Validation of NanoString® technologies for solid tumor clinical studies:

Robustness, precision and inter-site comparison

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Cerba Research
A referenced service provider by nanoString

Background

Cerba Research Montpellier (CRM) is a histopathology specialty lab, which is part of the larger Cerba Research group. Pioneers in multiplex immunofluorescence development on FFPE tissues, CRM recently acquired two NanoString® platforms to propose transcriptomic and proteomic analysis of samples to our clients. To be used in solid tumors clinical trials (and possibly in other therapy areas), CRM performed an internal validation of RNA analysis protocols for both bulk analysis with the nCounter or spatial analysis with the whole transcriptome atlas (WTA) with the GeoMx DSP platform.

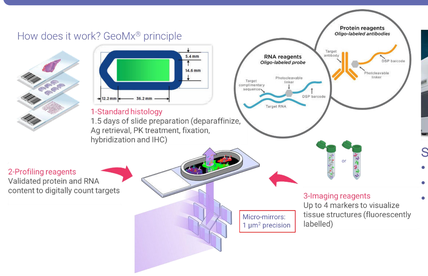
NanoString® technologies workflows at CRM

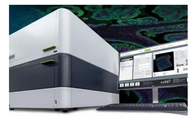
nCounter protocol

Day 0: RNA extraction and QC
- *2 curls of 10µm thickness*
- *IVD-labelled protocol for RNA extraction of FPPE samples*
- *RNA QCs with Agilent Tapestation 4150*

Day 1&2: nCounter®
- *1 cartridge = 12 samples*
- *15 min setup*
- *Overnight hybridization*
- *Load onto system*
- *Analyze results*

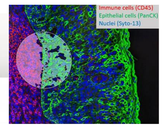
GeoMx DSP workflow





Study Design (CRM)
- Study design
- Protocol development
- Sample management, processing and data collection for GeoMx®

NGS (Cerba NL)
- Sample management (collection plates)
- NGS process and QCs
- Data processing
- Possibility of BioIT



GeoMx® Analysis (CRM)
- GeoMx® analysis, e.g.
  - QCs
  - Normalization
  - Analysis
- Report

Internal validation of bulk RNA analysis (nCounter)

Specificity on cell lines and FFPE samples

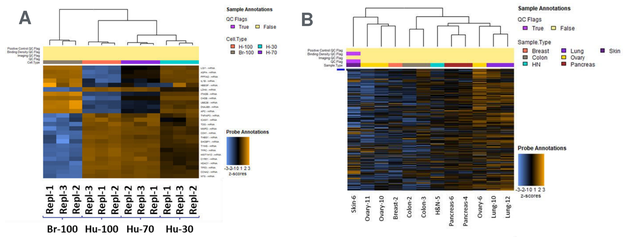
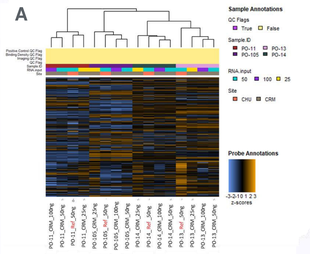


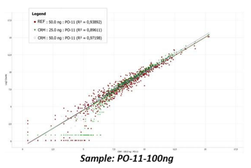
Figure 1: Unsupervised clustering of data was obtained with nSolver (V4.0.70) using the advanced analysis module. Only one sample was flagged (purple, top left of the panel) for high binding density. **A.** Data obtained on RNA from reference cell lines **B.** Data obtained on various FFPE samples from CRM biobank

Methods:
- *Two RNA references from cell lines (human (Hu) and brain (Br)) supplied by NanoString®.*
- *4 conditions tested in triplicate: 100% Hu; 70%Hu–30%Br (Hu-70); 30%Hu–70%Br (Hu-30) and 100% Br.*
- *Panel composed of 48 targets.*
- *Various FFPE samples analyzed with IO360 panel.*

Results:
- *Clustering of replicates and scatter plot analysis confirmed nCounter specificity on RNA references. Results correlate perfectly to NanoString's expected data (data not shown).*
- *FFPE matrix grouped by organs and can be separated according to tumor type (ovarian adenocarcinoma vs. papillary vs. serous).*

