EGFR activating and T790M resistance mutation in plasma exoRNA and cfDNA, detected with single-step isolation columns and targeted resequencing.

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Abstract #2618

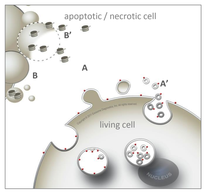
Background and Methods

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. In non-small cell lung cancer (NSCLC), patients with EGFR activating mutations often initially respond to treatment with tyrosine kinase inhibitor (TKI) therapy. As treatment progresses, patients often develop acquired mutations, including the T790M resistance mutation, rendering TKI therapy ineffective. There are currently several therapies in clinical development that target these acquired mutations. Unlike tissue-based approaches, biofluid-based molecular diagnostics enable the potential for serial, longitudinal monitoring, including real-time detection of drug resistance and acquired mutations.

Blood plasma contains at least two sources of circulating cell-free NA: RNA enclosed in exosomes (exoRNA), which are secreted by living cells through active metabolic processes (see Fig. 1), and circulating free DNA (cfDNA), from apoptotic/necrotic cells. However, tumor derived mutated sequences, including T790M, are often of very low abundance against a background of wild type, presenting mutation detection challenges in certain settings. For instance, cfDNA-only biofluid-based technologies have demonstrated challenges in detecting T790M in patients with intra-thoracic NSCLC. Therefore, efficient extraction of all available circulating NA and a highly sensitive mutation detection method are paramount to the development of clinically relevant liquid biopsies.

Here we used a single-step platform to isolate and analyze both exoRNA and cfDNA from plasma (ExoLution Plus) in combination with a quantitative next-generation sequencing (NGS) method detecting a panel of actionable mutations (**ExoDx** *Solid Tumor*). We applied a version of the assay to investigate the status of both the EGFR activating and acquired mutations of 47 NSCLC patients using a custom library preparation protocol and bioinformatics pipeline interrogating nine mutation hotspots within six genes.

Fig. 1 - 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').

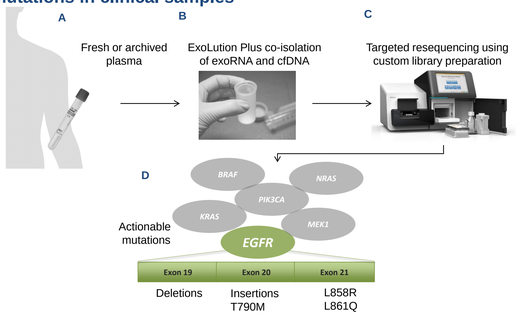
Fig. 2 - A single-step isolation platform for exoRNA and cfDNA from patient plasma samples

ExoLution Plus spin-column

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

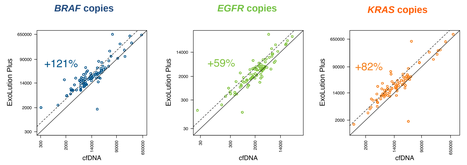
Workflow for co-isolation of exoRNA and cfDNA from biofluids using the ExoLution Plus technology platform. The ExoLution Plus platform employs a proprietary capture mechanism in a disposable spin-column format to enable routine parallel co-extraction of exoRNA and cfDNA from biofluids.

Fig. 3 - The ExoDx *Solid Tumor* Panel for monitoring circulating mutations in clinical samples



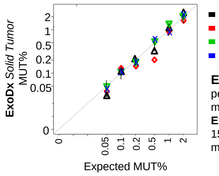
Workflow of the ExoDx *Solid Tumor* Panel: ExoLution Plus co-isolation of exoRNA and cfDNA from 0.5-4 mL of fresh or archived plasma, pre-filtered to exclude cellular material (**A** and **B**); Targeted enrichment, sequencing on the Illumina MiSeq™ platform of input material; Bioinformatics analysis including noise reduction and mutation calling (**C** and **D**).

Fig. 4 - Co-isolation of exoRNA + cfDNA yields more gene copies



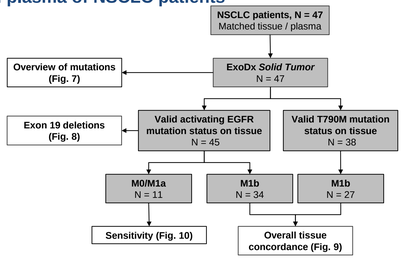
Comparing ExoLution Plus against extracting cfDNA only. Co-Isolating exoRNA+cfDNA (ExoLution Plus) in a large cohort of various cancer types, including NSCLC, approximately doubles the amount of detectable molecules in comparison to cfDNA only (dotted line = 100 % increase, each dot represents one patient).

