Assay Development for Droplet Digital PCR-based Detection of Clinically Targetable Mutations in Non-small Cell Lung Cancer

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Background

- Lung cancer is the leading cause of cancer-related death in America and across the world, accounting for nearly 1.6 million deaths per year.[1]
- Somatic mutations in the *EGFR*, *KRAS* or *TP53* gene are common in lung cancer, and *EGFR* mutational status is an important factor in choosing the optimal form of molecularly targeted therapy.[2]
- Liquid biopsy is the detection of tumor materials in blood and other body fluids, including the detection of cell-free DNA (cfDNA) and its component tumor derived DNA (termed circulating tumor DNA or ctDNA in the blood).
- ctDNA detection is less invasive than traditional tissue biopsy and usually has a faster turnaround time than tissue sequencing.
- While next generation sequencing (NGS)-based ctDNA detection is very sensitive in later stage disease, it is less sensitive for early-stage cancers.
- Droplet digital PCR (ddPCR) provides about a log higher sensitivity than NGS and may improve the diagnostic sensitivity of blood-based cfDNA detection.[3]
- Additionally, in early lung cancer, bronchoscopy and bronchoalveolar lavage (BAL) is used for diagnosis and staging.
- BAL samples the lung periphery by directly washing the tumor area with a sterile saline solution and is traditionally used for detecting tumor cells.
- However, BAL fluid (BALF) may be a better source of cfDNA for the detection of lung cancer mutations than blood, with equal or higher diagnostic sensitivity for early-stage disease.
- This project aims to detect and quantify targetable *EGFR* mutations in the blood or BALF of NSCLC patients using ddPCR.

Assay Development in Summer 2022

- Extract, quantify, pre-amplify, and quantitate control samples, prioritizing plasma over BAL, for the determination of assay background.
- Target: *EGFR* E746_A750del, the most common exon 19 deletion that is therapeutically targetable
- Patient selection:
  - All patients had suspected lung cancer and were consented under IRB Protocol 826909
  - Selected Patients
    - 2 positive control stage IV patients (known mutation by tissue sequencing)
    - 7 negative control stage IV patients with a KRAS G12 mutation, a known mutually exclusive mutations
    - 4 negative control stage IV patients lacking the mutation of interest
    - 10 negative control patients determined to be cancer-free on biopsy

Developed a Preliminary Lit Review for BALF utility in liquid biopsy:

Author (Year)	Genes	Summary
Lee, et. al (2020)	EGFR, L858R, Exon 19 in/del	In the early-stage group (stages I–IIIA; n = 38), there was a significant difference in EGFR detection between blood plasma (0.504) and BWF (0.768).[4]
Zhang et. al (2021)	Exon 19 del, L858R, L861Q, Exon 20	BWF showed a higher sensitivity in EGFR mutation testing than both plasma (100% [8/8] vs. 62.5% [5/8], p = 0.095) and bronchoscopy biopsy samples (92.5% [37/40] vs. 77.5% [31/40], p = 0.012) and identified EGFR mutations in 6 cases whose biopsy failed to establish a diagnosis.[5]
Sakamoto et. al (2015)	Exon 19 deletion, L858R mutation	The total diagnostic yield of EBUS was 91.0%. The positive concordance rates for detecting 19del and L858R with the ultrarapid PCR and PCR-invader methods were both 100%. Negative concordance rates were 97.2 and 98.1%, respectively.[6]

Methods

Stage (Overall)	EGFR/KRAS Variants by tissue NGS
IVB	EGFR E746_A750del
IV	EGFR E746_A750del
IV	EGFR L8585R
IV	EGFR L861Q
IV	KRAS G12A
IV	KRAS G12C
IV	KRAS G12D
IV	KRAS G12D
IVB	KRAS G12S
No cancer identified	NA
No cancer identified	NA
No cancer identified	NA
No cancer identified	NA
No cancer identified	NA
No cancer identified	NA
No cancer identified	NA
No cancer identified	NA
No cancer identified	NA
No cancer identified	NA
IV	None Detected
IV	None Detected
IV	None Detected
IV	None Detected