Inter-site verification and sensitivity test





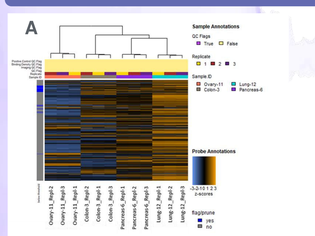
R^2 values	Ref vs. CRM	200ng vs. 100ng	200ng vs.50ng
PO-11	0.95	0.97	0.90
PO-13	0.90	0.97	0.92
PO-14	0.95	0.98	0.93
PO-105a	0.96	0.98	0.88
Mean	**0.94**	**0.98**	**0.91**

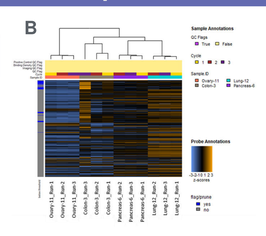
Figure 2: nCounter data from Inter-site analysis. **A.** Unsupervised clustering of data. **B.** Representative scatter plot analysis data obtained with 200ng RNA input. The X-axis is the reference and compared to data obtained with decreasing RNA quantities or the reference sample. Data are represented in Log2 and resume table of R^2 values extracted from scatter plot analysis.

Methods:
- *nCounter® data from 4 NSCLC FFPE samples run at a subcontracted lab (=Ref) vs. extraction and nCounter analysis at CRM.*
- *3 RNA input quantities per sample to evaluate sensitivity limits.*
- *Analyzed with PanCancer pathways panel (770 targets.*

Results:
- *Data from same sample clustered together.*
- *Scatter plot analysis confirmed high inter-site correlation (mean R^2 = 0,94).*
- ***Reduced RNA inputs showed high correlation with recommended quantities (mean R^2 = 0,91). Variations occurred mostly in low expressed genes.***

Precision tests of nCounter protocol





R^2 values	Ovary-11_1	Pancreas-6_1	Colon-3_1	Lung-12_1
Replicate_2	0.99	0.99	0.99	0.99
Replicate_3	0.99	0.99	0.99	0.99
Mean	**0.99**	**0.99**	**0.99**	**0.99**

R^2 values	Ovary-11_1	Pancreas-6_1	Colon-3_1	Lung-12_1
Run_2	0.99	0.99	0.98	0.99
Run_3	0.99	0.99	0.81	0.99
Mean	**0.99**	**0.99**	**0.89**	**0.99**

Figure 3: Precision of nCounter protocol: Results of **A.** Repeatability assessment and **B.** Reproducibility over 3 runs. Unsupervised clustering analysis of the data (on top) and resume table of R^2 values extracted from scatter plot analysis using first replicate (A.) or first run (B.) as reference data.

Methods:
- *RNA extracts from 4 FFPE tumor samples: ovary, colon, pancreas and lung. Analyzed with the PanCancer IO360 panel.*
- *Repeatability: RNA in triplicates in the same run.*
- *Reproducibility: same extract analyzed in 3 nCounter® runs.*

Results:
- *Clustering of replicates from intra- and inter-runs confirmed nCounter repeatability and reproducibility.*
- *Scatter plot analysis showed very high correlation between replicates or runs (mean R^2 > 0.99 or 0.97, respectively).*

Internal validation of spatial RNA analysis (GeoMx)

Specificity of GeoMx protocol

Methods:
- *Two FFPE samples: tonsil and colorectal cancer (CRM) (2 slides each).*
- *Morphological markers: PanCK / CD45 / Syto13 plus p53 IHC on serial CRC slide.*
- *Segment (=AOI) types: CD45 / PanCK-p53neg / PanCK-p53pos.*
- *Analyzed with WTA panel by NGS.*

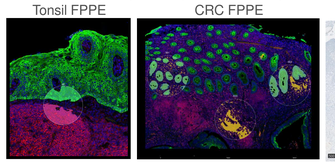
Analysis with GeoMx software:
- *Q3 (Third Quartile) normalization.*
- *Principal Component Analysis (PCA) plot.*
- *Differential expression analysis between PanCK and CD45 segments. Linear Mixed Model (LMM) statistical test to identify targets differentially expressed between PanCK and CD45 segments. Use Cancer Transcriptome Atlas (CTA) panel annotations to guide specificity analysis (expected localization available for our panel targets).*

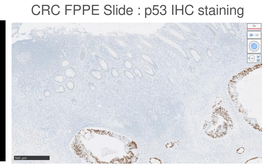
Results:

Principal component analysis:
- *Four populations of segments obtained with main separation criteria being segment types (PanCK or CD45) and organ.*
- *On CRC only, PanCK segments from p53 neg AOIs can be separated from p53 pos.*

