5237 High-throughput slide preparation for spatially-resolved, multiplexed quantification of protein or mRNA in tumor tissues with automation of GeoMx® DSP Assays on Leica Biosystems BOND RX

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Abstract

Characterization of the spatial distribution and abundance of proteins and mRNAs with morphological context within tissues enables a better understanding of biological systems in many research areas, including immunology and oncology. The GeoMx Digital Spatial Profiler (DSP) enables the highly-multiplexed detection of mRNA or protein targets within distinct regions of tissues, including FFPE. This assay utilizes in situ hybridization (ISH) probes or antibodies linked to indexing oligo barcodes via a photo-cleavable linker. The GeoMx DSP enables profiling of RNA or protein targets by releasing and collecting these barcodes from user-defined regions-of-interest. Manual preparation of FFPE tissue uses conventional immuno-fluorescence (IF) and ISH reagents, however it requires greater than two hours of hands-on-time over 2 days. Protocols have been developed to enable the automation of these tissue preparation protocols using the Leica Biosystems BOND RX and BOND RX^m Fully Automated Research Stainers. Here we describe protocols for the semi-automated preparation of GeoMx RNA and protein assays as well as the fully-automated preparation of GeoMx RNA assays. These protocols reduce the hands-on time of performing the assay to 72 minutes and 35 minutes for the semi- and fully-automated protocols, respectively. Signal-to-noise ratios (SNR) are highly concordant between serial sections processed manually and processed on the Leica BOND RX^m using the GeoMx Immune Cell Profiling Panel for protein (58-plex) and GeoMx Immune Pathways Panel for RNA (84-plex). Furthermore, automated assays show high concordance between the BOND RX and BOND RX^m instruments.

GeoMx and Leica Biosystems BOND RX Workflows

Leica Biosystems BOND RX

FFPE slide preparation

The Leica Biosystems BOND RX is a fully automated IHC and ISH stainer used for the high throughput and highly reproducible preparation of tissues such as formalin-fixed paraffin embedded (FFPE) samples. Here we show how the BOND RX system can be used to prepare slides for Nanostring GeoMx RNA and Protein assays. The GeoMx system consists of reagents, instrument, and software for the high-plex, high throughput profiling of protein or RNA in FFPE tissues. GeoMx detection reagents are RNA antisense oligo probes or antibodies conjugated to photocleavable indexing oligonucleotides (PC-oligo) for high-plex RNA or protein analysis. The GeoMx Digital Spatial Profiler Instrument (DSP) uses focused UV-light to release the PC-oligo from user-defined regions of interest (ROI).

To preform the GeoMx assays, 5 µm. FFPE sections are mounted on charged slides, baked, and prepared manually or on the Leica Biosystems BOND RX and BOND RX^m (Tables 1 and 2). Sections are then stained off-instrument with GeoMx detection reagents and fluorescent visualization markers. The DSP scans the entire tissue area generating a 4-channel fluorescent image to capture tissue morphology for selection of ROI. The DSP then cleaves the PC-oligos from a ROI, collects them using a microcapillary, and dispenses into a collection plate. PC-oligos are then quantified using with Nanostring nCounter® technology or Illumina NGS sequencing platforms. The GeoMx DSP software maintains the spatial relationship of the ROIs and the cleaved PC-oligos for mapping back gene quantitation onto the tissue image.

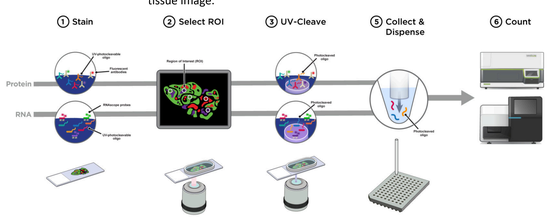

Figure 1. GeoMx DSP workflow

BOND RX automation reduces hands on time for GeoMx assays

Table 1: Detailed RNA Workflow

STEP	Reagent	Compatible BOND Reagent	Supplier	Manual Equipment
Deparaffinize*	Xylene (or Citrasolv)	BOND Dewax solution	Various	Coplin jars
	200 Proof Ethanol	-	Various	Coplin jars
Pretreat*	Tris EDTA pH 9.0	BOND ER2	Sigma	Pressure cooker or steamer
Protease digest*	Protease K	Enzyme Pretreat kit	Ambion	Water bath
Post-fixation*	10% NB-Formalin	-	Various	Fume hood
In situ hybridization (37C)	Hybridization buffer	-	NanoString	Hybridization Oven
	GeoMx RNA detection probes	-	NanoString	Water bath
Stringent wash (37C)	50% Formamide/ 2x SSC	-	Various	Water bath
Morphology Reagents	GeoMx Nuclei Stain	-	NanoString/Various	Staining Chamber

