Detection of EGFR activating and T790M resistance mutation in plasma of NSCLC patients using combined exosomal RNA and cfDNA capture.

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Introduction and Methods

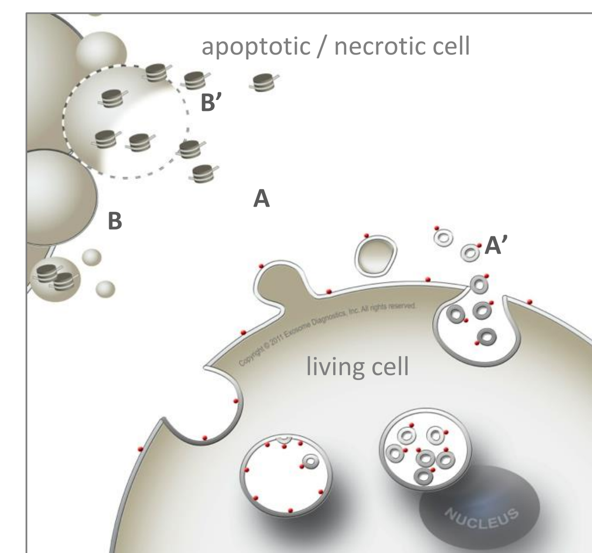
Initial responses to tyrosine kinase inhibitors (TKIs) in NSCLC patients harboring **EGFR activating mutations** are commonly observed, however patients inevitably progress as a consequence of acquired resistance (AR). Secondary mutations in the EGFR domains are thought to play a role in clinical resistance of a substantial portion of patients, and novel agents are in development in this setting.

Tissue based assays, requiring repeat biopsy, are unattractive, and detection of AR mutations in circulation would be an appealing alternative. Here we present data demonstrating the feasibility of detection of activating and AR EGFR mutations using a combined single-step **exosomal RNA (exoRNA) and cfDNA** approach from patient plasma to maximize sensitivity.

We applied our proprietary column-based method to co-isolate both exoRNA and cfDNA from 21 plasma samples (2 mL each) of lung cancer patients collected at the time of clinical resistance to EGFR TKI therapy, which were EGFR-genotyped on time-matched tissue from a repeat biopsy. The resulting high quality **plasma total nucleic acids** were subjected to reverse transcription and subsequent mutation analysis by targeted ultra-deep sequencing (UDS) of select genes.

We analyzed a **disease-specific gene panel** covering 9 mutation hotspots from 6 genes including EGFR mutations on exon 19, 20 and 21 in a qualitative and quantitative manner. The UDS data was generated using a custom library preparation method and bioinformatics pipeline to efficiently call the rare mutations.

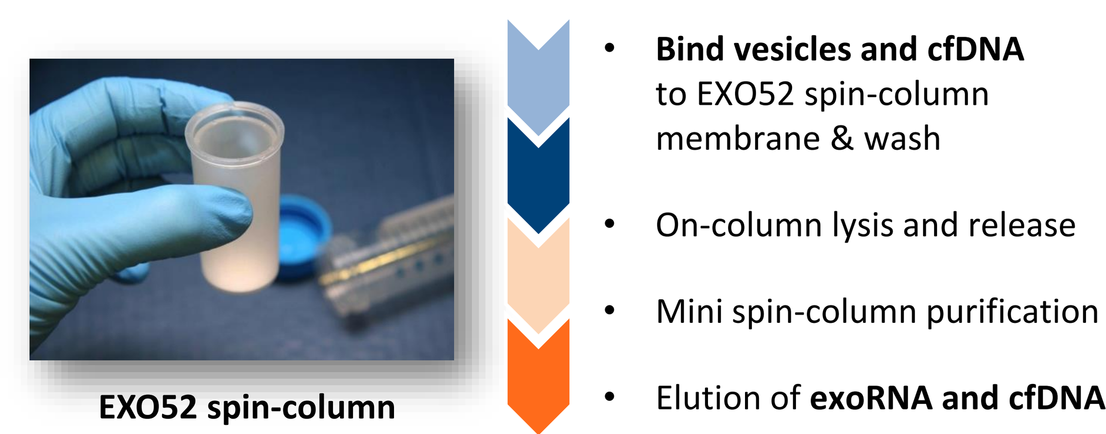
ExoRNA and cfDNA: Two distinct sources of cell-free nucleic acids in plasma



- **Extracellular vesicles**, about 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 will 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

