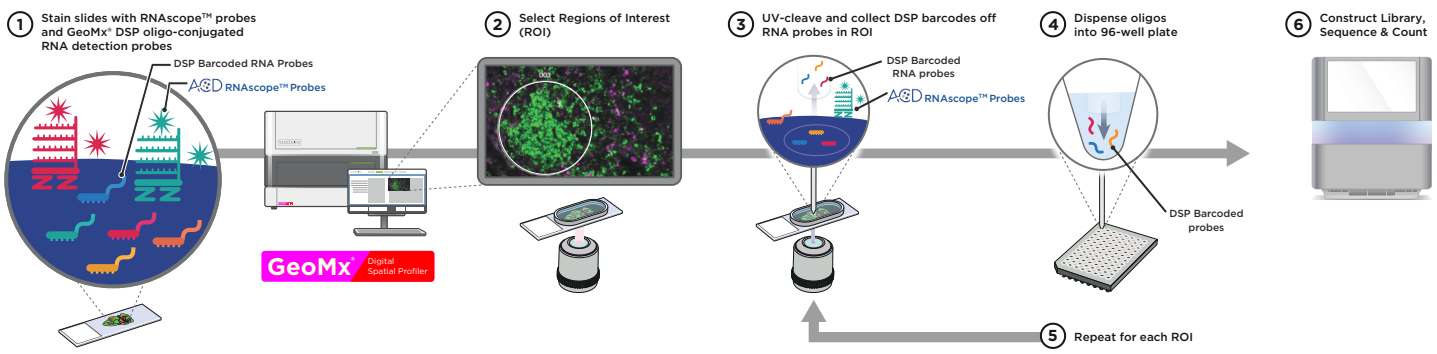


FIGURE 1. Overview of the Combined GeoMx DSP and RNAscope workflow.

RNAscope and GeoMx DSP Integrated Workflow

When using RNAscope as a morphology marker for GeoMx RNA assays, the two assays are performed in series with minimal alterations to each workflow. A user performs the RNAscope assay using either the manual assay (RNAscope Multiplex Fluorescent V2) or the automated assay for Leica Systems (RNAscope 2.5 LS Fluorescent Multiplex Assay). The RNAscope assay is performed first, followed by hybridization of GeoMx probes and finally, on instrument ROI selection and DSP barcode collection. The Illumina library preparation and sequencing can be performed any time after GeoMx DSP collection is finished (**FIGURE 1**).

The RNAscope workflows are performed according to the user manuals provided by ACD. The minor modification is to omit the nuclei marker DAPI, which is replaced with SYTO 13 after the GeoMx RNA assay. DAPI is not used in GeoMx assays as the emission wavelength is close to the absorption wavelength of the UV linker. RNAscope probes can be visualized with one to three fluorophores for each open channel on GeoMx DSP alongside the DNA stain. Target retrieval and protease digestion guidelines provided by ACD are comparable to those suggested for the GeoMx RNA assays. Further details can be found on NanoString's and ACD's websites.

