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Target Selector™ DNA EGFR kit for tissue demonstrates high sensitivity without the need for macro-dissection

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Introduction

Biocept's Target Selector™ ctDNA platform was originally developed to detect and track cancer-related mutations (EGFR, KRAS, BRAF) from a simple blood draw for use in analyzing biomarkers from circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). We have previously reported extensive analytical performance characteristics and clinical concordance of the Target Selector™ ctDNA assays. Here we demonstrate the expanded use of our Target Selector™ ctDNA assay on formalin-fixed paraffin-embedded tissue (FFPE). The high sensitivity of Target Selector™ DNA assays (LOD, 0.01-0.03%) allows molecular analysis of pathological tissue sections without tumor content enrichment by macro-dissection, potentially streamlining molecular testing processes in a cost-effective and time efficient manner. To test this kit, DNA was extracted from cancer patient FFPE tissue slides, analyzed by Target Selector™ qPCR assays, followed by Sanger sequencing. The mutational profile was compared to the pathological case report for each patient.

Materials and Methods

Unstained FFPE slides (5µm sections) and corresponding H&E slide from tumor cases with EGFR (L858R, Del19 or T790M), BRAF V600 or KRAS mutations (codon 12/13) were obtained from Discovery Life Sciences or Reprocell. The H&E slides were analyzed by in-house pathologist and marked for tumor content. The tumor-marked H&E slides were used to mark unstained slides for each case for macrodissection.

Figure 1. FFPE tissue from unstained slides was scraped for DNA extraction either from whole slide (non-macrodissected) or only from area marked by pathologist as tumor on corresponding H&E slide (macro-dissected).

Frozen slides from normal tissue 5µm sections (lung, colon or breast obtained from Origene) were also used as negative controls. DNA was extracted from the FFPE or normal tissue using QS GeneRead DNA FFPE treatment and QIAsymphony DSP DNA Mini kits on a QIAsymphony instrument (QIAGEN). Purified DNA was used in Target Selector™ reactions on QS5 followed by Sanger sequencing (3500Dx) to confirm mutations. EGFR exon 20 (WT) amplification was also performed in addition to mutant testing for normalization of the data to amplifiable DNA in the tissue slide samples.

Clinical characteristics of cancer patient cases

Sample number	Age, patient (years)	Gender	Tumor type	Mutation	Clinical stage	Age, FFPE slides (years)
1	69	F	NSCLC	L858R	I-A	20
2	79	M	NSCLC	L858R	III-A	14
3	79	M	NSCLC	L858R	III-A	14
4	60	F	NSCLC	L858R	Primary	12
5	79	M	NSCLC	L858R	III-A	14
6	58	M	NSCLC	L858R	I-B	6
7	53	F	NSCLC	L858R	I-A	4
8	59	M	NSCLC	L858R	II-A	6
9	72	F	NSCLC	L858R	I-B	5
10	52	F	NSCLC	L858R	I-B	5
11	56	M	NSCLC	L858R	I-A	5
12	36	M	NSCLC	T790M/L858R	I-B	1
13	49	M	NSCLC	T790M/L858R	I-A	1
14	51	M	NSCLC	T790M/L858R	I-A	1
15	45	M	NSCLC	T790M/L858R	I-A	3
16	67	F	NSCLC	T790M/L858R	I-B	2
17	68	F	NSCLC	Del19	I-A	16
18	48	F	NSCLC	Del19	I-A	7
19	55	M	NSCLC	Del19	II-A	5
20	68	F	NSCLC	Del19	I-A	6
21	61	F	NSCLC	Del19	I-B	6
22	Unknown	F	CRC	BRAF	II-A	23
23	Unknown	Unknown	CRC	BRAF	Unknown	23
24	42	M	CRC	BRAF	Unknown	24
25	Unknown	Unknown	CRC	BRAF	Unknown	24
26	57	M	Melanoma	BRAF	I-B	21
27	76	F	CRC	KRAS	Unknown	17
28	58	F	CRC	KRAS	Unknown	18
29	76	F	NSCLC	KRAS	II-A	15
30	81	F	CRC	KRAS	III-C	19

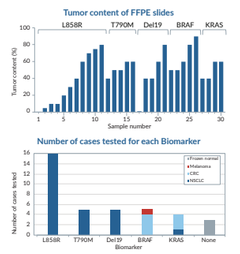
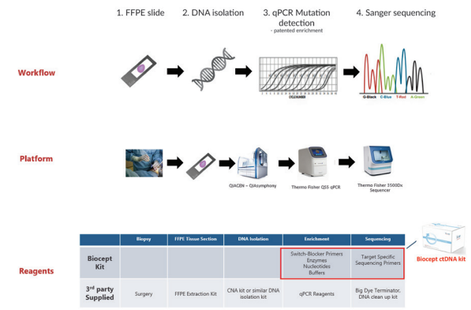