Table 1. Patient Characteristics Blood draws for lung cancer patients whose ctDNA was analyzed

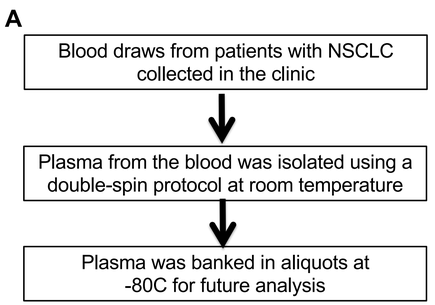

Figure 1. Plasma isolation and banking
A. Workflow for plasma collection and processing. **B.** Centrifugal blood yields separation of blood plasma which is then banked in a -80C fridge

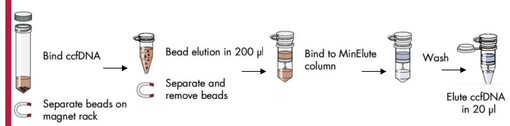

Figure 2. Cell-free DNA extraction from Plasma Workflow for cfDNA extraction from plasma with QIAamp® MinElute® ccfDNA Kit.

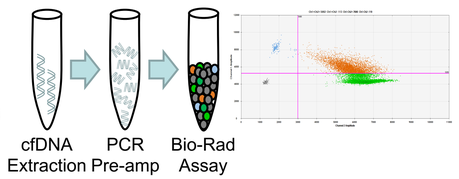

Figure 3. Droplet Digital PCR for EGFR Mutations. Workflow and sample analysis for ddPCR with a pre-amplification step to enhance detection of rare events.

Droplet Digital PCR Results

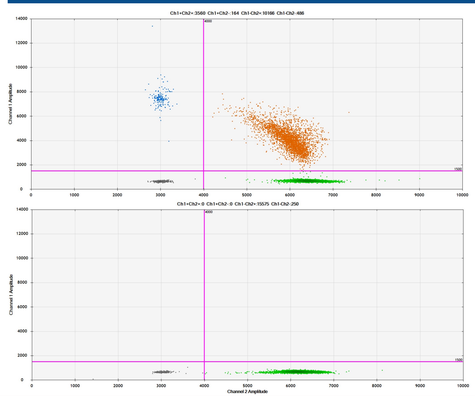

Figure 4. Positive Control ddPCR
This ddPCR result is of a patient with a known EGFR E746-A750 ddPCR-detected mutation

Figure 5. Negative Control ddPCR
This ddPCR result is of a patient with a known KRAS G12C ddPCR-detected mutation, which is mutually exclusively of EGFR mutation

Results

Sample	Plasma cfDNA Concentration (ng/µl)	Pre-amp Input (ng)
A	1.218	28.01
B	0.489	11.25
C	5.173	30.00
D	0.174	4.00
E	0.371	8.54
F	0.516	11.87
G	0.787	18.11
H	0.513	11.80
I	1.797	30.00
J	0.670	15.42
K	0.607	13.97
L	0.716	16.47
M	0.324	7.46
N	0.755	17.37
O	0.367	8.44
P	0.415	9.53
Q	0.854	19.65
R	0.377	8.67
S	0.398	9.16
T	0.634	14.57
U	0.233	5.36
V	1.153	26.51
W	0.563	12.94

Table 2. cfDNA concentrations and Pre Amplification Input for ddPCR detection of *EGFR* E746_A750

