Highly sensitive detection of low abundant somatic mutations in circulating exosomal RNA and cfDNA with next-generation sequencing.

Abstract #11061
Board #274

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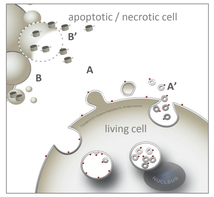
Background and Methods

Circulating nucleic acids (NA) in the bloodstream of cancer patients are of interest because of their potential to provide tumor mutation status without requiring a tissue sample. Blood plasma contains at least two sources of circulating cell-free NA: circulating free DNA (cfDNA), from apoptotic/necrotic cells, and RNA enclosed in exosomes (exoRNA), which are secreted by living cells through active metabolic processes. However, tumor derived mutated sequences are often of very low abundance against a background of wildtype. Therefore, efficient extraction of all available circulating NA as well as a highly sensitive mutation detection method, are paramount to the development of clinically relevant liquid biopsies.

We used a single-step isolation platform for both exoRNA and cfDNA from plasma (EXO52) in combination with a quantitative NGS method detecting a panel of actionable mutations (EXO1000). The current version of the assay uses a selection of nine mutation hotspots from six genes, a custom library preparation protocol, and bioinformatics pipeline. We analyzed mutations present in nucleic acids from plasma of patients with various types of cancer including NSCLC, CRC, and malignant melanoma (MM).

exoRNA and cfDNA: Two distinct sources of cell-free nucleic acids in plasma



- **Extracellular vesicles**, mainly 50-200 nm in size, are actively secreted by the cell and carry a snapshot of the body's transcriptome (exoRNA).
- **Circulating cell-free DNA** (cfDNA) is released by necrotic and apoptotic events in tumor and normal tissue.

Extracellular RNA and DNA in plasma. Exosomes are actively released by living cells directly from the plasma membrane (**A**) or via the multivesicular body pathway (A'), carrying RNA into circulation (exoRNA). Apoptotic or necrotic cells may release cell-free DNA (cfDNA) in apoptotic vesicles (B) or as free-circulating nucleosomes (B').

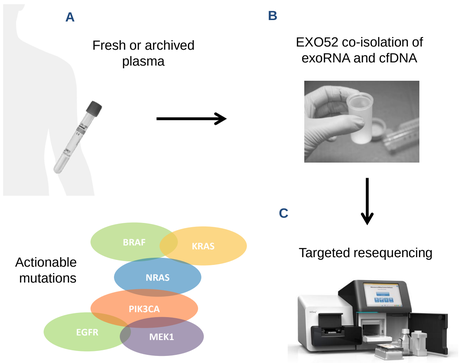
A single-step isolation platform for exoRNA and cell-free DNA from patient plasma samples

EXO52 spin-column

- **Bind vesicles and cfDNA** to EXO52 spin-column membrane & wash
- On-column lysis and release
- Mini spin-column purification
- Elution of **exoRNA and cfDNA**

Workflow for co-isolation of exoRNA and cfDNA from biofluids using EXO52 technology platform. The EXO52 platform employs a proprietary capture mechanism in a disposable spin-column format to enable routine parallel co-extraction of exoRNA and cfDNA from biofluids.

The EXO1000 Solid Tumor Panel for monitoring circulating mutations in clinical samples



Workflow of the EXO1000 Solid Tumor Panel: EXO52 co-isolation of exoRNA and cfDNA from 0.5-4 mL of fresh or archived plasma, pre-filtered with 0.8 µm to exclude cellular material (**A** and **B**); Targeted enrichment, sequencing on the Illumina MiSeq™ platform and absolute quantification of input material; Bioinformatic analysis that includes noise correction and calculation of mutant copy numbers (**C**).

A large cohort of EXO1000 liquid biopsies on tissue-matched plasma

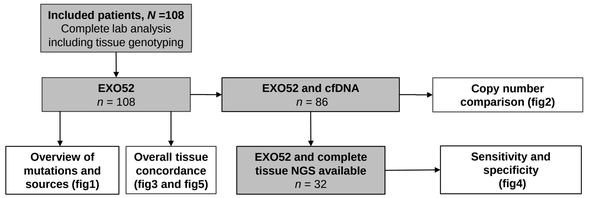
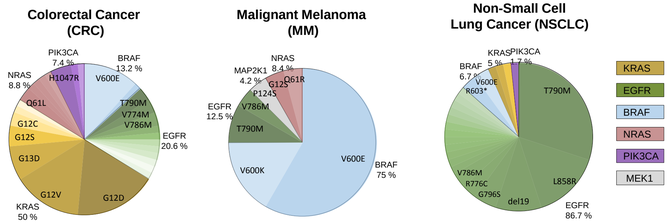


