Characterization of molecular targets of therapy in NSCLC utilizing a liquid biopsy

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Background

NSCLC patients commonly have insufficient tumor sample or high co-morbidities preventing access to tissue for molecular characterization. Molecular subtyping of NSCLC patients has identified molecular drivers of disease progression and enabled an era of precision medicine through the matching of driver mutations with targeted therapies. Currently, testing for EGFR (10-35%) and KRAS (15-25%) mutations and ALK (3-7%) rearrangements in NSCLC patients to determine differential sensitivity to TKIs has been incorporated into the standard of care. Recent demonstration of concordance in EGFR mutational status between ctDNA and tumor tissue in NSCLC patients highlights the utility of a liquid biopsies in a clinical setting. Additional candidate predictive markers, including CTC enumeration, CTC protein marker expression, detection of gene fusions (RET and ROS1) and amplifications (MET), may further enrich treatable patient populations for therapy sensitivity. We sought to develop a platform to enable diagnostic testing for clinically actionable NSCLC biomarkers from a single tube of blood utilizing a combination of protein and molecular CTC characterization with mutational analysis of cfDNA.

Methods

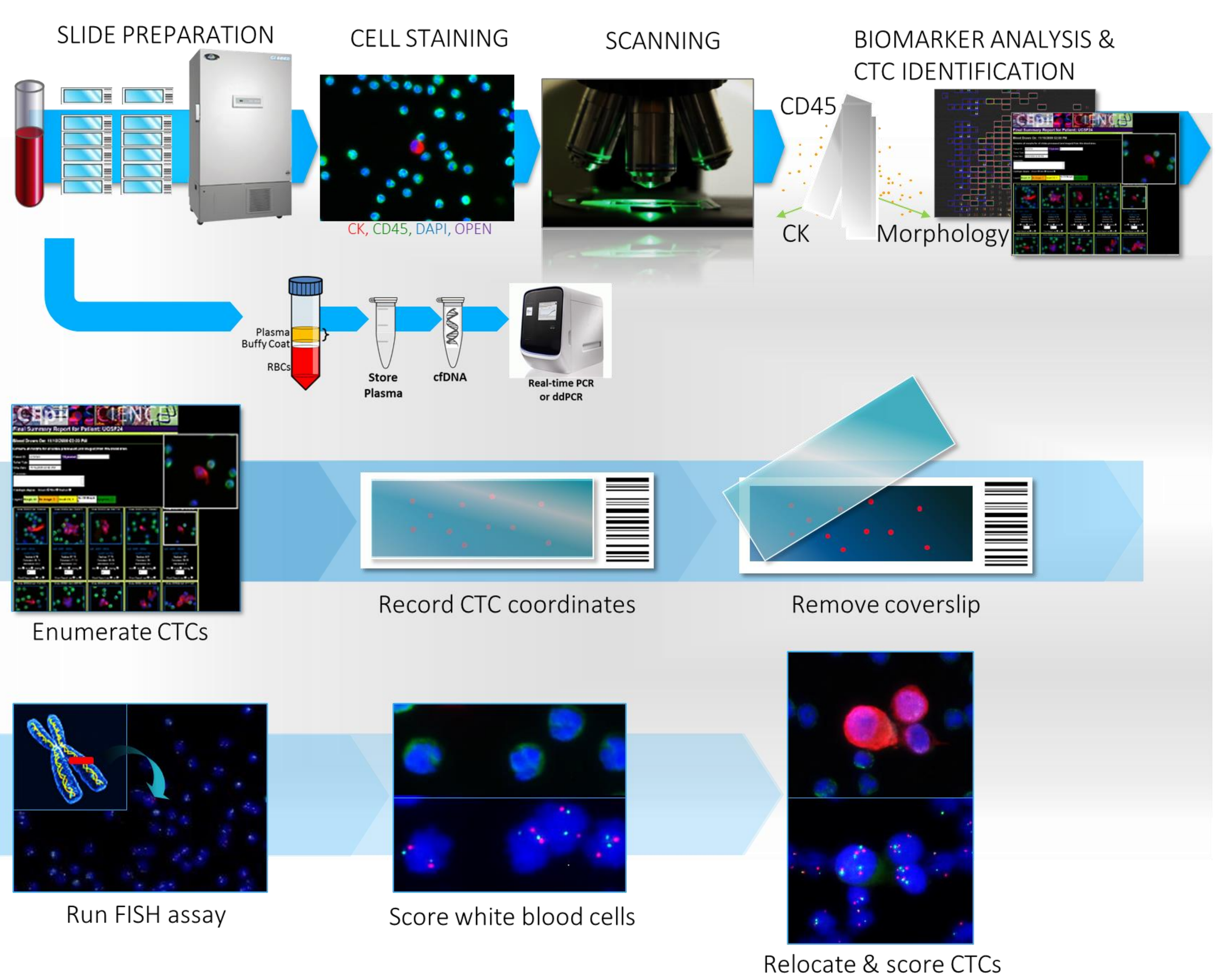


Figure 1. Schematic of Epic's CTC collection and detection process

1) Nucleated cells from blood sample placed onto slides
1a) cfDNA is extracted from Plasma and real-time PCR is performed
2) Slides stored in -80C biorepository
3) Slides stained with CK, CD45, DAPI and a biomarker of interest
4) Slides scanned
5) Multi-parametric digital pathology algorithms run
6) Software and human reader confirmation of CTCs & quantitation of biomarker expression
7) For FISH, coordinates are recorded and coverslip removed
8) FISH assay is performed
9) Regional WBCs are scored to assess normal
10) CTCs relocated and scored

Table 1. Study Population

Tube ID	Gene ID	Specific Mutation	Mutation Detection In Plasma	CTC/mL	CK-/mL	Apop/mL	Small/mL
2102	EGFR	ΔExon 19	Detected in ddPCR only	2	0	1	1
2632	EGFR	ΔExon 19	Detected in ddPCR only	11	0	0	1
3147	EGFR	ΔExon 19	Detected in real time and ddPCR	16	0	0	0
3678	EGFR	ΔExon 19	Detected in real time and ddPCR	1	0	1	0
3228	KRAS	Codon 61	G12C not detected in real time or ddPCR (not tested for Codon 61)	16	0	4	0
4198	KRAS	G12C	Detected in ddPCR only	1	0	1	0
4358	KRAS	G12C	Detected in real time and ddPCR	0	0	5	0

