EU Instand-NGS4P: Preanalytical Multimodal Workflows for NGS Research and Future Precision Cancer Care

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Introduction

The EU-funded Pre-Commercial Research project Instand-NGS4P (GA no. 874719) aims to improve cancer research by developing complete workflows for NGS, from specimen collection through data analysis and reporting. Multimodal testing involves analyzing multiple specimen types from one individual and/or multiple analytes (e.g., RNA, circulating cell-free DNA [ccfDNA], gDNA) from a single specimen. This can improve diagnostics by combining complementary results and increase sensitivity for earlier cancer detection. We developed 23 novel integrated NGS workflows for research purposes. Here, we present six example workflows that may inform future innovation in clinical applications.

Methods

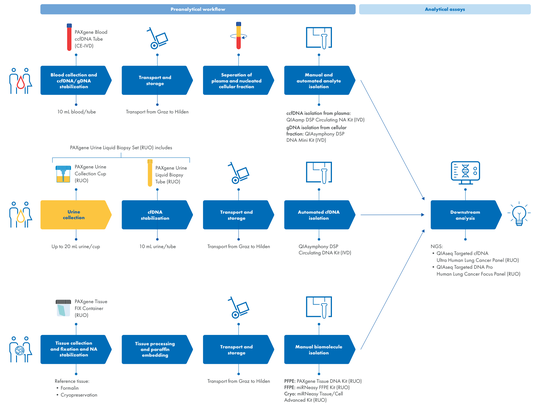
Different specimen types were obtained from seven lung cancer patients at the Medical University of Graz (Austria) under local ethics committee approval.

Whole blood specimens were collected and stabilized in PAXgene® Blood ccfDNA Tubes, urine specimens were collected and stabilized using the PAXgene Urine Liquid Biopsy Set and tumor tissue was preserved by PAXgene Tissue or formalin fixation followed by paraffin embedding (PFPE and FFPE) as well as cryopreservation.

Stabilized specimens were shipped to QIAGEN, Hilden, Germany. Blood ccfDNA and gDNA were extracted using the QIAamp® DSP Circulating NA Kit and QIAsymphony® DSP DNA Mini Kit, urine cell-free DNA (cfDNA) was isolated upon arrival at the laboratory and after additional storage using the QIAsymphony DSP Circulating DNA Kit and tissue gDNA was extracted using the PAXgene Tissue DNA Kit for PFPE samples, QIAamp DNA FFPE Advanced UNG Kit for FFPE samples and QIAamp DNA Mini Kit for cryopreserved samples.

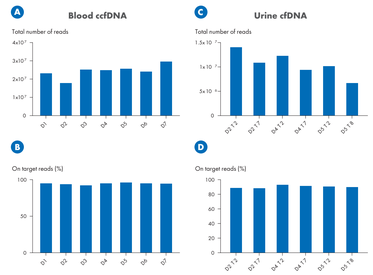
Mutation analysis of cfDNA was performed with the QIAseq® Targeted cfDNA Ultra Human Lung Cancer Panel, while gDNA was analyzed with QIAseq Targeted DNA Pro Human Lung Cancer Focus Panel. Sequencing runs were performed using the Illumina® NextSeq® 500/550 Mid Output Kit v2.5 (300 cycles).

Sample to Insight workflows



Preanalytical workflow. Human whole blood, urine and tumor tissue specimens from lung cancer patients were collected and stabilized using PAXgene® Blood ccfDNA Tubes, PAXgene Urine Liquid Biopsy Sets and PAXgene Tissue Systems, respectively, at the Medical University of Graz. Reference tissue material was stabilized by formalin fixation or freezing. After stabilization, specimens were transported to QIAGEN laboratories in Hilden, Germany. Blood ccfDNA, blood gDNA, urine cfDNA and tissue gDNA were extracted using the QIAamp® DSP Circulating NA Kit, QIAsymphony® DSP DNA Mini Kit, QIAsymphony DSP Circulating DNA Kit and PAXgene Tissue DNA Kit, respectively. Lung cancer mutation analyses were performed using the QIAseq® Targeted cfDNA Ultra Human Lung Cancer Panel for ccfDNA and cfDNA samples, and the QIAseq Targeted DNA Pro Human Lung Cancer Focus Panel was used for gDNA samples.