Fig. 1 - Overview of circulating mutations and sample sources



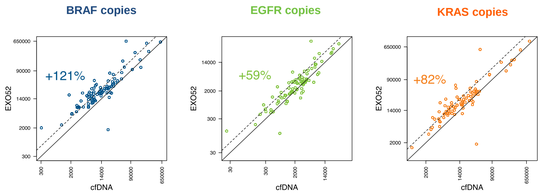
CRC: Prospectively collected plasma and time-matched tissue from late-stage CRC patients presenting for resection of the primary lesion. Tissue status of all mutations covered by EXO1000 determined by NGS.

MM: Plasma from late-stage malignant melanoma patients with active disease at the time of blood draw, who were BRAF genotyped by the institutional CLIA lab for eligibility to BRAF inhibitor treatment.

NSCLC: Plasma samples from Non-Small Cell Lung Cancer patients collected at the time of clinical resistance to EGFR TKI therapy, who were EGFR-genotyped on time-matched tissue from a repeat biopsy. EGFR tissue status from the institutional CLIA lab.

Circulating mutations found in various cancer types from different sample sources. Overview of the EXO1000 detected mutations (>0.1% above background) in the EXO52 isolated plasma samples used in this study.

Fig. 2 - Single-step co-isolation of exoRNA + cfDNA yields consistently greater gene copies



Absolute quantification of extracted gene copies. Comparison of two fractions isolated from 86 different patient samples: cell-free DNA (cfDNA) and exoRNA+cfDNA (EXO52). In these three genes, the added molecules from RNA are around 100% (dotted line)

Fig. 3 - High positive concordance with tissue in late-stage cancers

COSMIC Mutations

Cohort	Tissue	EXO1000	Positive Concordance
All Samples (*n*=94)	**116**	**98**	**84%**
CRC (*n*=38)	43	40	93%
NSCLC (*n*=28)	45	31	69%
Melanoma (*n*=20)	21	21	100%
Others (*n*=8)	7	6	86%

Positive concordance with tissue across complete dataset. Agreement of COSMIC mutations identified by EXO1000 liquid biopsy or tissue status as determined by CLIA lab facilities or NGS tissue genotyping (see fig1). The overall tissue concordance of 84% amongst all patients with late stage disease does not change significantly when considering the different cancer types individually.

Fig. 4 - High sensitivity and specificity in mCRC

Stage IIIc/IV, 18 patients

Plasma Mutations	Tissue Mutations +	Tissue Mutations -
+	24	197
-	5	11312

Sensitivity = 83%
Specificity = 98 %

Sensitivity and specificity in a subset of patients with complete NGS tissue status. Contingency table counting COSMIC mutations detected in tissue genotyping by NGS or EXO1000 assay on patient plasma. Patients with high-grade TNM stage show a sensitivity of 83% at a specificity of 98%.

Fig. 5 - Good sensitivity for challenging samples in NSCLC

Positive Concordance with Tissue

	EGFR L858R & del19	EGFR T790M
All stages	**81% (17/21)**	**75% (12/16)**
M0/M1a	67% (4/6)	40% (2/5)
M1b	87% (13/15)	82% (9/11)

Concordance of EGFR mutations in NSCLC. The positive concordance of EGFR mutations in metastatic (M1b) disease was 87% for the activating and 82% for the resistance mutation T790M. Patients with intra-thoracic disease (M0/M1a) has often been challenging to detect on cfDNA alone; however, by combining the exoRNA and DNA we achieved a 67% concordance for activating mutations and 40% for T790M.

Conclusions

- **Combined capture of exoRNA and cfDNA in plasma offers a major advance in the development of clinically relevant liquid biopsies.**
- **EXO52 technology platform provides a convenient and reproducible method for single-step co-isolation of all exoRNA and cfDNA from high volumes of biofluids.**
- **By combining exoRNA and cfDNA, EXO52 substantially increases the number of gene copies available for low abundant somatic mutation detection versus cfDNA alone.**
- **The EXO1000 Solid Tumor Panel provides excellent analytical performance for detection of actionable mutations in plasma across multiple cancers with immediate opportunity for clinical application.**

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