Biomarker Assay Development

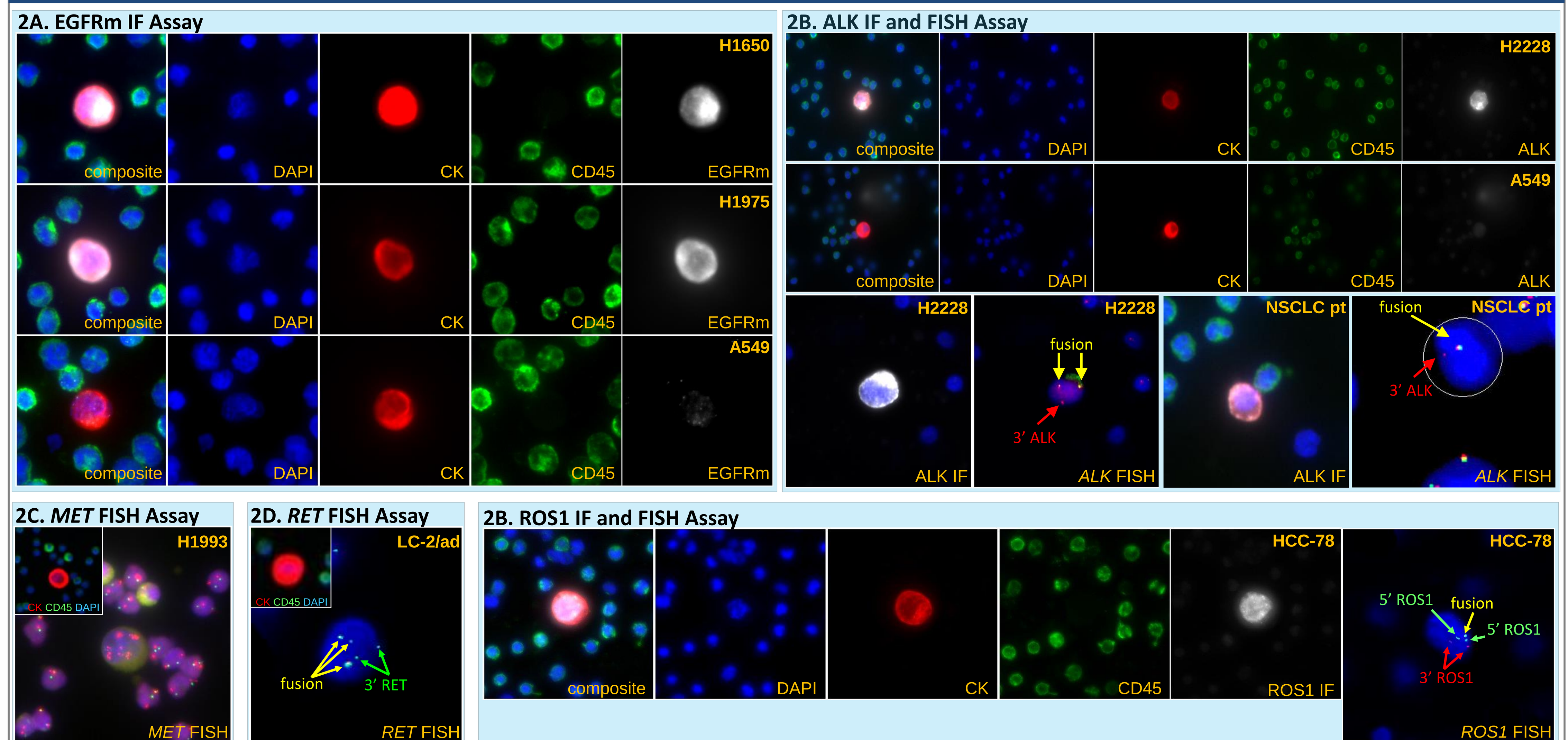


Figure 2. Biomarker Assay Development

A. H1650 (EGFR exon 19 deletion), H1975 (EGFR L858R) and A549 (wt EGFR) cell line cells stained using the Epic protein assay with an EGFRm specific antibody demonstrate specificity and sensitivity for this assay. This EGFRm antibody is specific for both L858R and Exon 19 deletion mutations.

B. H2228 (EML4-ALK, ALK over-expression) and A549 (ALK negative) cell line cells stained using the Epic protein assay demonstrate specificity and sensitivity for ALK protein expression. Following IF, individual H2228 cells were relocated and assayed via FISH. ALK rearrangement was determined by visualizing deletion of 5'ALK (**red** arrow). Intact ALK is seen as an adjacent red/green or yellow signal (**yellow** arrows). Clinical feasibility was determined in a NSCLC patient with ALK IF and *ALK* FISH. This patient had CTCs with ALK protein expression by IF and an *ALK* rearrangement confirmed by FISH, where patient WBCs had intact green/red or yellow signals.

C. H1993 (MET amplified) cell line stained with the Epic CTC assay (inset) followed by FISH assay for *MET* (**red**) and *CEP7* control (**green**), demonstrating *MET* amplification, where neighboring WBCs did not have amplification of *MET*.

D. LC-2/ad (*RET* rearranged) cell line cell stained using the Epic CTC assay (inset) followed by *RET* break apart FISH. A single 3' *RET* probe (**green**) was visualized indicating a *RET* rearrangement due to a deletion of the 5' *RET* probe (**red**).

