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Validation of Highly Sensitive TargetSelector™ ctDNA Assays for *EGFR*, *BRAF*, and *KRAS* Mutations

Shan-Fu Wu, PhD; Timothy T. Lu, PhD; Anh Pham; Jeffrey Chen; Tony Daher; Errin Samuelsz; Manisha Patel; Veena M. Singh, MD; Lyle J. Arnold, PhD; Jason C. Poole, PhD

Biocept, San Diego, CA, USA

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Background

Accurate detection of actionable mutations in patients with cancer is vital for targeted therapy. Compared to tissue biopsy, "liquid biopsy" offers a non-invasive and more systemic approach to identify tumor mutations by assessing circulating tumor DNA (ctDNA) released from tumor cells into peripheral blood. We have developed TargetSelector™ Real-Time PCR based assays to detect low frequency mutant alleles in ctDNA. The TargetSelector™ assays use a patented blocking approach to suppress amplification of excess WT DNA released from normal cells, while allowing specific amplification of mutants. Here we focus on five important targets: *EGFR* (Del19, L858, and T790), *BRAF* (V600), and *KRAS* (G12/G13), which are relevant to lung cancer, melanoma, and colorectal cancer.

TargetSelector™ ctDNA Assay Workflow & Diagram

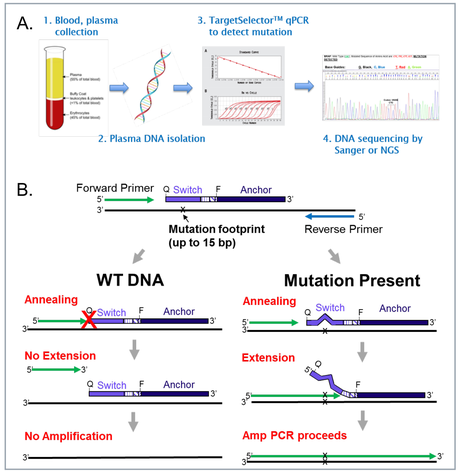


Figure 1: A. The TargetSelector™ ctDNA assays utilize qPCR followed by Sanger or NGS DNA sequencing to verify mutations.
B. The TargetSelector™ assays are targeted mutation tests which apply a blocker (switch + anchor) to block WT DNA amplification while allowing mutant DNA amplification. One specific blocker covers variants on a short stretch of target DNA (up to 15 bp for nucleotide variants).

Methods

The TargetSelector™ ctDNA assays apply a specific blocker to cover variants on a short stretch of target DNA (up to 15 bp for nucleotide variants). For example, one *KRAS* exon 2 blocker covers all variants on both G12 and G13 codon positions. DNA from cancer cell lines carrying the specific target mutations were used for analytical validation of the TargetSelector™ ctDNA assays incorporating the QuantStudio 5 (QS5) Real-Time PCR instrument (Thermo Fisher). Sanger or NGS DNA sequencing was subsequently performed to confirm the mutation. Analytical validation was conducted by 3 independent operators using 5 instruments across 5 days in Biocept's CLIA-certified and CAP-accredited laboratory. For ctDNA testing, whole blood samples were collected in CEE-Sure™ Blood Collection tubes and DNA extraction from plasma was performed using the QIAsymphony (QIAGEN).

Results

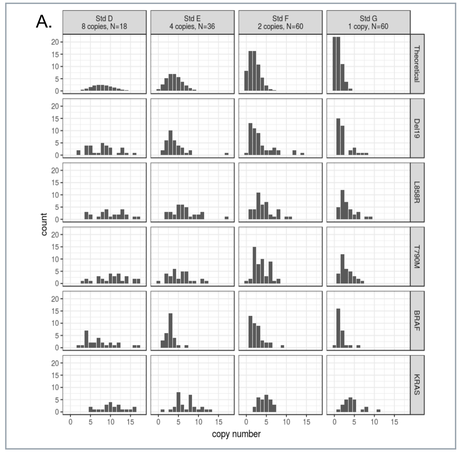
In total, we tested 3086 samples for *EGFR*, *BRAF* and *KRAS* TargetSelector™ ctDNA assays for analytical validation, with *EGFR* WT assay as the background reference. The inter-assay and intra-assay analyses showed r^2 >0.94, suggesting a consistent performance among operational variables. Each Biocept's TargetSelector™ ctDNA assay showed >99% analytical sensitivity and >99% analytical specificity. Samples tested from 20 healthy donors (100 tests in total) showed clinical specificity >99%.