High ccfDNA sequencing performance



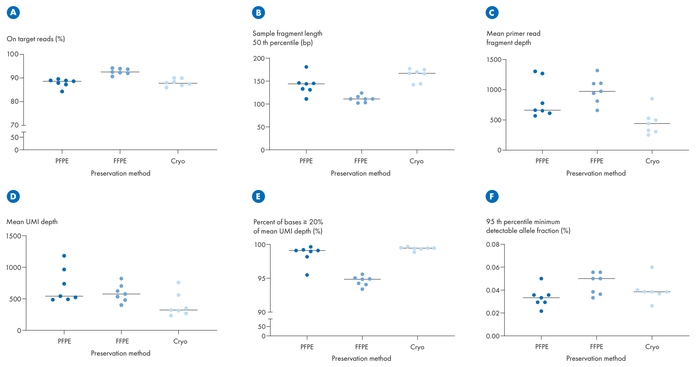
Sequencing performance. Sequencing quality parameters of cfDNA/ccfDNA libraries generated from lung cancer cfDNA/ccfDNA samples using the QIAseq Targeted cfDNA Ultra Kit in combination with the Lung Cancer Ultra Panel. The sequencing run was performed using an Illumina NextSeq 500/550 Mid Output Kit v2.5 (300 cycles). PAXgene Blood ccfDNA samples showed **A** similar numbers of reads and **B** a high target specificity. cfDNA samples were isolated directly after receipt of sample at QIAGEN R&D (T2) and after 5 and 6 days of storage (T7, T8). Most PAXgene Urine cfDNA samples showed **C** similar numbers of reads and **D** all samples demonstrated a high target specificity.

Storage does not interfere with mutation detection

	Donor 1		
	Mutation	2-day storage VAF	7-day storage VAF
Chr 7	7:55146655_C>T	52.1	32.95
	7:55161562_G>A	99.49	100
	7:55171181_T>A	51.35	59.49
	7:55181370_G>A	67.98	62.19
	7:55198724_T>C	89.27	91.95
Chr 10	7:116757518_A>G	62.28	65.27
	7:116771973_A>G	2.02	3.41
	10:43100520_A>G	48.15	48.75
	10:43111239_A>G	99.55	99.26
	10:43118395_G>T	100	100
Chr12	12:25245350_C>A	2.56	3.61
Chr17	17:7673792_G>C	1.56	2.32
	17:7673802_C>T	1.58	2.27
	17:7673806_C>T	1.23	2.29
	17:7676154_G>C	100	100
	17:39723335_A>G	45.11	48.38
	17:39727784_C>G	25.92	22

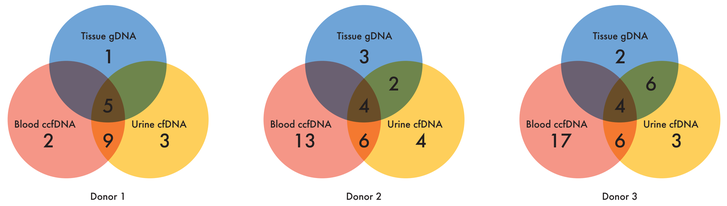
Impact of storage on mutation detection. Urine cfDNA was isolated 2 days after collection and stabilization in the PAXgene Urine Liquid Biopsy Set and after additional storage using the QIAsymphony DSP Circulating DNA Kit. Samples from both time points were implemented in QIAseq cfDNA Ultra library preparation using the QIAseq Targeted cfDNA Ultra Human Lung Cancer Panel to examine the stabilization of the mutation profile over time. One representative donor is shown here that demonstrates stabilization of mutation profiles, even after an additional 5 days' storage.

PAXgene Tissue gDNA shows higher fragment length and higher sensitivity for variant allele detection than FFPE gDNA



Comparison of sequencing quality parameters. A On-target reads, **B** sample fragment length, **C** mean primer read fragment depth, **D** mean UMI depth, **E** coverage uniformity and **F** LOD were compared between sequencing libraries generated from gDNA extracted from PFPE, FFPE and cryoconserved tissue taken from seven lung cancer patients (n=7). Fragment length was shortest and coverage uniformity lowest in FFPE samples (**B** and **E**). Limit of detection was detected to be lowest for PFPE samples (**F**).

Overlapping and unique variants in cancer-related genes found in different specimen types



Overlapping and complementary variants in cancer-related genes from tissue gDNA, blood ccfDNA and urine cfDNA of three representative lung cancer donors.

Conclusions

The developed workflows encompass:

- Specimen collection
- Stabilization
- Storage
- Analyte isolation
- Quality control (QC)
- Library preparation
- Sequencing

- A novel QC concept enables traceability and process standardization through systematic metadata documentation. Specimens yielded high-quality libraries and successful sequencing performance across all analyte runs.
- Comparative mutational profiling of blood ccfDNA, urine cfDNA and tissue DNA revealed both overlapping as well as complementary variants in key tumor genes. This gain in molecular information may lead to further development of multimodal liquid biopsy-based approaches with urine as a valuable complementing specimen type – also for non-urological malignancies.
- Our NGS workflows support multi- and single-mode testing for cancer research. They are developed in alignment with recognized international regulatory principles to enable comprehensive validation from sample collection to assay output.

Disclaimers

The PAXgene Blood ccfDNA Tube (CE-IVD) used in the studies is not available in the US. The product available in US is cat. no. 768115 which can be used for research use only. Not for use in diagnostic procedures. The PAXgene Urine Collection Cup, PAXgene Urine Liquid Biopsy Tube, PAXgene Tissue FIX Container, PAXgene Tissue DNA Kit, miRNeasy FFPE Kit, miRNeasy Tissue/Cells Advanced Mini Kit and QIAseq Panels are for research use only. Not for use in diagnostic procedures.

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Sample to Insight