	Run 1		Run 2	
Sample	Mut	WT	Mut	WT
Stage IV EGFR E46_A750del 1	26000	212000	7060	72800
Stage IV EGFR E46_A750del 2	0	91800		
Stage IV EGFR L8585R 1	0	Error	0	79200
Stage IV EGFR L861Q 1	0	41280		
Stage IV KRAS G12A 1	0	92200		
Stage IV KRAS G12C 1	0	127400	0	97600
Stage IV KRAS G12D 1	0	176000	0	101200
Stage IV KRAS G12D 2	0	109800		
Stage IV KRAS G12S 1	0	226000	0	60800
No Cancer Identified 1	0	131200	0	101600
No Cancer Identified 2	0	115800	0	93400
No Cancer Identified 3	0	153800	0	82000
No Cancer Identified 4	0	71200		
No Cancer Identified 5	0	140600	0	66000
No Cancer Identified 6	0	54000		
No Cancer Identified 7	0	75600		
No Cancer Identified 8	0	89200		
No Cancer Identified 9	0	46520		
No Cancer Identified 10	0	85600		
Stage IV None Detected 1	0	154200	0	74600
Stage IV None Detected 2	0	54600		
Stage IV None Detected 3	0	Error	0	75600
Stage IV None Detected 4	0	121200	0	97600

Table 3. Number of droplets generated for positive and negative controls for detection of the *EGFR* E746_A750 deletion mutation droplets and wild type droplets

Conclusions and Future Directions

- The assay used is specific at detecting positive and negative controls for the *EGFR* E746_A750 deletion mutation, however the small sample size should be noted.
- No false positive droplets were detected.
- Importantly, the assay detected mutant copies in positive control sample 1. Notably, it did not detect any in positive control; 2. however, this variant was missed by a commercial ctDNA assay as well, suggesting a very low variant allele fraction.
- Typically, assay thresholds are set three standard deviations above the mean number of false positive droplets. However, here we will use 3 positive droplets as the detection threshold.
- In the future, we intend to perform the same background analysis conducted on the blood plasma samples on the BALF controls. We also intend to perform this assay on plasma and BALF samples from stage II/III NSCLC patients to test hypotheses for greater sensitivities to determine assay sensitivity in the setting of earlier-stage disease.

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1. Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2022). Cancer statistics, 2022. *CA: a cancer journal for clinicians*, *72*(1), 7–33. https://doi.org/10.3322/caac.21708
2. Qin, K., Hou, H., Liang, Y. *et al.* Prognostic value of TP53 concurrent mutations for EGFR- TKIs and ALK-TKIs based targeted therapy in advanced non-small cell lung cancer: a meta-analysis. *BMC Cancer* 20, 328 (2020). https://doi.org/10.1186/s12885-020-06805-5
3. Nagasaka, M., Uddin, M. H., Al-Hallak, M. N., Rahman, S., Balasubramanian, S., Sukari, A., & Azmi, A. S. (2021). Liquid biopsy for therapy monitoring in early-stage non-small cell lung cancer. *Molecular cancer*, *20*(1), 82. https://doi.org/10.1186/s12943-021-01371-1
4. Lee, S.H., Kim, E.Y., Kim, T. *et al.* Compared to plasma, bronchial washing fluid shows higher diagnostic yields for detecting EGFR-TKI sensitizing mutations by ddPCR in lung cancer. *Respir Res* 21, 142 (2020). https://doi.org/10.1186/s12931-020-01408-x
5. Zhang, X., Li, C., Ye, M., Hu, Q., Hu, J., Gong, Z., Li, J., Zhao, X., Xu, Y., Zhang, D., Hou, Y., & Zhang, X. (2021). Bronchial Washing Fluid Versus Plasma and Bronchoscopy Biopsy Samples for Detecting Epidermal Growth Factor Receptor Mutation Status in Lung Cancer. *Frontiers in oncology*, *11*, 602402. https://doi.org/10.3389/fonc.2021.602402
6. Sakamoto, T., Kodani, M., Takata, M., Chikumi, H., Nakamoto, M., Nishii-Ito, S., Ueda, Y., Izumi, H., Makino, H., Touge, H., Takeda, K., Yamasaki, A., Yanai, M., Tanaka, N., Igishi, T., & Shimizu, E. (2015). A novel point-of-care system for high-speed real-time polymerase chain reaction testing for epidermal growth factor receptor mutations in bronchial lavage fluids after transbronchial biopsy in patients with non-small cell lung cancer. *International journal of oncology*, *46*(4), 1473–1480. https://doi.org/10.3892/ijo.2015.2875