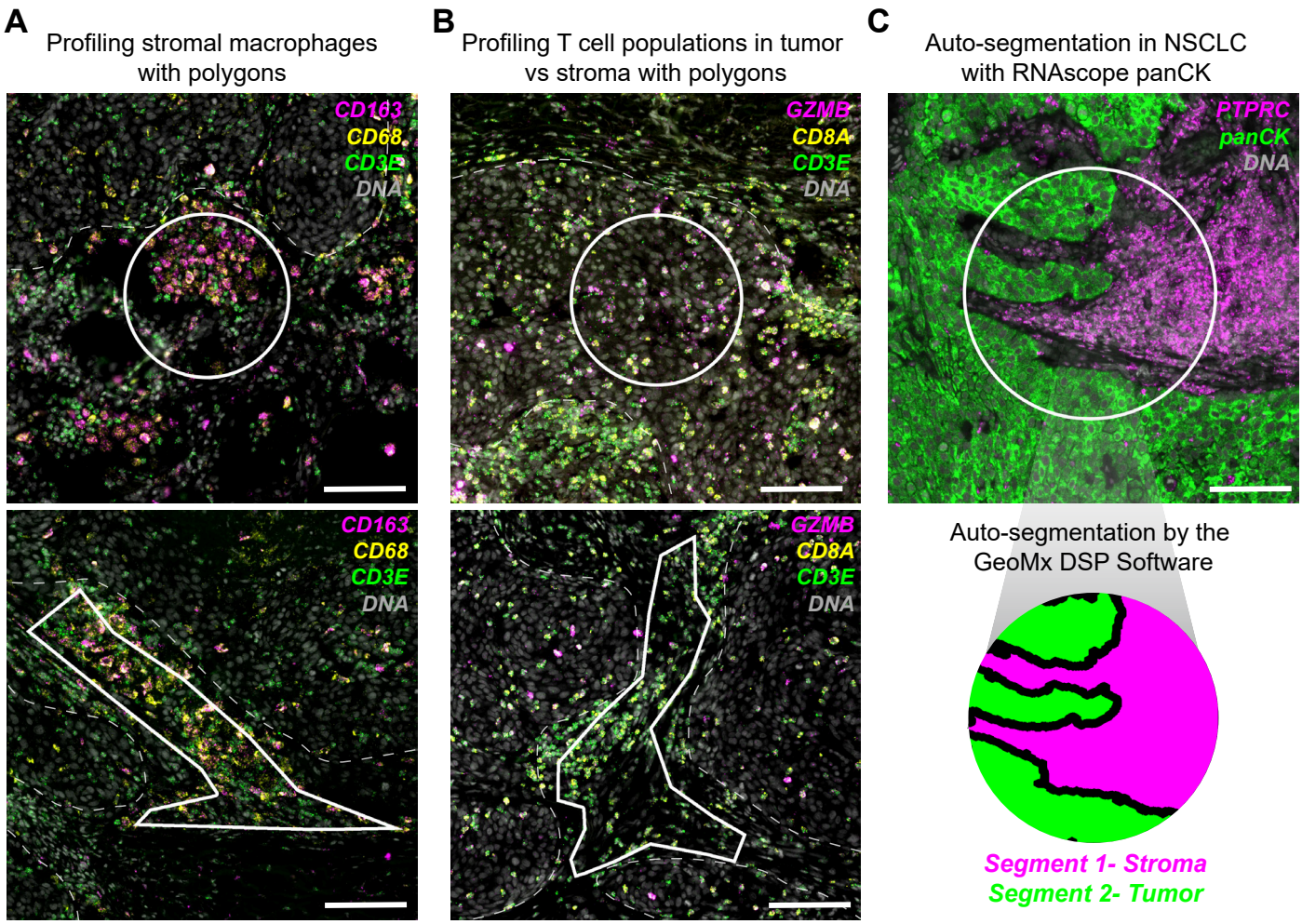


FIGURE 2. Examples of ROI selection using RNAscope probes in NSCLC. A) Stromal ROIs were used to profile different macrophage populations (CD163, CD68). 200 µm diameter circles were used when tumor morphology allowed, otherwise the GeoMx polygon tool was used (bottom). B) Cytotoxic T-cell populations were profiled in tumor (top) or stroma (bottom). C) Auto-segmentation was performed using the GeoMx auto-segmentation tool for the panCK-positive region (tumor, green) and the panCK-negative region (stroma, magenta). A-C) Scale bars are 100 µm, dashed lines are the tumor-stroma border, solid lines are the perimeter of each ROI.

Qualified RNAscope Probe Combinations for Single Molecule Visualization and ROI Selection

Using the protocols in series enables the use of any RNAscope probe in ACD's catalog. Immune cell type markers are particularly helpful in defining ROI for GeoMx DSP. Here, we selected ten combinations of RNAscope cell type markers that have been qualified for use with the GeoMx CTA (**TABLE 1**). While data was collected for all probe sets outlined in **TABLE 1**, we will focus on one particularly unique case study in this tech note. These probe combinations were chosen because they identify critical immune cell types and phenotypes in immunology that have historically been difficult to identify in tissue.