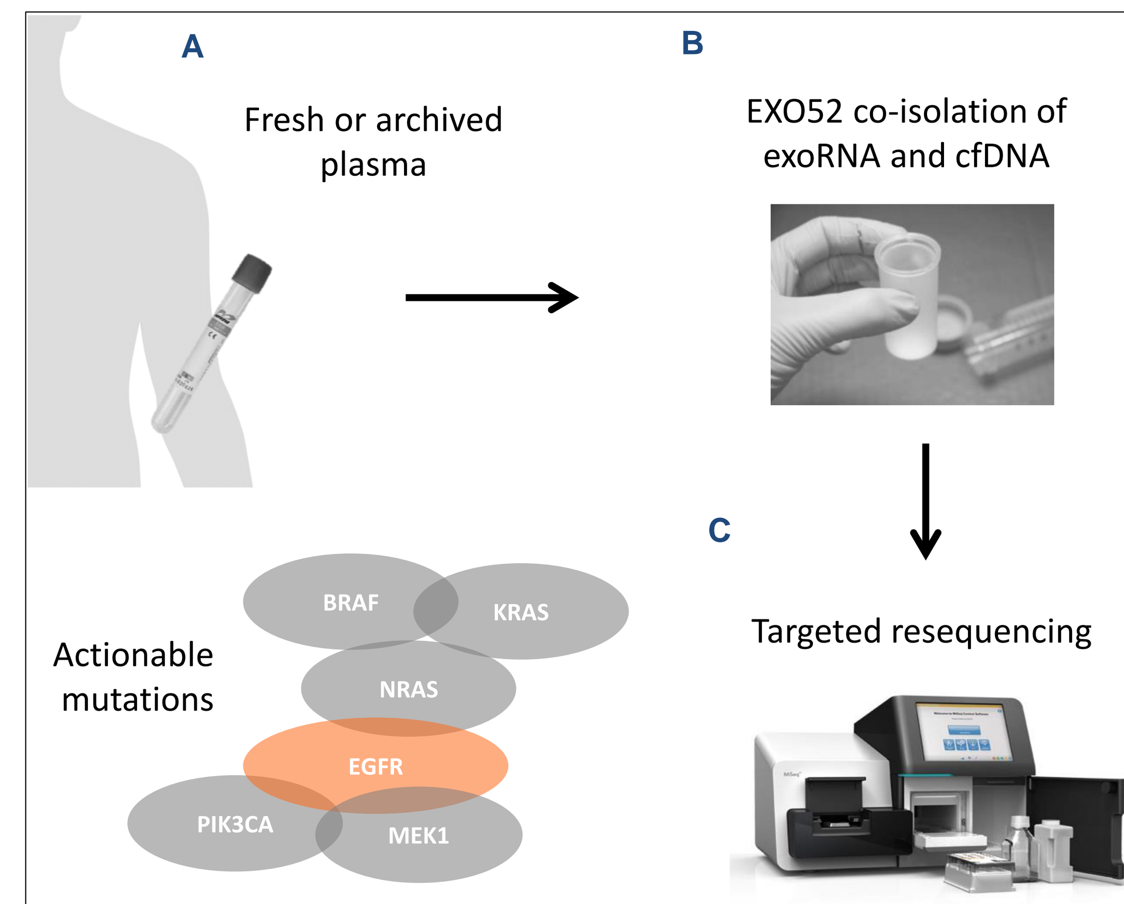


EXO52 spin-column

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

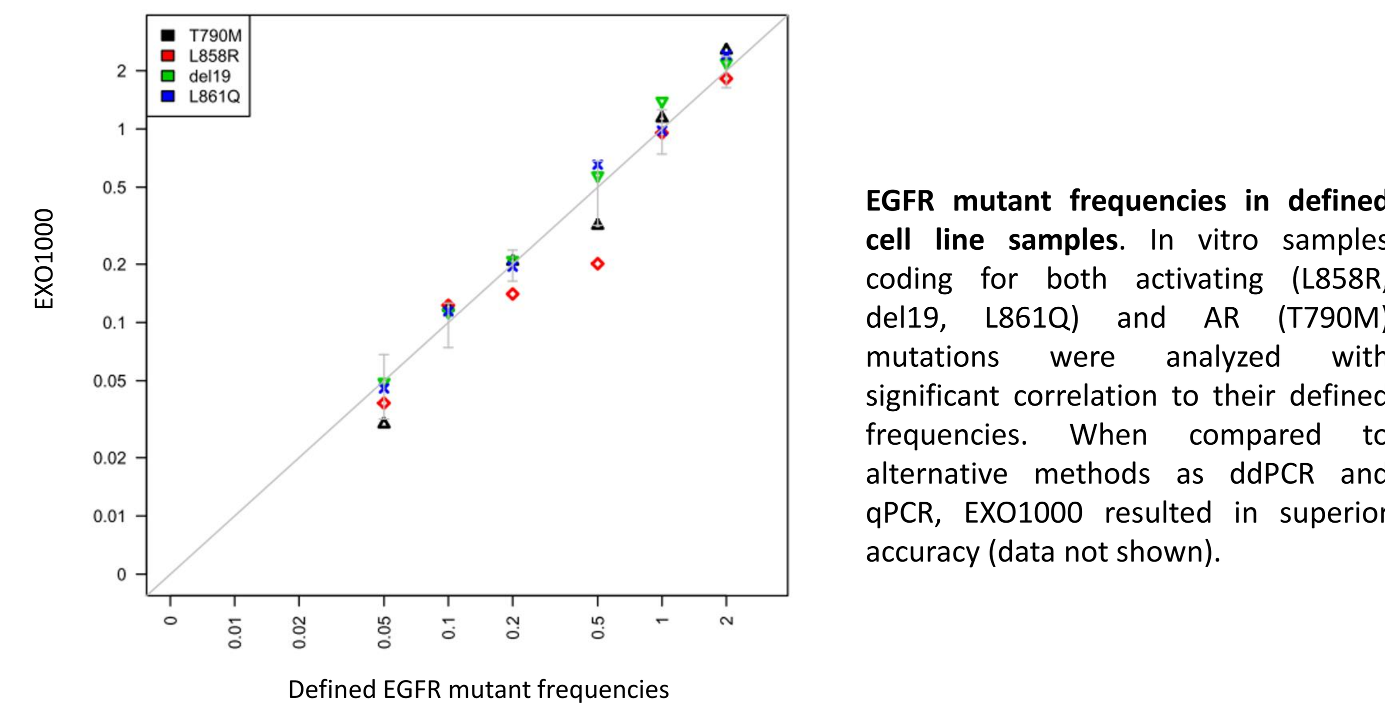
Workflow for co-isolation of exoRNA and cfDNA from plasma using EXO52 technology platform. The EXO52 platform employs a proprietary capture mechanism in a disposable spin-column format to enable routine parallel co-extraction of exoRNA and cfDNA from biofluids.

