High Sensitivity Detection of Rare EGFR Mutations with ctDNA using Target-Selector™ Assays

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Background

Determining the presence of mutated circulating tumor DNA sequences in the blood is an emerging technology termed "liquid biopsy" and is used to monitor tumor burden and to guide personalized treatment. Examples of critical mutations for lung cancer patients are activating mutations L858R and Del19 on EGFR exons 21 and 19 respectively, as well as the resistance mutation T790M on EGFR exon 20. Interrogating circulating tumor DNA for the presence of these mutations affords a more systemic (unlike tissue with it's inherent intra-tumoral heterogeneity), surrogate and non-invasive approach for EGFR mutational analysis in patients with lung cancer; however it can also be a great challenge due to the presence of excess wild-type sequences arising from normal necrotic and apoptotic cell material that has been shed into the bloodstream. To overcome these limitations, Biocept has developed the "Target-Selector™" Real-Time PCR based assays for the detection of EGFR mutations T790M, L858R and Del19. The Target-SelectorTM assays detect rare mutant alleles even in the presence of a large excess of wild-type (WT) DNA. By using a proprietary blocker to create a highly selective blockade of WT DNA while allowing mutant DNA templates to be amplified normally, the assay can detect down to 0.05% mutant allele frequency with high sensitivity and specificity. Subsequent Sanger sequencing of the Target-Selector™ assays can then identify any point mutation occurring in a short stretch of target DNA (8 or 10bp for L858R or T790M respectively), or in the case of deletions up to 24bp (spanning EGFR K745 to T751). Data from the clinical validation of these EGFR Target-Selector™ assays is presented.

Methods

Circulating nucleic acid was extracted from blood plasma and used in Target-Selector™ assays specific for the amplification of EGFR kinase mutations: (1) with deletion 746-750 in exon 19, (2) the L858R point mutation within exon 21, and (3) T790M within exon 20. Target-Selector™ uses forward and reverse primers as well as a Target-Selector™ probe to specifically block wild-type amplification and allow enrichment of mutant sequences. The Target-Selector™ probe serves as a wild-type blocker as well as a detection probe for amplification. Sanger sequencing of the amplified Target-Selector™ product is used to confirm presence of the mutation. Exon 19 deletion and L858R are the two most common EGFR activating mutations seen in patients with NSCLC, whereas T790M is associated with acquired resistance to TKI therapy.

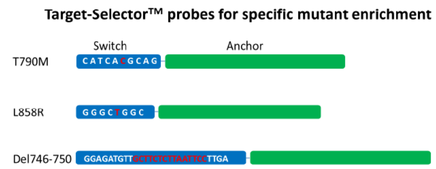

Fig. 1: Target-Selector™ probes consist of two domains: a switch domain which spans the mutation region of interest (marked in red for the individual mutations) and an anchor domain which allows efficient hybridization to target region. They also contain quencher and fluorescent label for detection of amplified product (not shown).

Methods

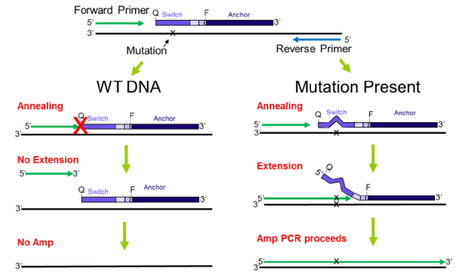

Fig. 2: Target-Selector™ probes block wild-type amplification but allow mutant amplification. When a mutation is found in the DNA template this leads to a reduction of the melting temperature of the switch portion of the Target-Selector™ probe for the mutant DNA template, which in turn allows the forward primer under the PCR cycling condition to extend through the switch portion. In the case of the wild-type DNA template the forward primer cannot extend through the switch portion. Once the temperature is increased during PCR cycling, the forward primer in the case of the wild-type falls off the wild-type DNA template without extending, but can complete extension through the mutant DNA template.

The Target-Selector™ probe allows the mutant to efficiently amplify in PCR reactions over a wide extension temperature window compared to the wild-type template which is efficiently blocked

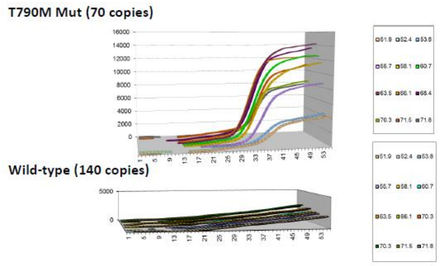

Fig. 3: Amplification temperature profile. Target-Selector™ assay was done using 500pg H1975 genomic DNA (70 copies T790M mutant) or 500pg LnCAP (140 copies wild-type) with increasing extension temperatures from 52°C to 72°C.

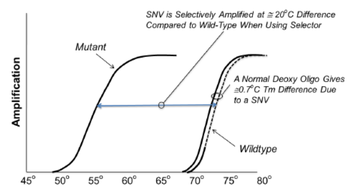

Fig. 4: The Target-Selector™ probe shows a 20°C window of preferential amplification of mutant template.