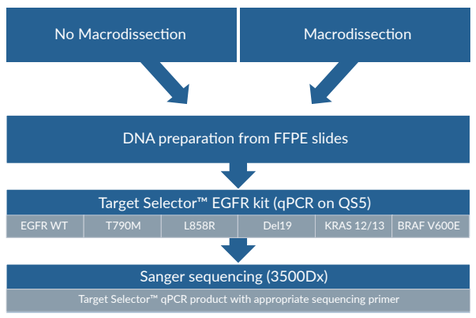
Figure 2. Summary of cancer patient cases analyzed with Target Selector™ assays using FFPE tissue slides. Clinical characteristics, tumor content and number of cases for each of the mutant EGFR L858R, del19, T790M, BRAF V600 and KRAS codon 12/13 Biomarker patient cases are indicated.

Technology

A. Target Selector™ kit workflow



B. Target Selector™ FFPE slide experimental procedure



C. Target Selector™ mutant enrichment

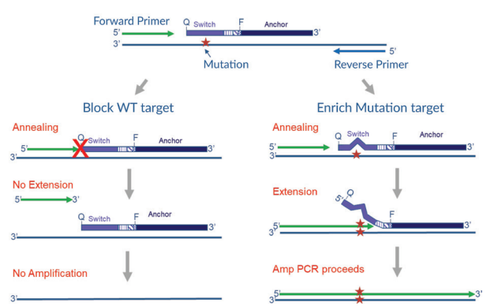
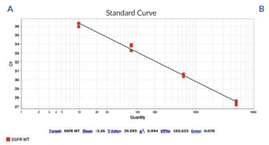


Figure 3: A) Target Selector™ kit assays utilize qPCR followed by DNA sequencing to verify mutations. B) For FFPE Target Selector™ kit assays FFPE DNA is prepared on QIAsymphony and used in Target Selector™ mutant enrichment. C) Target Selector™ mutant enrichment applies a blocker (switch + anchor) to block WT DNA amplification while allowing mutant DNA amplification.

Results

FFPE DNA represents challenging material for molecular analyses of cancer-related mutations because of DNA fragmentation due to long-term storage as well as crosslinks (DNA:DNA and DNA:protein) due to formalin treatment that have to be reversed. Also, hydrolytic cytosine deamination which converts cytosine to uracil has been identified as a major source for artifacts in FFPE tissue. To overcome these challenges: First, we used EGFR exon 20 (WT) amplification as a normalization factor for amplifiable DNA in FFPE DNA. To this end, 3ul of DNA purified from non-macrodissected or macrodissected FFPE slides and from frozen normal tissue slides was performed in EGFR WT Target Selector™. EGFR WT copy number was determined by performing on the same plate QPCR using a standard curve of serial dilutions with EGFR copy numbers of 5000, 625, 78 and 10 respectively. Second, the purification of FFPE DNA was done using an Uracil-N-Glycosylase treatment which eliminates uracil in U:G mismatches. Third, we established a cut-off for each Target Selector™ assay using the data from negative samples for each respective mutation Target Selector™ assay.



Patient case	Total copies EGFR (50ul)	
	Non-macro	Macro
Del19 #1	12688	3636
Del19 #2	51875	65986
Del19 #3	7343	3561
Del19 #4	19339	21915
Del19 #5	5151	4182
Del19 #6	10789	1946
L858R#1	7335	5965
L858R#2	637	353
L858R#3	34479	13712
L858R#4	22033	8446
L858R#5	1999	1846
L858R#6	51853	27294
L858R#7	49773	37203
L858R#8	2439	0
L858R#9	4453	223
L858R#10	10408	1513
T790M#1	7341	4561
T790M#2	3171	2900
T790M#3	4171	586
T790M#4	7044	3670
T790M#5	16052	6435
BRAF #1	15225	8593
BRAF #2	2863	935
BRAF #3	3804	1297
BRAF #4	17223	8733
BRAF #5	10957	3963
KRAS #1	4035	2378
KRAS #2	4032	2725
KRAS #3	8481	4302
KRAS #4	1626	2129
Normal colon	43236	N/A
Normal lung	37137	N/A
Normal breast	78776	N/A

Figure 4. (A) Standard curve which was used to quantify EGFR WT copies in FFPE DNA or normal tissue DNA. Serial dilution of genomic DNA from H1975 cell line corresponding to 5000, 625, 78 or 10 copies of EGFR respectively was used in EGFR WT Target Selector™. The standard curve was used to quantify the EGFR copies in DNA extracted from FFPE DNA or normal tissue DNA which were run on the same QPCR plate. **(B)** EGFR copies determined by Target Selector™ for FFPE DNA (from non-macro-dissected or macro-dissected tissue) or normal tissue. The total copies for each patient case are indicated. The total copies EGFR (50ul) were used in normalization of mutant data and analysis of mutant allele frequency for each of Target Selector™ assays tested.