The EXO1000 Solid Tumor Panel for monitoring circulating EGFR 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**).

High accuracy of EXO1000 – Analysis of EGFR mutations on *in vitro* samples



EGFR mutant frequencies in defined cell line samples. In vitro samples coding for both activating (L858R, del19, L861Q) and AR (T790M) mutations were analyzed with significant correlation to their defined frequencies. When compared to alternative methods as ddPCR and qPCR, EXO1000 resulted in superior accuracy (data not shown).

NSCLC patients analyzed for EGFR mutations

			exon 19 del	L858R	L861Q	T790M
1	M1b	plasma	+	-	-	+
		tissue	+	-	-	+
2	M1b (to be confirmed)	plasma	+	-	-	+
		tissue	+	-	-	invalid data
3	M1a or M0	plasma	-	+	-	-
		tissue	-	+	-	+
4	M1b	plasma	-	+	-	+
		tissue	-	+	-	+
5	M1a or M0	plasma	-	+	-	-
		tissue	+	-	-	+
6	M1b	plasma	-	-	-	+
		tissue	-	+	-	-
7	M1b	plasma	+	-	-	+
		tissue	+	-	-	+
8	M1a or M0	plasma	-	-	-	+
		tissue	+	-	-	-
9	M1b	plasma	-	+	-	-
		tissue	-	+	-	- (no tumor cells)
10	M1b	plasma	-	+	-	+
		tissue	-	+	-	+
11	M1b	plasma	-	+	-	+
		tissue	-	+	-	+
12	M1b	plasma	+	-	-	+
		tissue	+	-	-	+
13	M1a or M0	plasma	+	-	-	+
		tissue	+	-	-	+
14	M1b	plasma	-	+	-	+
		tissue	-	+	-	+
15	M1b	plasma	-	+	-	+
		tissue	-	+	-	+
16	M1b	plasma	-	+	-	+
		tissue	-	+	-	+
17	M1a or M0	plasma	+	-	-	+
		tissue	+	-	-	+
18	M1a or M0	plasma	+	-	-	-
		tissue	+	-	-	+
19	M1b	plasma	-	-	+	-
		tissue	-	-	+	+
20	M1b	plasma	-	-	-	-
		tissue	+	-	-	+
21	M1b	plasma	+	-	-	+
		tissue	+ (<10% tumor content)	-	-	- (<10% tumor content)
22	M1a or M0	plasma	+	-	-	-
		tissue		(<10% tumor content)		(<10% tumor content)

Positive tissue correlation

Positive for EXO1000 and negative for tissue

NSCLC patients analyzed for EGFR mutation – Concordance data

Positive Tissue Concordance

	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) have often been challenging to detect on cfDNA alone; however, by combining the exoRNA and cfDNA we achieved a 67% concordance for activating mutations and 40% for T790M.

Conclusions

- **EXO52 technology platform provides a convenient and reproducible method for single-step co-isolation of all exoRNA and cfDNA from high volumes of biofluids.**
- **The EXO1000 Solid Tumor Panel provides excellent analytical performance for detection of actionable mutations in plasma across multiple cancers including NSCLC with immediate opportunity for clinical application.**
- ***In vitro* data on EGFR-mutated cell lines confirm the accuracy of EXO1000 to analyze the frequencies of resistance and activating mutations (incl. deletions, substitutions).**
- **Patient data demonstrate the ability to detect activating and AR mutations on exoRNA and cfDNA co-isolated from plasma of lung cancer patients with high sensitivity**
- **Combined capture of exoRNA and cfDNA in plasma in a single step using the Exo52 technology offers a major advance in the development of clinically relevant liquid biopsies, with significant gains in sensitivity compared to cfDNA alone**

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