Decreasing amounts of H1975 standards were used in T790M Target-Selector™ qPCR reactions in the presence of 150ng LnCAP and the results plotted and compared to hypothetical results if 1%, 0.1% or 0.01% "break-through" amplification of wild-type is observed.

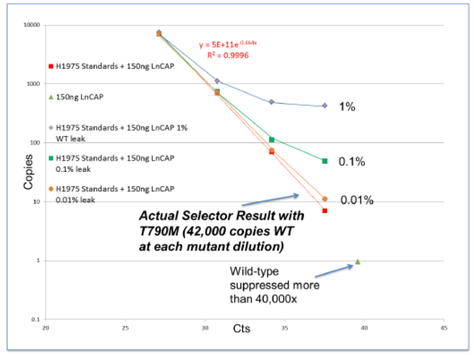

Fig. 5: T790M Target-Selector™ qPCR serial dilution showing the effect of "break-through" amplification at 42000 wild-type copies, if it were to occur.

Results

I. Analytical Data

Biocept's EGFR Target-Selector™ assays for T790M, L858R and Del746-750 have been optimized to reliably detect ~7 copies of mutant in a background of 14000 copies of wild-type (1:2000 or 0.05% mixture of mutant in wild-type). To test the analytical performance of the assays, 0.05% mixtures of mutant and wild-type genomic DNA templates (7 copies mutant in 14000 copies wild-type) were used as known positive and 0% mixtures of mutant (0 copies mutant in 14000 copies wild-type) as known negative genomic DNA templates in the respective Target-Selector™ assay. The results of the analytical data sets are shown below. EGFR Target-Selector™ assays show high sensitivity (>96%) and specificity (>98%).

	T790M Analytic	L858R Analytic	Del746-750 Analytic
N	100	100	100
Sensitivity	96%	100%	98%
Specificity	98%	100%	100%
NPV	96%	100%	98%
PPV	98%	100%	100%
Accuracy	97%	100%	99%

Table 1: Analytical performance of EGFR Target-SelectorTM assays: For the T790M Target-Selector™ assay 50 replicates of 50ng LnCAP genomic DNA (as negative) or 50 replicates of 50pg H1975 genomic DNA (as positive) in a mixture with 50ng LnCAP genomic DNA were tested. For the L858R Target-Selector™ assay 50 replicates of 50ng LnCAP genomic DNA (as negative) or 50 replicates of 50pg H1975 genomic DNA (as positive) in a mixture with 50ng LnCAP genomic DNA were tested. For the Del746-750 Target-SelectorTM assay 50 replicates of 50ng human placenta genomic DNA (from SIGMA, as negative) or 50 replicates of 50pg H1650 genomic DNA (as positive) in a mixture with 50ng human placenta genomic DNA were tested. H1650 is heterozygous for Del746-750, and H1975 is heterozygous for T790M and L858R mutations.

II. Clinical Data

The EGFR Target-Selector™ assays were used to detect presence of mutated T790M, L858R and Del19 sequences in blood plasma from lung cancer patients where tissue biopsy results were available. Plasma from in-house donors or breast cancer samples were used as negative control. Samples found positive using the Target-Selector™ assay were confirmed through Sanger sequencing. Droplet Digital PCR was used as an orthogonal method to evaluate discrepancies between plasma and tissue results.

	Positive	Negative	Concordance	69/74 (93%)
			Sensitivity	83%
True Positive	TP – 19	FN – 4	Specificity	98%
			Positive Predictive Value	95%
True Negative	FP – 1	TN – 50	Negative Predictive Value	93%
			Data Size (N)	74

Table 2: Comparison of blood plasma results by EGFR Target-Selector™ to tissue biopsy confirmed to contain EGFR mutation by a Reference lab. Overall concordance was 93%. One possible explanation for discrepancies between blood and tissue is the time between initial diagnosis to time of blood draw and analysis using Target-Selector™.

Conclusions

- Highly sensitive Target-Selector™ assays have been developed for the detection of activating EGFR mutations L858R and Del19 or resistance mutation T790M- which can detect the mutations with >0.05% mutant allele frequency in analytical validation experiments (LOD is 1 mutant copy in 14000 wild-type copies).
- Mutations were found in the circulating nucleic acid of plasma from lung cancer patient's matching tissue biopsy results performed in a Reference lab.
- Target-Selector™ assays have the advantage of allowing any mutation in a stretch of DNA (~10nt) to be enriched and specifically identified with Sanger sequencing.
- We identified T790M mutations in patient plasma with as low as 0.004% mutant allele frequency.
- Here we identified L858R mutations c.2573 T>G, c.2572_2573 CT>AG in patient plasma, as well as synonymous mutations in G857 c.2571 G>T and L858 c.2572 C>T.
- We identified Del19 mutations Del746-752 insV, Del747-750 insP, Del747-752 and Del746-750 in patient plasma.
- Using EGFR Target-Selector™ in a patient cohort of 74 samples we observed concordance to tissue biopsy results at 93%.

References

- Sundaresan T.K. et al., Detection of T790M, the acquired resistance EGFR mutation, by biopsy versus noninvasive blood-based analyses .Clin Cancer Res 2015
- Gazdar A.F., Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors. Oncogene 2009