RNAscope provides single molecule resolution for morphology driven ROI selection. Staining with RNAscope results in a punctate dot that identifies the location of every targeted transcripts. Users can then employ the various ROI selection modalities available in the GeoMx DSP software. For example, Geometric profiling can be used to assess tissue heterogeneity by profiling with standardized geometric shapes or user-defined polygons across distinct tissue regions (**FIGURES 2A,B**). Segment profiling through the GeoMx software or ImageJ scripts can segment on tissue images to profile unique biological compartments within an ROI based on RNAscope probes (**FIGURE 2C**). Contour profiling evaluates how proximity affects biological response and the local microenvironment around central structure or tissue boundary (not shown). The user can choose the profiling method that best suits their experimental needs. Tumor can be separated from stromal regions for high-plex analysis by segmenting on panCK. Alternatively, geometric ROI may be more appropriate to compare regions of viral infection versus uninfected cells. Combining RNAscope with GeoMx DSP for ROI selection uncovers important aspects of immune and tumor biology.

High-Plex RNA Profiling of Tumors with CTA

A non-small cell lung carcinoma (NSCLC) sample was identified in previous studies to have high expression of CD274, which encodes the key immune-checkpoint inhibitor programmed death-ligand 1 (PD-L1). In those studies, the cellular source of CD274 was not identified. To identify the source location of CD274 signal and further explore this sample, RNAscope probes for CD274, PDCD1 (PD-1), and CD3E were used for ROI selection and analysis with the GeoMx CTA. CD274 was found to be expressed predominantly in tumor cells based on nuclear morphology (**FIGURE 3A,B**). ROIs were placed in either the tumor or stroma while taking into consideration relative abundance of the RNAscope signal. Mean fluorescence intensity was measured per ROI for CD274 signal and shown to correlate with normalized counts for CD274 from the CTA assay (Pearson's R2 = 0.89; **FIGURE 3C**).

The high-plex data generated by the CTA allows for a variety of analyses to be performed in the DSP Data Analysis Suite, namely: unsupervised hierarchical clustering, differential gene expression, and pathway analysis. To demonstrate the depth of data generated and how expression patterns change with ROIs, unsupervised hierarchical cluster was performed on both ROIs and the top 600 targets. ROIs clustered predominantly by stroma and tumor, with some sub-clustering by CD274 signal. Targets fell into four predominant clusters based on their spatial expression with immune pathways being predominately represented in stromal ROIs and cell signaling, innate immunity, and immune response genes having higher expression in tumor ROI (**FIGURE 3D**).

To quantify differential gene expression in CD274-high and -low tumor regions, a linear mixed effects model was used to calculate fold-change (FC) and p-values for each gene. In these tumor regions, 360 genes are shown to be differentially expressed. Of genes with higher expression in CD274-high tumor, many were associated with interferon signaling, immune response, and key chemokines (**FIGURE 3E**). To quantify the gene expression at the pathway level, data was subject to a Gene Set Enrichment Analysis (GSEA). GSEA confirmed the higher expression of interferon signaling genes as well as demonstrating significant enrichment of interferon signaling pathways (alpha/beta and gamma) as well as chemokine and cytokine signaling pathways (**FIGURE 3F**).

Conclusion

The combined workflow of RNAscope and GeoMx DSP allows for the single molecule visualization and molecularly guided high-plex profiling of biologically driven ROI from FFPE and fresh frozen samples. Here we describe qualified RNAscope probe combinations selected for cell type markers shown to be compatible with the GeoMx CTA assay and present data from one of those sets from ACD's large catalogue of validated ISH probes. We show concordance between the RNAscope and GeoMx assays and demonstrate the detailed molecular analysis that can be performed with this type of high-plex spatial data. Additional information about the RNAscope and GeoMx DSP combined workflow, compatibility, concordance, and using RNAscope to confirm GeoMx DSP findings can be found in the white paper made available by NanoString Technologies[4].