E. HCC-78 (*ROS1* rearranged) cell line cell stained using the Epic protein assay demonstrates specificity and sensitivity for ROS1 protein expression. Following IF, individual HCC-78 cells were relocated and assayed via *ROS1* break apart FISH. *ROS1* rearrangement was determined by visualizing probe separation of the 5' *ROS1* probe (**green**) from the 3' *ROS1* probe (**red**), with a single red signal indicating a *ROS1* rearrangement.

cfDNA Mutation Detection by Real-time PCR

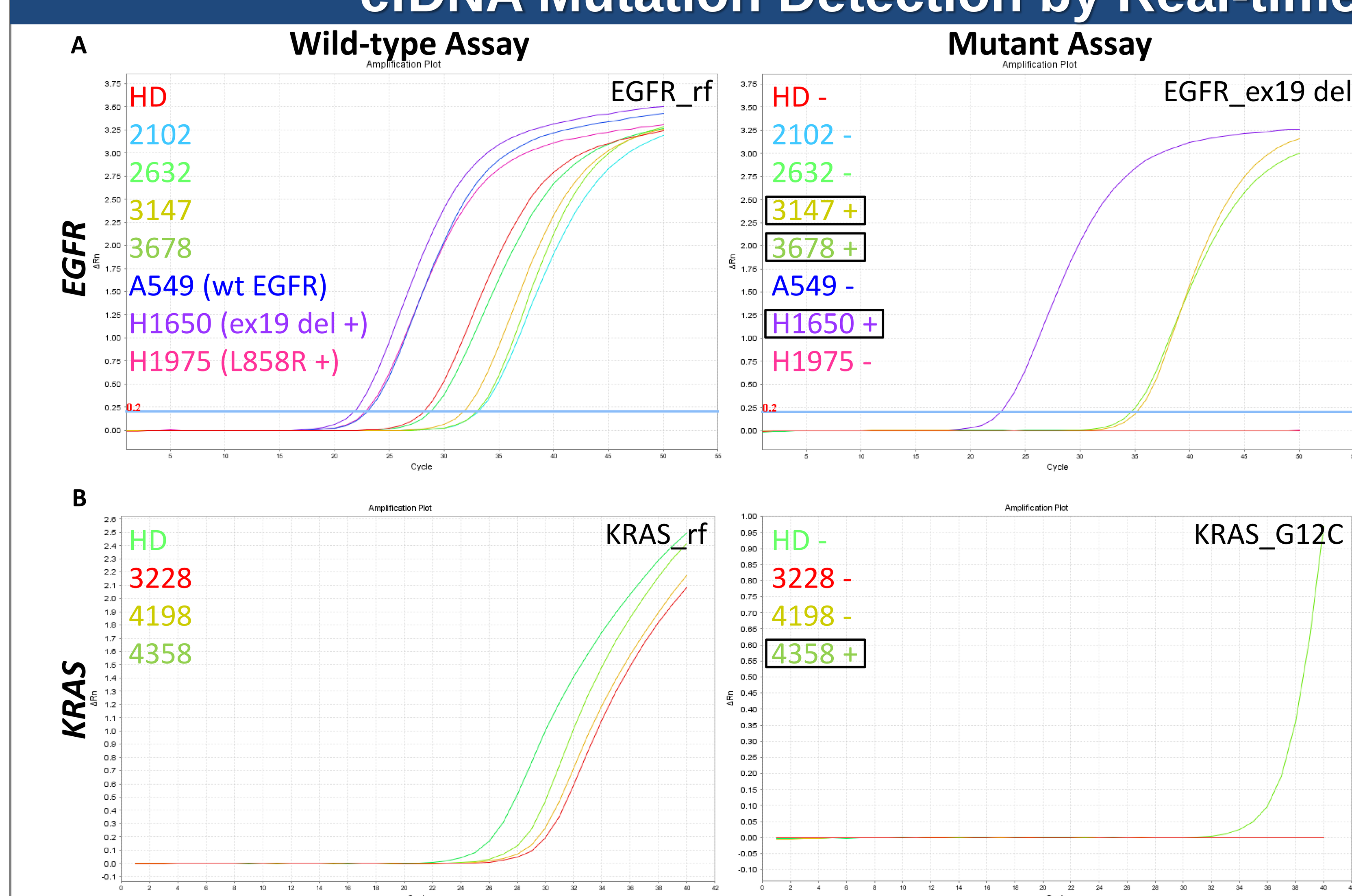


Figure 3. cfDNA mutation detection assays

A. Mutation detection assay for *EGFR*. All samples including the Healthy Donor (HD) and cell line controls were positive for the wild-type allele. 2 cfDNA samples were positive for the Exon 19 deletion allele, in addition to the H1650 cell line positive control. Exon 19 mutational status was confirmed by ddPCR in all patients with known Exon 19 deletions but not the HD (Table 2A).