DNA from 30 cancer patient cases (prepared from either non-macrodissected or macrodissected FFPE slides) or from 3 normal tissue slide was then used in Target Selector assays specific for mutations in Biomarkers: T790M, L858R, Del19, BRAF V600 or KRAS codon 12/13. A standard curve was used for each mutant assay to quantify the mutant copies and the ratio of mutant copies to EGFR WT copies was used to determine the mutant allele frequency. The data from negative samples for each mutant assay were tabulated to establish a cut-off and determine sensitivity and specificity for each assay using the cut-off value. The cut-off was set as the mean of the allele frequency (%) + 3*Standard deviations. All QPCR reactions showing amplification by Target Selector™ were analyzed by Sanger sequencing for confirmation of presence of mutant.

Results

Target Selector™ cut-off setting for FFPE tissue

Using the calculated cut-off of 1.7% for the T790M mutation the T790M Target Selector™ assay has a sensitivity of 100% (5/5 for both non-macrodissected and macrodissected) and a specificity of 96% (24/25) for non-macrodissected or 100% (25/25) for macro-dissected respectively. The cutoff setting for each of the Target Selector assays are shown below in Fig 5 and the accuracy, sensitivity and specificity for each assay based on these cutoffs is summarized in Fig 6.

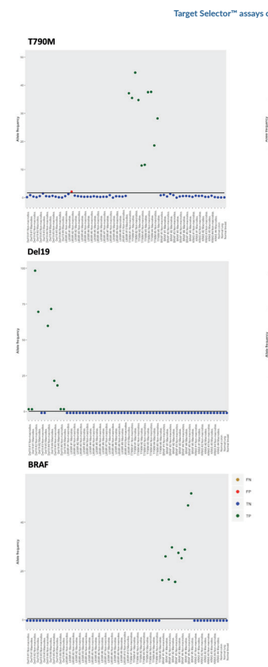


Figure 5. Target Selector™ cut-offs using negative cases (FFPE slides and frozen tissue) for T790M, L858R, Del19, KRAS and BRAF were set at 1.71%, 0.16%, 0.17%, 1.64% and 0.46% respectively (TP: True Positive; FP: False Positive; TN: True Negative; FN: False Negative). [Note: Del#3 has been confirmed as L858R case by vendor and confirmed in this study by Sanger sequencing.]

FFPE DNA Summary: Accuracy, Sensitivity and Specificity

Biomarker	Cut-off (%)	Accuracy		Sensitivity		Specificity	
		Non-macrodissected	Macrodissected	Non-macrodissected	Macrodissected	Non-macrodissected	Macrodissected
L858R	0.17	97% (29/30)	93% (28/30)	100% (16/16)	94% (15/16)	93% (13/14)	93% (13/14)
Del19	0.17	100% (30/30)	100% (30/30)	100% (5/5)	100% (5/5)	100% (25/25)	100% (25/25)
T790M	1.7	97% (29/30)	100% (30/30)	100% (5/5)	100% (5/5)	96% (24/25)	100% (25/25)
BRAF	0.46	100% (30/30)	100% (30/30)	100% (5/5)	100% (5/5)	100% (25/25)	100% (25/25)
KRAS	1.64	93% (28/30)	100% (30/30)	100% (4/4)	100% (4/4)	92% (24/26)	100% (26/26)
Total	N/A	97.3% (146/150)	98.7% (148/150)	100% (35/35)	97.1% (34/35)	96.5% (111/115)	99.1% (114/115)

Figure 6: Target Selector™ cut-off setting is indicated for each Biomarker and was used to determine accuracy, sensitivity and specificity of Target Selector™ assays using FFPE tissue (non-macrodissected or macrodissected).