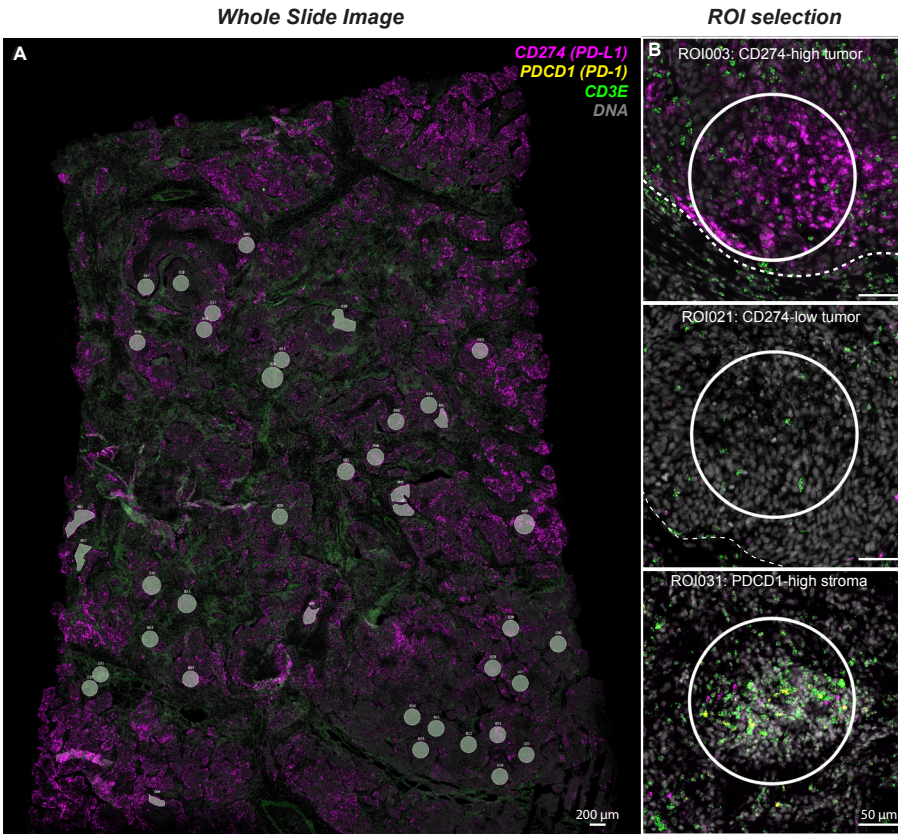
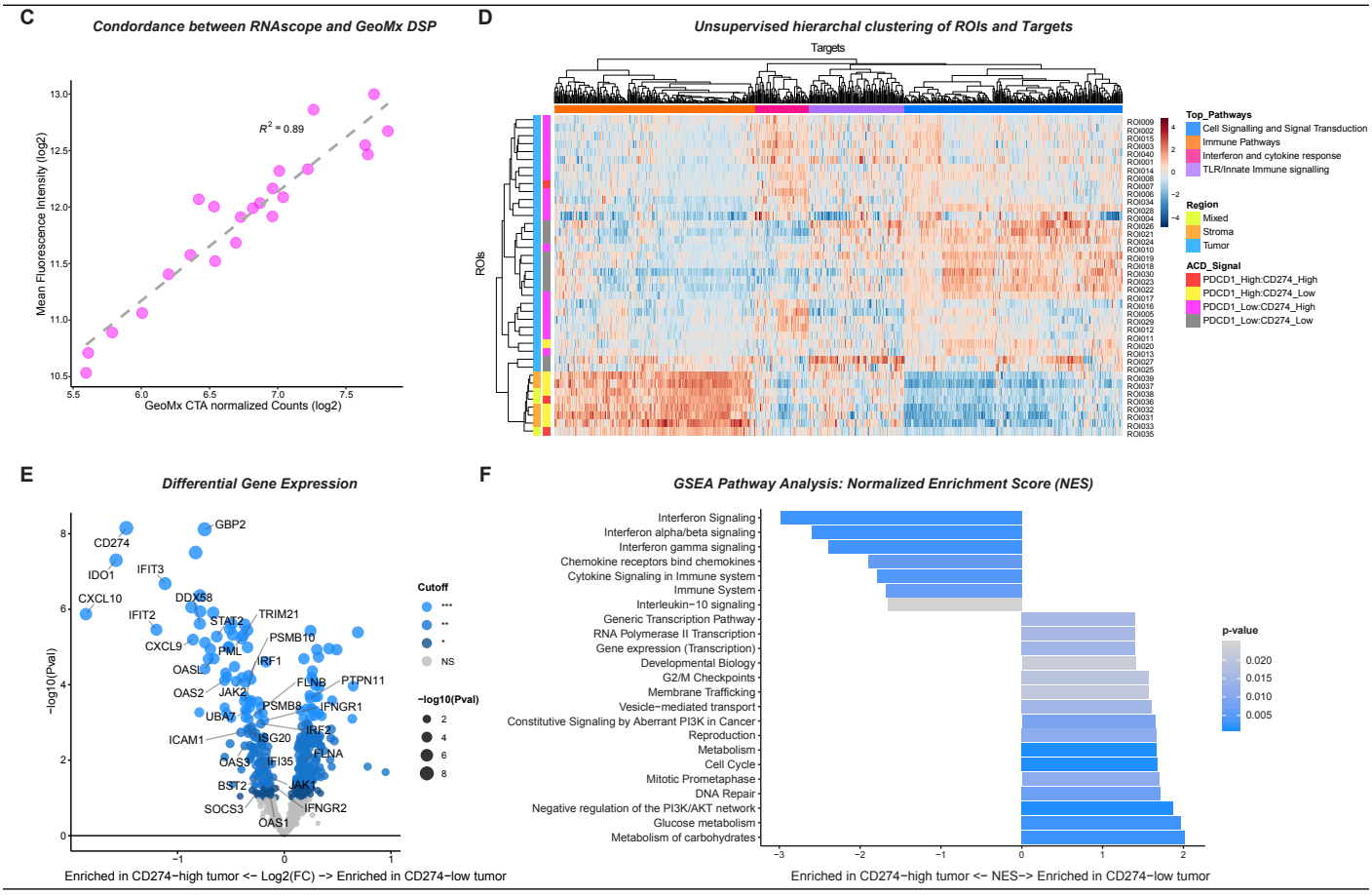