**automated by BOND RX in semi-automated protocol*

Table 1: The GeoMx RNA assay FFPE slide preparation protocol consist of multiple steps that have been optimized for GeoMx assays. The BOND RX system enables the automation of either all steps (fully-automated) or just steps preceding the overnight in situ hybridization (semi-automated). The semi-automated protocol is commercially available while the fully-automated protocol is still under development as a collaboration between Leica Biosystems and Nanostring. These protocols reduce the measured hands-on time of performing the assay from over 2 hours with the manual protocol to 72 minutes and 35 minutes for the semi- and fully-automated protocols, respectively. Furthermore, the automated assays reduce the equipment required for the GeoMx assays and are compatible with common BOND reagents. This protocol can be used for any GeoMx RNA assay, including the Cancer Transcriptome Atlas.

Table 2: Detailed Protein Workflow

STEP	Reagent	Compatible BOND Reagent	Supplier	Manual Equipment
Deparaffinize*	Xylene (or Citrasolv)	BOND Dewax solution	Various	Coplin jars
	200 Proof Ethanol	-	Various	Coplin jars
Antigen Retrieval*	Citrate Buffer pH 6.0	BOND ER1	Sigma	Pressure cooker
Blocking*	Buffer W		NanoString	Staining Chamber
Antibody Incubation	Buffer W and GeoMx antibodies		NanoString	Staining Chamber
Post-fixation	10% NB-Formalin	-	Sigma	Fume hood
Morphology Reagents	Buffer W and GeoMx Morphology Reagents	-	NanoString/Various	Staining Chamber

**automated by BOND RX*

Table 2: The GeoMx protein assay FFPE slide preparation protocol consist of multiple steps that have been optimized for GeoMx assays. The BOND RX system enables the automation of steps preceding the overnight antibody incubation at 4˚C. This protocol reduce the hands-on time of performing the assay from 2 hours to ~30 minutes, reduces the equipment required for the GeoMx assays, and is compatible with common BOND reagents.