Results

Target Selector™ assay tissue results Sanger sequencing summary

Biomarker	Patient case	Macro-dissected	Sanger Sequencing results				
			T790M	L858R	Del19	BRAF	KRAS
Del19	1	-	T790M	L858R	Del 747-753 ins Ser	V600 sym. mut.	N/A
		+	T790M	L858 and G857 sym. mut.	Del 747-753 ins Ser	N/A	N/A
	2	-	T790M	L858 and G857 sym. mut.	Del746-750	N/A	N/A
		+	T790M	L858 and G857 sym. mut.	Del746-750	V600 sym. mut.	N/A
	3	-	T790M	L858R	N/A	Not readable	[illegible]
		+	T790M	L858R	N/A	V600 sym. mut.	N/A
	4	-	T790M	L858R, G857 sym. mut.	Del 746-750	V600 sym. mut.	N/A
		+	T790M	L858 and G857 sym. mut.	Del 746-750	Not readable	N/A
	5	-	T790M	G857 sym mut.	Del 747-752 ins Ser	V600 sym. mut.	Not readable
		+	T790M	N/A	Del 747-752 ins Ser	K601 sym. mut.	Not readable
	6	-	T790M	G857V	Del747-753 ins Ser	N/A	[illegible]
		+	T790 sym. mut.	N/A	Del747-753 ins Ser	N/A	N/A
L858R	1	-	T790M	L858R	N/A	V600L	Not readable
		+	T790M	L858R	N/A	V600 sym. mut.	Not readable
	2	-	[illegible]	L858R	N/A	N/A	N/A
		+	T790M	L858R	N/A	N/A	N/A
	3	-	T790M	L858R	N/A	Not readable	N/A
		+	T790M	L858R	N/A	Not readable	N/A
	4	-	T790M	L858R	N/A	Not readable	N/A
		+	T790M	L858R	Del 746-750	N/A	N/A
	5	-	T790M	L858R	N/A	N/A	N/A
		+	T790M	L858R	N/A	N/A	N/A
	6	-	T790M	L858R	N/A	V600 sym. mut.	N/A
		+	T790M	L858R	N/A	N/A	N/A
	7	-	T790M	L858R	N/A	V600M	N/A
		+	T790M	L858R	N/A	V600L	Not readable
	8	-	T790M	L858R	N/A	N/A	N/A
		+	N/A	N/A	N/A	N/A	N/A
	9	-	T790M	L858R	N/A	V600M	N/A
		+	T790M	L858R	N/A	N/A	N/A
	10	-	T790M	L858R	N/A	V600E	N/A
		+	T790M	L858R	N/A	N/A	N/A
T790M	1	-	T790M	L858R	N/A	S602Y	G13S
		+	T790M	L858R	N/A	V600L	N/A
	2	-	T790M	L858R	N/A	N/A	N/A
		+	T790M	L858R	N/A	N/A	N/A
	3	-	T790M	L858R	N/A	N/A	N/A
		+	T790M	L858R	N/A	N/A	N/A
	4	-	T790M	L858R	Del746-Del750	S602Y	G12D
		+	T790M	L858R	N/A	N/A	Not readable
	5	-	T790M	L858R	N/A	V600 sym. mut.	N/A
		+	T790M	L858R	N/A	N/A	N/A
BRAF	1	-	T790M	L858 syn. mut.	N/A	V600E	N/A
		+	T790M	L858 syn. mut.	N/A	V600E	Not readable
	2	-	T790M	L858 syn. mut.	N/A	V600E	N/A
		+	T790M	N/A	N/A	V600E	N/A
	3	-	T790M	L858 syn. mut. and G857V	N/A	V600E	N/A
		+	N/A	N/A	N/A	V600E	N/A
	4	-	T790M	L858 syn. mut and L858F	N/A	V600E	N/A
		+	T790M	L858 syn. mut.	N/A	V600E	Not readable
	5	-	T790M	L858 and G857 sym. mut.	N/A	V600E	N/A
		+	T790M	L858 syn. mut.	N/A	V600E	N/A
KRAS	1	-	T790M	[illegible]	N/A	N/A	G12D
		+	T790M	N/A	N/A	N/A	G12D
	2	-	T790M	L858R, L858 syn. mut.	N/A	N/A	G13D
		+	T790M	[illegible]	N/A	N/A	G13D
	3	-	T790M	N/A	N/A	K601 sym. mut.	G12V
		+	T790M	L858R, L858 syn. mut, G857 syn. mut.	N/A	S602F	G12V
	4	-	T790M	L858 syn. mut.	N/A	N/A	G12D
		+	T790M	N/A	N/A	N/A	G12D
Normal	1	Normal colon	T790M	L858 syn. mut.	N/A	V600M	N/A
	2	Normal lung	T790M	L858 syn. mut.	N/A	V600E in/s Gly	Not readable
	3	Normal breast	T790M	L858 syn. mut.	N/A	V600M	N/A

True positive
False positive
True negative
False negative
N/A: Not applicable (No qPCR amplification detected)
syn. mut.: synonymous mutation

Conclusions

- Analysis of Target Selector™ mutation data obtained using FFPE DNA from non-macrodissected or macro-dissected tissue slides indicate that specific mutations for patient cases were detected with high sensitivity and specificity.
- Because of artifacts introduced by formaldehyde fixation and removal for FFPE DNA preparation, cutoffs were established for each of the mutant Target Selector™ assays based on FFPE tissue not containing the mutation or normal frozen tissue slides.
- FFPE DNA is a suitable input for Target Selector™ assays and allows mutation analysis without prior macro-dissection of FFPE tumor sections.

References

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2. Kim, S. S., et al. (2018) Droplet digital PCR-based EGFR mutation detection with an internal quality control index to determine the quality of DNA. Scientific reports, 8(1), 543. doi:10.1038/s41598-017-18642-x