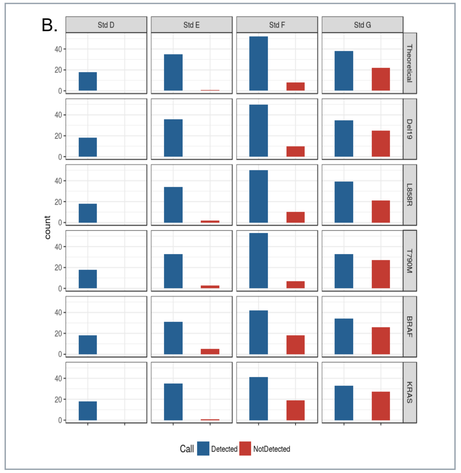
Analytical Validation of TargetSelector™ Assays

ctDNA Assay	Analytical Sensitivity (FN/TP)	Analytical Specificity (FP/TN)	LOD
***EGFR* Del19**	> 99% (0/120)	> 99% (1/112)	0.01%
***EGFR* L858**	> 99% (0/138)	> 99% (1/112)	0.02%
***EGFR* T790**	> 99% (0/138)	> 99% (0/112)	0.01%
***BRAF* V600**	> 99% (1/135)	> 99% (0/112)	0.01%
***KRAS* exon 2**	> 99% (0/136)	> 99% (0/112)	0.02%

Table 1: Each Biocept's TargetSelector™ ctDNA assay was analytically validated and showed >99% analytical sensitivity and >99% analytical specificity. Limit of detection (LOD) for each assay was tested in the presence of 14,000 WT copies, and showed sensitivity at 0.02% MAF or better. FN, false negative; TP, true positive; FP, false positive; TN, true negative.

Single Copy Sensitivity of TargetSelector™ Assays





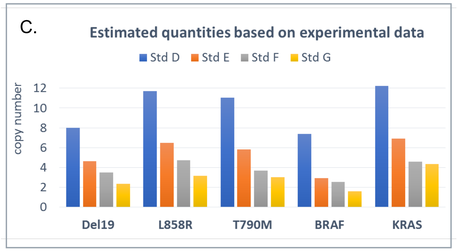


Figure 2: Detection sensitivity of each ctDNA assay was determined through a serial dilution of mutant copies (in the absence of WT copies). A. Histograms for the frequency of copy number detection occurrences.
B. Plots for the number of detection occurrences. The top rows show the theoretical profiles based on a Poisson distribution, with corresponding sample numbers (N) for each standard. The actual data of each assay recapitulate well with the theoretical model.
C. Based on the experimental dataset, we used the estimating method in statistics, maximum likelihood estimation, to estimate the mutant copy number at each standard level. Digital droplet PCR was used as an orthogonal method to confirm the level of the serial dilution. Detection of Std G (1 mutant copy) and the magnitude relationship of estimated copy numbers between standard levels demonstrated single mutant copy detection sensitivity.

Clinical Specificity of TargetSelector™ Assays

ctDNA Assay	Tested	Detected	Clinical Specificity
***EGFR* Del19**	20	0	> 99%
***EGFR* L858**	20	0	> 99%
***EGFR* T790**	20	0	> 99%
***BRAF* V600**	20	0	> 99%
***KRAS* exon 2**	20	0	> 99%

Table 2: Clinical specificity of each TargetSelector™ ctDNA assay was validated on DNA extracted from 4ml of healthy donor plasma samples. No mutation was detected from 20 healthy donors, suggesting >99% clinical specificity for each of our TargetSelector™ ctDNA assay.

Clinical Experience of TargetSelector™ ctDNA Assays using the QuantStudio 5

A.

All Samples	Tested	Detected	% of Detection
***EGFR* Del19**	1108	156	14%
***EGFR* L858**	1108	87	8%
***EGFR* T790**	1108	141	13%
***BRAF* V600**	760	24	3%
***KRAS* exon 2**	249	52	21%

B.

NSCLC Samples	Tested	Detected	% of Detection	Expected frequency
***EGFR* Del19 & L858**	487	124	26%	10 - 35%
***EGFR* T790**	487	69	14%	< 1 - 60%
***BRAF* V600**	292	6	2%	1 - 4%
***KRAS* exon 2**	60	9	15%	15 - 25%

Table 3: Whole blood samples from patients with cancer were collected in Biocept's CEE-Sure™ Blood Collection tubes. Plasma and ctDNA were isolated, and TargetSelector™ ctDNA assays were then run in Biocept's CLIA-certified and CAP-accredited laboratory. All samples shown here were tested using the QS5. A. Samples comprise various cancer types including lung, breast, colorectal, and melanoma cancers.
B. NSCLC (non-small cell lung cancer) samples are shown. Expected frequencies are referenced from "mycancergenome.com". Samples for *EGFR* T790M include both untreated and treated patients.

Conclusions

- Each Biocept's TargetSelector™ ctDNA assay shows >99% analytical sensitivity and >99% analytical specificity.
- TargetSelector™ ctDNA assays show single mutant copy detection based on experimental data compared to theoretical estimates, with sensitivity at 0.02% MAF or better in a background of excess WT DNA.
- Biocept's ctDNA assays detected no false positives from 20 healthy donors, and showed >99% clinical specificity.
- Implementation of the QuantStudio 5 qPCR platform into Biocept's TargetSelector™ ctDNA assays translated into high clinical sensitivity and fast turnaround time for patients in Biocept's CLIA-certified and CAP-accredited laboratory.