FIGURE 3. A 5 µm thick FFPE section of NSCLC was stained with three RNAscope probes (CD274, PDCD1, and CD3E), a nuclei stain, and the GeoMx CTA. A) RNAscope signal guided ROI selection throughout the tissue. Nuclei morphology was used to distinguish between tumor and stroma (dashed line indicates tumor-stroma border. Geometric ROIs (200 µm diameter circles) and polygon ROIs were used. B) ROIs were classified into three categories based on qualitative RNAscope signal. ROIs are 200 µm diameter circles. C) Mean fluorescence intensity for CD274 RNAscope signal was calculated in ImageJ and plotted against DSP counts for CD274 from the same ROI (Pearson's R-squared = 0.89). Only CD274-high regions are shown. D) Unsupervised hierarchical clustering of ROIs and targets identifies four main clusters of each. ROIs placed in tumor tend to cluster separately from stroma and mixed ROIs (both tumor and stroma). Gene expression falls into four main clusters. An over representation analysis was performed on genes within each cluster and the top pathways are annotated. E) Differential gene expression analysis between CD274-high and -low regions identified key genes in immune function. P-values and fold-changes (FC) were calculated with a linear mixed-effects model. Top-10 significant differentially expressed genes and those involved with interferon signaling are indicated with labels. F) Gene Set Enrichment Analysis (GSEA) identifies the top pathways by normalized enrichment score (NES) specific to each region.