Differential expression analysis:
- *CD45 enriched targets (and annotated) all localized in immune cells. Contains also Immunoglobulin-related genes (e.g. IGHA1, IGHG1, IGHG2, IGHG3, IGHG4, IGHM, IGKC, IGLL5, JCHAIN, FCMR or FCRL1).*
- *PanCK enriched targets contain keratin genes and annotations expected in tumor cells. Note that one target is annotated in immune cells but corresponds to CEACAM1 which is known to be enhanced in colon cancer.*

A PanCK (green), CD45 (red), DNA (Blue)





B

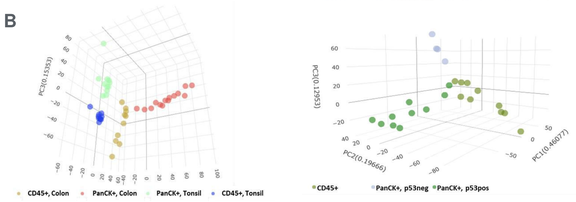
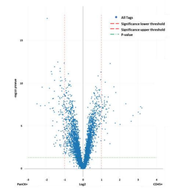


Figure 4: GeoMx specificity analysis. **A.** Representative images of morphology markers staining and p53 IHC on colorectal cancer (CRC) samples. **B.** PCA of all segments (left) or CRC only segments (right).



Segments type	# of target enriched	According to CTA panel annotation		
		# of target annotated	# of targets localized in Tumor	# of targets localized in Immune cells
PanCK+	96	27	26	1
CD45+	56	24	0	24

Figure 5: Differential expression analysis. Volcano plot after LMM statistical tests and summary table of expected localization analysis

Precision tests of GeoMx protocol

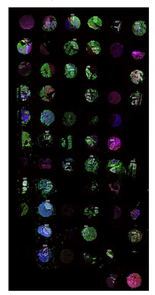
Methods:
- *Multi-organ tumor microarray (TMA) containing head & neck, colon, ovary, pancreas, skin and lung samples.*
- *Precision assessment on PanCK segments (1/sample).*
- *Run-1: Slide-1 (operator-1); Run-2: Slide-2 and -3 (Repeatability, Operator-2); Run-3: Slide-4 (Operator-2).*
- *Analyzed with WTA panel by NGS.*

Analysis with GeoMx software:
- *Selection of a "Repro-dataset" (16 samples)*
- *Q3 normalization.*
- *Correlation plot analysis.*

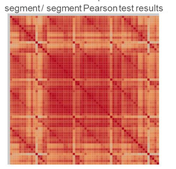
Results:
- *Correlation plot analysis module used to compare linear expression of targets between segments.*
- ***WTA RNA analysis with the GeoMx protocol has a mean correlation for repeatability and reproducibility respectively at 0,97 and 0,84. Our method's precision is properly validated for multi-solid FFPE tissues.***

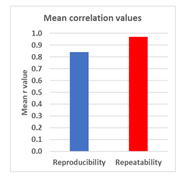
Figure 6:
A. Representative image of the TMA used for precision assessment stained with the morphology markers PanCK (green) and CD45 (red). **B.** Correlation plot obtained on Repro-dataset and summary graph of mean correlation values for repeatability and reproducibility of GeoMx RNA analysis protocol. **C.** Resume table of GeoMx internal protocol validation results.

A Multi-tumor TMA FPPE
PanCK (green), CD45 (red)



B Correlation plot: segment/ segment Pearson test results





C
Spatial transcriptomic analysis process on FFPE samples
Specificity verified on healthy tonsil and colorectal tumor FFPE samples

	GeoMx DSP RNA assay	# of samples tested
Repeatability	**r = 0.97**	N=16
Reproducibility	**r = 0.87**	N=16
Comparison wih bulk RNA analysis	**r = 0.77**	N=11

Conclusions

Based on the data performances, Cerba Research Montpellier robustly validated the use of both nCounter and GeoMx platforms for RNA analysis on clinical study samples from various solid tumors. We are confident that we can use NanoString® technologies for exploratory usage in solid tumor clinical trials.

Future Perspective

Setup and validation of protocol for spatial proteomic analysis (webinar planned for January 2024 and co-organized with NanoString®)

IO PROTEOME ATLAS 570+ GeoMx DSP