Automated assays show high concordance with the GeoMx manual assay

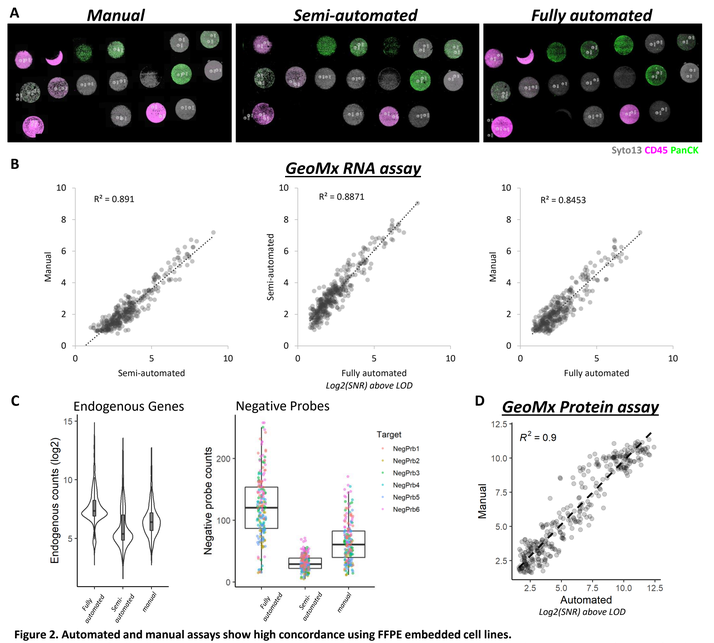

Figure 2. Automated and manual assays show high concordance using FFPE embedded cell lines.
A) FFPE sections of cell pellet arrays were either prepared manually or using the BOND RX^m. Antibodies against CD45 (magenta) and pan-cytokeratin (PanCK; green) and the GeoMx nuclei stain (gray) were used a morphology markers. This experiment was performed with both the GeoMx Immune Cell Profiling Panel for protein (58-plex) and GeoMx Immune Pathways Panel for RNA (84-plex) during subsequent experiments. Images shown are for the RNA assay. Duplicate 300 µm diameter circular ROIs were placed on each cell pellet. **B)** Signal to noise ratios (SNR) were calculated for each target per ROI. Counts above the limit of detection (LOD; average of negative controls plus 3 SD) are were plotted and a Pearson's correlation coefficient (R) was calculated between manual and automated slide preparations. For all assays, R^2 values were >0.84 showing strong concordance between preparation methods. **C)** The GeoMx Immune Pathways Panel for RNA consists of 78 targets against endogenous genes and 6 probes targeting sequences identified by the External RNA Controls Consortium as negative controls (termed negative probes). The fully automated protocol for RNA assays show slightly higher counts for endogenous genes as well as negative controls, suggesting further optimization and background reduction is necessary. Endogenous gene counts are comparable between manual and semi-automated assays while background is slightly lower for slides prepared with the semi-automated protocol. Thus, the semi-automated protocol does not increase background in the GeoMx RNA assays. **D)** SNR for protein targets above the LOD show high concordance between automate and manual slide preparation protocols (R^2 = 0.900).

BOND RX^m and BOND RX generate highly reproducible results

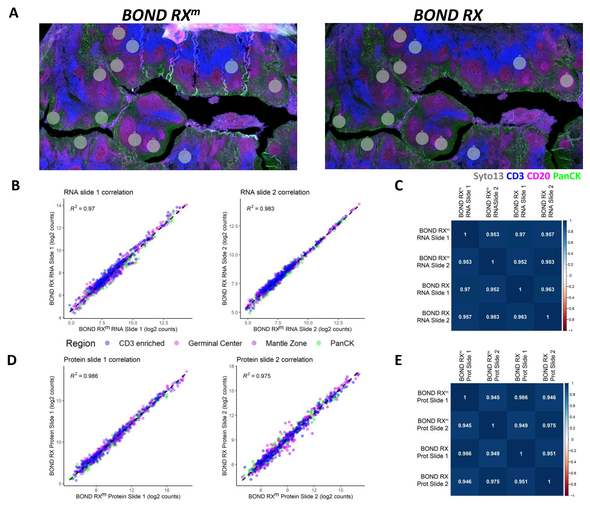

Figure 3. Leica Biosystems BOND RX^m and BOND RX instruments generally highly concordant results in serial sections. **A)** GeoMx RNA and Proteins assays were performed on eight serial sections of FFPE lymphatic tissue. The semi-automated slide preparation protocols were performed with either the BOND RX or BOND RX^m. Sections were stained with the GeoMx Immune Cell Profiling Panel for protein and GeoMx Immune Pathways Panel for RNA. Slides were stained with morphology markers for CD3E (blue), PanCK (green), CD20 (magenta), and the GeoMx nuclei stain (gray). To determine gene expression in varying regions of the tissue, four 300 µm diameter circular ROI were placed in either germinal centers (CD20 positive), mantle zones (CD20 and CD3 positive), CD3-enriched regions, or epithelium (PanCK positive). **B)** Counts from direct serial sections from the RNA assay show high concordance (R^2 > 0.97). Average fold change of counts is 0.995 ± 0.19 (mean ± SD) showing slides prepared with either instrument generate equivalent counts. **C)** Concordance of counts across all four serial sections show high concordance (R^2 > 0.95). **D)** Counts from direct serial sections from the Protein assay show high concordance (R^2 > 0.98). Average fold change of counts is 0.989 ± 0.34 (mean ± SD) showing slide prepared with either instrument generate equivalent counts. **E)** Concordance of counts across all four serial sections show high concordance (R^2 > 0.945).

Conclusions

- The Leica Biosystems BOND RX provides an automated solution for the preparation of slides for GeoMx RNA and Protein assays.
- Automation decreases hands on time.
- High concordance is shown between slides prepared manually or with the BOND RX.
- Initial experiments show full automation of the RNA assays generate highly concordant results with slightly higher background. Further optimization is underway in collaboration with Leica Biosystems.
- BOND RX^m and BOND RX platforms generate highly reproducible results.

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