Ordering information

GeoMx RNA assays are compatible with both the automated and manual RNAscope kits. See **TABLE 2** for ordering information. In order to use the automated Leica systems (BOND RX or RXm), users will need a Leica BOND Research Open Detection System, Leica BOND 30 mL Open Containers, and BOND Titration Kit in addition to standard Leica BOND buffers, covertiles, and cleaning kits. Ordering information for probe sets is available in **TABLE 1**.

Prequalified sets can be purchased in the configuration presented below. Probes can be combined in any combination, provided channel assignments do not overlap on a given slide. For example, GZMB could be ordered on channel three and combined with the CTLs (set 1) probe set. Probes are available on various channels from ACD. The GeoMx Cancer Transcriptome Atlas (CTA) can be ordered from Nanostring Technologies. See the CTA Product Bulletin for more information.

Contact GeoMxSupport@nanostring.com for more information

Probe Combination	Target	Channel	Manual Order #	Leica Order #	Tested Tissue
Immune checkpoint	PDCD1 (PD-1)	C1	602021	602028	NSCLC
	CD274 (PD-L1)	C2	600861-C2	600868-C2	
	CD3E	C3	553971-C3	553978-C3	
Tumor vs. Stroma	panCK	C1	404751	404758	NSCLC
	PTPRC (CD45)	C2	601991-C2	601998-C2	
	CD3E	C3	553971-C3	553978-C3	
CTLs	CD8	C1	560391	560398	NSCLC
	IFNG	C2	310501-C2	310508-C2	
	CD3E	C3	553971-C3	553978-C3	
CTLs	CD8	C1	560391	560398	Tonsil
	GZMB	C2	445971-C2	445978-C2	
Activated T regs	CD4	C1	605601	605608	Tonsil
	FOXP3	C2	418471-C2	418478-C2	
	TIGIT	C3	319791-C3	319798-C3	
Immune suppression	TGFB1	C1	400881	400888	Tonsil
	FOXP3	C2	418471-C2	418478-C2	
Macrophages	CD68	C1	560591	560598	Tonsil
	CD163	C2	417061-C2	417068-C2	
	CD3E	C3	553971-C3	553978-C3	
M1 Macrophages	CD68	C1	560591	560598	NSCLC
	CXCL10	C2	311851-C2	311858-C2	
M2 Macrophages	CD163	C1	417061	417068	TMA
	CCL22	C2	468701-C2	468708-C2	
	CD3E	C3	553971-C3	553978-C3	

Note: Channel 1 probes are ready-to-use (RTU) and the other channel probes are 50X concentrated stocks. When using a probe combination without a Channel 1 probe, use Blank Probe Diluent (#300041 for manual and #300048 for Leica).

		Manual Order #	Leica Order #
Control probes	positive control 3-plex	320861	320868
	negative control 3-plex	320871	320878

Product	Supplier	Catalog Number
Manual Assay		
RNAscope Multiplex Fluorescent Kit v2	Advanced Cell Diagnostics	323100
TSA Kits	Various Vendors	
RNAscope probes	See Table 1	
Automated		
RNAscope LS Multiplex Reagent Kit	Advanced Cell Diagnostics	322800
Leica BOND Research Detection System	Leica Biosciences	DS9455
Leica BOND 30 mL Open Containers	Leica Biosciences	OP309700
Leica BOND titration kit	Leica Biosciences	OPT9049
TSA Kits	Various Vendors	
RNAscope probes	See Table 1	

References:

1. Merritt CR, et al. 2020. Multiplex digital spatial profiling of proteins and RNA in fixed tissue. Nat. Biotech. In Press.

2. Wang, et al. 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn. Jan;14(1):22-9.

3. Anderson et al. 2016. Fully Automated RNAscope In Situ Hybridization Assays for Formalin-Fixed Paraffin-Embedded Cells and Tissues. J Cell Biochem. 2016 Oct;117(10):2201-8.

4. Merritt CR, et al. (2019). Molecularly Guided Highly Multiplexed Spatial Profiling with the RNAscope® and GeoMx™ Assays [White paper]. NanoString Technologies. http://www.nanostring.com/download_file/view/2038

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