Fig. 5 - ExoDx *Solid Tumor* accurately detects EGFR mutations



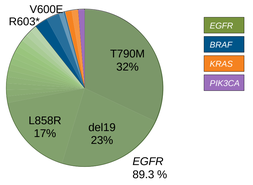
EGFR mutant frequencies in cell line samples. Mutant percentages of both activating (L858R, del19, L861Q) and resistance mutation (T790M) of *in vitro* samples were accurately recovered with **ExoDx** *Solid Tumor*. Low mutant copy samples (7.5 mutant copies in 15,000 wildtype copies; 0.05%) were consistently detected for all mutations.

Fig. 6 - Overview of ExoDx *Solid Tumor* liquid biopsies on tissue-matched plasma of NSCLC patients



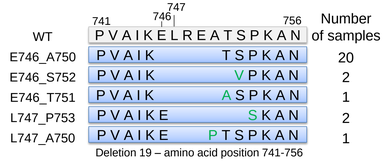
Overview of NSCLC samples analyzed with ExoDx *Solid Tumor*: Plasma samples from NSCLC patients, who were EGFR-genotyped on time-matched tissue from biopsy. Out of 47 samples, 45 had a valid tissue status. Tissue concordance was calculated for tumor stage M1b for activating and resistance mutations. A challenging group of patients with intra-thoracic disease (M0/M1a) was analyzed separately.

Fig. 7 - Overview of detected circulating mutations in NSCLC



Circulating mutations found in the analyzed NSCLC samples. Overview of the **ExoDx** *Solid Tumor* detected mutations (>0.1% after background correction) in the ExoLution Plus isolated plasma samples.

Fig. 8 - In-frame deletions in exon 19 occurring at different amino acid sites of EGFR



Deletions in Exon 19 of EGFR. Out of 47 samples, 26 harbored an EGFR exon 19 in-frame deletion, starting either at amino acid position 746 or 747; insertions are highlighted in green. Differentiating between deletions is important as response rates to TKIs differ (Chung et al., Clin Cancer Res; 2012).

Fig. 9 - High positive concordance for tumor stage M1b

ExoDx *Solid Tumor*	Tissue: L858R +	L858R -	del19 +	del19 -	T790M +	T790M -
+	**12**	0	**16**	0	**20**	0
-	1	2	2	2	4	3
Sensitivity:	**92.3** %		**88.9** %		**83.3** %	

Concordance of EGFR mutations in NSCLC. Plasma samples from NSCLC patients, who were EGFR-genotyped on time-matched tissue from biopsy. High sensitivity of the **ExoDx** *Solid Tumor* assay is stated for the most relevant mutations on EGFR.

Fig. 10 - High sensitivity for challenging samples in NSCLC

Positive Concordance with Tissue

Mutation	Tumor stage	Sensitivity
Activating *EGFR* mutations	M0/M1a	**72.7 % (8/11)**
	M1b	90.6 % (29/32)
	All stages	86.0 % (37/43)
T790M resistance mutation	All stages	64.7 % (22/34)

Concordance of EGFR mutations in NSCLC. The positive concordance of EGFR mutations in M1b disease was 90.3 % for the activating mutations. Patients with intra-thoracic disease (M0/M1a) are challenging to detect on cfDNA alone (*e.g.* Zhao et al., Respiration, 2013). By combining the exoRNA and DNA we achieved a 72.7 % concordance for activating mutations.

Conclusions

- **Detection of both activating and acquired resistance mutations to *EGFR* therapy in plasma offers a very promising alternative to tissue-based biopsy.**
- **ExoLution Plus substantially increases the number of gene copies available for low abundant somatic mutation detection, offering a major advance in the development of clinically relevant liquid biopsies.**
- **Combined plasma exoRNA/cfDNA isolation and analysis offers superior sensitivity enabling high sensitivity detection in challenging cases.**
- **The ExoDx *Solid Tumor* Panel provides excellent analytical performance for detection of actionable mutations in plasma of NSCLC with immediate opportunity for clinical application.**

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