Validation of a highly sensitive TargetSelector™ ctDNA assay for *ESR1* resistance mutations using an NGS enrichment strategy

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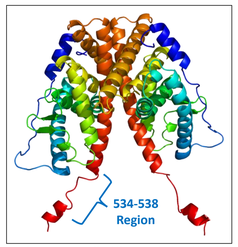
Background

Acquired resistance to anti-hormonal therapy is a common reason for treatment failure in advanced and metastatic breast cancer (mBC). Mutations in the *ESR1* gene, encoding the estrogen receptor (ER) protein, are often associated with this drug resistance. Accurate and timely *ESR1* mutation detection may enable early identification of progression and prediction of treatment failure. In contrast to using difficult and painful tissue biopsies, "liquid biopsy" offers a non-invasive and systemic approach to identify solid tumor mutations in peripheral blood by assessing circulating tumor DNA (ctDNA). Biocept has developed the highly sensitive TargetSelector™ NGS assay to detect low frequency mutant alleles in ctDNA using a patented blocker that suppresses excess wild-type WT alleles, while allowing amplification of tumor derived mutant templates.

ESR1 (Estrogen Receptor-α)

- The *ESR1* gene encodes the nuclear hormone receptor stimulated by estrogen. It is mostly commonly associated with breast cancer but has clinical relevance in endometrial, ovarian and other cancers.
- Abnormal ER expression occurs in >70% of invasive breast cancers, but *ESR1* mutations are rare in primary breast cancers at the time of diagnosis.
- Once patients have been treated with anti-estrogen therapy however, *ESR1* mutations have been identified in up to 55% of ER-positive metastatic breast cancers.
- The implication of these mutations in the resistance to anti-estrogen therapies have spurred efforts to develop therapies that antagonize ER activity in a different manner, one example being (fulvestrant) which stimulates *ESR1* protein degradation.

E380Q	4%
S463P	5%
V534E	3%
P535H	3%
L536Q	3%
L536R	3%
Y537C	5%
Y537S	28%
Y537N	13%
D538G	33%



Tanenbarum D.M. et al(1998) PNAS.USA **95**: 5998-6003

Figure 1: We focus our assay on a five-codon *ESR1* mutation hotspot that imparts tumor cell resistance to anti-hormonal therapy in mBC. The table shows the position and prevalence of these mutations of which over 90% are covered by the TargetSelector™ Assay (red oval and Figure 2C). Image on the right shows the location of the resistance markers within the protein complex.

Technology

TargetSelector™ NGS Assay Workflow & Diagram

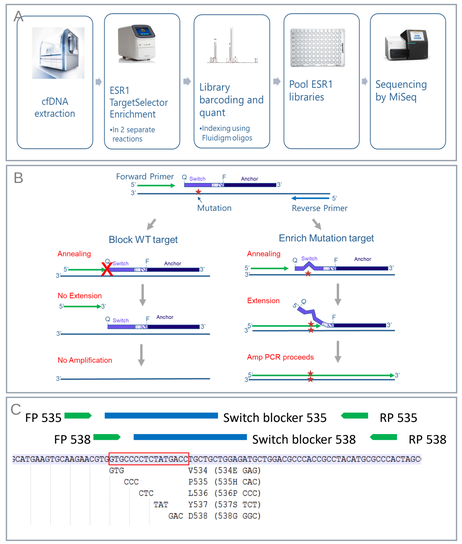


Figure 2: A) The TargetSelector™ assays utilize qPCR followed by DNA sequencing to verify mutations. B) The TargetSelector™ assays are targeted mutation tests which apply a blocker (switch + anchor) to block WT DNA amplification while allowing mutant DNA amplification. C) One specific blocker covers variants on a short stretch of target DNA (up to 15 bp for nucleotide variants).

Methods

We validated the TargetSelector™ assays by spiking low level gBlocks™ DNA fragments (IDT Inc.) carrying point mutations for each of the targeted *ESR1* sites, into WT human placental DNA. Mutant sequences were enriched via PCR and mutations were subsequently confirmed on the Illumina MiSeq down to a limit of detection (LOD) of 0.01-0.05% minor allele frequency (MAF). For ctDNA testing, whole blood was collected in Biocept CEE-Sure™ Blood Collection tubes, and total nucleic acid extraction from plasma was performed on the QIAsymphony (QIAGEN, Inc).

Results

Results Summary

- >600 samples tested during validation
- Fold enrichment of mutants between 4,000 and 22,000
- >99% analytical sensitivity (at 0.05% MAF)
- >97% analytical specificity
- Demonstrated single mutant copy detection at most positions
- Clinical specificity of 95% in testing of 20 healthy donors

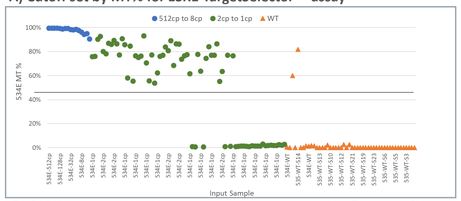
Target fold enrichment between 4,000 and 22,000

Pre-enrichment input			*NGS after TargetSelector enrichment*					
			534E			538G		
MT/WT copy INPUT	MT/WT % INPUT	MT fold enrichment	MT/WT reads OUTPUT	MT/WT % OUTPUT	MT fold enrichment	MT/WT reads OUTPUT	MT/WT % OUTPUT	MT fold enrichment
512/10,000	5.12%	N/A	208,005/711	29245%	5712	231,809/1,198	19350%	3779
128/10,000	1.28%	N/A	141,795/860	16483%	12877	193,400/2,410	8025%	6269
32/10,000	0.32%	N/A	102,203/1,520	6725%	21016	104,349/4,086	2554%	7980
8/10,000	0.08%	N/A	31,684/1,776	1784%	22300	46,967/7,870	597%	7459

Figure 3: Mutation targets were highly enriched by TargetSelector™ assays. All the reads here from NGS passed quality check (Q30 cutoff, 0.1% error rate) at each position. The *ESR1* mutation targets were enriched by the TargetSelector™ assay by at least 3000 fold.