B. Mutation detection assay for *KRAS*. All samples ,including HD, were positive for the wild-type allele. 1 cfDNA sample was positive for the G12C mutation. When analyzed by ddPCR, the *KRAS* G12C mutation was confirmed in all patients with known G12C SNPs but not patients with other mutations or the HD (Table 2B).

cfDNA Mutation Detection by ddPCR

Table 2A. *EGFR* ddPCR

Tube ID	ddPCR Assay	Copies/µL	Mutation Status
2102	EGFR_rf	4.5	EGFR Exon 19 deletion
	EGFR_ex19 del	0.15	
2632	EGFR_rf	1.98	EGFR Exon 19 deletion
	EGFR_ex19 del	0.3	
3147	EGFR_rf	2.59	EGFR Exon 19 deletion
	EGFR_ex19 del	1.63	
3678	EGFR_rf	0.64	EGFR Exon 19 deletion
	EGFR_ex19 del	0.08	
HD	EGFR_rf	24.7	wt EGFR
	EGFR_ex19 del	0	

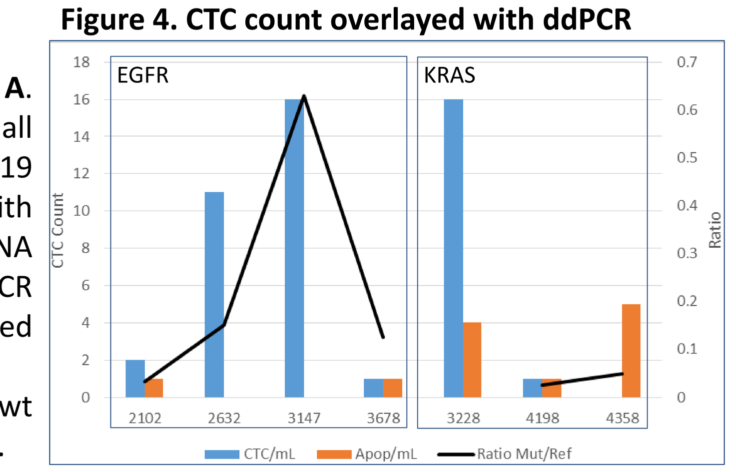
Table 2B. *KRAS* ddPCR

Tube ID	ddPCR Assay	Copies/µL	Mutation Status
3228	KRAS_rf	2.47	KRAS Codon 61
	KRAS_G12C	0	
4198	KRAS_rf	5.36	KRAS G12C
	KRAS_G12C	0.14	
4358	KRAS_rf	9.44	KRAS G12C
	KRAS_G12C	0.46	
HD	KRAS_rf	18.45	wt KRAS
	KRAS_G12C	0	

Table 2A and 2B. ddPCR (droplet digital PCR) assay for **A.** *EGFR* and **B.** *KRAS.* The wild-type allele was detected in all samples including the HD. The mutant allele, EGFR_ex19 del or KRAS_G12C, was detected in all samples with known corresponding mutations, including the cfDNA samples that did not amplify by standard real-time PCR (highlighted in gray). The mutant assay was not detected in the HD.

Figure 4. Patients with higher ratios of the mutant vs wt allele in cfDNA also possessed higher frequency of CTCs.