Establishing sensitivity and specificity cutoffs using mutationally enriched data

A) Cutoff set by MT% for *ESR1* TargetSelector™ assay



B) Additional cutoff selection criteria were added for On-target%

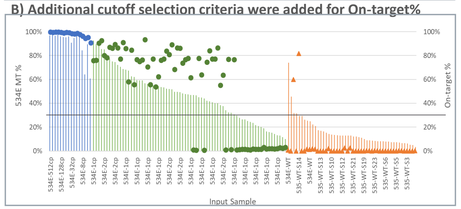
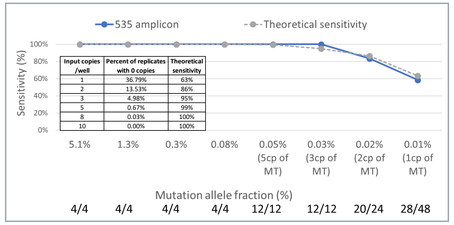


Figure 4: A) shows one example of setting a mutation specific cutoff for *ESR1* V534E. After enrichment the percentage of total high quality on target reads is expressed as a percentage (reads aligned to the amplicon region). The cutoff is set to 47% as indicated by the line.
B) An additional cutoff was set based on on-target %, (the percentage of reads aligned to the target amplicon). Setting the cutoff of on-target % at 30% (indicated by the line) improves the specificity.

Results continued

Single Copy Sensitivity of TargetSelector™ Assays



Input copies /well	Percent of replicates with 0 copies	Theoretical sensitivity
1	36.79%	63%
2	13.53%	86%
3	4.98%	95%
5	0.67%	99%
8	0.03%	100%
10	0.00%	100%

Figure 5: Serially diluted mutant input was applied to a 10,000 copy WT background for the analytical sensitivity test. Numbers below the plot indicate detected/total replicates. The detection rate is 100% from MAF 5% down to 0.03% (500 → 3 copies of MT).
The dotted line represents a theoretically perfect assay based on a Poisson distribution of samples (also shown in the embedded table). The mirroring between *ESR1* 535 assay and the theoretical sensitivity suggests single copy sensitivity and LOD at 0.01% MAF for *ESR1* 535 assay. The reportable range for the *ESR1* TargetSelector™ assay is >5.1% down to 0.01% MAF or a single copy depending on WT background.

Establishing Analytical Specificity

PER TARGET:			
Target Mutation	# of False variants	N	Analytical Specificity
534E	1	80	99%
535H	2	168	98%
536P	0	168	> 99%
537C	0	168	> 99%
537S	0	168	> 99%
537N	0	168	> 99%
538G	1	96	99%
TOTALS:			
Target Mutation	False Positive	N	Analytical Specificity
535 amplicon	3	168	98.2%
538 amplicon	1	184	99.5%
ALL ESR1	4	168	97.6%

Figure 6: The analytical specificity was determined for the *ESR1* TargetSelector™ assay based on WT DNA (sheared NA12878 DNA to 180bp, 33 ng or 10,000 copies input/reaction) or non-variant templates. Each individual variant of *ESR1* was tested. The analytical specificity of the *ESR1* TargetSelector™ assay showed 97% or better for each variant tested.

Results continued

Clinical Specificity and Clinical Experience of TargetSelector™ *ESR1* Assay

A.

Condition	Samples Tested	Spike in MT	Amplicon	Mutation Detected
Healthy	20	none	535 & 538	**1, Y537C (FP)**
Breast cancer, ER+	20	none	535 & 538	**2, D538G**
MT Spike in to healthy plasma	1	300 copies of 534E	535	**V534E**
MT Spike in to healthy plasma	1	300 copies of 537N	538	**Y537N**
Positive Control (0.3% MT)	1	534E & 537N in NA12878	535 & 538	**V534E & Y537N**
Negative Control	1	NA12878	535 & 538	**none**

Figure7: The clinical specificity was determined based on cfDNA extracted from 4ml of plasma samples from healthy donors. One false positive from 20 healthy samples yielded a clinical specificity of 95%. We also screened 20 breast cancer samples that tested ER positive in tissue or circulating tumor cells via immuno-histochemistry. The *ESR1* TargetSelector™ assay detected *ESR1* D538G mutations in 2 of these late-stage breast cancer samples. The high level of enrichment (Mutation (MT)% 95% & 99%) suggested they carry D538G at higher than 0.3% MAF. gBlocks™ DNA spiked into healthy plasma was used as positive controls and detected as expected.

Conclusions

1. Analytical Validation of our TargetSelector™ test shows single mutant copy detection and LOD at 0.03% or better in a background of excess WT DNA.
2. Ultra low copy mutation targets were enriched by the TargetSelector™ Assay >3000 fold.
3. The *ESR1* TargetSelector™ Assay maintains >99% sensitivity for MAF ranging from >5% to 0.03% (500 to 3 copies of MT).
4. We measured analytical specificity at >97% and clinical specificity 95% (N=20). Indicating the test is competent for use in a CLIA/CAP accredited laboratory.
1. In a cohort of 20 mBC patients we detected two D538G mutations at a MAF ~0.3%. Studies are underway to evaluate potential clinical applications of Biocept's highly sensitive Target Selector™ ctDNA *ESR1* mutation assays.