Figure 4. CTC count overlayed with ddPCR



Single Cell ddPCR

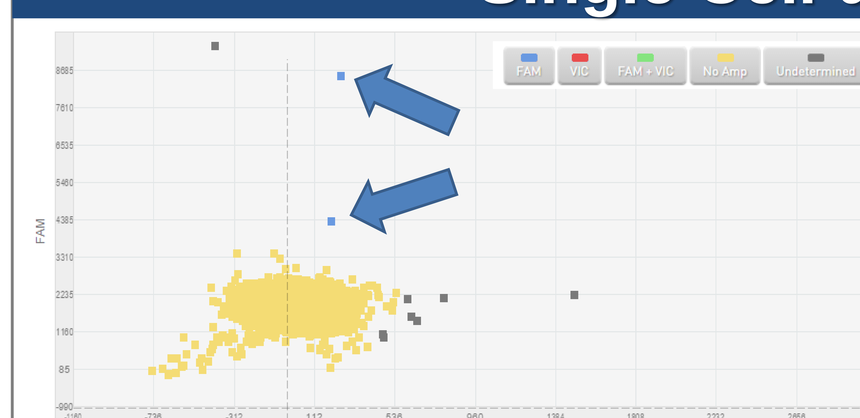


Figure 5. Single cell mutation detection by ddPCR

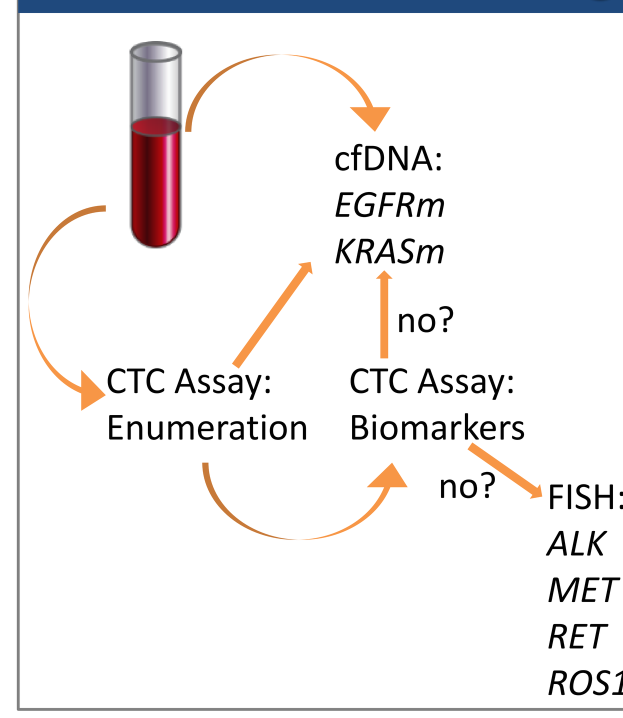
A single H1650 cell line cell, harboring a homozygous *EGFR* Exon 19 deletion, was lysed and analyzed directly by ddPCR for Exon 19. Two FAM-labeled amplicons (blue arrows) were detected, representative of the homozygous Exon 19 deletion.

Results

The combination CTC and cfDNA liquid biopsy test confirmed the EGFR, KRAS and ALK mutational status in NSCLC patient samples.

- ddPCR demonstrated higher sensitivity vs. real-time PCR in cfDNA
- EGFR mutational status was confirmed in 4/4 patients from cfDNA
- KRAS mutational status was confirmed in 2/3 patients from cfDNA
- Patients with higher levels of EGFRm cfDNA also possessed higher frequency of CTCs.
- ALK rearrangement confirmed by both IF and FISH from CTCs
- ALK and/or EGFRm protein was observed in traditional, apoptotic, small and CK- CTCs.

Conclusions



Characterization of NSCLC biomarkers is feasible utilizing a combination of protein and molecular endpoints starting from CTCs and cfDNA from a single tube of blood. These tests demonstrate sensitivity and specificity in patient selection for standard of care therapies or in the monitoring of residual disease following treatment. The identification of biomarker positive CTC subpopulations identifies unique tumor cell morphology and suggests evidence of epithelial plasticity. The use of both CTC and cfDNA analysis represent complementary approaches for evaluation of a larger, more robust biomarker panel at the protein and genomic level.